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Gaetano Santulli *Editor*

microRNA: Basic Science

From Molecular Biology to Clinical Practice

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microRNA: Basic Science

From Molecular Biology to Clinical Practice

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Preface

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

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

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

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

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

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

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

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

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

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

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

Victor Ambros *JCB* 2013;201:492

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

Gary Ruvkun *Nat Med* 2008;14:1041

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

Gaetano Santulli

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

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

Introduction

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

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Table 1.1 Characteristics of noncoding RNAs within the cell

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

(continued)

Table 1.1 (continued)

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

Biogenesis

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

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

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

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

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

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

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

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

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

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

Epigenetics and miRs: An Intricate Affair

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

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

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

The Emerging Functional Role of Micropeptidomics

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

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

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

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

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

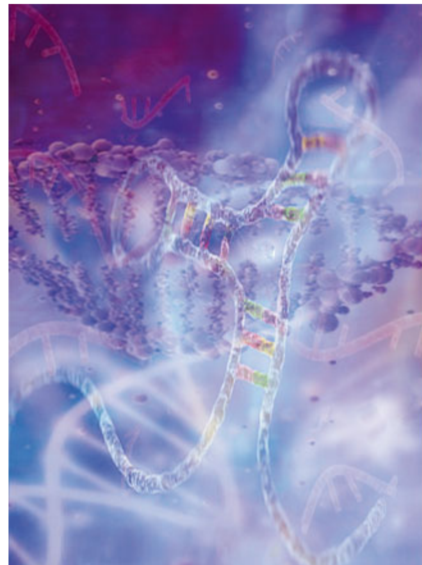

Fig. 1.1 Micropeptidomics: *multa paucis* or *hic sunt leones*?

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

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

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

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References

1. Bazzini AA, Johnstone TG, Christiano R, Mackowiak SD, Obermayer B, Fleming ES, Vejnar CE, Lee MT, Rajewsky N, Walther TC, Giraldez AJ. Identification of small ORFs in vertebrates using ribosome footprinting and evolutionary conservation. EMBO J. 2014;33:981–93.
2. Anderson DM, Anderson KM, Chang CL, Makarewich CA, Nelson BR, McAnally JR, Kasaragod P, Shelton JM, Liou J, Bassel-Duby R, Olson EN. A micropeptide encoded by a putative long noncoding RNA regulates muscle performance. Cell. 2015;160:595–606.
3. Lauressergues D, Couzigou JM, Clemente HS, Martinez Y, Dunand C, Becard G, Combier JP. Primary transcripts of microRNAs encode regulatory peptides. Nature. 2015;520:90–3.
4. Wronska A, Kurkowska-Jastrzebska I, Santulli G. Application of microRNAs in diagnosis and treatment of cardiovascular disease. Acta Physiol. 2015;213:60–83.
5. Ambros V. The functions of animal microRNAs. Nature. 2004;431:350–5.
6. Gan Z, Rumsey J, Hazen BC, Lai L, Leone TC, Vega RB, Xie H, Conley KE, Auwerx J, Smith SR, Olson EN, Kralli A, Kelly DP. Nuclear receptor/microRNA circuitry links muscle fiber type to energy metabolism. J Clin Invest. 2013;123:2564–75.
7. Ruvkun G, Giusto J. The Caenorhabditis elegans heterochronic gene lin-14 encodes a nuclear protein that forms a temporal developmental switch. Nature. 1989;338:313–9.
8. Ruvkun G, Ambros V, Coulson A, Waterston R, Sulston J, Horvitz HR. Molecular genetics of the Caenorhabditis elegans heterochronic gene lin-14. Genetics. 1989;121:501–16.
9. Dvinge H, Git A, Graf S, Salmon-Divon M, Curtis C, Sottoriva A, Zhao Y, Hirst M, Armisen J, Miska EA, Chin SF, Provenzano E, Turashvili G, Green A, Ellis I, Aparicio S, Caldas C. The shaping and functional consequences of the microRNA landscape in breast cancer. Nature. 2013;497:378–82.
10. Shen J, Xia W, Khotskaya YB, Huo L, Nakanishi K, Lim SO, Du Y, Wang Y, Chang WC, Chen CH, Hsu JL, Wu Y, Lam YC, James BP, Liu X, Liu CG, Patel DJ, Hung MC. EGFR modulates microRNA maturation in response to hypoxia through phosphorylation of AGO2. Nature. 2013;497:383–7.
11. Song SJ, Poliseno L, Song MS, Ala U, Webster K, Ng C, Beringer G, Brikbak NJ, Yuan X, Cantley LC, Richardson AL, Pandolfi PP. MicroRNA-antagonism regulates breast cancer

stemness and metastasis via TET-family-dependent chromatin remodeling. Cell. 2013;154: 311–24.

12. 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.
13. Gomez IG, MacKenna DA, Johnson BG, Kaimal V, Roach AM, Ren S, Nakagawa N, Xin C, Newitt R, Pandya S, Xia TH, Liu X, Borza DB, Grafals M, Shankland SJ, Himmelfarb J, Portilla D, Liu S, Chau BN, Duffield JS. Anti-microRNA-21 oligonucleotides prevent Alport nephropathy progression by stimulating metabolic pathways. J Clin Invest. 2015;125:141–56.
14. Epstein DM. Special delivery: microRNA-200-containing extracellular vesicles provide metastatic message to distal tumor cells. J Clin Invest. 2014;124:5107–8.
15. Sachdeva M, Mito JK, Lee CL, Zhang M, Li Z, Dodd RD, Cason D, Luo L, Ma Y, Van Mater D, Gladdy R, Lev DC, Cardona DM, Kirsch DG. MicroRNA-182 drives metastasis of primary sarcomas by targeting multiple genes. J Clin Invest. 2014;124:4305–19.
16. Santulli G, Wronska A, Uryu K, Diacovo TG, Gao M, Marx SO, Kitajewski J, Chilton JM, Akat KM, Tuschl T, Marks AR, Totary-Jain H. A selective microRNA-based strategy inhibits restenosis while preserving endothelial function. J Clin Invest. 2014;124:4102–14.
17. Bertero T, Lu Y, Annis S, Hale A, Bhat B, Saggar R, Saggar R, Wallace WD, Ross DJ, Vargas SO, Graham BB, Kumar R, Black SM, Fratz S, Fineman JR, West JD, Haley KJ, Waxman AB, Chau BN, Cottrill KA, Chan SY. Systems-level regulation of microRNA networks by miR-130/301 promotes pulmonary hypertension. J Clin Invest. 2014;124:3514–28.
18. Chao CH, Chang CC, Wu MJ, Ko HW, Wang D, Hung MC, Yang JY, Chang CJ. MicroRNA-205 signaling regulates mammary stem cell fate and tumorigenesis. J Clin Invest. 2014;124: 3093–106.
19. Alexander MS, Casar JC, Motohashi N, Vieira NM, Eisenberg I, Marshall JL, Gasperini MJ, Lek A, Myers JA, Estrella EA, Kang PB, Shapiro F, Rahimov F, Kawahara G, Widrick JJ, Kunkel LM. MicroRNA-486-dependent modulation of DOCK3/PTEN/AKT signaling pathways improves muscular dystrophy-associated symptoms. J Clin Invest. 2014;124:2651–67.
20. Latreille M, Hausser J, Stutzer I, Zhang Q, Hastoy B, Gargani S, Kerr-Conte J, Pattou F, Zavolan M, Esguerra JL, Eliasson L, Rulicke T, Rorsman P, Stoffel M. MicroRNA-7a regulates pancreatic beta cell function. J Clin Invest. 2014;124:2722–35.
21. Bang C, Batkai S, Dangwal S, Gupta SK, Foinquinos A, Holzmann A, Just A, Remke J, Zimmer K, Zeug A, Ponimaskin E, Schmiedl A, Yin X, Mayr M, Halder R, Fischer A, Engelhardt S, Wei Y, Schober A, Fiedler J, Thum T. Cardiac fibroblast-derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy. J Clin Invest. 2014;124:2136–46.
22. Li CJ, Cheng P, Liang MK, Chen YS, Lu Q, Wang JY, Xia ZY, Zhou HD, Cao X, Xie H, Liao EY, Luo XH. MicroRNA-188 regulates age-related switch between osteoblast and adipocyte differentiation. J Clin Invest. 2015;125:1509–22.
23. Murugaiyan G, da Cunha AP, Ajay AK, Joller N, Garo LP, Kumaradevan S, Yosef N, Vaidya VS, Weiner HL. MicroRNA-21 promotes Th17 differentiation and mediates experimental autoimmune encephalomyelitis. J Clin Invest. 2015;125:1069–80.
24. Santa-Maria I, Alaniz ME, Renwick N, Cela C, Fulga TA, Van Vactor D, Tuschl T, Clark LN, Shelanski ML, McCabe BD, Crary JF. Dysregulation of microRNA-219 promotes neurodegeneration through post-transcriptional regulation of tau. J Clin Invest. 2015;125:681–6.
25. Santulli G, Iaccarino G, De Luca N, Trimarco B, Condorelli G. Atrial fibrillation and microRNAs. Front Physiol. 2014;5:15.
26. Sardu C, Marfella R, Santulli G, Paolisso G. Functional role of miRNA in cardiac resynchronization therapy. Pharmacogenomics. 2014;15:1159–68.
27. van Rooij E, Olson EN. MicroRNA therapeutics for cardiovascular disease: opportunities and obstacles. Nat Rev Drug Discov. 2012;11:860–72.
28. Meijer HA, Kong YW, Lu WT, Wilczynska A, Spriggs RV, Robinson SW, Godfrey JD, Willis AE, Bushell M. Translational repression and eIF4A2 activity are critical for microRNA-mediated gene regulation. Science. 2013;340:82–5.

29. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 2010;466:835–40.
30. 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.
31. Yates LA, Norbury CJ, Gilbert RJ. The long and short of microRNA. Cell. 2013;153:516–9.
32. Yamamoto S, Niida S, Azuma E, Yanagibashi T, Muramatsu M, Huang TT, Sagara H, Higaki S, Ikutani M, Nagai Y, Takatsu K, Miyazaki K, Hamashima T, Mori H, Matsuda N, Ishii Y, Sasahara M. Inflammation-induced endothelial cell-derived extracellular vesicles modulate the cellular status of pericytes. Sci Rep. 2015;5:8505.
33. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116: 281–97.
34. Cullen BR. Transcription and processing of human microRNA precursors. Mol Cell. 2004; 16:861–5.
35. Roberts TC. The microRNA biology of the mammalian nucleus. Mol Ther Nucleic Acids. 2014;3, e188.
36. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the microprocessor complex. Nature. 2004;432:231–5.
37. Zeng Y, Yi R, Cullen BR. Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. EMBO J. 2005;24:138–48.
38. Shiohama A, Sasaki T, Noda S, Minoshima S, Shimizu N. Molecular cloning and expression analysis of a novel gene DGCR8 located in the DiGeorge syndrome chromosomal region. Biochem Biophys Res Commun. 2003;304:184–90.
39. Roth BM, Ishimaru D, Hennig M. The core microprocessor component DiGeorge syndrome critical region 8 (DGCR8) is a nonspecific RNA-binding protein. J Biol Chem. 2013;288: 26785–99.
40. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. Nature. 2007;448:83–6.
41. Alarcon CR, Lee H, Goodarzi H, Halberg N, Tavazoie SF. N6-methyladenosine marks primary microRNAs for processing. Nature. 2015;519:482–5.
42. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science. 2004;303:95–8.
43. Saxena A, Tabin CJ. miRNA-processing enzyme Dicer is necessary for cardiac outflow tract alignment and chamber septation. Proc Natl Acad Sci U S A. 2010;107:87–91.
44. Marasovic M, Zocco M, Halic M. Argonaute and Triman generate Dicer-independent priRNAs and mature siRNAs to initiate heterochromatin formation. Mol Cell. 2013;52(2): 173–83.
45. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008;9:102–14.
46. Wu PH, Isaji M, Carthew RW. Functionally diverse microRNA effector complexes are regulated by extracellular signaling. Mol Cell. 2013;52:113–23.
47. Pfaff J, Hennig J, Herzog F, Aebersold R, Sattler M, Niessing D, Meister G. Structural features of Argonaute-GW182 protein interactions. Proc Natl Acad Sci U S A. 2013;110:E3770–9.
48. Siomi H, Siomi MC. On the road to reading the RNA-interference code. Nature. 2009;457: 396–404.
49. von Brandenstein M, Richter C, Fries JW. MicroRNAs: small but amazing, and their association with endothelin. Life Sci. 2012;91:475–89.
50. Fabian MR, Sonenberg N. The mechanics of miRNA-mediated gene silencing: a look under the hood of miRISC. Nat Struct Mol Biol. 2012;19:586–93.
51. Kallen AN, Ma J, Huang Y. Does Lin28 antagonize miRNA-mediated repression by displacing miRISC from target mRNAs? Front Genet. 2012;3:240.
52. Papait R, Kunderfranco P, Stirparo GG, Latronico MV, Condorelli G. Long noncoding RNA: a new player of heart failure? J Cardiovasc Transl Res. 2013;6:876–83.

53. 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.
54. Dweep H, Sticht C, Pandey P, Gretz N. miRWalk--database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform. 2011;44:839–47.
55. Zacharewicz E, Lamon S, Russell AP. MicroRNAs in skeletal muscle and their regulation with exercise, ageing, and disease. Front Physiol. 2013;4:266.
56. Ambros V, Bartel B, Bartel DP, Burge CB, Carrington JC, Chen X, Dreyfuss G, Eddy SR, Griffiths-Jones S, Marshall M, Matzke M, Ruvkun G, Tuschl T. A uniform system for microRNA annotation. RNA. 2003;9:277–9.
57. Tycko B, Ashkenas J. Epigenetics and its role in disease. J Clin Invest. 2000;105:245–6.
58. Boulard M, Edwards JR, Bestor TH. FBXL10 protects Polycomb-bound genes from hypermethylation. Nat Genet. 2015;47:479–85.
59. Hohl M, Wagner M, Reil JC, Muller SA, Tauchnitz M, Zimmer AM, Lehmann LH, Thiel G, Bohm M, Backs J, Maack C. HDAC4 controls histone methylation in response to elevated cardiac load. J Clin Invest. 2013;123:1359–70.
60. Kraft M, Cirstea IC, Voss AK, Thomas T, Goehring I, Sheikh BN, Gordon L, Scott H, Smyth GK, Ahmadian MR, Trautmann U, Zenker M, Tartaglia M, Ekici A, Reis A, Dorr HG, Rauch A, Thiel CT. Disruption of the histone acetyltransferase MYST4 leads to a Noonan syndrome-like phenotype and hyperactivated MAPK signaling in humans and mice. J Clin Invest. 2011; 121:3479–91.
61. Leucci E, Patella F, Waage J, Holmstrom K, Lindow M, Porse B, Kauppinen S, Lund AH. MicroRNA-9 targets the long non-coding RNA MALAT1 for degradation in the nucleus. Sci Rep. 2013;3, 2535.
62. Swierczynski S, Klieser E, Illig R, Alinger-Scharinger B, Kiesslich T, Neureiter D. Histone deacetylation meets miRNA: epigenetics and post-transcriptional regulation in cancer and chronic diseases. Expert Opin Biol Ther. 2015;15:651–64.
63. Tu J, Liao JJ, Luk AC, Tang NL, Chan WY, Lee TL. MicroRNAs mediated targeting on the Yin-yang dynamics of DNA methylation in disease and development. Int J Biochem Cell Biol. 2015.
64. Greco CM, Condorelli G. Epigenetic modifications and noncoding RNAs in cardiac hypertrophy and failure. Nat Rev Cardiol. 2015;12:488–97.
65. Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, Schwind S, Pang J, Yu J, Muthusamy N, Havelange V, Volinia S, Blum W, Rush LJ, Perrotti D, Andreeff M, Bloomfield CD, Byrd JC, Chan K, Wu LC, Croce CM, Marcucci G. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood. 2009;113:6411–8.
66. Robaina MC, Mazzoccoli L, Arruda VO, Reis FR, Apa AG, de Rezende LM, Klumb CE. Deregulation of DNMT1, DNMT3B and miR-29s in Burkitt lymphoma suggests novel contribution for disease pathogenesis. Exp Mol Pathol. 2015;98:200–7.
67. Yuan JH, Yang F, Chen BF, Lu Z, Huo XS, Zhou WP, Wang F, Sun SH. The histone deacetylase 4/SP1/microrna-200a regulatory network contributes to aberrant histone acetylation in hepatocellular carcinoma. Hepatology. 2011;54:2025–35.
68. Lujambio A, Calin GA, Villanueva A, Ropero S, Sanchez-Cespedes M, Blanco D, Montuenga LM, Rossi S, Nicoloso MS, Faller WJ, Gallagher WM, Eccles SA, Croce CM, Esteller M. A microRNA DNA methylation signature for human cancer metastasis. Proc Natl Acad Sci U S A. 2008;105:13556–61.
69. Bueno MJ, Perez de Castro I, Gomez de Cedron M, Santos J, Calin GA, Cigudosa JC, Croce CM, Fernandez-Piqueras J, Malumbres M. Genetic and epigenetic silencing of microRNA-203 enhances ABL1 and BCR-ABL1 oncogene expression. Cancer Cell. 2008;13:496–506.
70. Hu W, Wang M, Yin H, Yao C, He Q, Yin L, Zhang C, Li W, Chang G, Wang S. MicroRNA-1298 is regulated by DNA methylation and affects vascular smooth muscle cell function by targeting connexin 43. Cardiovasc Res. 2015.
71. Long XR, He Y, Huang C, Li J. MicroRNA-148a is silenced by hypermethylation and interacts with DNA methyltransferase 1 in hepatocellular carcinogenesis. Int J Oncol. 2014;44: 1915–22.

72. Dai E, Yu X, Zhang Y, Meng F, Wang S, Liu X, Liu D, Wang J, Li X, Jiang W. EpimiR: a database of curated mutual regulation between miRNAs and epigenetic modifications. Database (Oxford). 2014;2014:bau023.
73. Santulli G, Marks AR. Essential roles of intracellular calcium release channels in muscle, brain, metabolism, and aging. Curr Mol Pharmacol. 2015;8:206–22.
74. Andrews SJ, Rothnagel JA. Emerging evidence for functional peptides encoded by short open reading frames. Nat Rev Genet. 2014;15:193–204.
75. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, Oyama R, Ravasi T, Lenhard B, Wells C, Kodzius R, Shimokawa K, Bajic VB, Brenner SE, Batalov S, Forrest AR, Zavolan M, Davis MJ, Wilming LG, Aidinis V, Allen JE, Ambesi-Impiombato A, Apweiler R, Aturaliya RN, Bailey TL, Bansal M, Baxter L, Beisel KW, Bersano T, Bono H, Chalk AM, Chiu KP, Choudhary V, Christoffels A, Clutterbuck DR, Crowe ML, Dalla E, Dalrymple BP, de Bono B, Della Gatta G, di Bernardo D, Down T, Engstrom P, Fagiolini M, Faulkner G, Fletcher CF, Fukushima T, Furuno M, Futaki S, Gariboldi M, Georgii-Hemming P, Gingeras TR, Gojobori T, Green RE, Gustincich S, Harbers M, Hayashi Y, Hensch TK, Hirokawa N, Hill D, Huminiecki L, Iacono M, Ikeo K, Iwama A, Ishikawa T, Jakt M, Kanapin A, Katoh M, Kawasawa Y, Kelso J, Kitamura H, Kitano H, Kollias G, Krishnan SP, Kruger A, Kummerfeld SK, Kurochkin IV, Lareau LF, Lazarevic D, Lipovich L, Liu J, Liuni S, McWilliam S, Madan Babu M, Madera M, Marchionni L, Matsuda H, Matsuzawa S, Miki H, Mignone F, Miyake S, Morris K, Mottagui-Tabar S, Mulder N, Nakano N, Nakauchi H, Ng P, Nilsson R, Nishiguchi S, Nishikawa S, Nori F, Ohara O, Okazaki Y, Orlando V, Pang KC, Pavan WJ, Pavesi G, Pesole G, Petrovsky N, Piazza S, Reed J, Reid JF, Ring BZ, Ringwald M, Rost B, Ruan Y, Salzberg SL, Sandelin A, Schneider C, Schonbach C, Sekiguchi K, Semple CA, Seno S, Sessa L, Sheng Y, Shibata Y, Shimada H, Shimada K, Silva D, Sinclair B, Sperling S, Stupka E, Sugiura K, Sultana R, Takenaka Y, Taki K, Tammoja K, Tan SL, Tang S, Taylor MS, Tegner J, Teichmann SA, Ueda HR, van Nimwegen E, Verardo R, Wei CL, Yagi K, Yamanishi H, Zabarovsky E, Zhu S, Zimmer A, Hide W, Bult C, Grimmond SM, Teasdale RD, Liu ET, Brusic V, Quackenbush J, Wahlestedt C, Mattick JS, Hume DA, Kai C, Sasaki D, Tomaru Y, Fukuda S, Kanamori-Katayama M, Suzuki M, Aoki J, Arakawa T, Iida J, Imamura K, Itoh M, Kato T, Kawaji H, Kawagashira N, Kawashima T, Kojima M, Kondo S, Konno H, Nakano K, Ninomiya N, Nishio T, Okada M, Plessy C, Shibata K, Shiraki T, Suzuki S, Tagami M, Waki K, Watahiki A, Okamura-Oho Y, Suzuki H, Kawai J, Hayashizaki Y, FANTOM Consortium, RIKEN Genome Exploration Research Group, Genome Science Group. The transcriptional landscape of the mammalian genome. Science. 2005;309:1559–63.
76. Lee LJ, Hughes TR, Frey BJ. How many new genes are there? Science. 2006;311:1709–11. author reply 1709–11.
77. Troy CS, MacHugh DE, Bailey JF, Magee DA, Loftus RT, Cunningham P, Chamberlain AT, Sykes BC, Bradley DG. Genetic evidence for Near-Eastern origins of European cattle. Nature. 2001;410:1088–91.
78. Rosenberg MI, Desplan C. Molecular biology. Hiding in plain sight. Science. 2010;329: 284–5.
79. Crappe J, Ndah E, Koch A, Steyaert S, Gawron D, De Keulenaer S, De Meester E, De Meyer T, Van Criekinge W, Van Damme P, Menschaert G. PROTEOFORMER: deep proteome coverage through ribosome profiling and MS integration. Nucleic Acids Res. 2015;43, e29.
80. Kondo T, Plaza S, Zanet J, Benrabah E, Valenti P, Hashimoto Y, Kobayashi S, Payre F, Kageyama Y. Small peptides switch the transcriptional activity of Shavenbaby during Drosophila embryogenesis. Science. 2010;329:336–9.
81. Martinho RG, Kunwar PS, Casanova J, Lehmann R. A noncoding RNA is required for the repression of RNApolII-dependent transcription in primordial germ cells. Curr Biol. 2004;14: 159–65.
82. Galindo MI, Pueyo JI, Fouix S, Bishop SA, Couso JP. Peptides encoded by short ORFs control development and define a new eukaryotic gene family. PLoS Biol. 2007;5, e106.
83. Hayden CA, Bosco G. Comparative genomic analysis of novel conserved peptide upstream open reading frames in Drosophila melanogaster and other dipteran species. BMC Genomics. 2008;9:61.

84. Hashimoto Y, Niikura T, Tajima H, Yasukawa T, Sudo H, Ito Y, Kita Y, Kawasumi M, Kouyama K, Doyu M, Sobue G, Koide T, Tsuji S, Lang J, Kurokawa K, Nishimoto I. A rescue factor abolishing neuronal cell death by a wide spectrum of familial Alzheimer's disease genes and Abeta. Proc Natl Acad Sci U S A. 2001;98:6336–41.
85. Guo B, Zhai D, Cabezas E, Welsh K, Nouraini S, Satterthwait AC, Reed JC. Humanin peptide suppresses apoptosis by interfering with Bax activation. Nature. 2003;423:456–61.
86. Ikonen M, Liu B, Hashimoto Y, Ma L, Lee KW, Niikura T, Nishimoto I, Cohen P. Interaction between the Alzheimer's survival peptide humanin and insulin-like growth factor-binding protein 3 regulates cell survival and apoptosis. Proc Natl Acad Sci U S A. 2003;100: 13042–7.
87. Washietl S, Findeiss S, Muller SA, Kalkhof S, von Bergen M, Hofacker IL, Stadler PF, Goldman N. RNAcode: robust discrimination of coding and noncoding regions in comparative sequence data. RNA. 2011;17:578–94.
88. Prokhorova IV, Osterman IA, Burakovsky DE, Serebryakova MV, Galyamina MA, Pobeguts OV, Altukhov I, Kovalchuk S, Alexeev DG, Govorun VM, Bogdanov AA, Sergiev PV, Dontsova OA. Modified nucleotides m(2)G966/m(5)C967 of Escherichia coli 16S rRNA are required for attenuation of tryptophan operon. Sci Rep. 2013;3:3236.
89. Selitsky SR, Baran-Gale J, Honda M, Yamane D, Masaki T, Fannin EE, Guerra B, Shirasaki T, Shimakami T, Kaneko S, Lanford RE, Lemon SM, Sethupathy P. Small tRNA-derived RNAs are increased and more abundant than microRNAs in chronic hepatitis B and C. Sci Rep. 2015;5:7675.
90. Katayama S, Tomaru Y, Kasukawa T, Waki K, Nakanishi M, Nakamura M, Nishida H, Yap CC, Suzuki M, Kawai J, Suzuki H, Carninci P, Hayashizaki Y, Wells C, Frith M, Ravasi T, Pang KC, Hallinan J, Mattick J, Hume DA, Lipovich L, Batalov S, Engstrom PG, Mizuno Y, Faghihi MA, Sandelin A, Chalk AM, Mottagui-Tabar S, Liang Z, Lenhard B, Wahlestedt C, Group RGER, Genome Science G, Consortium F. Antisense transcription in the mammalian transcriptome. Science. 2005;309:1564–6.
91. Mizuno T, Chou MY, Inouye M. A unique mechanism regulating gene expression: translational inhibition by a complementary RNA transcript (micRNA). Proc Natl Acad Sci U S A. 1984;81:1966–70.
92. Kim TK, Hemberg M, Gray JM, Costa AM, Bear DM, Wu J, Harmin DA, Laptewicz M, Barbara-Haley K, Kuersten S, Markenscoff-Papadimitriou E, Kuhl D, Bito H, Worley PF, Kreiman G, Greenberg ME. Widespread transcription at neuronal activity-regulated enhancers. Nature. 2010;465:182–7.
93. Orom UA, Derrien T, Beringer M, Gumireddy K, Gardini A, Bussotti G, Lai F, Zytnicki M, Notredame C, Huang Q, Guigo R, Shiekhattar R. Long noncoding RNAs with enhancer-like function in human cells. Cell. 2010;143:46–58.
94. Ranzani V, Rossetti G, Panzeri I, Arrigoni A, Bonnal RJ, Curti S, Gruarin P, Provasi E, Sugliano E, Marconi M, De Francesco R, Geginat J, Bodega B, Abrignani S, Pagani M. The long intergenic noncoding RNA landscape of human lymphocytes highlights the regulation of T cell differentiation by linc-MAF-4. Nat Immunol. 2015;16:318–25.
95. Han BW, Wang W, Li C, Weng Z, Noncoding ZPD, RNA. piRNA-guided transposon cleavage initiates Zucchini-dependent, phased piRNA production. Science. 2015;348:817–21.
96. Kang S, Kang D, Bakhtiar Ul Islam SM, Je S, Kim JH, Song JJ. Silencing Daxx increases the anti-tumor activity of a TRAIL/shRNA Bcl-xL-expressing oncolytic adenovirus through enhanced viral replication and cellular arrest. Cell Signal. 2015;27:1214–24.
97. Herrmann A, Priceman SJ, Kujawski M, Xin H, Cherryholmes GA, Zhang W, Zhang C, Lahtz C, Kowolik C, Forman SJ, Kortylewski M, Yu H. CTLA4 aptamer delivers STAT3 siRNA to tumor-associated and malignant T cells. J Clin Invest. 2014;124:2977–87.
98. Chu L, Su MY, Maggi Jr LB, Lu L, Mullins C, Crosby S, Huang G, Chng WJ, Vij R, Tomasson MH. Multiple myeloma-associated chromosomal translocation activates orphan snoRNA ACA11 to suppress oxidative stress. J Clin Invest. 2012;122:2793–806.
99. Taft RJ, Hawkins PG, Mattick JS, Morris KV. The relationship between transcription initiation RNAs and CCCTC-binding factor (CTCF) localization. Epigenetics Chromatin. 2011;4:13.

100. Lerner MR, Boyle JA, Hardin JA, Steitz JA. Two novel classes of small ribonucleoproteins detected by antibodies associated with lupus erythematosus. Science. 1981;211:400–2.
101. Wang I, Kowalski MP, Langley AR, Rodriguez R, Balasubramanian S, Hsu ST, Krude T. Nucleotide contributions to the structural integrity and DNA replication initiation activity of noncoding y RNA. Biochemistry. 2014;53:5848–63.
102. Davis CA, Ares Jr M. Accumulation of unstable promoter-associated transcripts upon loss of the nuclear exosome subunit Rrp6p in Saccharomyces cerevisiae. Proc Natl Acad Sci U S A. 2006;103:3262–7.
103. Zhang Y, Liu XS, Liu QR, Wei L. Genome-wide in silico identification and analysis of cis natural antisense transcripts (cis-NATs) in ten species. Nucleic Acids Res. 2006;34: 3465–75.
104. Li S, Liberman LM, Mukherjee N, Benfey PN, Ohler U. Integrated detection of natural antisense transcripts using strand-specific RNA sequencing data. Genome Res. 2013;23:1730–9.
105. Kapranov P, Cheng J, Dike S, Nix DA, Duttagupta R, Willingham AT, Stadler PF, Hertel J, Hackermuller J, Hofacker IL, Bell I, Cheung E, Drenkow J, Dumais E, Patel S, Helt G, Ganesh M, Ghosh S, Piccolboni A, Sementchenko V, Tammana H, Gingeras TR. RNA maps reveal new RNA classes and a possible function for pervasive transcription. Science. 2007;316:1484–8.
106. Affymetrix ENCODE Transcriptome Project, Cold Spring Harbor Laboratory ENCODE Transcriptome Project. Post-transcriptional processing generates a diversity of 5′-modified long and short RNAs. Nature. 2009;457:1028–32.
107. Kurokawa R. Promoter-associated long noncoding RNAs repress transcription through a RNA binding protein TLS. Adv Exp Med Biol. 2011;722:196–208.
108. Preker P, Almvig K, Christensen MS, Valen E, Mapendano CK, Sandelin A, Jensen TH. PROMoter uPstream Transcripts share characteristics with mRNAs and are produced upstream of all three major types of mammalian promoters. Nucleic Acids Res. 2011;39: 7179–93.
109. Guerrier-Takada C, Gardiner K, Marsh T, Pace N, Altman S. The RNA moiety of ribonuclease P is the catalytic subunit of the enzyme. Cell. 1983;35:849–57.
110. Howard MJ, Klemm BP, Fierke CA. Mechanistic studies reveal similar catalytic strategies for phosphodiester bond hydrolysis by protein-only and RNA-dependent ribonuclease P. J Biol Chem. 2015;290:13454–64.
111. Gold HA, Topper JN, Clayton DA, Craft J. The RNA processing enzyme RNase MRP is identical to the Th RNP and related to RNase P. Science. 1989;245:1377–80.
112. Mattijssen S, Hinson ER, Onnekink C, Hermanns P, Zabel B, Cresswell P, Pruijn GJ. Viperin mRNA is a novel target for the human RNase MRP/RNase P endoribonuclease. Cell Mol Life Sci. 2011;68:2469–80.
113. Varshney D, Vavrova-Anderson J, Oler AJ, Cowling VH, Cairns BR, White RJ. SINE transcription by RNA polymerase III is suppressed by histone methylation but not by DNA methylation. Nat Commun. 2015;6:6569.
114. Ohshima K, Koishi R, Matsuo M, Okada N. Several short interspersed repetitive elements (SINEs) in distant species may have originated from a common ancestral retrovirus: characterization of a squid SINE and a possible mechanism for generation of tRNA-derived retroposons. Proc Natl Acad Sci U S A. 1993;90:6260–4.
115. Feng J, Funk WD, Wang SS, Weinrich SL, Avilion AA, Chiu CP, Adams RR, Chang E, Allsopp RC, Yu J, et al. The RNA component of human telomerase. Science. 1995;269:1236–41.
116. Kannan R, Helston RM, Dannebaum RO, Baumann P. Diverse mechanisms for spliceosome-mediated 3′ end processing of telomerase RNA. Nat Commun. 2015;6:6104.
117. Fassan M, Dall'Olmo L, Galasso M, Braconi C, Pizzi M, Realdon S, Volinia S, Valeri N, Gasparini P, Baffa R, Souza RF, Vicentini C, D'Angelo E, Bornschein J, Nuovo GJ, Zaninotto G, Croce CM, Rugge M. Transcribed ultraconserved noncoding RNAs (T-UCR) are involved in Barrett's esophagus carcinogenesis. Oncotarget. 2014;5:7162–71.
118. Calin GA, Liu CG, Ferracin M, Hyslop T, Spizzo R, Sevignani C, Fabbri M, Cimmino A, Lee EJ, Wojcik SE, Shimizu M, Tili E, Rossi S, Taccioli C, Pichiorri F, Liu X, Zupo S, Herlea V,

Gramantieri L, Lanza G, Alder H, Rassenti L, Volinia S, Schmittgen TD, Kipps TJ, Negrini M, Croce CM. Ultraconserved regions encoding ncRNAs are altered in human leukemias and carcinomas. Cancer Cell. 2007;12:215–29.

119. St Laurent G, Shtokalo D, Dong B, Tackett MR, Fan X, Lazorthes S, Nicolas E, Sang N, Triche TJ, McCaffrey TA, Xiao W, Kapranov P. VlincRNAs controlled by retroviral elements are a hallmark of pluripotency and cancer. Genome Biol. 2013;14:R73.
120. Ferracin M, Gautheret D, Hubé D, Mani SA, Mattick JS, Andersson Ørom U, Santulli G, Slotkin RK, Szweykowska-Kulinska Z, Taube JH, Vazquez F, Yang JH. The Non-Coding RNA Journal Club: Highlights on Recent Papers. Non-Coding RNA 2015;1:87–93.

Chapter 2
The microRNA Machinery

Thomas C. Roberts

Abstract MicroRNAs (miRNAs) are short (~22 nucleotides) single-stranded RNA molecules that primarily function to negatively regulate gene expression at the post-transcriptional level. miRNAs have thus been implicated in the regulation of a wide variety of normal cell functions and pathophysiological conditions. The miRNA machinery consists of a series of protein complexes which act to: (1) cleave the precursor-miRNA hairpin from its primary transcript (i.e. DROSHA and DGCR8); (2) traffic the miRNA hairpin between nucleus and cytoplasm (i.e. XPO5); (3) remove the loop sequence of the hairpin by a second nucleolytic cleavage reaction (i.e. DICER1); (4) facilitate loading of the mature miRNA sequence into an Argonaute protein (typically AGO2) as part of the RNA-Induced Silencing Complex (RISC); (5) guide the loaded RISC complex to complementary, or semi-complementary, target transcripts and (6) facilitate gene silencing via one of several possible mechanisms.

Keywords Argonaute • AGO2 • Dicer • DICER1 • Exportin-5 • XPO5 • Drosha • microRNA • DGCR8

Introduction

Microribonucleic acids (microRNAs, miRNAs) are short (21–23 nucleotides), single-stranded, non-coding RNA molecules that are encoded in the genomes of higher organisms. miRNAs primarily function as post-transcriptional gene expression regulators [1] and miRNA-mediated regulation has been implicated in a wide variety of cellular processes and disease conditions. As such, miRNAs are of interest as potential therapeutic targets [2–4] and as disease biomarkers [5–7].

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miRNAs are embedded within hairpin structures in long (100s–1000s of nucleotides) primary-miRNA (pri-miRNA) transcripts that are transcribed by RNA Polymerase II [8]. pri-miRNAs are capped and polyadenylated transcripts that can be either long non-coding RNAs or conventional protein-coding mRNAs [9] (in which case the hairpin resides in the intronic sequence of the pre-mRNA [10]). The majority of miRNAs are transcribed from independent transcriptional units, although some miRNAs are arranged in polycistronic clusters [11]. For example, the lncRNA gene *MIR17HG* contains the miR-17-92 cluster implicated in tumorigenesis and other diseases [12].

The miRNA hairpin, termed the precursor-miRNA (pre-miRNA), is ~70 nucleotides in length and typically contains multiple bulges at mismatched nucleotides. The mature miRNA species is generated from the pri-miRNA by two sequential endonucleolytic processing reactions. The pre-miRNA is liberated from the primary transcript by the first RNase cleavage reaction, whereas the loop sequence is removed by the second cleavage reaction. These two processing stages are spatially separated such that they occur in the nucleus and cytoplasm, respectively [13]. Subsequently, the mature miRNA is then loaded into RISC (RNA-Induced Silencing Complex) where it acts to guide the complex to target mRNAs and silence their expression [14–18].

This chapter focuses on the canonical mode of miRNA maturation and function, focusing on the protein components which comprise this pathway. Notably, there are numerous caveats and exceptions. These include miRNAs processed from non-standard precursor RNA substrates, miRNAs which bypass various processing stages, and miRNAs which execute non-canonical functions in the nucleus or extracellular space. Such deviations from the canonical paradigm are reviewed elsewhere [19, 20].

While much of the seminal work on miRNAs was performed in model organisms such as *Drosophila melanogaster* and *Caenorhabditis elegans*, this chapter is primarily concerned with the human miRNA machinery for the purpose of medical relevance. HGNC gene symbols are used throughout with commonly used non-standard names in parentheses where applicable.

DROSHA, DGCR8

In human cells, the pre-miRNA hairpin is cleaved from the pri-miRNA transcript in the nucleus by the enzyme DROSHA (Drosha). DROSHA is 145–160 kDa protein [21] with predominantly nuclear localization [22, 23]. The importance of DROSHA is exemplified in the roundworm *Meloidogyne incognita*, where knock-down of Drosha results in embryonic lethality [24]. Similarly, a conditional *Drosha* knockout in postnatal murine testes revealed an essential role for *Drosha* in spermatogenesis [25]. DROSHA belongs to the type III ribonuclease class of enzymes, which characteristically introduce staggered cuts in their RNA substrate duplexes [26]. DROSHA contains two RNase III domains (RIIIDa and RIIIDb). These domains form an intramolecular dimer as they are arranged in close proximity in

three-dimensional space. As such, RIIIDa cleaves the 3′ strand of the hairpin whereas RIIIDb cleaves the 5′ strand [27]. Immunopurified DROSHA is capable of cleaving synthetic pri-miRNAs to generate pre-miRNAs in vitro [23]. Furthermore, silencing of DROSHA by RNA interference (RNAi) resulted in accumulation of pri-miRNAs and depletion of pre-miRNAs [23].

In vivo, DROSHA associates with a multitude of additional proteins including double-stranded RNA binding proteins (dsRBDs), hnRNPs, DEAD-box/DEAH-box RNA helicases and DGCR8 [21]. The *DGCR8* (DiGeorge Syndrome Critical Region 8) gene resides in a region of chromosome 22 that is deleted in sufferers of DiGeorge syndrome, a rare genetic disease with varied symptoms including congenital heart disease, learning difficulties, facial abnormalities and immune dysfunction [28, 29]. Immunoprecipitation of DGCR8 co-precipitated only DROSHA, and the resulting eluates exhibited robust pri-miRNA in vitro cleavage activity [21]. Similarly, recombinant DROSHA and DGCR8 generated in insect cells could reconstitute pri-miRNA cleavage activity when the two proteins were combined. However, either recombinant protein in isolation was insufficient for pri-miRNA processing [21]. Interestingly, DROSHA exhibited some level of non-specific RNase activity which was inhibited when DGCR8 was present. Depletion of DROSHA or DGCR8 by RNAi also abrogated pri-miRNA processing. Together, these studies demonstrated that the DROSHA–DGCR8 complex is necessary and sufficient for pri-miRNA processing [21]. This complex of the DROSHA and DGCR8 proteins is collectively known as the Microprocessor. Similar functional associations between DROSHA and DGCR8 homologues have also been demonstrated in *D. melanogaster* and *C. elegans* [30, 31] (Note: In these organisms, DGCR8 is called Pasha, partner of Drosha, or Pash-1).

Biochemical studies have dissected the substrate preference of DROSHA. A study by Zeng et al. suggested that DROSHA has a strong preference for pre-miRNA hairpins with large (≥10 nucleotide) unstructured terminal loop sequences [32]. However, Han et al. found that the loop sequence is dispensable for DROSHA processing, and that synthetic "hairpins" in which the loop is replaced by an extended duplex with open termini are easily processed [33]. Both studies identified the importance of single-stranded RNA regions flanking the base of the lower stem for recognition of the pre-miRNA by the Microprocessor complex [32, 33]. Thermodynamic analysis of hundreds of human and *D. melanogaster* pri-miRNA hairpins revealed that the DROSHA cleavage site is typically ~11 nucleotides (~1 helical turn) from the ssRNA–dsRNA stem junction and ~22 nucleotides (~2 helical turns) from the terminal loop, suggesting that DROSHA measures the distance from either the base of the stem or the loop in order to determine the cleavage site. Interestingly, pre-miR-30a does not effectively compete with pri-miR-30a, suggesting that the major site of pri-miRNA recognition by DGCR8 resides outside of the pre-miRNA hairpin structure [33].

Structures of intact DROSHA are currently lacking, although the C-terminal dsRBD has been solved by solution NMR [34]. This structure consists of the αβββα fold typical of dsRBDs (Fig. 2.1a). However, biochemical assays (EMSA and immunoprecipitation with radiolabelled synthetic pri-miRNA) failed to show RNA binding by DROSHA alone [33]. Furthermore, competition assays show that

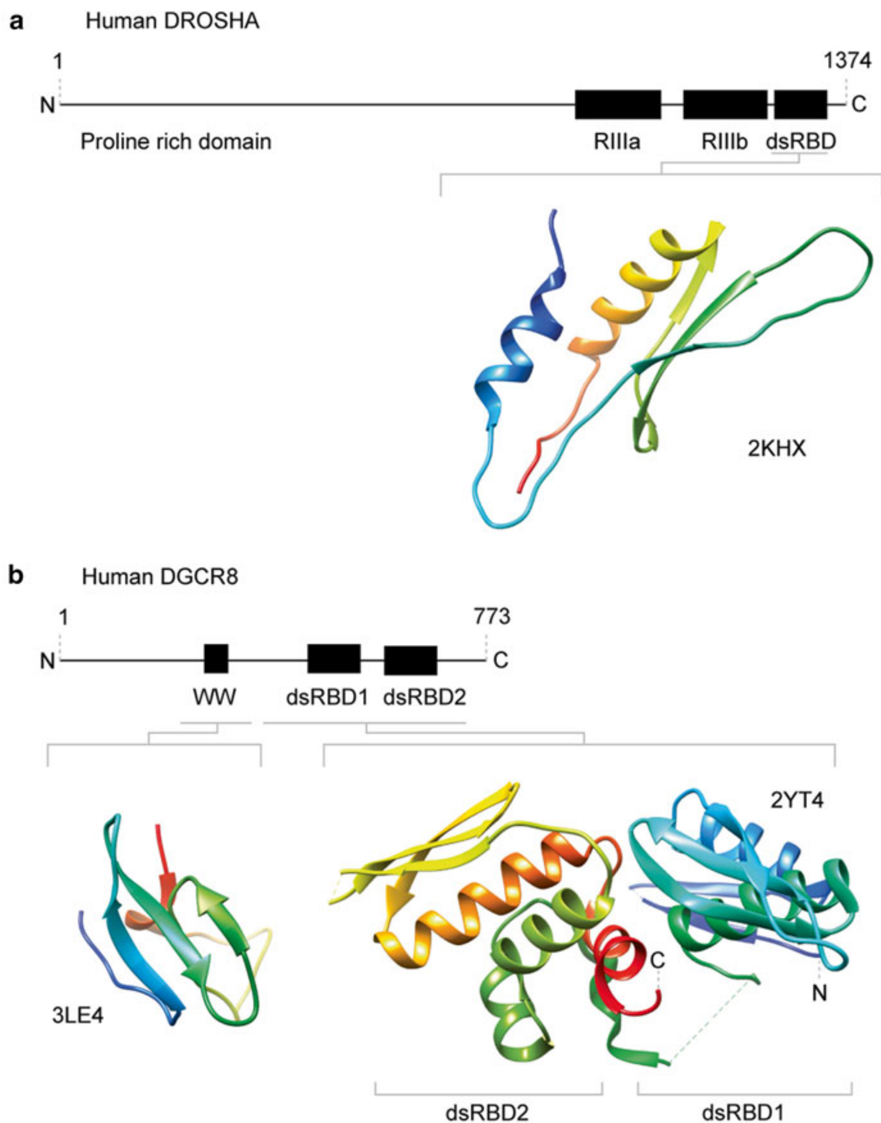

Fig. 2.1 Structures of DROSHA and DGCR8. (**a**) Domain structure of human DROSHA protein. A depiction of a solution NMR structure of the dsRNA binding domain (dsRBD) is shown (2KHX) [34]. (**b**) Domain structure of human DGCR8 protein and depictions of available crystal structures for the dimerization domain (3LE4) [36] and core domain (2YT4) [35]

processed pre-miRNAs rapidly dissociate from DGCR8, and that DROSHA exhibits low RNA binding activity [33]. As a result, it is likely that DGCR8 docks with the pri-miRNA by anchoring to the ssRNA–dsRNA stem junction in order to locate the cleavage site for DROSHA. DROSHA then associates with hairpin only transiently in order to perform catalytic pri-miRNA cleavage [33].

DGCR8 consists of a core region containing two dsRBDs near the C-terminus and a central WW motif which serves to mediate the interaction between DGCR8 and the proline-rich region within the N-terminal region of DROSHA. High-resolution crystal structures have been published for both of these domains [35, 36] (Fig. 2.1b). In the core domain structure (2.6 Å resolution), the two dsRBDs form a butterfly-like shape whereby the dsRBDs exhibit pseudo twofold symmetry [35]. Each dsRBD adopts an αβββα fold structure. A C-terminal helix and a flexible linker region act to bridge the two dsRBDs. Using FRET, the authors showed that pri-miRNA binding is unlikely to induce large-scale changes in DGCR8 conformation [35]. The structure of the dimerization domain (1.7 Å resolution) shows that the WW motif resides within a heme binding domain and forms a domain-swapped dimer structure [36]. DGCR8 has also been shown to bind to heme, which promotes self-dimerization [37]. Furthermore, in the absence of heme, the heme binding region inhibits the pri-miRNA processing activity of DGCR8, thereby acting as an autoinhibitory domain [37].

XPO5

The nuclear membrane which separates the nucleoplasm from the cytoplasm is punctuated by nuclear pores. The nuclear pore complex is a multi-protein structure that facilitates regulated trafficking of macromolecules through the nuclear pore. While small molecules can passively diffuse between compartments, larger macromolecules (e.g. the pre-miRNA hairpin) require active transport.

In human cells, the Karyopherin XPO5 (Exportin-5) [38] facilitates the transport of pre-miRNA hairpins though the nuclear pore complex and into the cytoplasm in a RAN-GTP-dependent manner [39–41]. XPO5 binds to dsRNA in a sequence-independent manner [41], but recognizes a minihelix motif that is common to pre-miRNAs and several other substrates including tRNA, Y1 RNA and the adenoviral VA1 RNA [42, 43]. Disruption of XPO5 function by RNAi, inhibition with antibodies or competition with VA1 RNA leads to a reduction in the levels of mature miRNAs [39–41, 44].

A common feature of the Exportins is that they take advantage of the gradient of RAN-GTP that exists across the nuclear envelope. This gradient is the result of the differential nucleocytoplasmic location of protein factors which regulate the GTP binding status of RAN. Specifically, RANGAP1 (RanGAP, RAN-GTPase Activating Protein) is cytoplasmic [45], and RCC1 (an RAN exchange factor) is nuclear [46]. In the nucleus, the XPO5 forms a trimeric complex with its pre-miRNA cargo and RAN-GTP. The complex is subsequently translocated through the nuclear pore complex. Once in the cytoplasm, the GTP is hydrolyzed to GDP which induces a conformational change in RAN with concomitant dissociation of the complex and release of the pre-miRNA hairpin. Binding of XPO5 to the pre-miRNA is dependent on the binding of the RAN-GTP in complex with GTP, as determined by electrophoretic mobility shift assay [40]. Furthermore, depletion of RAN-GTP by nuclear microinjection of Ran-GAP in *Xenopus* oocytes also inhibited miRNA export [41].

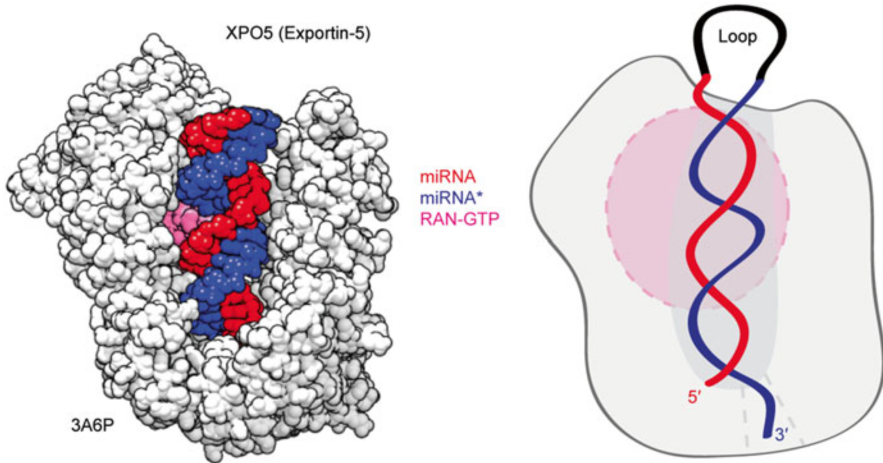

Fig. 2.2 Structure of XPO5. Depiction of the XPO5 (Exportin-5) structure. Structure (3A6P) [48] and a cartoon schematic are shown side by side. XPO5 protein is shown in *white* and RAN-GTP in *pink*. The pre-miRNA hairpin is shown bound to XPO5 with the 5′ strand in *red* and the 3′ strand in *blue*. The loop sequence (not visible in the crystal structure) is in *black*

The major determinants of pre-miRNA recognition by XPO5 are a helical stretch (~16 base pairs) and 3′ terminal overhangs (produced by DROSHA processing) [42, 43, 47]. As a result, XPO5 interacts with the majority of the pre-miRNA hairpin (excluding the terminal loop). Interestingly, the binding of XPO5 to a pre-miRNA has the additional effect of reducing degradation of the hairpin in the nucleus [47].

A high-resolution (2.9 Å) crystal structure of human XPO5 has been reported in complex with a fragment of canine Ran-GTP and the pre-miR-30a hairpin [48] (Fig. 2.2). XPO5 comprises multiple HEAT repeats and forms an overall “baseball mitt” shaped structure with Ran-GTP binding towards the top of the mitt. At the base of the mitt is a tunnel-like structure. The pre-miRNA hairpin forms an A-form helix that sits in the interior of the mitt (as if being held in the palm of the hand). The terminal nucleotides are oriented towards the base of the mitt with the 3′ overhang nucleotides inserted into the tunnel. The interior of the tunnel consists of several basic residues which make a number of close contact hydrogen bonds and salt bridges to the sugar-phosphate backbone of the overhang. Furthermore, an Arginine residue sidechain (Arg602) acts to sterically inhibit the double-stranded portion of the pre-miRNA from entering the tunnel. In silico modelling showed that a putative hairpin with a 5′ overhang would sterically clash with neighbouring HEAT repeat domains [48]. These observations explain the selectivity of XPO5 for cargo molecules with 3′ overhangs. In addition, the close contacts of XPO5 with the terminal nucleotides also explain why XPO5-bound pre-miRNAs are protected from nucleolytic degradation, as the terminal nucleotides are shielded by the surrounding protein.

The remainder of hairpin stem makes numerous contacts with the basic surface of the “mitt” interior [48]. This suggests that XPO5 primarily interacts with the

pre-miRNA hairpin via ionic interactions, which account for the sequence-independence of cargo recognition by XPO5. In further support, high ionic strength buffers promote dissociation of the pre-miRNA from XPO5 [48].

DICER1

In the cytoplasm, the pre-miRNA hairpin is subjected to a second cleavage reaction catalyzed by a second RNase III family member DICER1 (Dicer) which removes the terminal loop sequence [49]. DICER1 acts as a “molecular ruler” that cleaves the duplex at a defined distance from the terminus. (Similarly, human DICER1 will also progressively process long dsRNA molecules by cleaving ~22 nucleotide duplex fragments sequentially from their termini [50]).

Accuracy of DICER1 processing is important, as shifts in the site of cleavage will generate mature miRNAs with non-canonical seed sequences. This will lead to retargeting of the miRNA to a new set of mRNAs and/or potentially alter the strand selection preference [51]. Such an eventuality would effectively diminish the cellular concentration of correctly processed miRNAs and generate non-physiological miRNA–target interactions—with both outcomes being potentially detrimental, or at the very least wasteful, to the cell.

Dicer was initially discovered in *Drosophila* cell extracts where it was shown to cleave long dsRNA substrates into ~22 nucleotide siRNA duplexes [26]. Additionally, further studies showed that Dicer also generates mature miRNA species from let-7 precursors, thereby demonstrating its role in miRNA processing [52–54]. Genetic ablation of *Dicer1* in mice is lethal at the early stages of embryonic development on account of stem cell depletion [55]. (miRNAs are required for correct control of stem cell division [56].)

Human DICER1 is a ~218 kDa protein that is predominantly cytoplasmic and colocalizes with the endoplasmic reticulum marker calreticulin [49]. DICER1 consists of a number of conserved globular domains. The N-terminus of the protein contains three helicase domains (HEL1, HEL2 and HEL2i). The central region contains the platform domain and PAZ (Piwi/Argonaute/Zwille) domain. The PAZ domain is important for recognizing the RNA termini of the substrate duplex [57]. The C-terminal end contains a dsRBD and two catalytic RNase III-like domains (RIIIa and RIIIb) [49]. As with DROSHA, the two RNase III domains (RIIIa and RIIIb) of DICER1 are oriented in space such that they form an intramolecular dimer [58, 59]. As a result, DICER1 has a single catalytic centre at which both phosphodiester cleavage reactions occur (one on each strand of the substrate duplex). This configuration is responsible for the generation of the characteristic 2 nucleotide 3′ overhangs [58–60].

Human DICER1 requires the presence of Mg^{2+} for substrate cleavage but not binding, and catalytic activity is sensitive to ionic strength suggesting that electrostatic substrate–enzyme interactions are important for DICER1 functionality [49]. In contrast to *D. melanogaster* Dicer (Dcr-2) [61], human DICER1 does not require ATP for RNA cleavage to occur [49, 50].

There are currently no crystal structures of intact human DICER1 available, although a high-resolution (3.3 Å) structure of an intact Dicer from the protozoan *Giardia intestinalis* was published in 2006 [59]. This structure revealed that the PAZ domain is separated from the catalytic site by ~65 Å which corresponds with the length of dsRNA products (25–27 base pairs) generated by the G. *intestinalis* Dicer. Deletion of the PAZ domain in *G. intestinalis* results in loss of the size specificity of cleavage products [62] suggesting that the PAZ domain is required for molecular ruler functionality. Furthermore, the surface connecting the PAZ and RNase III domains contains a number of positively charged amino acid residues which, when substituted with alanine by site-directed mutagenesis, reduce the activity of Dicer [62]. These findings suggest that this region makes important electrostatic interactions between the substrate phosphodiester backbone and positively charged surface residues.

Several studies have utilized electron microscopy in order to determine structures for human DICER1 [63–65]. Most notably, Lau et al. showed that human DICER1 forms an "L"-shaped structure, and mapped the known globular domain structures to this general shape [65]. As such, the PAZ and platform domains were found to be tightly associated at the head of the "L," the RNase III domain was located centrally, and the helical domains formed the base, or "arm" of the L shape. Importantly, in the human DICER1 structure, the PAZ-RNase III domain distance is reduced relative to *G. intestinalis*, consistent with shorter human dsRNA cleavage products and "molecular ruler" functionality [65]. Furthermore, the helicase domains formed distinct lobes which the authors described as a "clamp" like structure. This configuration suggested that the substrate RNA duplex resides in a groove that runs vertically along the "L" shape [65].

Studies on reconstituted Dicer in *G. intestinalis* led to the so-called 3′ counting rule whereby the location of the Dicer cleavage event is determined by "counting" ~22 nucleotides along the duplex starting at the 3′ terminal nucleotide anchored in the PAZ domain. However, it was subsequently shown in human cells that the phosphorylated 5′ terminus of the pre-miRNA hairpin is also anchored in a binding pocket within the PAZ domain, and that a "5′ counting rule" predominates in humans [66]. (Interestingly this binding pocket is not conserved in *G. intestinalis*.) Additionally, a "loop counting rule" is also operative in human cells whereby DICER1 "counts back" from the loop (or bulged sequence) at the closed end of the hairpin [67]. Consequently, human DICER1 utilizes the 3′ counting, 5′ counting, and loop counting mechanisms in order to measure the length of the substrate duplex and precisely determine the site of cleavage.

AGO2, RISC

Following DICER1 processing, the miRNA duplex is passed to the RNA Induced Silencing Complex (RISC, or miRISC) and specifically to an Argonaute protein which constitutes the core of the complex [68]. Subsequently, one strand of the duplex is discarded to leave only a single-stranded mature ~23 nucleotide miRNA

bound to RISC. RISC loading is asymmetric such that the Argonaute protein shows a preference for the strand with the least thermodynamically stable (and therefore most easily unwound) 5′ terminus [69, 70]. However, loading is probabilistic, and the opposite "passenger" strand (often denoted as miRNA*) may be incorporated in a minority of cases. Furthermore, the termini of some miRNA duplexes are equally stable, leading to a mixture of 5′ arm- and 3′ arm-loaded RISC complexes.

The miRNA-loaded RISC complex is then able to scan the pool of cytoplasmic mRNAs for potential complementary targets. miRNAs primarily target the 3′ untranslated region (UTR) regions of mRNAs where they typically bind with imperfect complementarity [71, 72]. It has been estimated that >60% of human protein-coding transcripts have been under selective pressure to maintain miRNA binding sites [73], suggesting that miRNA-mediated gene regulation is a widespread phenomenon. The degree of miRNA-target base-pairing complementarity determines the fate of the target transcript. Perfect complementarity leads to target cleavage via the "slicer" activity of AGO2, analogous to small interfering RNA-induced RNAi [74, 75]. Slicer cleavage occurs in the target mRNA at the intervening scissile phosphate group opposite to nucleotides 10 and 11 in the mature miRNA sequence. In contrast, incomplete complementarity triggers mRNA silencing by distinct mechanisms which may involve translational repression, slicer-independent mRNA degradation and/or sequestration in cytoplasmic processing bodies (P-bodies) [76]. In this case, there is typically complete complementarity between the seed region (nucleotides 2–8) and the target mRNA with scattered base-pairing and bulged nucleotides in the remainder of the duplex [15]. Importantly, translational repression via sequestration in P-bodies is a reversible process suggesting that mRNAs can be stored in a translationally inactive form and then released in response to cell stress [77]. In humans, slicer-independent mechanisms of mRNA repression dominate on account of the majority of miRNAs lacking complementarity with their cognate targets around position 10/11. (The resulting bulge structure inhibits the slicer activity of AGO2 [78].) A detailed discussion of the mechanisms of RISC-mediated gene silencing is beyond the scope of this chapter and has been discussed elsewhere [1, 17, 79–81].

miRNAs can exert complex, combinatorial control over gene expression as one miRNA can target multiple mRNAs [82]. In this manner, one miRNA can target a family of transcripts with related functions in order to regulate a cellular process. For example, miR-29 family miRNAs repress a plethora of fibrosis-associated transcripts (collagens, fibrillins, elastin, fibronectin, etc.) [4]. Down-regulation of miR-29 is therefore often a feature of pathogenic fibrotic processes. On the other hand, a typical mRNA 3′ UTR contains binding sites for multiple miRNAs. This enables the transcript to integrate signals from different miRNAs, or to fine-tune expression (with the expression of the transcript inversely proportional to the number of miRNAs which are regulating it).

Transfer of the processed miRNA duplex to an Argonaute protein is accomplished by the RISC-loading complex (RLC), which consists primarily of an Argonaute protein, DICER1 (as discussed above) and TARBP2 (trans-activation response (TAR) RNA Binding Protein, also known as TRBP) [64, 83]. The direct association of DICER1 with an Argonaute protein promotes the transfer of the

miRNA duplex between the two proteins (TARBP2 also contributes to this process) [83, 84]. It has been suggested that binding by TARBP2 may allow the siRNA intermediate to stay associated with the RLC after release from DICER1 and may also help optimize the orientation of the siRNA for AGO2 loading [64]. Depletion of TARBP2 leads to a reduction in pre-miRNA processing [84], although TARBP2 is not required for DICER1 activity [83]. Instead, TARBP2 appears to be required for recruitment of AGO2 to the DICER1-miRNA complex [83]. Complexes of human DICER1 with TARBP2 [63] and with both TARBP2 and AGO2 have been solved by electron microscopy [64]. These studies suggest that AGO2 contacts the C-terminal region of DICER1, whereas TARBP2 interacts with the DExH/D domain [64].

In humans, there are four Argonaute proteins (AGO1–4). These homologous proteins appear to execute redundant functions in terms of miRNA-mediated gene silencing and have similar preferences for endogenous miRNAs or exogenous synthetic siRNAs [85]. Each Argonaute protein consists of four major domains: N-terminal (N), PAZ, MID and PIWI [86]. However, only AGO2 can mediate target mRNA "slicing" on account of an Asp-Glu-Asp-His (DEDH) catalytic tetrad at its active site [87, 88]. AGO2 has thus been called "the catalytic engine of RNAi" [78, 85]. The miRNA/siRNA-AGO2 complex is a multiple turnover enzyme such that after target cleavage, the loaded RISC complex can bind to another target and thereby induce multiple further gene silencing events [74]. In contrast, AGO1, AGO3 and AGO4, which lack the catalytic residues required for slicer functionality, mediate gene silencing via slicer-independent mechanisms only [85].

Ago2 knockout mice exhibit embryonic lethality [85, 89, 90], and transgenic mice that are homozygous for a catalytically deficient Ago2 die shortly after birth as a result of anaemia [91]. The slicer activity of Ago2 is uniquely required for Dicer-independent maturation of miR-451, an miRNA which is essential for haematopoiesis [91, 92].

In 2012, a high-resolution (2.3 Å) crystal structure of full length human AGO2 in complex with a heterogeneous mixture of guide RNAs was published by the McRae lab that revealed new insights into its function [93] (Fig. 2.3). AGO2 forms a bilobular structure with a central groove which accommodates the mature miRNA-target mRNA duplex. The first seven nucleotides are held in a well-defined, uniform conformation [93]. (The structure of AGO2 has also been likened to that of a duck, with the MID, PIWI and N domains forming the "body" and the PAZ domain the "head" of the "duck" [94].) AGO2 interacts with the guide RNA in a sequence-independent manner on account of multiple electrostatic interactions with the phosphate backbone and van der Waals interactions with the ribose sugar [93]. The guide RNA adopts an A-form conformation with nucleotides 2–6 "splayed out" such that their base-pairing surface is solvent accessible and available to interact with a target mRNA [93]. The 3′ terminal nucleotide was found to bind in a pocket residing within the PAZ domain. Interestingly, the guide RNA was found to kink after nucleotide 6, after which the A-form conformation of the guide was disrupted and the remainder of the nucleotides were less well ordered [93]. These observations provide a structural basis for the importance of seed sequence base-pairing as the primary determinant of miRNA-target recognition [15, 95]. The AGO2 structure also provided evidence of two tryptophan binding pockets within the PIWI domain which are a likely docking site for other RISC cofactors such as TNRC6A [93].

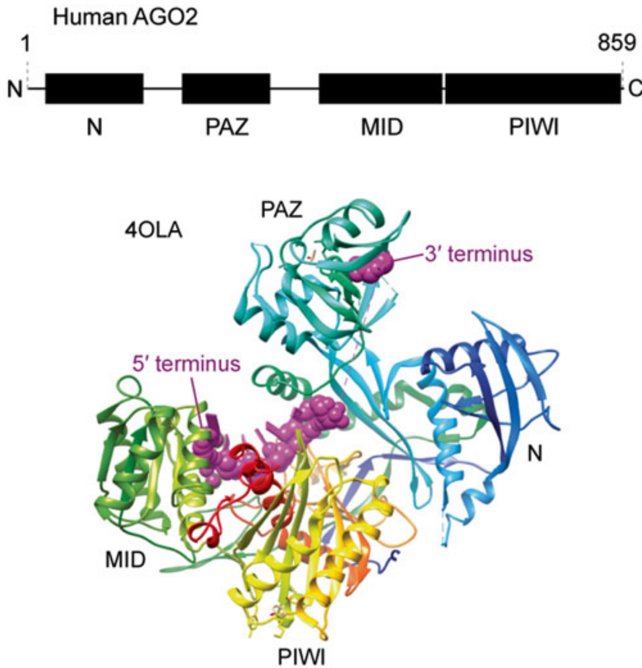

Fig. 2.3 Structure of AGO2. Domain organization for human AGO2 and depiction of a crystal structure of the full length protein (4OLA) [93]. The guide RNA is shown in *purple* (the middle portion of the guide RNA was too disordered to be resolved)

Subsequently, a similar structure (2.2 Å resolution) of human AGO2 in complex with miR-20a was published [94]. This structure clearly showed that the miRNA contacts multiple AGO2 subunits with the 5′ miRNA terminus bound in a pocket within the MID domain (consistent with previous studies [96, 97]) and that the 3′ terminal nucleotide bound in the PAZ domain [94]. Interestingly, this study also showed that miRNA binding stabilizes AGO2 such that it becomes resistant to proteolytic degradation [94].

A complete description of RISC components is currently lacking, although immunoprecipitation-mass spectrometry studies have identified a multitude of Argonaute-binding proteins [98–100]. Notably, a trinucleotide repeat containing protein partner, TNRC6A (also known as GW182), is required for miRNA function and is localized to P-bodies [100].

Conclusion

In summary, mature miRNAs are generated in a multi-step process requiring several enzymatic cleavage reactions and multiple protein cofactors. Biochemical and structural studies have revealed much of the mechanistic detail of how these

proteins operate. However, a complete understanding of how the miRNAs are loaded into the RISC complex, how RISC mediates gene silencing, and the regulatory control operating at each step is currently lacking. Future studies will likely shed light on these issues and potentially reveal new details of disease miRNA-associated pathophysiology, or improved methods for modulating miRNA function for therapeutic purposes.

References

1. Filipowicz W, Jaskiewicz L, Kolb FA, Pillai RS. Post-transcriptional gene silencing by siRNAs and miRNAs. Curr Opin Struct Biol. 2005;15:331–41.
2. Roberts TC, Wood MJA. Therapeutic targeting of non-coding RNAs. Essays Biochem. 2013; 54:127–45.
3. Cacchiarelli D, Incitti T, Martone J, Cesana M, Cazzella V, Santini T, Sthandier O, Bozzoni I. miR-31 modulates dystrophin expression: new implications for Duchenne muscular dystrophy therapy. EMBO Rep. 2011;12:136–41.
4. Wang L, Zhou L, Jiang P, Lu L, Chen X, Lan H, Guttridge DC, Sun H, Wang H. Loss of miR-29 in myoblasts contributes to dystrophic muscle pathogenesis. Mol Ther. 2012;20:1222–33.
5. Roberts TC, Godfrey C, McClorey G, Vader P, Briggs D, Gardiner C, Aoki Y, Sargent I, Morgan JE, Wood MJA. Extracellular microRNAs are dynamic non-vesicular biomarkers of muscle turnover. Nucleic Acids Res. 2013;41:9500–13.
6. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. Proc Natl Acad Sci U S A. 2008;105:10513–8.
7. Gilad S, Meiri E, Yogev Y, et al. Serum microRNAs are promising novel biomarkers. PLoS One. 2008;3, e3148.
8. Lee Y, Kim M, Han J, Yeom K-H, Lee S, Baek SH, Kim VN. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 2004;23:4051–60.
9. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA. 2004;10:1957–66.
10. Kim Y-K, Kim VN. Processing of intronic microRNAs. EMBO J. 2007;26:775–83.
11. Lagos-Quintana M, Rauhut R, Lendeckel W, Tuschl T. Identification of novel genes coding for small expressed RNAs. Science. 2001;294:853–8.
12. 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.
13. Lee Y, Jeon K, Lee J-T, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J. 2002;21:4663–70.
14. Olsen PH, Ambros V. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol. 1999; 216:671–80.
15. Doench JG, Sharp PA. Specificity of microRNA target selection in translational repression. Genes Dev. 2004;18:504–11.
16. Ohler U, Yekta S, Lim LP, Bartel DP, Burge CB. Patterns of flanking sequence conservation and a characteristic upstream motif for microRNA gene identification. RNA. 2004;10: 1309–22.
17. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 2010;466:835–40.
18. Gregory RI, Chendrimada TP, Cooch N, Shiekhattar R. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell. 2005;123:631–40.
19. Roberts TC. The microRNA biology of the mammalian nucleus. Mol Ther Nucleic Acids. 2014;3:e188

20. Roberts TC, Wood MJ. Non-canonical microRNA biogenesis and function. In: Arbuthnot P, Weinberg M, editors. Applied RNAi: from fundamental research to therapeutic applications. Norfolk: Caister Academic Press; 2014. p. 19–42.
21. Gregory RI, Yan K-P, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, Shiekhattar R. The Microprocessor complex mediates the genesis of microRNAs. Nature. 2004;432:235–40.
22. Wu H, Xu H, Miraglia LJ, Crooke ST. Human RNase III is a 160-kDa protein involved in preribosomal RNA processing. J Biol Chem. 2000;275:36957–65.
23. Lee Y, Ahn C, Han J, et al. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003;425:415–9.
24. Dalzell JJ, Warnock ND, Stevenson MA, Mousley A, Fleming CC, Maule AG. Short interfering RNA-mediated knockdown of drosha and pasha in undifferentiated Meloidogyne incognita eggs leads to irregular growth and embryonic lethality. Int J Parasitol. 2010;40: 1303–10.
25. Wu Q, Song R, Ortogero N, et al. The RNase III enzyme DROSHA is essential for microRNA production and spermatogenesis. J Biol Chem. 2012;287:25173–90.
26. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001;409:363–6.
27. Han J, Lee Y, Yeom K-H, Kim Y-K, Jin H, Kim VN. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev. 2004;18:3016–27.
28. Wilson DI, Burn J, Scambler P, Goodship J. DiGeorge syndrome: part of CATCH 22. J Med Genet. 1993;30:852–6.
29. Shiohama A, Sasaki T, Noda S, Minoshima S, Shimizu N. Molecular cloning and expression analysis of a novel gene DGCR8 located in the DiGeorge syndrome chromosomal region. Biochem Biophys Res Commun. 2003;304:184–90.
30. Denli AM, Tops BBJ, Plasterk RHA, Ketting RF, Hannon GJ. Processing of primary microRNAs by the microprocessor complex. Nature. 2004;432:231–5.
31. Landthaler M, Yalcin A, Tuschl T. The human DiGeorge syndrome critical region gene 8 and its D. melanogaster homolog are required for miRNA biogenesis. Curr Biol. 2004;14:2162–7.
32. Zeng Y, Yi R, Cullen BR. Recognition and cleavage of primary microRNA precursors by the nuclear processing enzyme Drosha. EMBO J. 2005;24:138–48.
33. Han J, Lee Y, Yeom K-H, Nam J-W, Heo I, Rhee J-K, Sohn SY, Cho Y, Zhang B-T, Kim VN. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell. 2006;125:887–901.
34. Mueller GA, Miller MT, Derose EF, Ghosh M, London RE, Hall TMT. Solution structure of the Drosha double-stranded RNA-binding domain. Silence. 2010;1:2.
35. Sohn SY, Bae WJ, Kim JJ, Yeom K-H, Kim VN, Cho Y. Crystal structure of human DGCR8 core. Nat Struct Mol Biol. 2007;14:847–53.
36. Senturia R, Faller M, Yin S, Loo JA, Cascio D, Sawaya MR, Hwang D, Clubb RT, Guo F. Structure of the dimerization domain of DiGeorge critical region 8. Protein Sci. 2010;19: 1354–65.
37. Faller M, Matsunaga M, Yin S, Loo JA, Guo F. Heme is involved in microRNA processing. Nat Struct Mol Biol. 2007;14:23–9.
38. Brownawell AM, Macara IG. Exportin-5, a novel karyopherin, mediates nuclear export of double-stranded RNA binding proteins. J Cell Biol. 2002;156:53–64.
39. Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science. 2004;303:95–8.
40. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 2003;17:3011–6.
41. Bohnsack MT, Czaplinski K, Gorlich D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA. 2004;10:185–91.
42. Gwizdek C, Ossareh-Nazari B, Brownawell AM, Doglio A, Bertrand E, Macara IG, Dargemont C. Exportin-5 mediates nuclear export of minihelix-containing RNAs. J Biol Chem. 2003;278: 5505–8.

43. Gwizdek C, Bertrand E, Dargemont C, Lefebvre JC, Blanchard JM, Singer RH, Doglio A. Terminal minihelix, a novel RNA motif that directs polymerase III transcripts to the cell cytoplasm. Terminal minihelix and RNA export. J Biol Chem. 2001;276:25910–8.
44. Lu S, Cullen BR. Adenovirus VA1 noncoding RNA can inhibit small interfering RNA and MicroRNA biogenesis. J Virol. 2004;78:12868–76.
45. Bischoff FR, Krebber H, Kempf T, Hermes I, Ponstingl H. Human RanGTPase-activating protein RanGAP1 is a homologue of yeast Rna1p involved in mRNA processing and transport. Proc Natl Acad Sci U S A. 1995;92:1749–53.
46. Bischoff FR, Ponstingl H. Catalysis of guanine nucleotide exchange on Ran by the mitotic regulator RCC1. Nature. 1991;354:80–2.
47. Zeng Y, Cullen BR. Structural requirements for pre-microRNA binding and nuclear export by Exportin 5. Nucleic Acids Res. 2004;32:4776–85.
48. Okada C, Yamashita E, Lee SJ, Shibata S, Katahira J, Nakagawa A, Yoneda Y, Tsukihara T. A high-resolution structure of the pre-microRNA nuclear export machinery. Science. 2009;326: 1275–9.
49. Provost P, Dishart D, Doucet J, Frendewey D, Samuelsson B, Rådmark O. Ribonuclease activity and RNA binding of recombinant human Dicer. EMBO J. 2002;21:5864–74.
50. Zhang H, Kolb FA, Brondani V, Billy E, Filipowicz W. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO J. 2002;21:5875–85.
51. Humphreys DT, Hynes CJ, Patel HR, Wei GH, Cannon L, Fatkin D, Suter CM, Clancy JL, Preiss T. Complexity of murine cardiomyocyte miRNA biogenesis, sequence variant expression and function. PLoS One. 2012;7, e30933.
52. Grishok A, Pasquinelli AE, Conte D, Li N, Parrish S, Ha I, Baillie DL, Fire A, Ruvkun G, Mello CC. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell. 2001;106:23–34.
53. Hutvágner G, McLachlan J, Pasquinelli AE, Bálint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science. 2001;293:834–8.
54. Ketting RF, Fischer SE, Bernstein E, Sijen T, Hannon GJ, Plasterk RH. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev. 2001;15:2654–9.
55. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, Elledge SJ, Anderson KV, Hannon GJ. Dicer is essential for mouse development. Nat Genet. 2003;35:215–7.
56. Hatfield SD, Shcherbata HR, Fischer KA, Nakahara K, Carthew RW, Ruohola-Baker H. Stem cell division is regulated by the microRNA pathway. Nature. 2005;435:974–8.
57. Ma J-B, Ye K, Patel DJ. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature. 2004;429:318–22.
58. Zhang H, Kolb FA, Jaskiewicz L, Westhof E, Filipowicz W. Single processing center models for human Dicer and bacterial RNase III. Cell. 2004;118:57–68.
59. MacRae IJ, Zhou K, Li F, Repic A, Brooks AN, Cande WZ, Adams PD, Doudna JA. Structural basis for double-stranded RNA processing by Dicer. Science. 2006;311:195–8.
60. Basyuk E, Suavet F, Doglio A, Bordonné R, Bertrand E. Human let-7 stem-loop precursors harbor features of RNase III cleavage products. Nucleic Acids Res. 2003;31:6593–7.
61. Cenik ES, Fukunaga R, Lu G, Dutcher R, Wang Y, Tanaka Hall TM, Zamore PD. Phosphate and R2D2 restrict the substrate specificity of Dicer-2, an ATP-driven ribonuclease. Mol Cell. 2011;42:172–84.
62. MacRae IJ, Zhou K, Doudna JA. Structural determinants of RNA recognition and cleavage by Dicer. Nat Struct Mol Biol. 2007;14:934–40.
63. Lau P-W, Potter CS, Carragher B, MacRae IJ. Structure of the human Dicer-TRBP complex by electron microscopy. Structure. 2009;17:1326–32.
64. Wang H-W, Noland C, Siridechadilok B, Taylor DW, Ma E, Felderer K, Doudna JA, Nogales E. Structural insights into RNA processing by the human RISC-loading complex. Nat Struct Mol Biol. 2009;16:1148–53.

65. Lau P-W, Guiley KZ, De N, Potter CS, Carragher B, MacRae IJ. The molecular architecture of human Dicer. Nat Struct Mol Biol. 2012;19:436–40.
66. Park J-E, Heo I, Tian Y, Simanshu DK, Chang H, Jee D, Patel DJ, Kim VN. Dicer recognizes the 5′ end of RNA for efficient and accurate processing. Nature. 2011;475:201–5.
67. Gu S, Jin L, Zhang Y, Huang Y, Zhang F, Valdmanis PN, Kay MA. The loop position of shRNAs and pre-miRNAs is critical for the accuracy of dicer processing in vivo. Cell. 2012;151:900–11.
68. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science. 2001;293:1146–50.
69. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell. 2003;115:209–16.
70. Schwarz DS, Hutvágner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. Cell. 2003;115:199–208.
71. Tokumaru S, Suzuki M, Yamada H, Nagino M, Takahashi T. let-7 regulates Dicer expression and constitutes a negative feedback loop. Carcinogenesis. 2008;29:2073–7.
72. Lai EC. Micro RNAs are complementary to 3′ UTR sequence motifs that mediate negative post-transcriptional regulation. Nat Genet. 2002;30:363–4.
73. Friedman RC, Farh KK-H, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19:92–105.
74. Hutvágner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. Science. 2002;297:2056–60.
75. Yekta S, Shih I-H, Bartel DP. MicroRNA-directed cleavage of HOXB8 mRNA. Science. 2004;304:594–6.
76. Zeng Y, Yi R, Cullen BR. MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. Proc Natl Acad Sci U S A. 2003;100:9779–84.
77. Bhattacharyya SN, Habermacher R, Martine U, Closs EI, Filipowicz W. Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell. 2006;125: 1111–24.
78. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, Teng G, Tuschl T. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell. 2004;15:185–97.
79. Pillai RS, Bhattacharyya SN, Filipowicz W. Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol. 2007;17:118–26.
80. Filipowicz W, Bhattacharyya SN, Sonenberg N. Mechanisms of post-transcriptional regulation by microRNAs: are the answers in sight? Nat Rev Genet. 2008;9:102–14.
81. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116: 281–97.
82. Krek A, Grün D, Poy MN, et al. Combinatorial microRNA target predictions. Nat Genet. 2005; 37:495–500.
83. Chendrimada TP, Gregory RI, Kumaraswamy E, Norman J, Cooch N, Nishikura K, Shiekhattar R. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature. 2005;436:740–4.
84. Haase AD, Jaskiewicz L, Zhang H, Lainé S, Sack R, Gatignol A, Filipowicz W. TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing. EMBO Rep. 2005;6:961–7.
85. Liu J, Carmell MA, Rivas FV, Marsden CG, Thomson JM, Song J-J, Hammond SM, Joshua-Tor L, Hannon GJ. Argonaute2 is the catalytic engine of mammalian RNAi. Science. 2004;305: 1437–41.
86. Swarts DC, Makarova K, Wang Y, Nakanishi K, Ketting RF, Koonin EV, Patel DJ, van der Oost J. The evolutionary journey of Argonaute proteins. Nat Struct Mol Biol. 2014;21: 743–53.
87. Rivas FV, Tolia NH, Song J-J, Aragon JP, Liu J, Hannon GJ, Joshua-Tor L. Purified Argonaute2 and an siRNA form recombinant human RISC. Nat Struct Mol Biol. 2005;12: 340–9.

88. Schürmann N, Trabuco LG, Bender C, Russell RB, Grimm D. Molecular dissection of human Argonaute proteins by DNA shuffling. Nat Struct Mol Biol. 2013;20:818–26.
89. Alisch RS, Jin P, Epstein M, Caspary T, Warren ST. Argonaute2 is essential for mammalian gastrulation and proper mesoderm formation. PLoS Genet. 2007;3, e227.
90. 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.
91. Cheloufi S, Dos Santos CO, Chong MMW, Hannon GJ. A dicer-independent miRNA biogenesis pathway that requires Ago catalysis. Nature. 2010;465:584–9.
92. Yang J-S, Maurin T, Robine N, Rasmussen KD, Jeffrey KL, Chandwani R, Papapetrou EP, Sadelain M, O'Carroll D, Lai EC. Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis. Proc Natl Acad Sci U S A. 2010;107: 15163–8.
93. Schirle NT, MacRae IJ. The crystal structure of human Argonaute2. Science. 2012;336: 1037–40.
94. Elkayam E, Kuhn C-D, Tocilj A, Haase AD, Greene EM, Hannon GJ, Joshua-Tor L. The structure of human Argonaute-2 in complex with miR-20a. Cell. 2012;150:100–10.
95. 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.
96. Ma J-B, Yuan Y-R, Meister G, Pei Y, Tuschl T, Patel DJ. Structural basis for 5′-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. Nature. 2005;434:666–70.
97. Frank F, Sonenberg N, Nagar B. Structural basis for 5′-nucleotide base-specific recognition of guide RNA by human AGO2. Nature. 2010;465:818–22.
98. Landthaler M, Gaidatzis D, Rothballer A, Chen PY, Soll SJ, Dinic L, Ojo T, Hafner M, Zavolan M, Tuschl T. Molecular characterization of human Argonaute-containing ribonucleoprotein complexes and their bound target mRNAs. RNA. 2008;14:2580–96.
99. Höck J, Weinmann L, Ender C, Rüdel S, Kremmer E, Raabe M, Urlaub H, Meister G. Proteomic and functional analysis of Argonaute-containing mRNA-protein complexes in human cells. EMBO Rep. 2007;8:1052–60.
100. Liu J, Rivas FV, Wohlschlegel J, Yates JR, Parker R, Hannon GJ. A role for the P-body component GW182 in microRNA function. Nat Cell Biol. 2005;7:1261–6.

Chapter 3
microRNAs in Mitochondria: An Unexplored Niche

Pedro M. Borralho, Cecília M.P. Rodrigues, and Clifford J. Steer

Abstract Mitochondria are pivotal organelles involved in the regulation of a myriad of crucial biological processes, including cell survival and cell death, rendering mitochondrial dysfunction a relevant step in numerous pathophysiological processes. MicroRNAs (miRNAs) are endogenous small noncoding RNAs that add a new layer of complexity to the control of gene expression. miRNAs function as master regulators and fine-tuners of gene expression, primarily via posttranscriptional mechanisms, and are increasingly demonstrated as a paramount class of endogenous molecules with relevant diagnostic, prognostic, and therapeutic applications. miRNAs and other RNA interference have recently been reported to be present in mitochondria from several species, and we are now beginning to unveil mitochondrial miRNA transport mechanisms, biological function and targets to ascertain their role in this unexplored niche. Here, we describe miRNA biogenesis and present key findings regarding miRNA localization to mitochondria, origin, putative biological function, and implications for human disease.

Keywords MicroRNA (miRNA) • Mitochondria • Mitochondria-associated miRNA (mitomiR)

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Introduction

Mitochondria play a central role in the regulation of many vital cellular processes, including, among others, cell viability, cell death, autophagy, energy metabolism, and calcium trafficking. They are also key organelles for the maintenance of cellular homeostasis, both in health and disease. Mitochondrial dysfunction or dysregulation is associated with a wide spectrum of diseases, such as cardiomyopathies, metabolic syndrome, obesity, neurodegenerative diseases, and cancer [1]. Human mitochondria harbor a ~16.6 kb circular intron-less genome, known as mitochondrial DNA (mtDNA), which encodes multiple noncoding RNAs, as well as 13 electron transport chain protein subunits. mtDNA replication and transcription initiate within a small noncoding region termed the D loop, and appear to be primarily regulated by proteins imported into mitochondria and expressed from nuclear genome encoded genes [1, 2]. Interestingly, mtDNA transcription, translation, and also the processing of transcripts is regulated by many types of noncoding RNAs, some of which are encoded in the nuclear genome and imported into mitochondria, while others are encoded in the mitochondrial genome [3]. Mitochondrial RNAs are transcribed as long polycistronic precursor transcripts from both DNA strands, and subsequently undergo bioprocessing that culminates in the release of coding and noncoding RNAs, including mRNAs, tRNAs, and rRNAs [2].

MicroRNAs (miRNAs) are a growing class of endogenous noncoding RNAs, which operate as master fine-tuners of genome-wide gene expression, primarily via posttranscriptional mechanisms [4–7]. miRNAs have the capability to regulate the expression of multiple target genes and also signaling pathways involved in key cellular processes [5], including, among others, cell differentiation [8, 9], cell growth and proliferation [10, 11], and apoptosis [10–12], as well as mitochondrial function (reviewed in [3, 13–17]). Resulting from this pivotal role in the cellular environment, it is not surprising that deregulation of a single miRNA may result in the malfunction of key cellular mechanisms, contributing to disease onset and/or progression. In result of this impact on the (de)regulation of health and disease, modulation of miRNA expression and/or function has attracted growing interest as therapeutic targets and tools in multiple disease processes, being increasingly demonstrated as a relevant therapeutic strategy in human disease [7, 10, 12, 18].

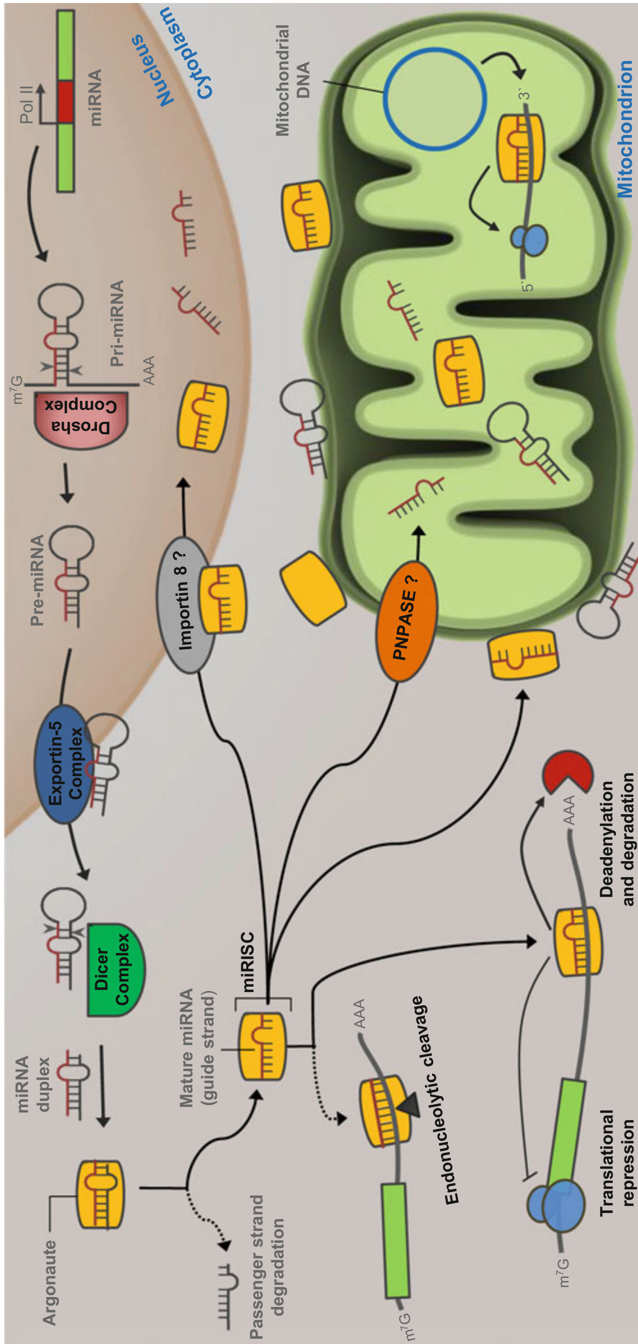
miRNA Biogenesis and Mechanism(s) of Action

The discovery of miRNAs more than two decades ago, forged a novel and expanding field of science. These small nucleic acids significantly impacted and altered our understanding of gene expression, expanding the intricacy and complexity of gene regulation, while at the same time, providing a novel toolset for therapeutic intervention. miRNAs were initially identified in the nematode *C. elegans*, where the gene regulating larval development timing, lin-4, was expressed as 22- and 61-nucleotide (nt) long

small RNA transcripts, displaying complementarity to the 3′ untranslated region (3′ UTR) of lin-14. Importantly, it was shown that negative regulation of lin-14 resulted from lin-4-mediated posttranscriptional regulation, which did not alter mRNA expression, but decreased lin-14 protein [19, 20]. This groundbreaking and paramount finding remained unrecognized for many years, until the discovery of let-7 miRNA, which was found to display complementarity to the 3′ UTR of several genes [21]. Since then, a massive research effort has exponentially increased our understanding of miRNA biogenesis and mechanisms of action, and significantly expanded our knowledge on the influence of miRNAs in processes of both health and disease. These facts are translated by the identification, to date, of 2588 mature miRNAs in humans, expressed from 1881 precursors. In addition, many more miRNAs were also identified in other organisms, including mammals, worms, fish, and plants, bringing the global number of known miRNAs to a total of 35,828 mature miRNA products expressed from 28,645 hairpin precursor miRNAs, in 223 species (www.miRbase.org; release 21, June 2014). It is therefore not surprising that the initial prediction of miRNA posttranscriptional regulation of over 30 % of human genes [22] is now recognized as a gross underestimate. In fact, it has been suggested that miRNAs may indeed regulate over 60 % of human genes [23]. This clearly highlights the intricacy of gene expression and its regulation, in addition to also identifying a growing spectrum of therapeutic opportunities.

miRNAs are endogenously encoded single-stranded noncoding RNAs (ssRNAs) 22 nt in length, whose main function is to negatively regulate (silence) gene expression at the posttranscriptional level [24]. However, in certain instances, miRNAs have also been reported to upregulate translation of certain proteins [25, 26]. Endogenously encoded miRNAs undergo several bioprocessing steps to generate a mature and biologically functional miRNA, with the vast majority of miRNAs resulting from processing by the canonical miRNA biogenesis pathway (Fig. 3.1). Nevertheless, a smaller number of miRNAs are produced by alternative miRNA biogenesis pathways, termed noncanonical pathways. These include miRtrons which are miRNAs resulting from the bioprocessing of debranched introns mimicking the structural features of pre-miRNAs, which enter the miRNA-processing pathway without requiring Drosha-mediated cleavage [27]; and simtrons which are mirtron-like miRNAs, splicing-independent entities which do not require canonical miRNA components for their biogenesis [28]. The vast majority of miRNAs resulting from the canonical pathway are transcribed by RNA polymerase II, originating a primary miRNAs (pri-miRNAs), whose length can range from hundreds to thousands of nucleotides. Similarly to mRNAs, pri-miRNAs are 5′-7-methyl-guanosine capped and 3′-polyadenylated RNAs [29]. However, a small number of miRNAs found interspersed among repetitive elements are transcribed by RNA polymerase III [30]. Pri-miRNAs often originate from intergenic regions, where the miRNA can alternatively reside within an intron or exon of a noncoding transcript. Interestingly, many mammalian miRNAs are found to reside within introns of protein-coding genes, displaying a similar transcription pattern to that of the protein-coding gene [31, 32].

pri-miRNAs typically harbor a 33 base pair stem, a terminal loop that forms the hairpin, and a flanking ssRNA region [33]. Subsequent to their transcription, and still within the nucleus, pri-miRNAs are subject to enzymatic cleavage mediated by


Pol II
miRNA
Nucleus
Cytoplasm
Mitochondrial DNA
3'
5'
Mitochondrion
m^{7}G
AAA
Pri-miRNA
Drosha Complex
Pre-miRNA
Importin 8 ?
Exportin-5 Complex
PNPASE ?
Dicer Complex
miRNA duplex
Argonaute
Mature miRNA (guide strand)
miRISC
Passenger strand degradation
Endonucleolytic cleavage
Deadenylation and degradation
Translational repression

Drosha (a RNAse III endonuclease), and its cofactor double-stranded RNA-(dsRNA)-binding protein DiGeorge syndrome critical region gene 8 (DGCR8), which together form a large protein complex, 650 kDa in size, termed microprocessor [34]. This enzymatic cleavage takes place at the stem of the hairpin structure, and leads to the release of a double-stranded precursor miRNA (pre-miRNA) of 60–70 bp in length, with the mature miRNA sequence being located within one of the arms of this hairpin RNA product. During the processing of pri-miRNA by the microprocessor, DGCR8 assists Drosha, functioning as a molecular anchor by binding to the stem of the pri-miRNA, allowing it to cleave the pri-miRNA at exactly 11 bp from the stem to ssRNA junction. This elaborate bioprocessing gives rise to the

Fig. 3.1 miRNA transcription that proceeds via the canonical pathway is primarily mediated by RNA polymerase II, giving rise to a 5′-capped, 3′-polyadenylated primary miRNA (pri-miRNA), harboring hairpin stem-loop structure(s). Mature miRNAs originate from within one of the stem-loop arms. After being transcribed, the pri-miRNA is then processed by the nuclear RNase III endonuclease Drosha, producing a double-stranded hairpin structure, termed precursor miRNA (pre-miRNA). Subsequently, these pre-miRNAs are actively exported into the cytoplasm, via the Exportin-5 complex, where they are substrates of RNase III endonuclease Dicer. The pre-miRNA is cleaved near the terminal loop of the hairpin, thus producing miRNA duplexes, which are subsequently loaded onto miRNA-induced silencing complexes (miRISCs). This process is then followed by unwinding of the duplex and strand selection, leading to the transfer of the mature antisense miRNA (guide strand) to Argonaute (AGO) proteins in miRISC. Generally, the passenger strand is rapidly degraded. After miRISC:mature miRNA assembly, the ~22 nt mature miRNA is able to guide the miRISC assembly to target mRNAs, inducing their posttranscriptional silencing, either by translational repression and/or mRNA degradation. In the rare case of near or perfect miRNA complementarity to the target mRNA, the transcript may also be endonucleolyticly cleaved. Importantly, in addition to their well known cytoplasmic localization, mature miRNAs are also present in additional subcellular compartments and organelles, including the mitochondria and the nucleus. However, the mechanisms regulating the trafficking of miRNAs to and from mitochondria are mostly unknown. Nevertheless, it is possible that the 3′/5′ exoribonuclease and poly-A polymerase, PNPASE, located in mitochondrial intermembrane space, or additional yet unidentified proteins, may be involved in this process. It is well established that PNPASE was shown to play a pivotal role in the import of small RNA components into the matrix. The biological role(s) of mature miRNAs within mitochondria is yet to be firmly established. However, a mitochondria-localized miRNA (mitomiR) (miR-1) was shown to enhance the translation of mtDNA-encoded transcripts. This process requires specific miR:mRNA basepairing, AGO2, and also detachment of AGO2 from its functional partner GW182, which is excluded from mitochondria. Nevertheless, the precise molecular mechanisms of miRNA/AGO2 complex leading to enhanced mitochondrial translation remain unclear. It has been suggested that the three critical requirements for converting miRNA-dependent translational repression to activation, include (a) lack of the cap at 5′ end; (b) absence of a typical poly(A) tail at 3′ end; and (c) detachment of GW182 from Ago protein. The mitochondrion fulfills all three requirements, since mitochondrial transcripts resemble mRNAs in prokaryotic cells, having no 5′ cap, long poly(A) tails, and no evidence of GW182. There is also a growing interest in the putative mechanism(s) by which mature miRNAs are imported back into the nucleus. Of note, importin 8 has been shown to physically associate with Ago2 in regulating the transport of mature miRNAs into the cell nucleus. Importantly, in addition to mature miRNAs, pre-miRNA are also present in mitochondria, suggesting that mitochondria may provide a platform allowing the assembly of signaling complexes involved in the control of transcriptional repression, reinforcing the pivotal biological relevance of this organelle in the regulation of health and disease

production of pre-miRNAs [33], which are intermediate structures containing a 5′ phosphate and a 2-nt 3′ overhang [34, 35]. Interestingly, Drosha is also responsible for the negative regulation of its cofactor DGCR8, by cleaving RNA hairpins within one of the exons of DGCR8 mRNA. This relevant regulation clearly illustrates the stricking effect of a miRNA hairpin located within an exon of a protein-coding gene, where Drosha processing destabilizes the transcript, leading to downregulation of protein synthesis. In addition, it suggested the ability to downregulate protein expression via a miRNA-independent and microprocessor-dependent mRNA pathway [36].

Subsequent to its release from pri-miRNA, pre-miRNA is exported from the nucleus to the cytoplasm primarily by exportin-5 (Exp5), a member of the nuclear receptor family, and mediated by the recognition of the 2-nt 3′ overhang arising from Drosha cleavage. Exp-5 binds its nuclear pre-miRNA cargo in a Ran guanosine triphosphate (Ran-GTP)-dependent manner, and following hydrolysis of GTP, releases pre-miRNAs within the cytoplasm [37–39], where they are substrates for the RNase III endonuclease Dicer. This bioprocessing step originates a miRNA duplex resulting from Dicer-mediated cleavage near the terminal loop of the pre-miRNA hairpin, at the opposite end from Drosha cleavage site [40]. miRNA duplexes are subsequently loaded onto Argonaute (AGO) proteins, where in association with dsRNA-binding proteins, TAR (HIV trans-activator RNA) RNA-binding protein (TRBP), and possibly the protein kinase R-activating protein (PACT), form the RNA-induced silencing complex (RISC) [4, 41, 42].

Since miRNAs function as ssRNA, unwinding of the miRNA duplex and strand selection process must take place, thus allowing the final production of the functional, mature, miRNA. During this process, one strand from the RNA duplex remains bound to AGO as the mature miRNA (guide strand), while the other strand (passenger strand) is usually removed and rapidly degraded [24]. The process of strand selection is dependent on relative thermodynamic stability of the two strands of the duplex intermediate [43, 44]. There are four Argonaute proteins in humans, AGO1-4, which can bind miRNAs, and support their posttranscriptional gene regulation. The catalytic component of RISC is AGO-2, also known as Slicer, which is the only known human AGO capable of cleaving mRNAs [45]. miRNA-mediated gene silencing occurs due the guidance of the RISC complex to target mRNAs effected by mature miRNAs within miRISC, which in turn leads to their posttranscriptional silencing.

The key feature of target recognition in animals is the perfect, or near perfect, base-pairing between the miRNA seed sequence (miRNA nucleotides at positions 2–7 on the miRNA 5′ end) and the miRNA target sites within 3′-UTR of mRNA transcripts [5, 6, 46, 47]. Therefore, it is not surprising that one miRNA is predicted to interact, and may in fact regulate, hundreds of different target genes, due to the requirement for miRNA targeting being merely complementarity to a small number of nucleotides [23, 48]. In mammals, miRNA-mediated gene silencing occurs due to either translational repression and/or mRNA degradation as a result of miRNA recognition, binding to target mRNA and degree of complementarity. When miRNA silencing induces target mRNA degradation, it is not the result of AGO cleavage,

but rather due to target mRNA deadenylation, decapping, and exonucleolytic degradation [7, 46, 47]. This process is thought to occur in processing bodies (P-bodies), and involves AGO, GW182, and the cellular decapping and deadenylation machinery, CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. P-bodies are intracellular foci, where translationally inactive mRNAs are often stored and may ultimately undergo decay. miRNAs, mRNA targets, and AGO proteins have all been identified within these foci [46, 47, 49]. In the case of target mRNA endonucleolytic cleavage, it may occur when the miRNA binds to its target mRNA with nearly perfect complementarity [5, 6, 50].

Recently, several reports have provided an additional layer of complexity to miRNA function and subcellular localization. Somewhat unexpectedly, mature miRNAs were detected not only in the cytoplasm, where they exert their traditional biological function, but also in other subcellular organelles and compartments, including mitochondria (Table 3.1) [2, 51–58], the nucleus [59, 60], and nucleolus [61, 62]. This has significantly expanded our knowledge on microRNA biology, further underscoring the potential relevance of miRNAs in the regulation of health and disease.

Mitochondria-Localized miRNAs and Their Biological Role

Mitochondria are cellular organelles found in eukaryotic cells, which harbor their own mtDNA. It is known that replication and transcription of mtDNA are regulated by noncoding RNAs and proteins, expressed from both the nuclear and mitochondrial genomes. Interestingly, the mitochondrial genome only encodes for 13 proteins, but up to 1500 proteins may be found in mitochondria [2, 63]. This discrepancy in numbers clearly indicates that the vast majority of these proteins are imported into mitochondria, following their synthesis in the cytoplasm [64]. In contrast, the majority of noncoding RNAs present in mitochondria are encoded and expressed from mtDNA [65]. Nevertheless, there is growing evidence to suggest that mitochondria also traffic multiple RNA species. In this regard, many relevant nuclear-encoded RNAs are known to be imported into mitochondria, including nuclear-encoded 5S rRNA [66], the most abundant RNA found in mitochondria, tRNAs [67], and also the RNA components of RNAse MRP and RNAse P [68]. Further, mitochondria also export tRNAs encoded by mtDNA into the cytoplasm [69], indicating that the process of RNA trafficking to and from mitochondria is dynamic and bidirectional. Importantly, mitochondria are known to contain multiple families of noncoding RNAs, including miRNA, snoRNA, snRNA, srpRNA, piRNA, as well as repeat associated small RNAs [2, 55].

Multiple mechanisms have been described for protein import into mitochondria [63, 70]. In this regard, understanding protein–protein interactions and their role in mitochondrial function [71] will significantly contribute to understanding target gene regulation by mitochondrial miRNAs (mitomiRs), and their impact on signaling pathways. Over the last several years, the mechanisms regulating mitochondrial

Table 3.1 miRNAs detected in mitochondria

Species	Source	Detection	miRNAs detected	Validation	miRNAs validated	References
Human	143B cells (osteosarcoma)	Deep sequencing	(hsa-miR)-16, -103, -146a	qRT-PCR	(hsa-miR)-16, -103, -146a	[2]
Human	Myotubes	qRT-PCR	(hsa-miR)-let-7b, -let-7g, -19b, -20a,-23a, -23b, -24, -34a, -92a, -93, -103,-106a,-107, -125a-5p, -125b, -127-3p, -133a, -133b, -134, -149, -151-5p, -181a, -193b, -197, -199a-5p, -210, -221, -324-3p, -324-5p, -365,-423-3p, -484, -486-5p, -490-3p, -501-3p, -503, -532-3p, -542-5p, -574-3p, -598, -675*, -720, -1974, -1979, -miRPlus-D1033; (hsa-) pre-miR-let7b, pre-miR-302a	In situ hybridization	hsa-miR-365 (hsa-) pre-miR-let7b, pre-miR-302a	[52]
Human	HeLa cells (cervical cancer)	Deep sequencing	HeLa: 428 known, 196 putative novel miRNAs	qRT-PCR	HeLa and HEK293: (hsa-miR)-let-7b, let-7g, -107, -181a, -221, -320a	[55]
	HEK293 cells (embryonic kidney)		HEK293: 327 known, 13 putative novel miRNAs			
Human	HeLa cells (cervical cancer)	Microarray	(hsa-miR)-328, -494, -513a-5p, -638, -1201, -1246, -1275, -1908, -1978 -1972, -1973, -1974, -1977	qRT-PCR	(hsa-miR)-494, -1275, -1974	[51]
Human	C2C12 myoblasts and myotubes	qPCR	miR-1		miR-1	[58]
Mouse	Heart					
Rat	Heart	Microarray	hsa-miR-181c	qRT-PCR in situ hybridization	hsa-miR-181c	[56]

Rat	Liver	Microarray	(rno-miR)-21, -130a, -130b, 140*, -290, -320, -494, -671	qRT-PCR	(rno-miR)-130a, -130b, -140*, -320, -494	[54]
			(mmu-miR)-202, -705, -709, -721, -761, -763			
			(hsa-miR)-198, -765			
Rat	Hippocampus	Microarray	(mmu-miR)-10a, -142-3p, -142-5p, -146a, -150, -155, -202-3p, -223, -302a, 339-5p, -741, (rno-miR)-339-3p, -344-5p	qRT-PCR	(mmu-miR)-142-3p, -142-5p, -146a, -155, -223	[57]
Mouse	Liver	Microarray	(mmu-miR)-122, -134, -155, -202-5p, -223, -494, -680, -705, -709, -720	qRT-PCR	(mmu-miR)-122, -134, -155, -202-5p, -223, -494, -680, -705, -709, -720	[53]

Comprehensive studies performed in human, rat, and mouse, leading to the identification and validation of individual miRNAs in the mitochondrial niche (mitomiRs). The full relevance of these mitomiRs is not entirely understood, but it has been demonstrated that mitochondrial-located miRNAs do play functional roles within this niche, as illustrated by the nuclear-encoded miR-181c, which following translocation to the mitochondria targets cytochrome c oxidase subunit 1 (mt-cox1), a mitochondrial-encoded gene [56]. In addition, miR-1 enters the mitochondrial niche, where it unexpectedly activates the translation of ND1 and COX1 mitochondrial-encoded transcripts, in contrast to its known transcriptional inhibitory role in the cytoplasm [58]

RNA trafficking are emerging [72–74], but not those specifically underlying miRNA transport to and from mitochondria. In this regard, polynucleotide phosphorylase (PNPASE), a 3′-to-5′ exoribonuclease and poly-A polymerase located in the mitochondrial intermembrane space, appears to play a key role in the mitochondrial import of small RNA components necessary for mtDNA replication and mtRNA processing. In addition, PNPASE regulates mitochondrial homeostasis and adenine nucleotide levels, at least partly by regulating RNA import, and via components of the electron transport chain [72, 73]. Collectively, such studies may suggest that PNPASE is involved in the trafficking of mitochondrial miRNAs, but does not exclude the possible involvement of additional, yet unidentified, RNA transport mechanisms. Interestingly, the identification of hundreds of mature miRNAs in the cell nucleus raises the possibility of their potential involvement in the transcriptional regulation of gene expression [59]. In this regard it is known that importin 8 physically associates with Ago2, regulating the nuclear import of mature miRNAs [75]. Therefore, the potential functional role(s) of mature miRNAs within the cell nucleus has established a heretofore unrecognized regulation of gene expression [60, 76].

Mitochondria are organelles of pivotal relevance for cellular homeostasis, and changes affecting mitochondria function may contribute to disease. For example, mutations in mtDNA affecting mt-tRNA$^{Leu(UUR)}$ cause a rare neurodegenerative disease, termed MELAS syndrome, in which patient cybrid cells exhibit elevated levels of oxidative stress [77]. In turn, oxidative stress was shown to mediate the increased expression of miR-9/9*, leading to the posttranscriptional negative regulation of mt-tRNA-modification enzymes GTPBP3, MTO1, and TRMU, which contributes to the MELAS phenotype. This study demonstrated that mt-DNA disease can directly affect miRNA expression, where miR-9/9* was found to be a crucial player in mitochondria-to-nucleus signaling. Most notably, miR-9/9* regulates the expression of nuclear genes in response to changes in the functional state of mitochondria.

Together with the wide number of existing nuclear- and mitochondrial-encoded RNAs and proteins, mitochondria may represent a novel unexplored and extremely relevant subcellular niche for miRNA-mediated gene regulation. In this regard, RNAi components were already found to localize to mitochondria. The first evidence of such an event was provided almost a decade ago, with the identification of the interaction of human Ago2 with mitochondrial tRNA^{met} [69]. In addition, Ago2 and Ago3 were recently found to co-localize to mitochondria, adding to these initial observations, and increasing the potential relevance of mitochondria as a novel subcellular site for RNAi-mediated gene silencing [51, 53, 55]. AGO2 and Dicer were also recently shown to be present, and enriched, in mitochondrial matrix and outer mitochondrial membrane of mitochondria isolated from rat brain [57]. An additional study in adult mouse heart, C2C12 myoblasts, and C2C12 myotubes also confirmed that AGO2 was present in highly purified mitochondria and mitoplasm preparations. Interestingly, neither AGO1 nor AGO3 was detectable in these preparations, suggesting that AGO2 may be selectively imported into the mitochondria [58].

Less than a decade ago, by using sequence analysis, small noncoding RNAs were identified both in mitochondria and chloroplasts [78]. Since that initial report, miRNAs were shown to be present in this novel subcellular niche, in studies using mitochondria isolated from multiple organisms and sources, including rat liver [54], cardiac myocytes [56], and hippocampus [57]; mouse liver [53], heart and C2C12 myoblasts and myotubes [58]; human skeletal primary muscle cells [52], and the cancer cell lines Hela [51, 55], HEK293 [55], and 143B cells [2]. Interestingly, precursor miRNAs have also been identified in mitochondria [52] (Fig. 3.1).

Currently, there is a growing interest in the identification of miRNAs in mitochondria and in exploring their roles in the (de)regulation of cellular homeostasis. Highlighting these efforts, our laboratory has recently identified the presence of nuclear-encoded mature miRNAs in highly purified and intact mitochondria isolated from rat liver [54]. To allow the identification of such miRNAs, it was critical to ensure the highest degree of purity in the isolated mitochondria, eliminating the potential contamination arising from cytosolic components containing miRNAs, such as endoplasmic reticulum, Golgi, and free ribosomes.

To address this critical concern, isolated and purified rat liver mitochondria were carefully processed to ensure that they were devoid of contaminating RNAs from the cytosol or other cellular organelles and compartments. This was achieved by performing extensive purification steps coupled with the microarray detection of mature miRNAs, followed by their validation by northern blot, which undoubtedly confirmed the presence of mature miRNAs in highly purified mitochondria [54]. Our detailed methods for isolation of highly purified mitochondria and for the coupled extraction of total RNA and protein are available, and may allow for downstream evaluation of miRNA and protein expression in mitochondria, thus increasing the relevance of the experimental data [79].

Our initial efforts led to the identification of a unique profile of mature miRNAs in isolated mitochondria, composed by 15 distinct microRNAs. To further understand the provenance of such miRNAs, we performed functional analysis using TargetScan, Miranda, and Ingenuity Pathway Analysis, which showed that these miRNAs were not targeting mitochondrial genes nor nuclear RNAs encoding mitochondrial proteins. Rather, and perhaps not surprisingly, this analysis indicated that mitochondria-associated miRNAs were involved in the regulation of crucial biological processes such as proliferation, differentiation, and apoptosis [54]. Subsequently, in the following year, an additional miRNA signature profile comprising 20 miRNAs was reported in intact mitochondria isolated from mouse liver [53]. Most notably, the enrichment in these miRNAs was shown to be independent of the total cellular miRNA abundance, suggesting that mitochondria, in fact, harbor a characteristic and distinctive miRNA population, involved in the regulation of mitochondria-specific, and also of general cellular functions. Interestingly, in silico analysis performed by several target-predicting algorithms indicated that putative targets of mitomiRs miR-705, miR-494, and miR-202-5p are related to mitochondrial specific functions, such as transcription factor A (Tfam) and tryptophanyl-tRNA synthetase (WARS).

Specifically, the first studies reporting the detection of mitochondrial miRNAs in human cells occurred only recently, in mitochondria isolated from human skeletal primary muscle cells [52] and HeLa cancer cell line [51]. In mitochondria isolated from human skeletal muscle, 46 miRNAs were identified, and reported as being potentially involved in myogenesis, inflammation, fibrosis, oncogenesis, and tumor suppression. Importantly, these authors identified for the first time human precursor miRNAs localized to mitochondria, since they were able to detect, and validate by in situ hybridization and confocal microscopy, the mitochondrial localization of pre-miR-302a and pre-miR-let-7a [52].

Importantly, in silico analysis of the putative targets of miR-302a and miR-let-7a resulted in a wide number of sequences mapping to the mitochondrial genome, with many of these sequences lying within mitochondrial regulatory genes. Interestingly, several putative miRNA target genes displayed multiple miRNA recognition/target sequences. In this regard, the gene ND6 was found to harbor 38 putative miRNA target sites, which was twice as many as in the genes CYTB and ND1. Further, additional genes, including COX1, ND4L, and ND4, also displayed multiple potential miRNA-binding sites, highlighting the potential relevance of miRNA gene expression regulation in mitochondrial function. Furthermore, miRNA let-7b was predicted to target several potential mitochondrial gene products, including ATP6, ATP8, COX2, and ND5, in addition to other let-7 family members, which also displayed putative targets. Collectively, certain miRNAs detected within mitochondria, including let-7 family members (let-7b, c, d, e, f, i) and also miR-133a, could target mitochondrial mRNAs, thus playing a role in the regulation of mitochondrial gene expression [52].

Thirteen nuclear-encoded and highly abundant miRNAs were identified in mitochondria isolated from human HeLa cancer cells, in a separate, and confirmatory study [51]. The detection of the miRNAs was performed following a rigorous mitochondria isolation protocol, which included RNAse A treatment of isolated mitochondria to remove possible contaminating miRNAs associated with or bound to the outer mitochondrial membrane. Further, in silico analysis of the putative targets of 4 of these 13 mitochondrial localized miRNAs, miR-328, miR-494, miR-513, and miR-638, suggested their involvement in the regulation of mitochondrial homeostasis.

Recently, deep sequencing analysis of RNA, extracted from mitochondria isolated from 143B human osteosarcoma cells and treated with RNAse A to prevent contamination from non-mitochondrial RNAs, provided an unprecedented knowledge of the human mitochondrial transcriptome. This study showed that mitochondria harbor 3 % of the whole-cell small RNA population, containing a diverse population of small RNAs, including miRNAs [2]. Further, purified mitochondrial RNA deep sequencing indicated that a large number of reads aligned only to the nuclear genome, suggesting that these nuclear-encoded RNAs were subsequently imported into mitochondria. Nevertheless, some concern was raised regarding validity of this data, since a strong enrichment was found in genes associated with protein translation, suggesting potential contamination with ribosomes attached to the outer mitochondrial membrane. To address this potential problem, the authors

compared RNA content from whole mitochondria with that of mitoplasts (mitochondrial extracts devoid of outer mitochondrial membrane). Matched sequencing of these RNA preparations demonstrated a selective depletion of nuclear-encoded tRNAs and mRNAs in mitoplasts, suggesting that most of the nuclear-encoded RNAs found in mitochondria were indeed associated with the outer mitochondrial membrane, and not actually within this organelle. Importantly, the miRNAs showing the highest enrichment in whole mitochondria preparations, miR-146a, miR-103, and miR-16, also showed depletion in mitoplasts.

An additional study made use of RNA deep sequencing to evaluate small RNA populations extracted from mitochondria isolated from HEK293 and HeLa human cancer cells. Once again, the results confirmed the presence of unique small RNA populations associated with mitochondria [55], while putative novel miRNAs from unannotated small RNA sequences were characterized. In fact, this study demonstrated association of 428 known and 196 putative novel miRNAs to mitochondria of HEK293 cells, and 327 known and 13 putative novel miRNAs to mitochondria of HeLa cells. The in silico analysis of potential targets indicated that miRNAs associated with mitochondria may be involved in the regulation of critical cellular processes in which mitochondria play significant roles, including apoptosis, cell cycle, RNA turnover, and nucleotide metabolism. Further, in silico analysis to identify the subcellular provenance of such miRNAs suggested that known miRNAs mapped to mitochondrial genome, namely hsa-miR-4461, hsa-miR-4463, hsa-miR-4484, and hsa-miR-4485, which aligned at positions corresponding to ND4L, ND5, L-ORF, and 16S rRNA mitochondrial genes, respectively. Furthermore, this analysis also indicated that seven putative novel miRNAs align to noncoding regions, tRNA, 12S rRNA, and also to coding regions within ATP6, ND2, HVRI, COI, CytB, and ND1 genes [55]. Importantly, mitochondrial transcript targeting by miRNAs was already experimentally validated in rat cardiac myocytes for miR-181c. Following translocation to mitochondria, the nuclear-encoded miRNA was demonstrated to negatively regulate the expression of cytochrome c oxidase subunit 1 (mt-cox1), a mitochondrial-encoded gene [56]. A follow-up study recently evaluated the impact of miR-181c in vivo administration in rats, using a miR-181c expression vector packaged in lipid-based nanoparticles for systemic delivery [80]. The administration of this nanovector resulted in reduced exercise capacity, with signs of heart failure, as a consequence of miR-181c targeting the 3′-end of mt-cox1.

The global expression of miRNAs localized in mitochondria, and also in the cytosol, was recently evaluated using TaqMan® RT-qPCR Array Rodent MicroRNA Card A (V2.0). Using RNA extracted from rat hippocampus mitochondria, 285 out of 381 miRNAs present in the Array, were detected in both mitochondria and cytosol [57]. As expected, most of these miRNAs were much more abundant in the cytosol compared to the mitochondrial niche. However, a subset of miRNAs were found to be at least 1.5-fold more abundant in mitochondria, including mmu-miR-741, mmu-miR-142-5p, mmu-miR-302a, mmu-miR-142-3p, rno-miR-339-3p, mmu-miR-10a, mmu-miR-146a, mmu-miR-202-3p, mmu-miR-150, mmu-miR-339-5p, and rno-miR-344-5p. Among these mitochondria-enriched miRNAs, miR-142-5p, miR-142-3p, and miR-146a displayed significantly higher mitochondrial

levels compared to levels in the cytosol, and this data was validated by TaqMan® single-tube RT-qPCR assays using rat hippocampal and rat cortical astrocyte, mitochondrial and cytosolic preparations. In addition, the relevance of mitochondria-located miRNAs was also highlighted following the identification of altered expression of hippocampal mitochondria-associated miRNAs after severe traumatic brain injury (TBI). The authors induced TBI in rats, by surgical and controlled cortical impact (CCI) injury. The hippocampus from both injured and naïve animals was collected 12 h post-injury, and processed for the detection of mitochondrial and cytosolic miRNAs by TaqMan® RT-qPCR Array. Interestingly, TBI reduced the levels of most mitochondria-associated miRNAs, including the mitochondria-enriched miRNAs miR-142-3p, miR-142-5p, and miR-146a, which in turn increased in the cytosol. In contrast, the levels of several mitochondria-associated miRNAs were found to increase following TBI, including miR-155 and miR-223, which are known to play a role in inflammatory processes. This study showed dramatically that mitochondria-localized miRNAs are dynamically regulated by disease and play an important role in regulating the response to TBI [57].

Interestingly, not only do mitochondria harbor miRNAs within their mitoplasm, but they may also constitute a platform of signaling complexes involved in the regulation of transcriptional repression, thus significantly expanding the relevance of mitochondria-localized miRNAs. In this regard, nuclear-encoded miRNAs are associated with the outer mitochondrial membrane [2, 55], together with RNAi components, Ago2, and Ago3 [51, 53, 55] (Fig. 3.1). Indeed, there may be an even more critical function for miRNAs associated with the outer membrane of mitochondria than within its space. To elucidate in detail mitochondria-localized miRNA dynamics and their associated proteins in the various submitochondrial compartments, further studies will be necessary to understand the full relevance of miRNAs within this cellular niche. Interestingly, mitochondria are also known to interact with P-bodies, involved in mRNA decay, storage, and RNA interference, including miRNA-mediated effects [81, 82]. In addition, it was demonstrated that exposure to carbonyl cyanide *p*-chlorophenylhydrazone, which dysregulates mitochondrial function, leads to a significant decrease in miRNA-mediated activity, possibly by interfering with RISC assembly in association with Ago2 delocalization from P-bodies. It was also suggested that RNAi defects may be involved in pathologies associated with mitochondrial deficiencies [82].

A recent study has extended the initial observation that miRNAs may positively regulate translation [25, 26]. In this regard, miR-1, which is specifically induced during myogenesis, was unexpectedly reported to efficiently enter mitochondria where it stimulates, rather than represses, the translation of specific mitochondrial genome-encoded transcripts. This process was shown to require specific miR:mRNA base pairing and AGO2, but not GW182, which was excluded from the mitochondria [58]. These findings reveal a unique function of miRNAs in mitochondrial translation, selectively activating mtDNA-encoded mRNAs. The findings suggest a highly coordinated myogenic program via miR-1-mediated translational stimulation in the mitochondria and repression in the cytoplasm, thus representing a new mitochondrial regulatory pathway, and a potentially powerful approach to therapeutically modulating mtDNA expression.

Although many questions remain unanswered, it is now clearly established that miRNAs and RISC complex proteins are associated with, and also localized within mitochondria. Importantly, the final destination of these miRNAs may be key to their biological function, which may include the regulation of mitochondria-encoded mRNA or nuclear-encoded mRNA/protein complexes, and additional heretofore unrecognized roles. In this regard, it is feasible to envision that miRNAs associated to the outer membrane may putatively play a role in the regulation of mRNA/protein levels at distant subcellular sites. The role of mitochondria extends far beyond their function in energy metabolism, inflammation, or apoptosis [3, 16], and the active cross talk of mitochondria with Golgi, nuclear membrane, and P-bodies, highlights the relevance of miRNAs in both normal and pathological conditions.

Mitochondria are extremely motile cellular organelles, whose dynamics carry important biological impacts, and this is particularly relevant to neurons [83, 84]. Neurons are polarized, post-mitotic, and long-lived cells, displaying a small cell body, branched dendrites, and a thin axon, which can be extremely long. Due to these unique morphological features, neurons face several challenges to accomplish their energy homeostasis, requiring specialized mechanisms to efficiently relocate mitochondria to where energy is in high demand. Such subcellular location includes axonal branches and synaptic terminals, which during neuronal development and in response to synaptic activity, undergo dynamic remodeling, resulting in significant alteration in the dynamics of mitochondrial trafficking. Therefore, efficient regulation of mitochondrial trafficking and anchoring is essential to the recruitment and relocation of mitochondria within the cell, to quickly address changes in metabolic requirements, and also to remove and replace aged and/or damaged mitochondria [84].

There is growing evidence suggesting that mitochondria are also transferred between cells, via tunneling nanotubes and exosomes, thereby playing a role on target cells [85–87]. Importantly, in highly heterogeneous, cross-resistant, and radiorefractory cell populations, obtained by selection following exposure to anticancer agents, the occurrence of a number of atypical recurrent cell types was reported [88]. Interestingly, some of these resistant tumor cells displayed nuclear encapsulation via mitochondrial aggregation in the nuclear perimeter in response to cytostatic insults, which is thought to confer imperviousness to drugs, thus allowing for long periods of dormancy, until nuclear eclosion. This phenomenon was correlated with an increase in both intracellular and intercellular mitochondrial traffic as well as with the uptake of free extracellular mitochondria.

Collectively, these key findings underscore the potential importance of mitochondrial miRNAs and their impact in numerous cell processes. In this regard, mitochondria intracellular and intercellular dynamics may be biologically relevant, since mitochondria are key regulators of cell survival and death, in addition to playing important regulatory roles in signaling pathways elicited by alterations to homeostasis [89]. Despite lacking experimental confirmation, it is conceivable that following miRNA transport within or along with mitochondria, a local enrichment in mitochondrial miRNAs may occur, which upon organelle damage may lead to the release of mitochondrial miRNAs and RNAi proteins. In turn, this could lead to a local shift in transcript targeting, which translates into a putative biological effect.

Importantly, insults affecting organism homeostasis may alter organ, tissue, as well as mitochondrial-associated miRNA expression. This is clearly illustrated by the treatment of mice with streptozotocin, which induced mitochondria dysfunction and type 1 diabetes, and also significantly altered the expression patterns of liver miRNAs, and mitochondria-associated miRNAs, suggesting the potential involvement of miRNAs in mitochondrial dysfunction [53].

In addition, some mitomiRs are also involved in the regulation of cell aging and in inflamm-aging, namely miR-146a, miR-34a, and miR-181a. Further, Ingenuity Pathway Analysis of aging-related mitomiR targets suggested that several resident mitochondrial proteins involved in energy metabolism, mitochondrial transport, and apoptosis are targeted by such miRNAs. This negatively impacts on mitochondrial function and function in aging cells, which may induce or contribute to the inflammatory response and to age-related diseases [90].

Interestingly, a new layer of complexity regarding mitochondria noncoding RNAs is also emerging. In addition to mitochondrial miRNAs, a unique family of mitochondrial long ncRNAs (ncmtRNAs) comprising sense and antisense members, containing stem-loop structures, and also found in the cytosol and nucleus, display differential expression from cancer to normal cells [91]. Normal proliferating cells express sense (SncmtRNA) and antisense (ASncmtRNA) transcripts, and in contrast, tumor cells display downregulation of ASncmtRNAs, regardless of tissue of origin. In addition, the functional significance of ASncmtRNAs in cancer cells was confirmed by the demonstration that ASncmtRNAs knockdown induced apoptotic cell death mediated by surviving inhibition in several tumor cell lines, whereas it did not affect the viability of normal cells. Further, translational inhibition of surviving was found to possibly be mediated by microRNAs generated by dicing of the double-stranded stem of the ASncmtRNAs. These results suggest that the downregulation of ASncmtRNAs may be promising targets for cancer therapy, and warrant further study.

Undoubtedly, mitomiRs, ncmtRNAs, and other mitochondrial noncoding RNAs play relevant roles in the regulation of the fine balance between health and disease, and it is of pivotal importance to fully understand what is the role of the mitochondrial niche in the universal language of “competing endogenous RNA” (ceRNA), and how they putatively modulate the alphabet of microRNA response elements (MREs) [92, 93].

Conclusions

Currently, there is an expanding body of evidence supporting the role of miRNAs in mitochondria functional regulation. Somewhat unexpectedly, it is also becoming increasingly clear that several distinct subcellular niches and compartments, including mitochondria and the nucleus, are extremely relevant sites of miRNA action. Notwithstanding, it remains unclear whether certain mitochondria-localized miRNAs are encoded by mtDNA or in nuclei and later imported into mitochondria.

Nevertheless and despite their subcellular origin, the full biological and pathophysiological implications of mitomiRs are incompletely explored, and beginning to emerge. It is conceivable that mitochondria-localized miRNAs may function as post-transcriptional regulators of the mitochondrial genome. Additional studies will undoubtedly confirm whether the mitochondrial genome is a source, a target, or both for miRNAs localized to mitochondria. Growing evidence suggests that the mitochondrial niche provides both a miRNA source and a regulatory target for mitochondria-localized miRNAs. It will be interesting to ascertain if mitochondria function as a storage and/or carrier for miRNAs and proteins, including RNAi regulatory proteins, localized at, or enclosed within, this organelle. In this regard, mitochondria-associated proteins differ among cell type and tissue, depending on a multitude of factors influencing the local environment, including energy and metabolic requirements. In line with this notion, it is becoming increasingly evident that mitochondria-localized miRNA may also be cell type-specific and regulated by intracellular and extracellular environments. Extending our current knowledge base on mitochondria miRNA transport/trafficking mechanisms will expand the relevance of miRNAs in the (de)regulation of cellular homeostasis. Importantly, there is a pressing requirement for additional studies to fully elucidate the spectrum of mitochondria-localized/associated miRNAs in health and disease, as well as to grasp the full extent of their biological implications and impact, which will undoubtedly expand the growing list of therapeutic targets for intervention.

References

1. Nunnari J, Suomalainen A. Mitochondria: in sickness and in health. Cell. 2012;148(6):1145–59. PubMed.
2. Mercer TR, Neph S, Dinger ME, Crawford J, Smith MA, Shearwood AM, et al. The human mitochondrial transcriptome. Cell. 2011;146(4):645–58. PubMed Pubmed Central PMCID: 3160626.
3. Tomasetti M, Neuzil J, Dong L. MicroRNAs as regulators of mitochondrial function: role in cancer suppression. Biochim Biophys Acta. 2014;1840(4):1441–53. PubMed.
4. Graves P, Zeng Y. Biogenesis of mammalian microRNAs: a global view. Genomics Proteomics Bioinformatics. 2012;10(5):239–45. PubMed.
5. Huntzinger E, Izaurralde E. Gene silencing by microRNAs: contributions of translational repression and mRNA decay. Nat Rev Genet. 2011;12(2):99–110. PubMed.
6. Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. Nat Rev Genet. 2012;13(4):271–82. PubMed.
7. Pereira DM, Rodrigues PM, Borralho PM, Rodrigues CM. Delivering the promise of miRNA cancer therapeutics. Drug Discov Today. 2013;18(5–6):282–9. PubMed.
8. Aranha MM, Santos DM, Solá S, Steer CJ, Rodrigues CM. miR-34a regulates mouse neural stem cell differentiation. PLoS One. 2011;6(8):e21396. PubMed Pubmed Central PMCID: 3153928.
9. Aranha MM, Santos DM, Xavier JM, Low WC, Steer CJ, Solá S, et al. Apoptosis-associated microRNAs are modulated in mouse, rat and human neural differentiation. BMC Genomics. 2010;11:514. PubMed Pubmed Central PMCID: 2997008.
10. Borralho PM, Kren BT, Castro RE, da Silva IB, Steer CJ, Rodrigues CM. MicroRNA-143 reduces viability and increases sensitivity to 5-fluorouracil in HCT116 human colorectal cancer cells. FEBS J. 2009;276(22):6689–700. PubMed.

11. Borralho PM, Simões AE, Gomes SE, Lima RT, Carvalho T, Ferreira DM, et al. miR-143 overexpression impairs growth of human colon carcinoma xenografts in mice with induction of apoptosis and inhibition of proliferation. PLoS One. 2011;6(8):e23787. PubMed Pubmed Central PMCID: 3162002.
12. Castro RE, Ferreira DM, Afonso MB, Borralho PM, Machado MV, Cortez-Pinto H, et al. miR-34a/SIRT1/p53 is suppressed by ursodeoxycholic acid in the rat liver and activated by disease severity in human non-alcoholic fatty liver disease. J Hepatol. 2013;58(1):119–25. PubMed.
13. Bandiera S, Mategot 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. PubMed.
14. Bienertova-Vasku J, Sana J, Slaby O. The role of microRNAs in mitochondria in cancer. Cancer Lett. 2013;336(1):1–7. PubMed.
15. Li P, Jiao J, Gao G, Prabhakar BS. Control of mitochondrial activity by miRNAs. J Cell Biochem. 2012;113(4):1104–10. PubMed Pubmed Central PMCID: 3325319.
16. Sripada L, Tomar D, Singh R. Mitochondria: one of the destinations of miRNAs. Mitochondrion. 2012;12(6):593–9. PubMed.
17. Duarte FV, Palmeira CM, Rolo AP. The role of microRNAs in mitochondria: small players acting wide. Genes. 2014;5(4):865–86. PubMed Pubmed Central PMCID: 4276918.
18. Oberg AL, French AJ, Sarver AL, Subramanian S, Morlan BW, Riska SM, et al. miRNA expression in colon polyps provides evidence for a multihit model of colon cancer. PLoS One. 2011;6(6):e20465. PubMed Pubmed Central PMCID: 3111419.
19. 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. PubMed.
20. 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. PubMed.
21. 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. PubMed.
22. 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(1):15–20. PubMed.
23. Friedman RC, Farh KK, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2009;19(1):92–105. PubMed Pubmed Central PMCID: 2612969.
24. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116(2): 281–97. PubMed.
25. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science. 2007;318(5858):1931–4. PubMed.
26. Vasudevan S. Posttranscriptional upregulation by microRNAs. Wiley Interdiscip Rev RNA. 2012;3(3):311–30. PubMed.
27. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. Nature. 2007;448(7149):83–6. PubMed Pubmed Central PMCID: 2475599.
28. Havens MA, Reich AA, Duelli DM, Hastings ML. Biogenesis of mammalian microRNAs by a non-canonical processing pathway. Nucleic Acids Res. 2012;40(10):4626–40. PubMed Pubmed Central PMCID: 3378869.
29. Cai X, Hagedorn CH, Cullen BR. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA. 2004;10(12):1957–66. PubMed Pubmed Central PMCID: 1370684.
30. Borchert GM, Lanier W, Davidson BL. RNA polymerase III transcribes human microRNAs. Nat Struct Mol Biol. 2006;13(12):1097–101. PubMed.
31. Baskerville S, Bartel DP. Microarray profiling of microRNAs reveals frequent coexpression with neighboring miRNAs and host genes. RNA. 2005;11(3):241–7. PubMed Pubmed Central PMCID: 1370713.
32. Rodriguez A, Griffiths-Jones S, Ashurst JL, Bradley A. Identification of mammalian microRNA host genes and transcription units. Genome Res. 2004;14(10A):1902–10. PubMed Pubmed Central PMCID: 524413.

33. 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. PubMed.
34. Gregory RI, Yan KP, Amuthan G, Chendrimada T, Doratotaj B, Cooch N, et al. The microprocessor complex mediates the genesis of microRNAs. Nature. 2004;432(7014):235–40. PubMed.
35. Denli AM, Tops BB, Plasterk RH, Ketting RF, Hannon GJ. Processing of primary microRNAs by the microprocessor complex. Nature. 2004;432(7014):231–5. PubMed.
36. Han J, Pedersen JS, Kwon SC, Belair CD, Kim YK, Yeom KH, et al. Posttranscriptional cross-regulation between Drosha and DGCR8. Cell. 2009;136(1):75–84. PubMed Pubmed Central PMCID: 2680683.
37. Bohnsack MT, Czaplinski K, Gorlich D. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA. 2004;10(2):185–91. PubMed Pubmed Central PMCID: 1370530.
38. Lund E, Guttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science. 2004;303(5654):95–8. PubMed.
39. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 2003;17(24):3011–6. PubMed Pubmed Central PMCID: 305252.
40. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature. 2001;409(6818):363–6. PubMed.
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. PubMed Pubmed Central PMCID: 2944926.
42. Lee Y, Hur I, Park SY, Kim YK, Suh MR, Kim VN. The role of PACT in the RNA silencing pathway. EMBO J. 2006;25(3):522–32. PubMed Pubmed Central PMCID: 1383527.
43. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell. 2003;115(2):209–16. PubMed.
44. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. Cell. 2003;115(2):199–208. PubMed.
45. Peters L, Meister G. Argonaute proteins: mediators of RNA silencing. Mol Cell. 2007;26(5):611–23. PubMed.
46. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. Cell. 2009;136(4):642–55. PubMed Pubmed Central PMCID: 2675692.
47. Liu X, Fortin K, Mourelatos Z. MicroRNAs: biogenesis and molecular functions. Brain Pathol. 2008;18(1):113–21. PubMed.
48. Griffiths-Jones S, Saini HK, van Dongen S, Enright AJ. miRBase: tools for microRNA genomics. Nucleic Acids Res. 2008;36(Database issue):D154–8. PubMed Pubmed Central PMCID: 2238936.
49. Behm-Ansmant I, Rehwinkel J, Doerks T, Stark A, Bork P, Izaurralde E. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev. 2006;20(14):1885–98. PubMed Pubmed Central PMCID: 1522082.
50. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol. 2009;10(2):126–39. PubMed.
51. Bandiera S, Ruberg S, Girard M, Cagnard N, Hanein S, Chretien D, et al. Nuclear outsourcing of RNA interference components to human mitochondria. PLoS One. 2011;6(6):e20746. PubMed Pubmed Central PMCID: 3113838.
52. 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. PubMed Pubmed Central PMCID: 3102686.
53. Bian Z, Li LM, Tang R, Hou DX, Chen X, Zhang CY, et al. Identification of mouse liver mitochondria-associated miRNAs and their potential biological functions. Cell Res. 2010;20(9): 1076–8. PubMed.
54. Kren BT, Wong PY, 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. PubMed Pubmed Central PMCID: 3972804.

55. Sripada L, Tomar D, Prajapati P, Singh R, Singh AK, Singh R. Systematic analysis of small RNAs associated with human mitochondria by deep sequencing: detailed analysis of mitochondrial associated miRNA. PLoS One. 2012;7(9):e44873. PubMed Pubmed Central PMCID: 3439422.
56. 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. PubMed Pubmed Central PMCID: 3390752.
57. Wang WX, 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. PubMed Pubmed Central PMCID: 4346439.
58. 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. PubMed Pubmed Central PMCID: 4119298.
59. Park CW, Zeng Y, Zhang X, Subramanian S, Steer CJ. Mature microRNAs identified in highly purified nuclei from HCT116 colon cancer cells. RNA Biol. 2010;7(5):606–14. PubMed Pubmed Central PMCID: 3073257.
60. Zisoulis DG, Kai ZS, Chang RK, Pasquinelli AE. Autoregulation of microRNA biogenesis by let-7 and Argonaute. Nature. 2012;486(7404):541–4. PubMed Pubmed Central PMCID: 3387326.
61. Li ZF, Liang YM, Lau PN, Shen W, Wang DK, Cheung WT, et al. Dynamic localisation of mature microRNAs in human nucleoli is influenced by exogenous genetic materials. PLoS One. 2013;8(8):e70869. PubMed Pubmed Central PMCID: 3735495.
62. Politz JC, Hogan EM, Pederson T. MicroRNAs with a nucleolar location. RNA. 2009;15(9): 1705–15. PubMed Pubmed Central PMCID: 2743059.
63. Herrmann JM, Longen S, Weckbecker D, Depuydt M. Biogenesis of mitochondrial proteins. Adv Exp Med Biol. 2012;748:41–64. PubMed.
64. Neupert W, Herrmann JM. Translocation of proteins into mitochondria. Annu Rev Biochem. 2007;76:723–49. PubMed.
65. Entelis NS, Kolesnikova OA, Martin RP, Tarassov IA. RNA delivery into mitochondria. Adv Drug Deliv Rev. 2001;49(1–2):199–215. PubMed.
66. Entelis NS, Kolesnikova OA, Dogan S, Martin RP, Tarassov IA. 5 S rRNA and tRNA import into human mitochondria. Comparison of in vitro requirements. J Biol Chem. 2001;276(49): 45642–53. PubMed.
67. Alfonzo JD, Soll D. Mitochondrial tRNA import—the challenge to understand has just begun. Biol Chem. 2009;390(8):717–22. PubMed.
68. Puranam RS, Attardi G. The RNase P associated with HeLa cell mitochondria contains an essential RNA component identical in sequence to that of the nuclear RNase P. Mol Cell Biol. 2001;21(2):548–61. PubMed Pubmed Central PMCID: 86618.
69. Maniataki E, Mourelatos Z. Human mitochondrial tRNAMet is exported to the cytoplasm and associates with the Argonaute 2 protein. RNA. 2005;11(6):849–52. PubMed Pubmed Central PMCID: 1370769.
70. Dudek J, Rehling P, van der Laan M. Mitochondrial protein import: common principles and physiological networks. Biochim Biophys Acta. 2013;1833(2):274–85. PubMed.
71. Gu Z, Li J, Gao S, Gong M, Wang J, Xu H, et al. InterMitoBase: an annotated database and analysis platform of protein-protein interactions for human mitochondria. BMC Genomics. 2011;12:335. PubMed Pubmed Central PMCID: 3142533.
72. Wang G, Chen HW, Oktay Y, Zhang J, Allen EL, Smith GM, et al. PNPASE regulates RNA import into mitochondria. Cell. 2010;142(3):456–67. PubMed Pubmed Central PMCID: 2921675.
73. Wang G, Shimada E, Koehler CM, Teitell MA. PNPASE and RNA trafficking into mitochondria. Biochim Biophys Acta. 2012;1819(9–10):998–1007. PubMed Pubmed Central PMCID: 3267854.
74. Wang G, Shimada E, Zhang J, Hong JS, Smith GM, Teitell MA, et al. Correcting human mitochondrial mutations with targeted RNA import. Proc Natl Acad Sci U S A. 2012;109(13):4840–5. PubMed Pubmed Central PMCID: 3323963.

75. Wei Y, Li L, Wang D, Zhang CY, Zen K. Importin 8 regulates the transport of mature microRNAs into the cell nucleus. J Biol Chem. 2014;289(15):10270–5. PubMed Pubmed Central PMCID: 4036152.
76. Salmanidis M, Pillman K, Goodall G, Bracken C. Direct transcriptional regulation by nuclear microRNAs. Int J Biochem Cell Biol. 2014;54:304–11. PubMed.
77. Meseguer S, Martinez-Zamora A, Garcia-Arumi E, Andreu AL, Armengod ME. The ROS-sensitive microRNA-9/9* controls the expression of mitochondrial tRNA-modifying enzymes and is involved in the molecular mechanism of MELAS syndrome. Hum Mol Genet. 2015;24(1): 167–84. PubMed.
78. 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. PubMed Pubmed Central PMCID: 1557801.
79. Borralho PM, Steer CJ, Rodrigues CM. Isolation of mitochondria from liver and extraction of total RNA and protein: analyses of microRNA and protein expressions. Methods Mol Biol. 2015;1241:9–22. PubMed.
80. 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. PubMed Pubmed Central PMCID: 4014556.
81. Ernoult-Lange M, Benard M, Kress M, Weil D. P-bodies and mitochondria: which place in RNA interference? Biochimie. 2012;94(7):1572–7. PubMed.
82. Huang L, Mollet S, Souquere S, Le Roy F, Ernoult-Lange M, Pierron G, et al. Mitochondria associate with P-bodies and modulate microRNA-mediated RNA interference. J Biol Chem. 2011;286(27):24219–30. PubMed Pubmed Central PMCID: 3129203.
83. Du H, Guo L, Yan S, Sosunov AA, McKhann GM, Yan SS. Early deficits in synaptic mitochondria in an Alzheimer's disease mouse model. Proc Natl Acad Sci U S A. 2010;107(43):18670–5. PubMed Pubmed Central PMCID: 2972922.
84. Sheng ZH. Mitochondrial trafficking and anchoring in neurons: new insight and implications. J Cell Biol. 2014;204(7):1087–98. PubMed Pubmed Central PMCID: 3971748.
85. Ahmad T, Mukherjee S, Pattnaik B, Kumar M, Singh S, Kumar M, et al. Miro1 regulates intercellular mitochondrial transport & enhances mesenchymal stem cell rescue efficacy. EMBO J. 2014;33(9):994–1010. PubMed Pubmed Central PMCID: 4193933.
86. Lou E, Fujisawa S, Morozov A, Barlas A, Romin Y, Dogan Y, et al. Tunneling nanotubes provide a unique conduit for intercellular transfer of cellular contents in human malignant pleural mesothelioma. PLoS One. 2012;7(3):e33093. PubMed Pubmed Central PMCID: 3302868.
87. Spees JL, Olson SD, Whitney MJ, Prockop DJ. Mitochondrial transfer between cells can rescue aerobic respiration. Proc Natl Acad Sci U S A. 2006;103(5):1283–8. PubMed Pubmed Central PMCID: 1345715.
88. Diaz-Carballo D, Gustmann S, Jastrow H, Acikelli AH, Dammann P, Klein J, et al. Atypical cell populations associated with acquired resistance to cytostatics and cancer stem cell features: the role of mitochondria in nuclear encapsulation. DNA Cell Biol. 2014;33(11):749–74. PubMed Pubmed Central PMCID: 4216482.
89. Galluzzi L, Kepp O, Kroemer G. Mitochondria: master regulators of danger signalling. Nat Rev Mol Cell Biol. 2012;13(12):780–8. PubMed.
90. Rippo MR, Olivieri F, Monsurro V, Prattichizzo F, Albertini MC, Procopio AD. MitomiRs in human inflamm-aging: a hypothesis involving miR-181a, miR-34a and miR-146a. Exp Gerontol. 2014;56:154–63. PubMed.
91. Vidaurre S, Fitzpatrick C, Burzio VA, Briones M, Villota C, Villegas J, et al. Down-regulation of the antisense mitochondrial non-coding RNAs (ncRNAs) is a unique vulnerability of cancer cells and a potential target for cancer therapy. J Biol Chem. 2014;289(39):27182–98. PubMed Pubmed Central PMCID: 4175353.
92. Salmena L, Poliseno L, Tay Y, Kats L, Pandolfi PP. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell. 2011;146(3):353–8. PubMed Pubmed Central PMCID: 3235919.
93. Yuan Y, Liu B, Xie P, Zhang MQ, Li Y, Xie Z, et al. Model-guided quantitative analysis of microRNA-mediated regulation on competing endogenous RNAs using a synthetic gene circuit. Proc Natl Acad Sci U S A. 2015;112(10):3158–63. PubMed.

Chapter 4
microRNAs Distinctively Regulate Vascular Smooth Muscle and Endothelial Cells: Functional Implications in Angiogenesis, Atherosclerosis, and In-Stent Restenosis

Gaetano Santulli

Abstract Endothelial cells (EC) and vascular smooth muscle cells (VSMC) are the main cell types within the vasculature. We describe here how microRNAs (miRs)—noncoding RNAs that can regulate gene expression via translational repression and/or post-transcriptional degradation—distinctively modulate EC and VSMC function in physiology and disease. In particular, the specific roles of miR-126 and miR-143/145, master regulators of EC and VSMC function, respectively, are deeply explored. We also describe the mechanistic role of miRs in the regulation of the pathophysiology of key cardiovascular processes including angiogenesis, atherosclerosis, and in-stent restenosis post-angioplasty. Drawbacks of currently available therapeutic options are discussed, pointing at the challenges and potential clinical opportunities provided by miR-based treatments.

Keywords Endothelium • Restenosis • VSMC • Neointima • Stent • DES • Circulation • Thrombosis • miR-126 • miR-143/145 • Diabetes • Myocardin • Myoregulin • Atherosclerosis • Angiogenesis • Inflammation • Angioplasty

Introduction

Endothelial cells (EC) and vascular smooth muscle cells (VSMC) constitute the main cell types within the vasculature [1, 2]. The endothelium forms the inner thin layer that represents an interface between circulating fluid in the lumen and the rest

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of the vessel wall. The endothelial monolayer lines the entire circulatory system, from the smallest capillaries to the heart [3]. The main functions of EC include regulation of vascular tone, fluid filtration, neutrophil recruitment, hormone trafficking, and hemostasis [4–6].

VSMC are able to contract or relax in response to various stimuli, and are thereby responsible for the redistribution of the blood within the body to areas where it is needed, including tissues with temporarily enhanced oxygen consumption [7–11]. Their main function is to regulate the caliber of the blood vessels: excessive vasoconstriction might lead to hypertension, whereas excessive vasodilation can induce hypotension (as in shock) [12, 13].

Coding or Noncoding: *Whether "Tis Nobler in the Mind to Suffer…"*

MicroRNAs (miRs) are small, generally noncoding RNAs, that regulate gene expression via post-transcriptional degradation or translational repression [14]. Unquestionably, miRs are fundamental regulators of numerous biological processes. See Chaps. 1 and 2 of this book for more detailed information on biological aspects of miRs.

More than 30,000 mature miR products have been identified (~200 in the human genome), and the number of published miR sequences continues to increase rapidly [15].

Importantly, some transcripts that originally appeared to be lncRNAs may actually encode micropeptides. For instance, a conserved micropeptide, named myoregulin, encoded by RNA that had been misannotated as noncoding, has been recently identified by Eric Olson [16]. The fact that putative noncoding RNA may actually harbor hidden micropeptides had been recently suggested by genome-wide analyses [17].

Consequently, scanning the microproteome embedded in "noncoding RNA," whose coding capacity has been somehow ignored in gene annotations [18, 19], will certainly represent a main field of research in the next future, revisiting previously established annotations of noncoding RNAs and hopefully leading to the discovery of new mechanistically important micropeptides.

Fine Tuning of Vascular Biology via MicroRNA

Vascular endothelium plays a key role in regulating)vessel biology and homeostasis. Alteration of its function can be the basis of cardiovascular disorders including hypertension, atherosclerosis, and impairment of the angiogenetic processes [20–22]. Several events are involved in angiogenesis, including EC division, selective degradation of the basal membrane, and the surrounding extracellular matrix, with the subsequent EC migration with the formation of neovessels [7].

Similar events take place during embryogenesis, however in this context blood vessels are generated from the differentiation of EC precursors, the angioblasts, which associate to eventually form primitive vessels in a process known as vasculogenesis [23]. Endothelial maturation is finely guided by signals from other cell types [23]. Indeed, development of the vascular architecture involves the strict association between EC and mural cells, including VSMC and pericytes [24]. Under physiological conditions, the communication between these cell types leads to the maturation and stabilization of the vessel. These processes involve the action of different growth factors and heterotypic cellular interactions [25].

miR-143/145 vs. miR-126, Master Regulators of VSMC and EC Function

miR-143/145

A major advance in the smooth muscle) biology field occurred with the report of the essential role of a bicistrionic unit encoding for miR-143 and miR-145 in the regulation of smooth muscle physiology [26–41]. The miR-143/145 gene is located on chromosome 5 in humans and chromosome 18 in mice and rats. miR-143 and miR-145 are distinct in sequence, yet transcribed together as one primary cluster. Being controlled by serum response factor (together with myocardin) and Nkx2.5, expression of miR-143/145 seems to occur primarily in VSMCs and is critical for maintaining their contractile phenotype [2, 26]. Its expression pattern correlates with a differentiated phenotype as defined by alpha-smooth muscle actin, smooth muscle myosin heavy chain, and calponin expression [39, 42, 43].

The joint suppression of specific target mRNAs by miR-143/145 contributes to a contractile phenotype, as demonstrated in mice with genetic deficiency of miR-143/145, which display reduced vascular tone and blood pressure control [44]. miR-143/145$^{-/-}$ mice also have a tendency to develop neointimal lesions. Aortic VSMCs from the double knockout mouse lack myofilamentous cytoskeletal organization and have diffuse actin staining. A proteomic analysis of aortas from global miR-143/145$^{-/-}$ mice revealed angiotensin-converting enzyme (ACE-1) as a target of the miR-143/145 cluster. Strikingly, pharmacological inhibition by ACE inhibitors or angiotensin 1 receptor blockers partially reverses vascular dysfunction normalizing gene expression in miR-143/145$^{-/-}$ mice [45].

At the ultrastructural electron microscopic analysis, miR-143/145-deficient VSMC exhibit show greater presence of rough endoplasmic reticulum and less focal adhesions compared with wild-type cells. These differences have been associated with impaired contractility of femoral artery rings in response to angiotensin II and phenylephrine. Of note, the functional relevance of miR-143/145 in human vessel pathology had been suggested by the observed downregulation of the miR-143/145 cluster in human aortic aneurysms compared with normal aortic tissue [46].

As mentioned above, the cardiac muscle- and smooth muscle-specific transcriptional factor myocardin has been shown to up-regulate miR-143/145 expression [47, 48]. Similarly, the introduction of miR-143/145 potentiates myocardin-induced smooth muscle marker gene expression, calcium fluxes, and myofilament formation in response to endothelin-1. The repressor of myocardin-directed smooth muscle gene expression KLF4, which binds myocardin, has been identified as a potential target of miR-145 in murine VSMC [29]. However, human KLF4 mRNA contains no binding site for miR-143 and only a low-probability site for miR-145, whereas KLF5 (which has protein sequences highly conserved across human, mouse, and rat) contains a binding site for miR-145. In vivo experiments revealed that vascular injury causes downregulation of miR-145, which up-regulates KLF5 and inhibits myocardin, leading to VSMC dedifferentiation and increased neointimal lesion formation [26, 49]. Notably, PDGF receptor α, fascin, and protein kinase C ε, essential regulators of podosome formation, were revealed as direct targets of miR-143 and miR-145 [50].

Various reports revealed that cells can release miRs which can then exert their specific effects by modulating processes in recipient cells. Thus, miRs have intercellular signal transduction capabilities. Most recently, Climent and colleagues elegantly demonstrated that VSMC communicate with EC via miR-143 and miR-145: cell-to-cell VSMC/EC contacts induce the activation of miR-143/145 transcription in VSMC, promoting the transfer of these miRNAs to the endothelium [51]. In particular, VSMC can deliver miR-143/145 to EC via fine intercellular tubes, named membrane nanotubes or tunneling nanotubes [51]. Indeed, the level of miR-143/145, but not that of its precursor molecule (pri-miR-143/145), rose substantially in EC when these cells were cultured together with VSMC. A specific molecular pathway has been elucidated, in which secretion of transforming growth factor-β by EC stimulates the transfer of miR-143/145 from VSMC to EC, where VSMC-derived miR-143/145 represses hexokinase II and integrin β8 and thereby the angiogenic potential of the recipient cell [51]. Notably, the expression of miR-143/145 in EC could not be achieved by the simple transfer of conditioned medium or VSMC-derived exosomes and was not sensitive to gap junction uncoupling agents (both exosomes and gap junctions had been reported as potential routes for intercellular transfer of miRs). Instead, the transfer of miR-143/145 was sensitive to latrunculin A, an inhibitor of the formation of tunneling nanotubes, tiny membrane connections that cultured cells form among each other. The intercellular transfer of miRs through tunneling nanotubes had been previously reported in ovarian cancer [52]. High-resolution imaging allowed the direct visualization of tunneling nanotubes between EC and VSMC and the transport of miRs within them [51].

miR-126

miR-126 is encoded by)intron 7 of the EGF-like domain 7 (EGFL7) gene, a.k.a. vascular endothelial-statin (VE-statin), which is under the transcriptional control of the E-26 family of transcription factors ETS1/2 [53–65]. In resting conditions, ETS1 is expressed at a very low level whereas during angiogenesis or re-endothelialization, it is transiently expressed at high levels. During replicative senescence an increased

expression of ETS1 could induce the increasing of miR-126 expression. Interestingly, one of the main targets of mir-126 is the host gene EGFL7, which regulates the proper spatial organization of ECs within each sprout and influences their collective movement. The cardiovascular phenotype of EGFL7-deficient mice is recapitulated by the ablation of miR-126, causing ruptured blood vessels, multifocal hemorrhages, and systemic edema (~40 % of mir-126 KO mice die embryonically) [57].

miR-126 has been extensively studied in plasma and circulating cells because its expression is very high in EC [2], endothelial progenitor cells (EPCs), and platelets [59, 66–80]. Most recently, miR-126 has been identified as an efficient marker in the detection and purification of EC [81]. This miR plays a critical role in modulating vascular development and homeostasis, targeting specific mRNAs including the Sprouty-related protein 1 (SPRED-1), CXCL12, SDF-1, and phosphoinositol-3 kinase regulatory subunit 2 (PIK3R2) [82–87]. Confirming its key role in maintaining vascular integrity, amidst the other targets of miR-126 there is a key mediator of leukocyte adhesion and inflammation: vascular cell adhesion molecule 1 (VCAM-1). miR-126 has also been related with the endothelial dysfunction associated with the development of diabetes and its complications [88].

Circulating miR-126 acts as an intercellular messenger mainly released by EC and internalized primarily by monocytes and VSMC [57]. Its transfer modulation may be an important strategy to prevent or delay endothelial dysfunction [88–92]. A significant increase in circulating miR-126 has been detected in patients with acute myocardial infarction and angina whereas miR-126 downregulation has been reported in plasma from patients with diabetes, heart failure, or cancer [14]. Hence, circulating miR-126 can be modulated by diverse stimuli inducing dissimilar cellular fates in different cell types [60, 85, 93–97]. Most recently, Italian researchers demonstrated that circulating miR-126-3p (see Chap. 1 for miR nomenclature) can be considered a biomarker of physiological endothelial senescence in normoglycemic elderly subjects and appears to underlie a mechanism that may be disrupted in aged diabetic patients [98]. Intriguingly, diabetes mellitus leads to dysregulated activation of ETS, which blocks the functional activity of progenitor cells and their commitment toward the endothelial cell lineage [99].

The Phenotypic Switch of Vascular Smooth Muscle Cells: A Key Event in Atherosclerosis and Restenosis

VSMC can modulate their phenotype) in response to the environmental stimuli via a process characterized by decreased gene expression of VSMC contractile markers and increased proliferation, migration, and matrix synthesis [100]. Such a phenotypic switch represents one of the main cellular events underlying various VSMC-related pathological conditions, including hypertension, atherosclerosis, post-angioplasty restenosis, and angiogenesis. Unraveling the mechanisms involved in VSMC phenotypic switch is an important step toward better understanding the pathophysiology of these disorders and ultimately designing therapeutic agents for their treatment and prevention.

The switch between the contractile (differentiated, quiescent) and synthetic (dedifferentiated, proliferative) VSMC phenotypes are orchestrated via a synergistic molecular regulatory network. Overall, contractile smooth muscle proteins contain an evolutionarily conserved *cis*-elements, the CArG box (CC(A/T)$_6$GG) in the promoter–enhancer region of the gene. This has been demonstrated for α-smooth muscle actin, desmin, smooth muscle myosin heavy chain, SM22-α, and calponin.

The serum response factor (SRF), a ubiquitously expressed transcription factor, binds the CArG box driving transcription. The interaction between SRF and CArG boxes in response to environmental changes is generally coordinated by the interaction of SRF with additional transcription factors, divided in co-activators (myocardin, PRX1, and GATA factors) and co-repressors (ELK-1, KLF4, YY1, and Foxo factors).

Virtually all of the VSMC-specific contractile protein genes alongside with many other genes involved in migration, proliferation, and extracellular matrix production during the process of VSMC phenotypic switch, containing CArG boxes within their promoters. The balance between those positive and negative SRF cofactors finely regulates the dynamic VSMC gene expression in response to environmental signals. Amidst these regulatory components, myocardin, which binds SRF in a 1:2 stoichiometric ratio inducing CArG-dependent VSMC gene transcription, is widely considered to be the master gene regulating VSMC differentiation.

The TGF-β and bone morphogenetic proteins (BMPs) also contribute to VSMC phenotypic switch by promoting VSMC contractility enhancing the expression of the contractile apparatus [101]. In particular, TGF-β superfamily of growth factors triggers VSMC differentiation by post-transcriptionally increasing the expression of a subset of miRs including miR-21. Treatment of VSMC with TGF-β and BMP4 results in change in the expression of miRs, by finely regulating DROSHA complex [102].

Intriguingly, the TGF-β signaling pathway is also under the control of miRs. Other studies demonstrated that the inhibition of miR-26a promote VSMC apoptosis and phenotypic switch to a contractile status while inhibiting proliferation and migration [103]. Intriguingly, miR-24 is involved into both TGF-β and PDGF-BB signaling pathways, which respectively represent the distinguishing trigger of VSMC differentiation and proliferation [104]: PDGF-BB induces miR-24 expression, which subsequently leads to Tribbles-like protein-3 (TRB3) downregulation by a post-transcriptional effect, with a decrease in BMP and TGFb signaling, promoting VSMC proliferation. On the contrary, inhibition of miR-24 enhances TRB3 expression and impairs VSMC proliferative activity [104]. Of note, TGF-β Signaling has been also implied in the mineralization of VSMC induced by oxidized LDL [105].

The specific roles of miRs in the regulation of VSMC phenotypic switch are summarized in Table 4.1.

Several miRs have been shown to modulate VSMC proliferation and migration, key aspects in the pathogenesis of atherosclerosis. For instance, miR-133 [136] and miR-136 [137] promote whereas miR-365 [117] and miR-1298 (which has been shown to be regulated by DNA methylation [138]) inhibit VSMC proliferation and migration.

Table 4.1 miRNAs involved in the VSMC phenotypic switch

	Effect of injury on miRNA level	Main targets	References
Pro-contractile miRNAs (*quiescent, differentiated state*)			
miR-1	Reduction	PIM1, KLF4	[106, 107]
miR-10	Unknown	HDAC4, MAP3K7, β-TRC	[108, 109]
miR-15b	Increase	MAP2K4	[110]
miR-18a-5p	Increase	Syndecan4	[111]
miR-21	Increase	PDCD4	[112, 113]
miR-132	Increase	LRRFIP1	[114]
miR-133a	Reduction	MSN, SP-1	[115, 116]
miR-143/145	Reduction	FRA1, Elk1, CALMK, KLF4, ACE, KLF5, MRTFB, myocardin	[26, 42, 49]
miR-365	Reduction	Cyclin D1	[117]
miR-424/322	Increase	STIM1, calumenin	[118]
miR-490-3p	Reduction	PAPP-A	[119]
miR-638	Unknown	NOR1	[120]
miR-663	Unknown	JunB	[121, 122]
Pro-synthetic miRNAs (*proliferative*, *dedifferentiated state*)			
miR-21	Increase	PTEN, Bcl-2	[102, 123, 124]
miR-24	Reduction	TRB3	[104, 125, 126]
miR-26a	Increase	SMAD1/4	[103, 127, 128]
miR-31	Increase	LATS2, CREG	[129–131]
miR-146	Increase	HuR, NF-κB	[132]
miR-221/222	Increase	p27, p57, c-kit	[102, 133–135]

βTRC β transducin repeat-containing gene, *CREG* cellular repressor of E1A-stimulated genes, *Elk1* ETS domain-containing protein-1, *KLF4* Kruppel-like factor 4, *Trb3* Tribbles-like protein-3, *LATS2* large tumor suppressor homolog 2, *LRRFIP1* leucine-rich repeat in Flightless-1 interacting protein-1, *MAP2K4* mitogen-activated protein kinase kinase 4, *MAP3K7* mitogen-activated kinase kinase kinase 7, *MSN* moesin, *NOR1* orphan nuclear receptor, *PAPP-A* pregnancy-associated plasma protein A, *PDCD4* Programmed Cell Death 4, *SMAD* small mother against decapentaplegic, *STIM1* stromal-interacting molecule 1, *TRB3* Tribbles-like protein-3

Importantly, the role of VSMC varies depending on the stage of atherosclerotic disease, playing a maladaptive role in early lesion development and progression and a beneficial adaptive role within the fibrous cap by stabilizing advanced plaques in the face of end-stage disease events such as plaque rupture [139]. Indeed, VSMC can synthesize components of the fibrous cap, which separates circulating blood from the thrombogenic plaque interior [140]. In humans, ruptured atherosclerotic plaques show less VSMC compared with stable lesions, indicating an active contribution of VSMC to plaque stability [139]. Promoting a quiescent VSMC phenotype might lead to increased fibrous cap integrity, and in this respect, miRs represent an opportunity for positive VSMC regulation (see Chap. 5 of this book for a systematic overview of miRs involved in the atherosclerotic process).

Mechanistic Importance of Endothelium in Restenosis and in Vascular Remodeling

Stent implantation and/or balloon angioplasty, interventions that are used routinely to treat coronary artery disease, lead to mechanical EC damage. A normal operative endothelium is crucial due to its participation in the regulation of vascular tone, alongside with its role in suppressing intimal hyperplasia by inhibiting inflammation, thrombus formation, and VSMC proliferation and migration [2, 141, 142]. Thus, the endothelium provides a selectively permeable barrier that protects against potentially detrimental circulating factors [143–145]. Endothelial denudation and medial wall injury are generally considered the initial effects of angioplasty-induced injury [146–149].

Given the essential role of EC in suppressing inflammation and thrombosis [150, 151] and overall in controlling vascular tone and function [4], the restoration of a healthy endothelial layer is an imperative therapeutic goal in order to prevent restenosis and to avoid the detrimental consequences of in-stent thrombosis [2, 152].

Re-endothelialization of injured arteries occurs naturally via outgrowth of local EC [153]. The recruitment of circulating bone marrow-derived endothelial progenitor cells in this process is controversial [154–156], and the actual contribution of this cell population continues to be uncertain [157].

The effect of stent deployment on EC behavior remains poorly understood. Unquestionably, re-endothelialization of injured coronary arteries is affected by the presence of a stent since such a structure provides a nonphysiological surface for adhesion and generates perturbations in blood flow [158, 159].

The issue of EC repair has been somehow brought into sharp relief in the era of drug-eluting stents (DES), which can release cytostatic compounds that inhibit cell cycle progression [160, 161]. Albeit DES are associated with reduced restenosis rates via inhibition of VSMC proliferation [162–164], they have also been linked to lethal late-stage thrombotic events, which are associated with EC injury [165–167]. Ergo, there is an urgent need to develop new therapeutic interventions to promote EC repair in stented arteries and thereby reduce the incidence of late thrombosis and avoid serious risks associated with prolonged administration of systemic antiplatelet treatments [168, 169], as discussed in detail in the section “Angioplasty, Stents, and miRs” of this chapter.

The precise mechanisms of endothelial repair following angioplasty-related injury have been the focus of a plethora of studies. As mentioned above, the potential regenerative capacity of endothelial progenitor cells remains controversial [170–172], and current research focuses on the complex interaction of circulating cells and mature vessel-wall residual EC. In this context, the emerging functional role of microparticles, tiny membrane fragments of activated and apoptotic cells, has been recently investigated: in brief, EC injury triggers the release of EC-derived microparticles, which act as important carriers of bioactive molecules playing crucial roles in cell–cell cross talk. Indeed, microparticles can trigger antiapoptotic effects on EC, and are able to transfer microRNAs, such as miR-126, to target EC, ultimately enhancing endothelial repair mechanisms [170].

Under hyperglycemic conditions, EC microparticles exhibit reduced regenerative capacity, suggesting that hyperglycemia not only directly harms the endothelium, but also indirectly promotes vascular damage by altering endogenous vascular regeneration mechanisms [170]. Analysis of microRNA-126 level in patients with stable coronary artery disease confirmed that diabetes mellitus reduces microRNA-126 expression in circulating microparticles. Moreover, genetic downregulation of microRNA-126 reduces endothelial microparticle-mediated EC repair both in vivo and in vitro [170, 173].

The endothelium plays a fundamental role in angiogenesis [20–22, 25, 174] and numerous studies investigated the role of miRs in regulating this process. For simplicity, the most important miRs heretofore implicated in EC-mediated angiogenetic process are reported in Table 4.2, alongside with their target gene(s) and function(s).

Table 4.2 Proangiogenic and antiangiogenic miRNAs

	Targets	Main effect	References
Pro-angiogenic miRNAs			
miR-9	SOCS5	Enhanced EC migration	[175]
miR-10a	MAP3K7; HOXA1; bTRC	Anti-inflammatory	[109, 176, 177]
miR-23/27	SEMA6A; SPROUTY2	Pro-angiogenic	[178]
miR-126	PI3KR2; SPRED1	Pro-angiogenic	[57, 89, 98, 179–182]
	VCAM1; SDF1	Anti-inflammatory	
miR-130	HOXA5, GAX	Migration and proliferation	
miR-210	EFNA3	Pro-angiogenic	[183]
miR-217	SIRT1-FOXO/eNOS	Pro-angiogenic	[184]
miR-424	HIF-1α	Chemotaxis, proliferation	[185]
Anti-angiogenic miRNAs			
miR-17	JAK-1	Anti-angiogenic	[186]
miR-21	RhoB	Antiproliferative	[187]
	PPARγ	Pro-inflammatory	[188]
miR-24	GATA-2;	Anti-angiogenic	[189]
	PAK4	Pro-apoptotic	[190]
miR-92a	SIRT1; ITGA5	Anti-angiogenic	[191]
	KLF4 and MKK4	Inhibited re-endothelialization	[192]
miR-200	Ets-1; IL-8; CXCL1	Anti-angiogenic	[193, 194]
miR-221/222	STA5a; c-KIT; eNOS	Anti-angiogenic	[195–197]
miR-492	eNOS	Anti-angiogenic	[198]
14q32 miR cluster (329, 487b, 494, 495)	Multiple neovascularization genes	Anti-angiogenic	[199]

EFNA3 Ephrin-A3, *eNOS* Endothelial Nitric Oxide Synthase, *JAK1* Janus kinase 1, *KLF4* Kruppel-like factor 4, *PPAR-γ* Peroxisome proliferator-activated receptor gamma, *RhoB* Ras homolog gene family, member B, *SIRT1* sirtuin (silent mating type information regulation 2 homolog) 1, *SOCS5* suppressor of cytokine signaling 5, *PI3KR2* phosphoinositol-3 kinase regulatory subunit 2, *SEMA6A* Semaphorin-6A, *VCAM-1* vascular cell adhesion molecule 1

Angioplasty, Stents, and miRs

Coronary artery disease represents a leading cause of mortality worldwide [200–203]. Percutaneous coronary intervention (PCI) is one of the most commonly performed interventions [204], representing the main option for revascularization in a series of cardiovascular disorders, from unstable angina and myocardial infarction, to multi-vascular disease [204].

Milestones in Interventional Cardiology

Recurrent lumen narrowing has been a substantial limitation of open and percutaneous methods of arterial reconstruction from their inception.

The term restenosis, commonly used since 1950 in reference to recurrent cardiac valvular stenosis [205], was later adopted to define lumen re-narrowing after open arterial reconstruction such as carotid endarterectomy.

With the development of peripheral angioplasty by Dotter in the 1960s [206] and then the application of percutaneous approach to angioplasty of renal, coronary, and iliac arteries by Gruentzig and colleagues [207, 208] the problem of restenosis grew exponentially: as angioplasty became widely adopted in the 1980s, restenosis after the intervention was reported in up to 60 % of patients [209, 210].

A major breakthrough in the field was the introduction of bare metal stents (BMS), which revolutionized interventional cardiology preventing the elastic recoil of the treated vessels [211] and, more importantly, significantly reducing the phenomenon of restenosis, as demonstrated by two milestone prospective randomized clinical trials [212, 213]: compared with angioplasty, stenting massively reduced restenosis, reaching percentages of 22 %.

However, the major drawback of this procedure is the induction of proliferation/migration and subsequent accumulation of VSMC [214], macrophages [215], and lymphocytes [216] in the arterial wall, eventually leading to restenosis [217].

Once the molecular mechanisms underlying the restenosis process were better comprehended (and mainly attributable to the proliferation of VSMC), DES were introduced in the clinical scenario, in order to deliver in situ a drug that can inhibit cell proliferation [218, 219].

Nowadays, millions of procedures to intervene on occlusive vascular lesions are performed worldwide each year (~700,000 angioplasties are performed annually in the USA) and 70–90 % of all angioplasty patients receive a stent, inserted permanently at the site of the vascular blockage to form an internal scaffolding that keeps the angioplastied vessel from closing.

The introduction of DES significantly reduced rates of restenosis [218, 220, 221]. Though, concerns have been raised over the long-term safety of the DES, with particular reference to stent thrombosis, essentially due to impaired re-endothelization caused by the nonselective antiproliferative properties of DES

[222–224]. As such, when the obstacle of restenosis seemed finally overcome, enthusiasm and euphoria were tempered by epidemiologic data reporting that DES did not ameliorate mortality rates when compared to BMS [225]. Basic research revealed that the essential cause of the increased mortality observed in patients receiving DES, despite the achieved prevention of restenosis, was mainly attributable to the fact that the antiproliferative drugs eluted by the stents were nonselective, inhibiting not only the proliferation and migration of the "bad" cells responsible for restenosis (VSMC), but also the growth and mobility of the "good" EC, utterly indispensable for the healing of the vessel following the stent implantation [226]. The lack of endothelial coverage eventually leads to an increased risk of thrombosis, with catastrophic clinical consequences for the patients.

Drugs eluted by the stents currently available in clinical practice (Fig. 4.1) are not able to differentiate EC from VSMC, T-cells or macrophages [227–229], and the inhibition of proliferation and migration affects all these cellular types [228, 230, 231], leading to an increased risk for late thrombosis, due to delayed/incomplete re-endothelization [204, 214, 232]. Thus, impaired endothelial coverage after angioplasty prolongs the window of vulnerability to thrombosis, requiring thereby a prolonged dual antiplatelet therapy.

Several vasculoprotective approaches have been proposed to overcome the restenosis problem after angioplasty, preserving endothelial function [233–235]. However, vascular restenosis and thrombosis continue to be a major problem of interventional cardiology, limiting the effectiveness of revascularization procedures. The ideal eluting stent should display a selective antiproliferative effect on VSMC, macrophages, and T-lymphocytes, without affecting EC [204].

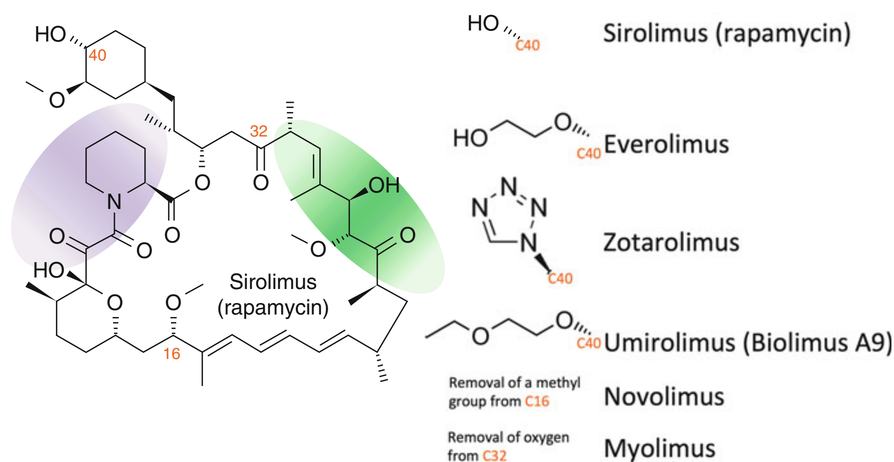

Fig. 4.1 Chemical modifications of the macrocyclic ring in order to obtain drugs with potent antiproliferative and immunosuppressive effects, including rapamycin (sirolimus), everolimus, zotarolimus, umirolimus (biolimus A9), novolimus, and myolimus. *Purple*: FKBP12 (a.k.a. Calstabin1) binding site; *Green*: mTOR binding site

Since EC injury is a fundamental element in the pathophysiology of atherogenesis, understanding EC repair is of critical importance in order to develop novel therapeutic strategies to preserve endothelial integrity and vascular health. In this sense, miRs and their intrinsic properties represent a wonderful opportunity to obtain a specific attenuation of neointimal formation.

Several miRs have been implicated in modification of vessel restenosis after interventional endothelial injury: Antisense knockdown of miR-21, which is moderately increased after vessel injury [123], can blunt the formation of neointimal lesions in response to balloon injury of the carotid artery. Inducing miR-221 in VSMCs on PDGF-β stimulation causes $p27^{Kip1}$ inhibition [236], thereby increasing VSMC proliferation contributing to the formation of a neointimal lesion after arterial injury. Overexpression of miR-145 promotes neointima formation in response to balloon injury [237].

The fundamental role of miRs in the restenosis process has been also confirmed by Baker and colleagues, who identified multiple miRs, including miR-21, miR-146, and miR142-3p, aberrantly expressed in stented pig arteries. Using a mouse vascular stent model, they demonstrate that the knockout of miR-21 can attenuate neointimal formation post-stenting modulating inflammation and VSMC response [238].

Harnessing the EC-specific expression of miR-126, we were able to obtain in one fell swoop both the inhibition of restenosis, attacking VSMC, and the prevention of restenosis and thrombosis, preserving the endothelial function [2].

A major challenge in the field is the delivery of miR-based therapies. In preclinical studies, antagomiRs and miR mimetics have been successfully [239–243] delivered systemically (intravenously injected) showing beneficial effects on cardiac remodeling; however, they are preferentially targeted to the liver, spleen, and kidney. The specific application of miR-based agents to the vasculature, for instance during cardiac catheterization for angioplasty, can be considered as an effective therapeutic strategy.

Potential alternatives [93, 244–250] to the direct intravascular delivery (which ideally could be combined to the new generation bioresorbable stents with biodegradable scaffolds [251–255]) include the stabilization of the miR-based agent (various chemical modifications of the nucleotides can enhance stability in vivo, for instance by using cholesterol-conjugated, 2′-*O*-methyl-modified antagomiRs) or the conjugation to targeting molecules including antibodies, peptides, or other bioactive molecules, which may promote the specific homing of the miR-based drug to the site of the injury.

Conclusion

Accumulating evidence establishes that miRs are becoming one of the most fascinating areas of biology, given their fundamental roles in several pathophysiological processes. The relative role of different miRs in vascular biology as direct or indirect post-transcriptional regulators of genes implied in structural remodeling,

inflammation, angiogenesis, atherosclerosis, in-stent restenosis, and thrombosis indicate that miRs may serve as promising drug targets or potential biomarkers in prevention and management of vascular disorders.

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References

1. Iaccarino G, Ciccarelli M, Sorriento D, Cipolletta E, Cerullo V, Iovino GL, et al. AKT participates in endothelial dysfunction in hypertension. Circulation. 2004;109:2587–93.
2. 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.
3. Cimpean A, Ribatti D, Raica M. A historical appraisal of angiogenesis assays since Judah Folkman and before…. In: Santulli G, editor. Angiogenesis: insights from a systematic overview. New York: Nova Science; 2013. p. 31–50.
4. Santulli G, Cipolletta E, Sorriento D, Del Giudice C, Anastasio A, Monaco S, et al. CaMK4 gene deletion induces hypertension. J Am Heart Assoc. 2012;1, e001081.
5. Santulli G, Trimarco B, Iaccarino G. G-protein-coupled receptor kinase 2 and hypertension: molecular insights and pathophysiological mechanisms. High Blood Press Cardiovasc Prev. 2013;20:5–12.
6. Sorriento D, Ciccarelli M, Santulli G, Campanile A, Altobelli GG, Cimini V, et al. The G-protein-coupled receptor kinase 5 inhibits NFkappaB transcriptional activity by inducing nuclear accumulation of IkappaB alpha. Proc Natl Acad Sci U S A. 2008;105:17818–23.
7. Santulli G. Angiopoietin-like proteins: a comprehensive look. Front Endocrinol. 2014;5:4.
8. Santulli G. β-blockers in diabetic patients with heart failure. JAMA Intern Med. 2015; 175(4):657.
9. Santulli G. Effects of low-carbohydrate and low-fat diets. Ann Intern Med. 2015;162:392.
10. Perino A, Ghigo A, Ferrero E, Morello F, Santulli G, Baillie GS, et al. Integrating cardiac PIP(3) and cAMP signaling through a PKA anchoring function of p110gamma. Mol Cell. 2011;42: 84–95.
11. Santulli G. Adrenal signaling in heart failure: something more than a distant ship's smoke on the horizon. Hypertension. 2014;63:215–6.
12. van Lieshout JJ, Wieling W, Karemaker JM, Eckberg DL. The vasovagal response. Clin Sci. 1991;81:575–86.
13. Santulli G, Ciccarelli M, Trimarco B, Iaccarino G. Physical activity ameliorates cardiovascular health in elderly subjects: the functional role of the beta adrenergic system. Front Physiol. 2013;4:209.
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.
15. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 2014;42:D68–73.
16. Anderson DM, Anderson KM, Chang CL, Makarewich CA, Nelson BR, McAnally JR, et al. A micropeptide encoded by a putative long noncoding RNA regulates muscle performance. Cell. 2015;160:595–606.
17. Andrews SJ, Rothnagel JA. Emerging evidence for functional peptides encoded by short open reading frames. Nat Rev Genet. 2014;15:193–204.
18. Carninci P, Kasukawa T, Katayama S, Gough J, Frith MC, Maeda N, et al. The transcriptional landscape of the mammalian genome. Science. 2005;309:1559–63.

19. Lee LJ, Hughes TR, Frey BJ. How many new genes are there? Science. 2006;311:1709–11; author reply-1711.
20. Santulli G, Ciccarelli M, Palumbo G, Campanile A, Galasso G, Ziaco B, et al. In vivo properties of the proangiogenic peptide QK. J Transl Med. 2009;7:41.
21. Ciccarelli M, Santulli G, Campanile A, Galasso G, Cervero P, Altobelli GG, et al. Endothelial alpha1-adrenoceptors regulate neo-angiogenesis. Br J Pharmacol. 2008;153:936–46.
22. Santulli G, Basilicata MF, De Simone M, Del Giudice C, Anastasio A, Sorriento D, et al. Evaluation of the anti-angiogenic properties of the new selective alphaVbeta3 integrin antagonist RGDechiHCit. J Transl Med. 2011;9:7.
23. Berthod F. Fibroblasts and endothelial cells: the basic angiogenic unit. In: Santulli G, editor. Angiogenesis: insights from a systematic overview. New York: Nova Science; 2013. p. 145–58.
24. Yamamoto S, Niida S, Azuma E, Yanagibashi T, Muramatsu M, Huang TT, et al. Inflammation-induced endothelial cell-derived extracellular vesicles modulate the cellular status of pericytes. Sci Rep. 2015;5:8505.
25. Matarese A, Santulli G. Angiogenesis in chronic obstructive pulmonary disease: a translational appraisal. Transl Med UniSa. 2012;3:49–56.
26. Cordes KR, Sheehy NT, White MP, Berry EC, Morton SU, Muth AN, et al. miR-145 and miR-143 regulate smooth muscle cell fate and plasticity. Nature. 2009;460:705–10.
27. Iio A, Nakagawa Y, Hirata I, Naoe T, Akao Y. Identification of non-coding RNAs embracing microRNA-143/145 cluster. Mol Cancer. 2010;9:136.
28. Boucher JM, Peterson SM, Urs S, Zhang C, Liaw L. The miR-143/145 cluster is a novel transcriptional target of Jagged-1/Notch signaling in vascular smooth muscle cells. J Biol Chem. 2011;286:28312–21.
29. Davis-Dusenbery BN, Chan MC, Reno KE, Weisman AS, Layne MD, Lagna G, et al. Down-regulation of Kruppel-like factor-4 (KLF4) by microRNA-143/145 is critical for modulation of vascular smooth muscle cell phenotype by transforming growth factor-beta and bone morphogenetic protein 4. J Biol Chem. 2011;286:28097–110.
30. Batliner J, Buehrer E, Fey MF, Tschan MP. Inhibition of the miR-143/145 cluster attenuated neutrophil differentiation of APL cells. Leuk Res. 2012;36:237–40.
31. Iio A, Takagi T, Miki K, Naoe T, Nakayama A, Akao Y. DDX6 post-transcriptionally down-regulates miR-143/145 expression through host gene NCR143/145 in cancer cells. Biochim Biophys Acta. 1829;2013:1102–10.
32. Medrano S, Sequeira-Lopez ML, Gomez RA. Deletion of the miR-143/145 cluster leads to hydronephrosis in mice. Am J Pathol. 2014;184:3226–38.
33. Dahan D, Ekman M, Larsson-Callerfelt AK, Turczynska K, Boettger T, Braun T, et al. Induction of angiotensin-converting enzyme after miR-143/145 deletion is critical for impaired smooth muscle contractility. Am J Physiol Cell Physiol. 2014;307:C1093–101.
34. Kent OA, McCall MN, Cornish TC, Halushka MK. Lessons from miR-143/145: the importance of cell-type localization of miRNAs. Nucleic Acids Res. 2014;42:7528–38.
35. Chivukula RR, Shi G, Acharya A, Mills EW, Zeitels LR, Anandam JL, et al. An essential mesenchymal function for miR-143/145 in intestinal epithelial regeneration. Cell. 2014;157:1104–16.
36. Kojima S, Enokida H, Yoshino H, Itesako T, Chiyomaru T, Kinoshita T, et al. The tumor-suppressive microRNA-143/145 cluster inhibits cell migration and invasion by targeting GOLM1 in prostate cancer. J Hum Genet. 2014;59:78–87.
37. Zhao W, Zheng XL, Peng DQ, Zhao SP. Myocyte enhancer factor 2A regulates hydrogen peroxide-induced senescence of vascular smooth muscle cells via microRNA-143. J Cell Physiol. 2015;230:2202–11.
38. Bhattachariya A, Dahan D, Ekman M, Boettger T, Braun T, Sward K, et al. Spontaneous activity and stretch-induced contractile differentiation are reduced in vascular smooth muscle of miR-143/145 knockout mice. Acta Physiol (Oxf). 2015. doi:10.1111/apha.12536.
39. Vengrenyuk Y, Nishi H, Long X, Ouimet M, Savji N, Martinez FO, et al. Cholesterol loading reprograms the MicroRNA-143/145-myocardin axis to convert aortic smooth muscle cells to a dysfunctional macrophage-like phenotype. Arterioscler Thromb Vasc Biol. 2015;35:535–46.

40. Koo S, Martin G, Toussaint LG. MicroRNA-145 promotes the phenotype of human glioblastoma cells selected for invasion. Anticancer Res. 2015;35:3209–15.
41. Avgeris M, Mavridis K, Tokas T, Stravodimos K, Fragoulis EG, Scorilas A. Uncovering the clinical utility of miR-143, miR-145 and miR-224 for predicting the survival of bladder cancer patients following treatment. Carcinogenesis. 2015;36:528–37.
42. Riches K, Alshanwani AR, Warburton P, O'Regan DJ, Ball SG, Wood IC, et al. Elevated expression levels of miR-143/5 in saphenous vein smooth muscle cells from patients with Type 2 diabetes drive persistent changes in phenotype and function. J Mol Cell Cardiol. 2014;74: 240–50.
43. Sala F, Aranda JF, Rotllan N, Ramirez CM, Aryal B, Elia L, et al. MiR-143/145 deficiency attenuates the progression of atherosclerosis in Ldlr-/-mice. Thromb Haemost. 2014;112: 796–802.
44. Engelhardt S, Leierseder S. Coinciding functions for miR-145 in vascular smooth muscle and cardiac fibroblasts. J Mol Cell Cardiol. 2013;65:105–7.
45. 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.
46. 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.
47. Wang D, Chang PS, Wang Z, Sutherland L, Richardson JA, Small E, et al. Activation of cardiac gene expression by myocardin, a transcriptional cofactor for serum response factor. Cell. 2001;105:851–62.
48. Wang DZ, Li S, Hockemeyer D, Sutherland L, Wang Z, Schratt G, et al. Potentiation of serum response factor activity by a family of myocardin-related transcription factors. Proc Natl Acad Sci U S A. 2002;99:14855–60.
49. Liu X, Cheng Y, Yang J, Qin S, Chen X, Tang X, et al. Flank sequences of miR-145/143 and their aberrant expression in vascular disease: mechanism and therapeutic application. J Am Heart Assoc. 2013;2, e000407.
50. Quintavalle M, Elia L, Condorelli G, Courtneidge SA. MicroRNA control of podosome formation in vascular smooth muscle cells in vivo and in vitro. J Cell Biol. 2010;189:13–22.
51. Climent M, Quintavalle M, Miragoli M, Chen J, Condorelli G, Elia L. TGFbeta triggers miR-143/145 transfer from smooth muscle cells to endothelial cells, thereby modulating vessel stabilization. Circ Res. 2015;116:1753–64.
52. Thayanithy V, Dickson EL, Steer C, Subramanian S, Lou E. Tumor-stromal cross talk: direct cell-to-cell transfer of oncogenic microRNAs via tunneling nanotubes. Transl Res. 2014;164: 359–65.
53. 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.
54. Guo C, Sah JF, Beard L, Willson JK, Markowitz SD, Guda K. The noncoding RNA, miR-126, suppresses the growth of neoplastic cells by targeting phosphatidylinositol 3-kinase signaling and is frequently lost in colon cancers. Genes Chromosomes Cancer. 2008;47:939–46.
55. Kuhnert F, Mancuso MR, Hampton J, Stankunas K, Asano T, Chen CZ, et al. Attribution of vascular phenotypes of the murine Egfl7 locus to the microRNA miR-126. Development. 2008;135:3989–93.
56. Musiyenko A, Bitko V, Barik S. Ectopic expression of miR-126*, an intronic product of the vascular endothelial EGF-like 7 gene, regulates prostein translation and invasiveness of prostate cancer LNCaP cells. J Mol Med (Berl). 2008;86:313–22.
57. 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.
58. Zhang J, Du YY, Lin YF, Chen YT, Yang L, Wang HJ, et al. The cell growth suppressor, mir-126, targets IRS-1. Biochem Biophys Res Commun. 2008;377:136–40.

59. Zhao C, Li Y, Zhang M, Yang Y, Chang L. miR-126 inhibits cell proliferation and induces cell apoptosis of hepatocellular carcinoma cells partially by targeting Sox2. Hum Cell. 2015;28: 91–9.
60. Khella HW, Scorilas A, Mozes R, Mirham L, Lianidou E, Krylov SN, et al. Low expression of miR-126 is a prognostic marker for metastatic clear cell renal cell carcinoma. Am J Pathol. 2015;185:693–703.
61. Yang Y, Song KL, Chang H, Chen L. Decreased expression of microRNA-126 is associated with poor prognosis in patients with cervical cancer. Diagn Pathol. 2014;9:220.
62. Bijkerk R, van Solingen C, de Boer HC, de Vries DK, Monge M, van Oeveren-Rietdijk A, et al. Silencing of miRNA-126 in kidney ischemia reperfusion is associated with elevated SDF-1 levels and mobilization of Sca-1+/Lin- progenitor cells. Microrna. 2014;3:144–9.
63. Lonvik K, Sorbye SW, Nilsen MN, Paulssen RH. Prognostic value of the MicroRNA regulators dicer and Drosha in non-small-cell lung cancer: co-expression of Drosha and miR-126 predicts poor survival. BMC Clin Pathol. 2014;14:45.
64. Liu R, Gu J, Jiang P, Zheng Y, Liu X, Jiang X, et al. DNMT1-microRNA126 epigenetic circuit contributes to esophageal squamous cell carcinoma growth via ADAM9-EGFR-AKT signaling. Clin Cancer Res. 2015;21:854–63.
65. Jiang L, Tao C, He A, He X. Overexpression of miR-126 sensitizes osteosarcoma cells to apoptosis induced by epigallocatechin-3-gallate. World J Surg Oncol. 2014;12:383.
66. 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.
67. Feng R, Chen X, Yu Y, Su L, Yu B, Li J, et al. miR-126 functions as a tumour suppressor in human gastric cancer. Cancer Lett. 2010;298:50–63.
68. Harnprasopwat R, Ha D, Toyoshima T, Lodish H, Tojo A, Kotani A. Alteration of processing induced by a single nucleotide polymorphism in pri-miR-126. Biochem Biophys Res Commun. 2010;399:117–22.
69. Meister J, Schmidt MH. miR-126 and miR-126*: new players in cancer. ScientificWorldJournal. 2010;10:2090–100.
70. Oglesby IK, Bray IM, Chotirmall SH, Stallings RL, O'Neill SJ, McElvaney NG, et al. miR-126 is downregulated in cystic fibrosis airway epithelial cells and regulates TOM1 expression. J Immunol. 2010;184:1702–9.
71. Sun Y, Bai Y, Zhang F, Wang Y, Guo Y, Guo L. miR-126 inhibits non-small cell lung cancer cells proliferation by targeting EGFL7. Biochem Biophys Res Commun. 2010;391:1483–9.
72. Chen JJ, Zhou SH. Mesenchymal stem cells overexpressing MiR-126 enhance ischemic angiogenesis via the AKT/ERK-related pathway. Cardiol J. 2011;18:675–81.
73. 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.
74. Cui W, Li Q, Feng L, Ding W. MiR-126-3p regulates progesterone receptors and involves development and lactation of mouse mammary gland. Mol Cell Biochem. 2011;355:17–25.
75. Donnem T, Lonvik K, Eklo K, Berg T, Sorbye SW, Al-Shibli K, et al. Independent and tissue-specific prognostic impact of miR-126 in nonsmall cell lung cancer: coexpression with vascular endothelial growth factor-A predicts poor survival. Cancer. 2011;117:3193–200.
76. Li XM, Wang AM, Zhang J, Yi H. Down-regulation of miR-126 expression in colorectal cancer and its clinical significance. Med Oncol. 2011;28:1054–7.
77. Li Z, Chen J. In vitro functional study of miR-126 in leukemia. Methods Mol Biol. 2011;676: 185–95.
78. Ren G, Kang Y. A one-two punch of miR-126/126* against metastasis. Nat Cell Biol. 2013;15:231–3.
79. Schmidt Y, Simunovic F, Strassburg S, Pfeifer D, Stark GB, Finkenzeller G. miR-126 regulates platelet-derived growth factor receptor-alpha expression and migration of primary human osteoblasts. Biol Chem. 2015;396:61–70.

80. Togliatto G, Trombetta A, Dentelli P, Gallo S, Rosso A, Cotogni P, et al. Unacylated ghrelin induces oxidative stress resistance in a glucose intolerance and peripheral artery disease mouse model by restoring endothelial cell miR-126 expression. Diabetes. 2015;64:1370–82.
81. Miki K, Endo K, Takahashi S, Funakoshi S, Takei I, Katayama S, et al. Efficient detection and purification of cell populations using synthetic microRNA switches. Cell Stem Cell. 2015; 16:699–711.
82. Feng X, Tan W, Cheng S, Wang H, Ye S, Yu C, et al. Upregulation of microRNA-126 in hepatic stellate cells may affect pathogenesis of liver fibrosis through the NF-kappaB pathway. DNA Cell Biol. 2015;34(7):470–80.
83. Rohde JH, Weigand JE, Suess B, Dimmeler S. A universal aptamer chimera for the delivery of functional microRNA-126. Nucleic Acid Ther. 2015;25:141–51.
84. Shibayama Y, Kondo T, Ohya H, Fujisawa S, Teshima T, Iseki K. Upregulation of microRNA-126-5p is associated with drug resistance to cytarabine and poor prognosis in AML patients. Oncol Rep. 2015;33:2176–82.
85. Hu J, Zeng L, Huang J, Wang G, Lu H. miR-126 promotes angiogenesis and attenuates inflammation after contusion spinal cord injury in rats. Brain Res. 2015;1608:191–202.
86. Nakano M, Fukushima Y, Yokota S, Fukami T, Takamiya M, Aoki Y, et al. CYP2A7 pseudogene transcript affects CYP2A6 expression in human liver by acting as a decoy for miR-126. Drug Metab Dispos. 2015;43:703–12.
87. Meng Q, Wang W, Yu X, Li W, Kong L, Qian A, et al. Upregulation of microRNA-126 contributes to endothelial progenitor cell function in deep vein thrombosis via its target PIK3R2. J Cell Biochem. 2015;116(8):1613–23.
88. Mocharla P, Briand S, Giannotti G, Dorries C, Jakob P, Paneni F, et al. AngiomiR-126 expression and secretion from circulating CD34(+) and CD14(+) PBMCs: role for proangiogenic effects and alterations in type 2 diabetics. Blood. 2013;121:226–36.
89. Meng S, Cao JT, Zhang B, Zhou Q, Shen CX, Wang CQ. Downregulation of microRNA-126 in endothelial progenitor cells from diabetes patients, impairs their functional properties, via target gene Spred-1. J Mol Cell Cardiol. 2012;53:64–72.
90. Prattichizzo F, Giuliani A, Ceka A, Rippo MR, Bonfigli AR, Testa R, et al. Epigenetic mechanisms of endothelial dysfunction in type 2 diabetes. Clin Epigenetics. 2015;7(1):56.
91. Tian HS, Zhou QG, Shao F. Relationship between arterial atheromatous plaque morphology and platelet-associated miR-126 and miR-223 expressions. Asian Pac J Trop Med. 2015;8:309–14.
92. Zhang T, Li L, Shang Q, Lv C, Wang C, Su B. Circulating miR-126 is a potential biomarker to predict the onset of type 2 diabetes mellitus in susceptible individuals. Biochem Biophys Res Commun. 2015;463(1–2):60–3.
93. Endo-Takahashi Y, Negishi Y, Nakamura A, Ukai S, Ooaku K, Oda Y, et al. Systemic delivery of miR-126 by miRNA-loaded bubble liposomes for the treatment of hindlimb ischemia. Sci Rep. 2014;4:3883.
94. 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;4:175–84.
95. Yamaguchi T, Iijima T, Wakaume R, Takahashi K, Matsumoto H, Nakano D, et al. Underexpression of miR-126 and miR-20b in hereditary and nonhereditary colorectal tumors. Oncology. 2014;87:58–66.
96. Yan T, Cui K, Huang X, Ding S, Zheng Y, Luo Q, et al. Assessment of therapeutic efficacy of miR-126 with contrast-enhanced ultrasound in preeclampsia rats. Placenta. 2014;35:23–9.
97. Goerke SM, Kiefer LS, Stark GB, Simunovic F, Finkenzeller G. miR-126 modulates angiogenic growth parameters of peripheral blood endothelial progenitor cells. Biol Chem. 2015; 396:245–52.
98. Olivieri F, Bonafe M, Spazzafumo L, Gobbi M, Prattichizzo F, Recchioni R, et al. Age- and glycemia-related miR-126-3p levels in plasma and endothelial cells. Aging. 2014;6:771–87.

99. McAuley AK, Dirani M, Wang JJ, Connell PP, Lamoureux EL, Hewitt AW. A genetic variant regulating miR-126 is associated with sight threatening diabetic retinopathy. Diab Vasc Dis Res. 2015;12:133–8.
100. Schwartz SM. Smooth muscle migration in atherosclerosis and restenosis. J Clin Invest. 1997;100:S87–9.
101. Lagna G, Ku MM, Nguyen PH, Neuman NA, Davis BN, Hata A. Control of phenotypic plasticity of smooth muscle cells by bone morphogenetic protein signaling through the myocardin-related transcription factors. J Biol Chem. 2007;282:37244–55.
102. Davis BN, Hilyard AC, Lagna G, Hata A. SMAD proteins control DROSHA-mediated microRNA maturation. Nature. 2008;454:56–61.
103. 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.
104. Chan MC, Hilyard AC, Wu C, Davis BN, Hill NS, Lal A, et al. Molecular basis for antagonism between PDGF and the TGFbeta family of signalling pathways by control of miR-24 expression. EMBO J. 2010;29:559–73.
105. Yan J, Stringer SE, Hamilton A, Charlton-Menys V, Gotting C, Muller B, et al. Decorin GAG synthesis and TGF-beta signaling mediate Ox-LDL-induced mineralization of human vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2011;31:608–15.
106. Nasser MW, Datta J, Nuovo G, Kutay H, Motiwala T, Majumder S, et al. Down-regulation of micro-RNA-1 (miR-1) in lung cancer. Suppression of tumorigenic property of lung cancer cells and their sensitization to doxorubicin-induced apoptosis by miR-1. J Biol Chem. 2008;283:33394–405.
107. Xie C, Huang H, Sun X, Guo Y, Hamblin M, Ritchie RP, et al. MicroRNA-1 regulates smooth muscle cell differentiation by repressing Kruppel-like factor 4. Stem Cells Dev. 2011;20:205–10.
108. Huang H, Xie C, Sun X, Ritchie RP, Zhang J, Chen YE. miR-10a contributes to retinoid acid-induced smooth muscle cell differentiation. J Biol Chem. 2010;285:9383–9.
109. 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 U S A. 2010;107:13450–5.
110. Kim S, Kang H. miR-15b induced by platelet-derived growth factor signaling is required for vascular smooth muscle cell proliferation. BMB Rep. 2013;46:550–4.
111. Kee HJ, Kim GR, Cho SN, Kwon JS, Ahn Y, Kook H, et al. miR-18a-5p microRNA increases vascular smooth muscle cell differentiation by downregulating syndecan4. Korean Circ J. 2014;44:255–63.
112. Cheng Y, Zhang C. MicroRNA-21 in cardiovascular disease. J Cardiovasc Transl Res. 2010;3:251–5.
113. Young MR, Santhanam AN, Yoshikawa N, Colburn NH. Have tumor suppressor PDCD4 and its counteragent oncogenic miR-21 gone rogue? Mol Interv. 2010;10:76–9.
114. Choe N, Kwon JS, Kim JR, Eom GH, Kim Y, Nam KI, et al. The microRNA miR-132 targets Lrrfip1 to block vascular smooth muscle cell proliferation and neointimal hyperplasia. Atherosclerosis. 2013;229:348–55.
115. Kinoshita T, Nohata N, Fuse M, Hanazawa T, Kikkawa N, Fujimura L, et al. Tumor suppressive microRNA-133a regulates novel targets: moesin contributes to cancer cell proliferation and invasion in head and neck squamous cell carcinoma. Biochem Biophys Res Commun. 2012;418:378–83.
116. Qiu T, Zhou X, Wang J, Du Y, Xu J, Huang Z, et al. MiR-145, miR-133a and miR-133b inhibit proliferation, migration, invasion and cell cycle progression via targeting transcription factor Sp1 in gastric cancer. FEBS Lett. 2014;588:1168–77.
117. Kim MH, Ham O, Lee SY, Choi E, Lee CY, Park JH, et al. MicroRNA-365 inhibits the proliferation of vascular smooth muscle cells by targeting cyclin D1. J Cell Biochem. 2014;115:1752–61.
118. Merlet E, Atassi F, Motiani RK, Mougenot N, Jacquet A, Nadaud S, et al. miR-424/322 regulates vascular smooth muscle cell phenotype and neointimal formation in the rat. Cardiovasc Res. 2013;98:458–68.

119. Sun Y, Chen D, Cao L, Zhang R, Zhou J, Chen H, et al. MiR-490-3p modulates the proliferation of vascular smooth muscle cells induced by ox-LDL through targeting PAPP-A. Cardiovasc Res. 2013;100:272–9.
120. Li P, Liu Y, Yi B, Wang G, You X, Zhao X, et al. MicroRNA-638 is highly expressed in human vascular smooth muscle cells and inhibits PDGF-BB-induced cell proliferation and migration through targeting orphan nuclear receptor NOR1. Cardiovasc Res. 2013;99:185–93.
121. Li P, Zhu N, Yi B, Wang N, Chen M, You X, et al. MicroRNA-663 regulates human vascular smooth muscle cell phenotypic switch and vascular neointimal formation. Circ Res. 2013;113:1117–27.
122. Korff T, Pfisterer L, Schorpp-Kistner M. miR-663 and the miRaculous vascular smooth muscle phenotypic switch. Circ Res. 2013;113:1102–5.
123. Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, et al. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circ Res. 2007;100:1579–88.
124. Silvestri P, Rigattieri S, Loschiavo P. Does the effect of microRNAs in vascular neointimal formation depend on cell cycle phase? Circ Res. 2008;102(9):e101; author reply e102.
125. Davis-Dusenbery BN, Wu C, Hata A. Micromanaging vascular smooth muscle cell differentiation and phenotypic modulation. Arterioscler Thromb Vasc Biol. 2011;31:2370–7.
126. Talasila A, Yu H, Ackers-Johnson M, Bot M, van Berkel T, Bennett MR, et al. Myocardin regulates vascular response to injury through miR-24/-29a and platelet-derived growth factor receptor-beta. Arterioscler Thromb Vasc Biol. 2013;33:2355–65.
127. Icli B, Wara AK, Moslehi J, Sun X, Plovie E, Cahill M, et al. MicroRNA-26a regulates pathological and physiological angiogenesis by targeting BMP/SMAD1 signaling. Circ Res. 2013;113:1231–41.
128. Bai Y, Wang L, Sun L, Ye P, Hui R. Circulating microRNA-26a: potential predictors and therapeutic targets for non-hypertensive intracerebral hemorrhage. Med Hypotheses. 2011;77:488–90.
129. Liu X, Cheng Y, Chen X, Yang J, Xu L, Zhang C. MicroRNA-31 regulated by the extracellular regulated kinase is involved in vascular smooth muscle cell growth via large tumor suppressor homolog 2. J Biol Chem. 2011;286:42371–80.
130. Liu X, Sempere LF, Ouyang H, Memoli VA, Andrew AS, Luo Y, et al. MicroRNA-31 functions as an oncogenic microRNA in mouse and human lung cancer cells by repressing specific tumor suppressors. J Clin Invest. 2010;120:1298–309.
131. Wang J, Yan CH, Li Y, Xu K, Tian XX, Peng CF, et al. MicroRNA-31 controls phenotypic modulation of human vascular smooth muscle cells by regulating its target gene cellular repressor of E1A-stimulated genes. Exp Cell Res. 2013;319:1165–75.
132. Cheng HS, Sivachandran N, Lau A, Boudreau E, Zhao JL, Baltimore D, et al. MicroRNA-146 represses endothelial activation by inhibiting pro-inflammatory pathways. EMBO Mol Med. 2013;5:949–66.
133. Togliatto G, Trombetta A, Dentelli P, Rosso A, Brizzi MF. MIR221/MIR222-driven post-transcriptional regulation of P27KIP1 and P57KIP2 is crucial for high-glucose- and AGE-mediated vascular cell damage. Diabetologia. 2011;54:1930–40.
134. Kothapalli D, Castagnino P, Rader DJ, Phillips MC, Lund-Katz S, Assoian RK. Apolipoprotein E-mediated cell cycle arrest linked to p27 and the Cox2-dependent repression of miR221/222. Atherosclerosis. 2013;227:65–71.
135. Mackenzie NC, Staines KA, Zhu D, Genever P, Macrae VE. miRNA-221 and miRNA-222 synergistically function to promote vascular calcification. Cell Biochem Funct. 2014;32:209–16.
136. Gao S, Wassler M, Zhang L, Li Y, Wang J, Zhang Y, et al. MicroRNA-133a regulates insulin-like growth factor-1 receptor expression and vascular smooth muscle cell proliferation in murine atherosclerosis. Atherosclerosis. 2014;232:171–9.
137. Zhang CF, Kang K, Li XM, Xie BD. MicroRNA-136 promotes vascular muscle cell proliferation through the ERK1/2 pathway by targeting PPP2R2A in atherosclerosis. Curr Vasc Pharmacol. 2014;13(3):405–12.

138. Hu W, Wang M, Yin H, Yao C, He Q, Yin L, et al. MicroRNA-1298 is regulated by DNA methylation and affects vascular smooth muscle cell function by targeting connexin 43. Cardiovasc Res. 2015;107:534–45.
139. Weissberg PL, Clesham GJ, Bennett MR. Is vascular smooth muscle cell proliferation beneficial? Lancet. 1996;347:305–7.
140. O'Sullivan JF, Martin K, Caplice NM. Microribonucleic acids for prevention of plaque rupture and in-stent restenosis: "a finger in the dam". J Am Coll Cardiol. 2011;57:383–9.
141. Vanhoutte PM. Endothelium-derived free radicals: for worse and for better. J Clin Invest. 2001;107:23–5.
142. Harrison DG. Cellular and molecular mechanisms of endothelial cell dysfunction. J Clin Invest. 1997;100:2153–7.
143. Iaccarino D, Politi L, Rossi R, Sgura F, Monopoli D, Modena MG, et al. Rationale and study design of the OISTER trial: optical coherence tomography evaluation of stent struts re-endothelialization in patients with non-ST-elevation acute coronary syndromes—a comparison of the intrEpide tRapidil eluting stent vs. taxus drug-eluting stent implantation. J Cardiovasc Med (Hagerstown). 2010;11:536–43.
144. Mei H, Campbell JM, Paddock CM, Lertkiatmongkol P, Mosesson MW, Albrecht RM, et al. Regulation of endothelial cell barrier function by antibody-driven affinity modulation of PECAM-1. J Biol Chem. 2014;289(30):20836–44.
145. Pannekoek WJ, Post A, Bos JL. Rap1 signaling in endothelial barrier control. Cell Adh Migr. 2014;8(2):100–7.
146. Gadang V, Konaniah E, Hui DY, Jaeschke A. Mixed-lineage kinase 3 deficiency promotes neointima formation through increased activation of the RhoA pathway in vascular smooth muscle cells. Arterioscler Thromb Vasc Biol. 2014;34:1429–36.
147. Popma JJ, Topol EJ. Factors influencing restenosis after coronary angioplasty. Am J Med. 1990;88:16N–24.
148. Faxon DP, Sanborn TA, Haudenschild CC. Mechanism of angioplasty and its relation to restenosis. Am J Cardiol. 1987;60:5B–9.
149. Chaabane C, Otsuka F, Virmani R, Bochaton-Piallat ML. Biological responses in stented arteries. Cardiovasc Res. 2013;99:353–63.
150. De Caterina R, Massaro M, Scoditti E, Annunziata CM. Pharmacological modulation of vascular inflammation in atherothrombosis. Ann N Y Acad Sci. 2010;1207:23–31.
151. Esmon CT. Inflammation and thrombosis. J Thromb Haemost. 2003;1:1343–8.
152. Rhee JW, Wu JC. Advances in nanotechnology for the management of coronary artery disease. Trends Cardiovasc Med. 2013;23:39–45.
153. Otsuka F, Finn AV, Yazdani SK, Nakano M, Kolodgie FD, Virmani R. The importance of the endothelium in atherothrombosis and coronary stenting. Nat Rev Cardiol. 2012;9:439–53.
154. Hagensen MK, Raarup MK, Mortensen MB, Thim T, Nyengaard JR, Falk E, et al. Circulating endothelial progenitor cells do not contribute to regeneration of endothelium after murine arterial injury. Cardiovasc Res. 2012;93:223–31.
155. Tsuzuki M. Bone marrow-derived cells are not involved in reendothelialized endothelium as endothelial cells after simple endothelial denudation in mice. Basic Res Cardiol. 2009;104: 601–11.
156. Cevese A. Totipotent stem cells could do everything … or else nothing: the case of vascular reendothelialization. Cardiovasc Res. 2012;93:211–2.
157. Pearson JD. Endothelial progenitor cells—hype or hope? J Thromb Haemost. 2009;7: 255–62.
158. Jimenez JM, Prasad V, Yu MD, Kampmeyer CP, Kaakour AH, Wang PJ, et al. Macro- and microscale variables regulate stent haemodynamics, fibrin deposition and thrombomodulin expression. J R Soc Interface. 2014;11(94):20131079.
159. Alexander RW. Getting stents to go with the flow. J Clin Invest. 2004;113:1532–4.
160. Liuzzo JP, Ambrose JA, Coppola JT. Sirolimus- and taxol-eluting stents differ towards intimal hyperplasia and re-endothelialization. J Invasive Cardiol. 2005;17:497–502.

161. Simard T, Hibbert B, Ramirez FD, Froeschl M, Chen YX, O'Brien ER. The evolution of coronary stents: a brief review. Can J Cardiol. 2014;30:35–45.
162. Palmerini T, Biondi-Zoccai G, Della Riva D, Stettler C, Sangiorgi D, D'Ascenzo F, et al. Stent thrombosis with drug-eluting and bare-metal stents: evidence from a comprehensive network meta-analysis. Lancet. 2012;379:1393–402.
163. Di Lorenzo E, Carbone G, Sauro L, Casafina A, Capasso M, Sauro R. Bare-metal stents versus drug-eluting stents for primary angioplasty: long-term outcome. Curr Cardiol Rep. 2011; 13:459–64.
164. Wessely R. New drug-eluting stent concepts. Nat Rev Cardiol. 2010;7:194–203.
165. Huang KN, Grandi SM, Filion KB, Eisenberg MJ. Late and very late stent thrombosis in patients with second-generation drug-eluting stents. Can J Cardiol. 2013;29:1488–94.
166. Siddiqi OK, Faxon DP. Very late stent thrombosis: current concepts. Curr Opin Cardiol. 2012;27:634–41.
167. McFadden EP, Stabile E, Regar E, Cheneau E, Ong AT, Kinnaird T, et al. Late thrombosis in drug-eluting coronary stents after discontinuation of antiplatelet therapy. Lancet. 2004; 364:1519–21.
168. Rossini R, Baroni M, Musumeci G, Gavazzi A. Oral antiplatelet therapy after drug-eluting stent implantation: adherence and side-effects. J Cardiovasc Med (Hagerstown). 2013;14: 81–90.
169. Dehmer GJ, Smith KJ. Drug-eluting coronary artery stents. Am Fam Physician. 2009; 80:1245–51.
170. Jansen F, Yang X, Hoelscher M, Cattelan A, Schmitz T, Proebsting S, et al. Endothelial microparticle-mediated transfer of MicroRNA-126 promotes vascular endothelial cell repair via SPRED1 and is abrogated in glucose-damaged endothelial microparticles. Circulation. 2013;128:2026–38.
171. Deb A, Patterson C. Hard luck stories: the reality of endothelial progenitor cells continues to fall short of the promise. Circulation. 2010;121:850–2.
172. Hagensen MK, Shim J, Thim T, Falk E, Bentzon JF. Circulating endothelial progenitor cells do not contribute to plaque endothelium in murine atherosclerosis. Circulation. 2010;121: 898–905.
173. Jansen F, Yang X, Proebsting S, Hoelscher M, Przybilla D, Baumann K, et al. MicroRNA expression in circulating microvesicles predicts cardiovascular events in patients with coronary artery disease. J Am Heart Assoc. 2014;3, e001249.
174. Santulli G. Angiogenesis: insights from a systematic overview. New York: Nova Science; 2013.
175. Zhuang G, Wu X, Jiang Z, Kasman I, Yao J, Guan Y, et al. Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. EMBO J. 2012;31:3513–23.
176. Qin B, Yang H, Xiao B. Role of microRNAs in endothelial inflammation and senescence. Mol Biol Rep. 2012;39:4509–18.
177. 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.
178. Zhou Q, Gallagher R, Ufret-Vincenty R, Li X, Olson EN, Wang S. Regulation of angiogenesis and choroidal neovascularization by members of microRNA-23~27~24 clusters. Proc Natl Acad Sci U S A. 2011;108:8287–92.
179. 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.
180. 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;70:4631–44.
181. Taverna S, Amodeo V, Saieva L, Russo A, Giallombardo M, De Leo G, et al. Exosomal shuttling of miR-126 in endothelial cells modulates adhesive and migratory abilities of chronic myelogenous leukemia cells. Mol Cancer. 2014;13:169.

182. Liu LY, Wang W, Zhao LY, Guo B, Yang J, Zhao XG, et al. Mir-126 inhibits growth of SGC-7901 cells by synergistically targeting the oncogenes PI3KR2 and Crk, and the tumor suppressor PLK2. Int J Oncol. 2014;45:1257–65.
183. Wang Z, Yin B, Wang B, Ma Z, Liu W, Lv G. MicroRNA-210 promotes proliferation and invasion of peripheral nerve sheath tumor cells targeting EFNA3. Oncol Res. 2013;21: 145–54.
184. 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.
185. 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;19:74–82.
186. Katz G, Pobezinsky LA, Jeurling S, Shinzawa M, Van Laethem F, Singer A. T cell receptor stimulation impairs IL-7 receptor signaling by inducing expression of the microRNA miR-17 to target Janus kinase 1. Sci Signal. 2014;7:ra83.
187. Jin C, Zhao Y, Yu L, Xu S, Fu G. MicroRNA-21 mediates the rapamycin-induced suppression of endothelial proliferation and migration. FEBS Lett. 2013;587:378–85.
188. Zhou J, Wang KC, Wu W, Subramaniam S, Shyy JY, Chiu JJ, et al. MicroRNA-21 targets peroxisome proliferators-activated receptor-alpha in an autoregulatory loop to modulate flow-induced endothelial inflammation. Proc Natl Acad Sci U S A. 2011;108:10355–60.
189. Fiedler J, Jazbutyte V, Kirchmaier BC, Gupta SK, Lorenzen J, Hartmann D, et al. MicroRNA-24 regulates vascularity after myocardial infarction. Circulation. 2011;124:720–30.
190. Zhou Q, Anderson C, Zhang H, Li X, Inglis F, Jayagopal A, et al. Repression of choroidal neovascularization through actin cytoskeleton pathways by microRNA-24. Mol Ther. 2014; 22:378–89.
191. Ohyagi-Hara C, Sawada K, Kamiura S, Tomita Y, Isobe A, Hashimoto K, et al. miR-92a inhibits peritoneal dissemination of ovarian cancer cells by inhibiting integrin alpha5 expression. Am J Pathol. 2013;182:1876–89.
192. Chen Z, Wen L, Martin M, Hsu CY, Fang L, Lin FM, et al. Oxidative stress activates endothelial innate immunity via sterol regulatory element binding protein 2 (SREBP2) transactivation of microRNA-92a. Circulation. 2015;131:805–14.
193. Chan YC, Khanna S, Roy S, Sen CK. miR-200b targets Ets-1 and is down-regulated by hypoxia to induce angiogenic response of endothelial cells. J Biol Chem. 2011;286:2047–56.
194. Pecot CV, Rupaimoole R, Yang D, Akbani R, Ivan C, Lu C, et al. Tumour angiogenesis regulation by the miR-200 family. Nat Commun. 2013;4:2427.
195. Liu X, Cheng Y, Yang J, Xu L, Zhang C. Cell-specific effects of miR-221/222 in vessels: molecular mechanism and therapeutic application. J Mol Cell Cardiol. 2012;52:245–55.
196. Nicoli S, Knyphausen CP, Zhu LJ, Lakshmanan A, Lawson ND. miR-221 is required for endothelial tip cell behaviors during vascular development. Dev Cell. 2012;22:418–29.
197. Poliseno L, Tuccoli A, Mariani L, Evangelista M, Citti L, Woods K, et al. MicroRNAs modulate the angiogenic properties of HUVECs. Blood. 2006;108:3068–71.
198. Patella F, Leucci E, Evangelista M, Parker B, Wen J, Mercatanti A, et al. MiR-492 impairs the angiogenic potential of endothelial cells. J Cell Mol Med. 2013;17:1006–15.
199. Welten SM, Bastiaansen AJ, de Jong RC, de Vries MR, Peters EA, Boonstra MC, et al. Inhibition of 14q32 microRNAs miR-329, miR-487b, miR-494, and miR-495 increases neovascularization and blood flow recovery after ischemia. Circ Res. 2014;115:696–708.
200. Santulli G. Coronary heart disease risk factors and mortality. JAMA. 2012;307:1137.
201. Santulli G. Epidemiology of cardiovascular disease in the 21st century: updated numbers and updated facts. J Cardiovasc Dis (JCvD). 2013;1:1–2.
202. Weintraub WS, Daniels SR, Burke LE, Franklin BA, Goff Jr DC, Hayman LL, et al. Value of primordial and primary prevention for cardiovascular disease: a policy statement from the American Heart Association. Circulation. 2011;124:967–90.

203. Lloyd-Jones D, Adams RJ, Brown TM, Carnethon M, Dai S, De Simone G, et al. Heart disease and stroke statistics–2010 update: a report from the American Heart Association. Circulation. 2010;121:e46–215.
204. Stefanini GG, Holmes Jr DR. Drug-eluting coronary-artery stents. N Engl J Med. 2013;368:254–65.
205. Rao PS, Thapar MK, Kutayli F. Causes of restenosis after balloon valvuloplasty for valvular pulmonary stenosis. Am J Cardiol. 1988;62:979–82.
206. Friedman SG. Charles dotter: interventional radiologist. Radiology. 1989;172:921–4.
207. Gruentzig AR. Percutaneous transluminal coronary angioplasty. Semin Roentgenol. 1981;16:152–3.
208. Meier B, Gruentzig AR, Goebel N, Pyle R, von Gosslar W, Schlumpf M. Assessment of stenoses in coronary angioplasty. Inter- and intraobserver variability. Int J Cardiol. 1983;3:159–69.
209. Nobuyoshi M, Kimura T, Nosaka H, Mioka S, Ueno K, Yokoi H, et al. Restenosis after successful percutaneous transluminal coronary angioplasty: serial angiographic follow-up of 229 patients. J Am Coll Cardiol. 1988;12:616–23.
210. Holmes Jr DR, Vlietstra RE, Smith HC, Vetrovec GW, Kent KM, Cowley MJ, et al. Restenosis after percutaneous transluminal coronary angioplasty (PTCA): a report from the PTCA registry of the National Heart, Lung, and Blood Institute. Am J Cardiol. 1984;53:77C–81.
211. Sigwart U, Puel J, Mirkovitch V, Joffre F, Kappenberger L. Intravascular stents to prevent occlusion and restenosis after transluminal angioplasty. N Engl J Med. 1987;316:701–6.
212. Serruys PW, de Jaegere P, Kiemeneij F, Macaya C, Rutsch W, Heyndrickx G, et al. A comparison of balloon-expandable-stent implantation with balloon angioplasty in patients with coronary artery disease. Benestent Study Group. N Engl J Med. 1994;331:489–95.
213. Fischman DL, Leon MB, Baim DS, Schatz RA, Savage MP, Penn I, et al. A randomized comparison of coronary-stent placement and balloon angioplasty in the treatment of coronary artery disease. Stent Restenosis Study Investigators. N Engl J Med. 1994;331:496–501.
214. Marx SO, Totary-Jain H, Marks AR. Vascular smooth muscle cell proliferation in restenosis. Circ Cardiovasc Interv. 2011;4:104–11.
215. Park YM, Febbraio M, Silverstein RL. CD36 modulates migration of mouse and human macrophages in response to oxidized LDL and may contribute to macrophage trapping in the arterial intima. J Clin Invest. 2009;119:136–45.
216. Suzuki N, Angiolillo DJ, Monteiro C, Shuja S, Futamatsu H, Kawaguchi R, et al. Variable histological and ultrasonic characteristics of restenosis after drug-eluting stents. Int J Cardiol. 2008;130:444–8.
217. Jukema JW, Verschuren JJW, Ahmed TAN, Quax PH. Restenosis after PCI. Part 1: pathophysiology and risk factors. Nat Rev Cardiol. 2012;9:53–62.
218. Marks AR. Sirolimus for the prevention of in-stent restenosis in a coronary artery. N Engl J Med. 2003;349:1307–9.
219. Santulli G, Totary-Jain H. Tailoring mTOR-based therapy: molecular evidence and clinical challenges. Pharmacogenomics. 2013;14:1517–26.
220. Moses JW, Leon MB, Popma JJ, Fitzgerald PJ, Holmes DR, O'Shaughnessy C, et al. Sirolimus-eluting stents versus standard stents in patients with stenosis in a native coronary artery. N Engl J Med. 2003;349:1315–23.
221. Stone GW, Ellis SG, Cox DA, Hermiller J, O'Shaughnessy C, Mann JT, et al. A polymer-based, paclitaxel-eluting stent in patients with coronary artery disease. N Engl J Med. 2004;350:221–31.
222. Rollini F, Aprile A, Politi L, Sangiorgi GM. Evaluation of re-endothelization extent at mid-term follow-up after drug eluting balloon plus bare metal stent implantation during primary coronary angioplasty: insight from OCT imaging. Minerva Cardioangiol. 2011;59:109–12.
223. Jakabcin J, Bystron M, Spacek R, Veselka J, Kvasnak M, Kala P, et al. The lack of endothelization after drug-eluting stent implantation as a cause of fatal late stent thrombosis. J Thromb Thrombolysis. 2008;26:154–8.

224. Tamburino C, Ussia GP, Zimarino M, Galassi AR, De Caterina R. Early restenosis after drug-eluting stent implantation: a putative role for platelet activation. Can J Cardiol. 2007;23:57–9.
225. Wijns WC, Krucoff MW. Increased mortality after implantation of first generation drug-eluting stents: seeing the smoke, where is the fire? Eur Heart J. 2006;27:2737–9.
226. Joner M, Finn AV, Farb A, Mont EK, Kolodgie FD, Ladich E, et al. Pathology of drug-eluting stents in humans: delayed healing and late thrombotic risk. J Am Coll Cardiol. 2006;48:193–202.
227. Cassese S, Kastrati A. New-generation drug-eluting stents for patients with myocardial infarction. JAMA. 2012;308:814–5.
228. Moss SC, Lightell Jr DJ, Marx SO, Marks AR, Woods TC. Rapamycin regulates endothelial cell migration through regulation of the cyclin-dependent kinase inhibitor p27Kip1. J Biol Chem. 2010;285:11991–7.
229. Lange RA, Hillis LD. Second-generation drug-eluting coronary stents. N Engl J Med. 2010;362:1728–30.
230. Sun J, Marx SO, Chen HJ, Poon M, Marks AR, Rabbani LE. Role for p27(Kip1) in vascular smooth muscle cell migration. Circulation. 2001;103:2967–72.
231. Poon M, Marx SO, Gallo R, Badimon JJ, Taubman MB, Marks AR. Rapamycin inhibits vascular smooth muscle cell migration. J Clin Invest. 1996;98:2277–83.
232. Wenaweser P, Daemen J, Zwahlen M, van Domburg R, Juni P, Vaina S, et al. Incidence and correlates of drug-eluting stent thrombosis in routine clinical practice. 4-year results from a large 2-institutional cohort study. J Am Coll Cardiol. 2008;52:1134–40.
233. Kipshidze N, Dangas G, Tsapenko M, Moses J, Leon MB, Kutryk M, et al. Role of the endothelium in modulating neointimal formation: vasculoprotective approaches to attenuate restenosis after percutaneous coronary interventions. J Am Coll Cardiol. 2004;44:733–9.
234. Yu PJ, Ferrari G, Pirelli L, Gulkarov I, Galloway AC, Mignatti P, et al. Vascular injury and modulation of MAPKs: a targeted approach to therapy of restenosis. Cell Signal. 2007;19:1359–71.
235. Torella D, Gasparri C, Ellison GM, Curcio A, Leone A, Vicinanza C, et al. Differential regulation of vascular smooth muscle and endothelial cell proliferation in vitro and in vivo by cAMP/PKA-activated p85alphaPI3K. Am J Physiol Heart Circ Physiol. 2009;297:H2015–25.
236. Davis BN, Hilyard AC, Nguyen PH, Lagna G, Hata A. Induction of microRNA-221 by platelet-derived growth factor signaling is critical for modulation of vascular smooth muscle phenotype. J Biol Chem. 2009;284:3728–38.
237. Cheng Y, Liu X, Yang J, Lin Y, Xu DZ, Lu Q, et al. MicroRNA-145, a novel smooth muscle cell phenotypic marker and modulator, controls vascular neointimal lesion formation. Circ Res. 2009;105:158–66.
238. McDonald RA, Halliday CA, Miller AM, Diver LA, Dakin RS, Montgomery J, et al. Reducing in-stent restenosis: therapeutic manipulation of miRNA in vascular remodeling and inflammation. J Am Coll Cardiol. 2015;65:2314–27.
239. 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;105:13027–32.
240. Care A, Catalucci D, Felicetti F, Bonci D, Addario A, Gallo P, et al. MicroRNA-133 controls cardiac hypertrophy. Nat Med. 2007;13:613–8.
241. Lin Z, Murtaza I, Wang K, Jiao J, Gao J, Li PF. miR-23a functions downstream of NFATc3 to regulate cardiac hypertrophy. Proc Natl Acad Sci U S A. 2009;106:12103–8.
242. Bonauer A, Carmona G, Iwasaki M, Mione M, Koyanagi M, Fischer A, et al. MicroRNA-92a controls angiogenesis and functional recovery of ischemic tissues in mice. Science. 2009;324:1710–3.
243. 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;456:980–4.

244. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005;438:685–9.
245. Weiler J, Hunziker J, Hall J. Anti-miRNA oligonucleotides (AMOs): ammunition to target miRNAs implicated in human disease? Gene Ther. 2006;13:496–502.
246. Stenvang J, Kauppinen S. MicroRNAs as targets for antisense-based therapeutics. Expert Opin Biol Ther. 2008;8:59–81.
247. Baumann V, Winkler J. miRNA-based therapies: strategies and delivery platforms for oligonucleotide and non-oligonucleotide agents. Future Med Chem. 2014;6:1967–84.
248. Zhang Y, Wang Z, Gemeinhart RA. Progress in microRNA delivery. J Control Release. 2013;172:962–74.
249. Luck ME, Muljo SA, Collins CB. Prospects for therapeutic targeting of MicroRNAs in human immunological diseases. J Immunol. 2015;194:5047–52.
250. Muthiah M, Park IK, Cho CS. Nanoparticle-mediated delivery of therapeutic genes: focus on miRNA therapeutics. Expert Opin Drug Deliv. 2013;10:1259–73.
251. Puricel S, Arroyo D, Corpataux N, Baeriswyl G, Lehmann S, Kallinikou Z, et al. Comparison of everolimus- and biolimus-eluting coronary stents with everolimus-eluting bioresorbable vascular scaffolds. J Am Coll Cardiol. 2015;65:791–801.
252. Sato K, Latib A, Panoulas VF, Kawamoto H, Naganuma T, Miyazaki T, et al. Procedural feasibility and clinical outcomes in propensity-matched patients treated with bioresorbable scaffolds vs new-generation drug-eluting stents. Can J Cardiol. 2015;31:328–34.
253. Raungaard B, Jensen LO, Tilsted HH, Christiansen EH, Maeng M, Terkelsen CJ, et al. Zotarolimus-eluting durable-polymer-coated stent versus a biolimus-eluting biodegradable-polymer-coated stent in unselected patients undergoing percutaneous coronary intervention (SORT OUT VI): a randomised non-inferiority trial. Lancet. 2015;385:1527–35.
254. Karanasos A, Simsek C, Gnanadesigan M, van Ditzhuijzen NS, Freire R, Dijkstra J, et al. OCT assessment of the long-term vascular healing response 5 years after everolimus-eluting bioresorbable vascular scaffold. J Am Coll Cardiol. 2014;64:2343–56.
255. Muthiah M, Islam MA, Cho CS, Hwang JE, Chung IJ, Park IK. Substrate-mediated delivery of microRNA-145 through a polysorbitol-based osmotically active transporter suppresses smooth muscle cell proliferation: implications for restenosis treatment. J Biomed Nanotechnol. 2014;10:571–9.

Chapter 5
Mechanistic Role of MicroRNAs in Coupling Lipid Metabolism and Atherosclerosis

Jan Novák, Veronika Olejníčková, Nikola Tkáčová, and Gaetano Santulli

Abstract MicroRNAs (miRNAs, miRs) represent a group of powerful and versatile posttranscriptional regulators of gene expression being involved in the fine control of a plethora of physiological and pathological processes. Besides their well-established crucial roles in the regulation of cell cycle, embryogenesis or tumorigenesis, these tiny molecules have also been shown to participate in the regulation of lipid metabolism. In particular, miRs orchestrate cholesterol and fatty acids synthesis, transport, and degradation and low-density and high-density lipoprotein (LDL and HDL) formation. It is thus not surprising that they have also been reported to affect the development and progression of several lipid metabolism-related disorders including liver steatosis and atherosclerosis. Mounting evidence suggests that miRs might represent important "posttranscriptional hubs" of lipid metabolism, which means that one miR usually targets 3'-untranslated regions of various mRNAs that are involved in *different* steps of *one* precise metabolic/signaling pathway, e.g., one miR targets mRNAs of enzymes important for cholesterol synthesis, degradation, and transport. Therefore, changes in the levels of one key miR affect *various* steps of *one* pathway, which is

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thereby promoted or inhibited. This makes miRs potent future diagnostic and even therapeutic tools for personalized medicine. Within this chapter, the most prominent microRNAs involved in lipid metabolism, e.g., miR-27a/b, miR-33/33*, miR-122, miR-144, or miR-223, and their intracellular and extracellular functions will be extensively discussed, in particular focusing on their mechanistic role in the pathophysiology of atherosclerosis. Special emphasis will be given on miR-122, the first microRNA currently in clinical trials for the treatment of hepatitis C and on miR-223, the most abundant miR in lipoprotein particles.

Keywords Lipid metabolism • HDL • LDL • Cholesterol • Atherosclerosis • miR-33 • miR-122 • miR-223

Introduction

Lipid metabolism is a multifaceted process including lipid synthesis from their basic components (i.e., basically acetyl-CoA), accumulation, distribution to specific tissues of the organism, and also degradation and excretion [1]. The complex machinery of lipid metabolism is driven by a set of step-limiting enzymes and transporters that are finely modulated according to the needs of the organisms [2]. The discovery that these crucial components are targeted by microRNAs (miRNAs, miRs) grabbed our attention towards the exploiting of miRs as regulators of lipid metabolism with subsequent potential use in the treatment of lipid metabolism-related disorders, including dyslipidemia, atherosclerosis, and nonalcoholic fatty liver disease [1, 3, 4]. Within this chapter, we will provide a basic overview of the most important components of lipid and lipoprotein metabolism and then we will more deeply focus on the functional role of key miRs.

Lipid Metabolism in the Nutshell

Acetyl-CoA represents a well-established precursor molecule for both cholesterol and fatty acids synthesis. Depending on the cholesterol status, i.e., low- or high-cholesterol levels, metabolic pathways leading to cholesterol or fatty acids synthesis, respectively, are activated alongside with pathways leading to cholesterol uptake or efflux. Sterol regulatory element binding proteins (SREBPs) [5, 6] and liver X receptors (LXRs) [7] are among the most important modulators of such processes. Three SREBPs have been identified hitherto, namely SREBP-1a, SREBP-1c (two isoforms originating from *SREBP-1* gene), and SREBP-2 (originating from *SREBP-2* gene), whereas two LXRs isoforms (LXRα and LXRβ) are known [5–7].

If the cholesterol level is low, SREBP-2 is activated, leading to upregulation of critical enzymes involved in cholesterol synthesis (about 20 various steps and reactions), with 3-hydroxy-3-methylglutaryl-coenzyme-A reductase (HMGCoA-R) representing the rate-limiting enzyme. Furthermore, the expression of low-density

lipoprotein receptor (LDLr) is upregulated both by SREBP-2 and SREBP-1a, thus enhancing cholesterol uptake [8].

Conversely, high cholesterol level triggers the activation of LXRs, which forms heterodimers with retinoid X receptors (RXRs) and subsequently activates genes involved in cholesterol efflux and also SREBP-1c, which further promotes formation of triglycerides and phospholipids with, e.g., fatty acid synthase (FAS) being one of the designed targets [7]. Furthermore, if there is too much cholesterol in the cell, HMGCoA-R and LDLr levels are downregulated in order to prevent cholesterol accumulation [7, 8].

Another way to affect cholesterol uptake is via scavenger receptors that bind LDL and modified oxidized LDL (oxLDL) [9]. Last but not least, cholesterol can be partly excreted from the organism as bile acids. Intriguingly, bile acids themselves were recently shown to act as signaling molecules via specific binding of farnesoid X receptor (FXR) [10]. Once FXR is activated, it creates heterodimers with RXRs which in turn further activate expression of enzymes involved in cholesterol degradation and excretion [10].

MicroRNAs Involvement in Lipid Metabolism

MicroRNAs act as negative posttranscriptional regulators of gene expression affecting also the expression of genes involved in lipid metabolism [1, 3]. One miR usually targets more mRNAs that are often connected in the same metabolic pathway and via their targets, miRs are also able to regulate expression of many other miRs [11, 12]. Moreover, each mRNA is usually targeted via several miRs, thus enabling a fine-tuning of the targeted mRNA expression [11, 13]. Some miRs show pleiotropic expression in almost all tissues, while others act more in a tissue-dependent manner [12, 14, 15]. Additionally, during their biogenesis, miRs can be transcribed as individual miRs from their genes having their own promoters, but quite often, they are embedded within the introns of the protein coding genes being transcribed together with their host [16–18], as outlined in detail in Chaps. 1 and 2 of this book. Moreover, during the last step of miR maturation, miR duplex is dissociated giving rise to mature miR and passenger miR* strand. Initially, only mature miRs were believed able to exert a function while passengers strands were simply degraded; however, it has been shown that even passenger miR* strand can exert crucial functions [19]. Furthermore, overcoming the borders of the cell membrane, miRs can be found also in extracellular fluids either bound to proteins or being packaged in microvesicles/exosomes and also in lipoprotein particles—as such, miRs may serve as stable biomarkers and also as novel means of intercellular communication [20, 21].

The most abundant miR in the liver, miR-122, is involved in countless metabolic pathways within the hepatocytes, reflects liver damage if present in serum/plasma, and is mechanistically involved in the pathogenesis of hepatitis C virus infection by targeting its 5-UTR [22–25]. Another key lipid metabolism miR, miR-33, was found to be hidden within the SREBP genes—miR-33a/33a* in the *SREBP-2* gene and miR-33b/33b* within the *SREBP-1* gene [18]. Interestingly, both mature and passenger strands of miR-33 have been shown to affect lipid and even glucose metabolism [19].

Last but not least, miR-223, besides affecting lipid metabolism on the cellular level, is the best example of miR highly abundant in HDL particles [26], microvesicles and exosomes [27] and even platelets [28]. The exact roles of the three abovementioned and other miRs will be described in more detail in this chapter. Returning to key regulatory molecules of lipid metabolism, i.e., SREBP, LXR, RXR, and FXR, various miRs have been shown to control their expression (Fig. 5.1). In particular, SREBP was predicted and confirmed to be targeted by miR-122 [4, 29]. In prostate cancer tumor cells, miR-185/342 was shown to target SREBP, which, alongside with a decrease in tumorigenicity, led also to downregulation of SREBP-targeted genes, i.e., fatty acid synthase (FASN) and HMGCoA-R [30]. Importantly, miR-185 was shown to significantly decrease SREBP-2 level, while expression of miR-185 itself is regulated by SREBP-1c [30], creating an autoregulatory loop. Other miRs targeting SREBP were also identified in osteosarcoma cell lines (miR-29a) and adrenal glands (miR-132) [31]. All of these miRs represent potentially strong modulators of lipid, and generally of the overall metabolism of the cell.

LXRα was revealed to be targeted by miR-613 whose upregulation caused inhibition of lipogenesis in HepG2 cells [32] and also triggered lipid accumulation, i.e., decreased cholesterol efflux, in THP1 macrophages (this effect was partly shown to be mediated by targeting ABCA1) [33]. Mice lacking miR-155, another miR targeting LXRα, exhibited increased cholesterol accumulation in the liver resulting in hepatic steatosis [34]. Conversely to miR-613, miR-206 has been shown to target LXRα; however, miR-206 increase promotes cholesterol efflux from THP macrophages [35]. LXR increases the expression of miR-613 [36] and decreases the expression of miR-206 [35] in another autoregulatory feedback loop.

Looking one step above LXR and SREBP, expression of both genes was shown to be regulated by sirtuin 1 (SIRT1) [37, 38], which is a member of the sirtuin family of deacetylases involved in the regulation of innumerable cellular processes, including cellular metabolism [39]. SIRT1 increases the level of LXR [38] and decreases SREBP [40]; remarkably, SIRT1 itself is targeted by miR-34a [41, 42], miR-132 [43], miR-204-5p [44], miR-499 [45], and many others.

FXR was shown to be targeted by miR-92 [46] and miR-412 [47] in gastric cancer and hepatocellular carcinoma cells, respectively, promoting proliferation and invasiveness of both tumors. FXR negatively regulates the expression of several miRs (e.g., miR-199-3a [48] or 29a [49]) and such a negative regulation seems to be beneficiary—downregulation of FXR in human tumors is thus predictive for worse patient prognosis [46, 47]. In the context of lipid regulation, FXR was shown to upregulate the expression of miR-144, one of the ABCA1 regulators, resulting in cholesterol accumulation within the cells [50]. Further investigations are definitely needed to reveal all FRX-regulating and FXR-regulated miRs.

Fig. 5.1 (continued) AMP-activated protein kinase, *ACC* acyl-CoA carboxylase, *GPAM* mitochondrial glycerol-3-phosphate, *GPC-6* glucose-6-phosphatase, *PCK1* phosphoenolpyruvate carboxykinase; *IRS-2* insulin receptor substrate 2, *ALDO-A* aldolase A, *ApoE3* apolipoprotein E3, *ApoA1* apolipoprotein A1, *ANGPTL3* angiopoietin-like 3, *PPAR* peroxisome proliferator-activated receptor α, *CEBP* CCAAT/enhancer-binding protein α

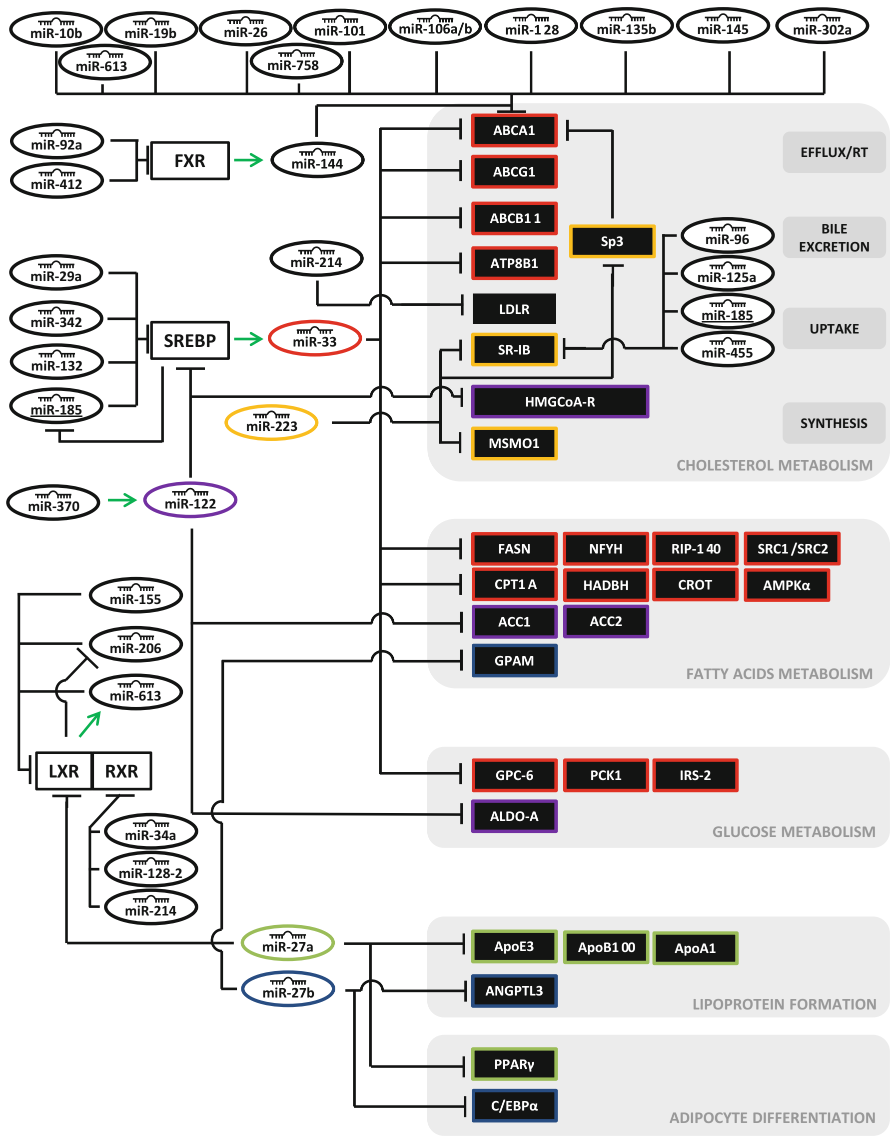

Fig. 5.1 MicroRNAs in lipid metabolism. The figure summarizes the main miRs involved in lipid metabolism. *Blunted arrows* represent inhibition, *classical arrows* represent activation. miRs are marked in *circles*, their targets are marked in *squares*. Detailed description is provided in the text. *FXR* Farnesoid X receptor, *SREBP* sterol regulatory element binding protein, *LXR* liver X receptor, *RXR* retinoid X receptor, *miR* microRNA, *RT* reverse transport; *ABCA1* ATP-binding cassette transporter A1, *ABCG1* ATP-binding cassette transporter G1, *ABCB11* ATP-binding cassette, sub-family B member 11, *LDLR* low density lipoprotein receptor, *SR-IB* scavenger receptor type I class B, *HMGCoA-R* hydroxymethyl-glutaryl coenzyme A reductase, *MSMO1* methylsterol monooxygenase 1, *FASN* fatty acid synthase, *NFYH* nuclear transcription factor Y, *RIP-140* receptor-interacting protein 140, *SRC* steroid receptor coactivator, *CPT1A* carnitine palmitoyltransferase 1A, *HADBH* hydroxyacyl-CoA-dehydrogenase, *CROT* carnitine O-octaniltransferase, *AMPK*

Lastly, RXRα is targeted by miR-34a [51] (similarly to LXRα [41, 42]) and by miR128-2 [52]. Looking at other fundamental molecules, also LDLR expression was shown to be targeted by miR-214 [53], while other miRs (miR-125a and miR-455 [54]) regulate scavenger receptor class B type I (SR-BI) expression. Other molecules involved in lipid metabolism besides those mentioned in previous paragraphs are also regulated by miRs (e.g., MTP, protein important for lipoprotein formation, is regulated by miR-30c [55], or Lectin-like oxidized LDL receptor-1 (LOX-1), one of the scavenger receptors, is targeted by let-7g [56]). Collectively, these data suggest a strong potential of miRs in the posttranscriptional regulation of lipid metabolism-related genes and prompt potential usage of selected miRs as tools to monitor and treat metabolic, cardiovascular and especially cardiometabolic disorders, including atherosclerosis [57].

miR-33a/b: In the Center of Metabolic Pathways

Expression of miR-33a/b

As mentioned above, miR-33 family comprises two members named miR-33a and miR-33b, embedded within the intron 16 of *SREBP-2* and intron 17 of *SREBP-1* genes [58, 59], respectively. Similarly to the majority of intronic miRs, miR-33a/b expression is coordinated with the expression of the host genes [60]. However, unlike many other miRs, in the case of miR-33 both strands are used in mRNA targeting; indeed following pre-miR-33 dicing, miR-33 and miR-33* are formed and both miRs create RISCs with Ago proteins, subsequently resulting in suppression of genes involved in lipid metabolism [19].

miR-33a/b in Cholesterol Metabolism and Atherosclerosis

miR33a/b have been shown to target multiple genes involved in individual steps of cholesterol metabolism, particularly in reverse cholesterol transport from peripheral tissues and cholesterol excretion into the bile. ATP-binding cassette (ABC) transporter A1, ABCA1, was confirmed as miR-33 target in various animal models and cell lines. As a direct consequence of ABCA1 targeting, cholesterol accumulation or cholesterol efflux into Apo-A1 (with subsequent HDL formation) occurs, depending on whether miR-33 levels are upregulated or downregulated, respectively [18, 19, 58–65]. Antagonism of miR-33 in experimental animal models generally results in increased cholesterol efflux to ApoA1, thus increasing serum HDL and decreasing cholesterol levels in peripheral tissues, especially in peripheral macrophages (and atherosclerotic plaques), where also ABCG1 targeting has been ascertained to affect reverse cholesterol transport [58, 59]. The overall result is increased plaque stability, improved blood lipid profile, and inhibition of atherosclerosis progression [19, 62–64, 66, 67].

Strikingly, the effects of miR-33 antagonism were observed not only in mice, that have only one miR-33 isoform (specifically miR-33b) [62, 64, 66, 67], but also in nonhuman primates, which, similarly to human, express both miR-33 isoforms [19, 63].

Together with ABCA1 and ABCG1, the latter one being targeted only in murine models and not in humans [58, 59], other important cholesterol-related targets have been identified for miR-33: Nieman-Pick Protein C1 (NPC1), lysosomal protein involved in the intracellular distribution of cholesterol between lysosomes and other parts of the cell [19, 68] and ABCB11/ATP8B1 transporters that are both responsible for cholesterol excretion in the bile [65]. Inhibition of miR-33 thus also causes increased cholesterol secretion into the bile, thereby lowering cholesterol levels—a strategy that would be of potential use in the clinical scenario in patients with hypercholesterolemia.

miR-33a/b in Fatty Acids Metabolism

Various studies revealing the role of miR-33 in lipid metabolism further focused on miR-33 targets involved in fatty acids synthesis and oxidation [18, 19, 63]. The first identified targets include three important enzymes critical for β-oxidation: carnitine-O-octanoyltransferase (CROT), carnitine palmitoyltransferase 1A (CPT1A), and hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase/enoyl-CoA hydratase beta subunit (HADHB) [18, 19]. Other targets were then identified in studies conducted on nonhuman primates, where therapeutic inhibition of miR-33, except of its known effect on HDL and LDL levels, also affected VLDL and circulating triglycerides level, a phenotype that was not observed in mice [63]. This was partly caused by activation of β-oxidation (through previously identified CROT, CPT1A, HADHB, and the novel target protein kinase AMP-activated α1 (PRKAA1)) and inhibition of fatty acid synthesis (by targeting fatty acid synthase (FAS), ATP citrate lyase (ACLY), or Acetyl-CoA carboxylase 1 (ACACA)) [63]. Hypothetically, targeting Sirtuin-6 (SIRT6), a protein known to be involved in triglycerides metabolism, by miR-33 may also affect triglycerides metabolism [69], similarly to targeting AMPKα, which is known to stimulate β-oxidation and even ketogenesis in the liver [69].

To sum it up, inhibition of miR-33a increases β-oxidation and decreases fatty acid synthesis, eventually resulting in ameliorated circulating lipids profile, slowing down the progression of atherosclerosis. These findings may result in the introduction of anti-miR-33 specific treatment in the future.

miR-33 in Glucose and Energy Metabolism

Captivatingly, also glucose metabolism, insulin signaling, and energy metabolism were shown to be affected in anti-miR-33 treated cells. Ramirez and colleagues identified phosphoenolpyruvate carboxykinase (PCK1) and glucose-6-phosphatase

(G6PC) as miR-33 putative targets. Both of these enzymes are essential for hepatic gluconeogenesis [70]. Another putative target, insulin receptor substrate-2 (IRS-2), was confirmed as miR-33 target by Fernandez-Hernando's group [19]—IRS-2, together with IRS-1, are both important for transduction of insulin signaling over the cell membrane and their blockade thus interferes with insulin effects on the cells [71].

Recently, miR-33 function was also studied in pancreatic islets, unveiling that it induces cholesterol accumulation by targeting ABCA1 [72]. Cholesterol accumulation further leads to a decrease in glucose-stimulated insulin secretion, supporting a connection among miR-33 function, hypercholesterolemia, pancreatic β cell dysfunction, and insulin resistance [72]. A thorough overview of miR and β cell dynamics is presented in Chap. 6. Most recently, miR-33 was clearly shown to be involved in the pathophysiology of atherosclerosis (as expected considering its roles in lipid metabolism and macrophage function) [73]. Moreover, it represents a major regulator of cell cycle [74] and is also involved in the self-renewal of hematopoietic stem cells via p53 targeting [75].

miR-122: Liver-Specific miR with Pleiotropic Effect on Hepatic Functions

Regulation of miR-122 Expression

In 2002, miR-122 was identified as a liver-specific miR, representing approximately 70 % of all liver miRs [14]. During embryogenesis, level of miR-122 rapidly increases and around 50,000 copies are present in the average hepatic cell before birth [76]. In humans, miR-122 is located on chromosome 18 (hr18:54269286–54269370, NCBI36/hg1) and initially it was suggested that miR-122 was transcribed from the *hcr* gene which encodes for the noncoding RNA in the woodchuck (gi: 51212); however, a 7506 bps long primary transcript was further described as a primary sequence for miR-122 [77]. Furthermore, hepatocyte nuclear factor 4α (HNF4alfa) was shown to regulate miR-122 expression by binding to the promoter region of this primary transcript [77]. Other regulatory factors include Rev-ERB2α, an orphan nuclear receptor that is responsible for circadian variation of miR-122 levels; however, circadian rhythmicity does not seem to significantly affect miR-122 function [78]. Of note, miR-370, which transfection is known to alter triglycerides accumulation in the liver, positively regulates miR-122 expression [29]. Last but not least, miR-122 is regulated at posttranscriptional level by 3'adenylation. Indeed, germ line development-2 (GLD-2) knockout animals present with low hepatic miR-122 levels, a phenotype mainly attributable to the absence of GLD-2-mediated adenylation and stabilization [79].

Targets of miR-122 and Lipid Metabolism

Heretofore, studies investigating the roles of miR-122 in lipid metabolism used either antisense oligonucleotides (ASO) [22, 80, 81] or gene knockout approaches [25, 82]. Moreover, just indirect measurements (usually gene expression profiling) are performed to quantify the effects of miR-122 loss. Thus, only a few targets have been validated and more analyses are needed to reveal whether miR-122 targets lipid metabolism directly through many targets or indirectly through a few key targets, such as SREBP [83].

In nonalcoholic steatohepatitis [4] and hepatocellular carcinoma (HCC, [84]), miR-122 level was found to be downregulated, which contributed to the pathophysiology of both disorders. In knockout animals, the deletion of miR-122 led to accumulation of lipids in the liver (resembling steatohepatitis) and also to increased fibrosis (resembling cirrhosis) and to formation of tumor-like lesions (resembling HCC) [25, 82]—the absence of miR-122 thus definitely alters liver metabolism and makes liver more susceptible to the development of mentioned diseases.

On the other hand, even in the gene knockout animals, level of circulating HDL and VLDL is lower compared to control animals and this is essentially due to altered hepatic secretion of these lipoproteins [25, 82]. Similarly, cholesterol levels decrease after the introduction of ASO [22, 80, 81], even in the African green monkeys in which also no signs of toxicity connected with locked nucleic acid (LNA) administration was observed [81]. Moreover, it was shown that after ASO introduction, fatty acids and cholesterol synthesis was decreased and liver steatosis was reversed in high-fat diet-induced obesity model [22].

Indirect targets of miR-122 involved in lipid metabolism were repeatedly suggested including SREBP-1c and SREBP-2 [4, 29], which further makes identification of miR-122-related lipid metabolism targets more difficult due to SREBPs pleiotropic effects on lipid metabolism. Among others let us name fatty acid synthase [4, 22, 29], aldolase-A [80], ATP-citrate lyase (ACLY), acyl-CoA carboxylase (ACC1, ACC2) [22, 29], HMGCR [4], and many others. Altogether, miR-122 is predicted to have more than 100 various targets and potential usage of miR-122 antagonism in the treatment of lipid disorders may represent a novel therapeutic approach in the future after all security issues are solved.

miR-122 in HCV Infection: Miravirsen

Besides the roles of miR-122 in the normal liver functions, its involvement in the replication of hepatitis C virus resulted in the development of the first miR based therapeutics called miravirsen, i.e., miR-122 suppression therapy [85, 86].

Within the genome of HCV, there is a conserved 5UTR region that was shown to be targeted by miR-122 [87]. However, although miR binding usually leads to the

degradation of targeted mRNA, in case of HCV RNA, miR-122 binding is necessary to stabilize this RNA molecule and to promote its replication [86]. Miravirsen was designed as a 15 nct long LNA-modified anti-miR, and it binds to the 5'UTR of miR-122 thus inhibiting its function [88].

In the recently performed phase 2a clinical trial involving seven international centers, miravirsen application led to a decrease in HCV RNA levels and after 14 weeks, viral RNA was even not detected in five patients (out of 36). Moreover, no adverse events were observed and no escape mutation in HCV genome was detected [86]. As long as miR-122 binding site in HCV genome seems to be conserved across HCV genotypes, these results make miravirsen a very promising therapeutic tool against HCV infection [24].

Circulating miR-122 as a Disease/Damage-Related Biomarker

Besides the therapeutic potential in HCV or dyslipidemia treatment, it was shown that circulating miR-122 levels are increased in obese patients and that there are correlations connecting miR-122 levels to BMI, levels of ALT, TG, and HDL-cholesterol and moreover, after adjustment for confounding factors, elevated miR-122 levels were shown to be a risk factor for insulin resistance [89]. Similarly, miR-122 levels are increased in plasma of patients with nonalcoholic steatohepatitis [90, 91]. Moreover, since circulating miRs may be found in the protein complexes or in exosomes, Bala and colleagues studied the different liver injury models and revealed that in inflammatory diseases, miR-122 levels are increased in exosomal fraction of the plasma, while in drug-induced injury, miR-122 levels are higher predominantly in protein-fraction suggesting exosomal miR-122 to act as a mean of intercellular communication [91]. Last but not least, circulating levels of miR-122 are also increased in hyperlipidemia patients and they correlate with the levels of cholesterol, triglycerides, and LDL-c; in the subset of patients with hyperlipidemia and coronary artery disease (CAD), miR-122 levels also correlated with CAD severity [92]. Specificity for circulating miR-122 increase still definitely needs to be determined; however, in the combination with altered extracellular expression of other miRs, miR-122 may serve even as a diagnostic or prognostic tool in the future.

miR-223: "The Messenger"

Regulation of miR-223 Expression

The gene encoding for miR-223 is located on the X-chromosome; miR-223 was originally connected with the hematopoiesis and the function of granulocytes [93]. Transcription factors regulating miR-223 expression have already been identified and include transcription factor binding to PU-box 1 (PU.1), CCAAT-enhancer-binding

proteins α and β (C/EBP α and β), and nuclear factor I-A (NFI-A) [94, 95]. The first two mentioned transcription factors increase miR-223 expression whereas the third one decreases miR-223 expression; moreover, NFI-A itself is an miR-223 target, thus creating a negative regulatory feedback loop [94, 95]. In relation to lipid metabolism, high intracellular cholesterol levels were also shown to increase miR-223 expression [96].

miR-223 and Lipid Metabolism

Within the cells, miR-223 was described as an important regulator of cholesterol homeostasis [96] cooperating side by side with miR-122 and miR-33a/b. As mentioned above, miR-223 level is increased in the presence of high intracellular cholesterol; in order to prevent cholesterol overload, miR-223 (a) inhibits HDL cellular uptake via targeting scavenger receptor IB (SR-IB), (b) inhibits cholesterol synthesis by targeting HMGCoA-R and methylsterol monooxygenase 1 (MSMO1), and (c) increases cholesterol efflux by targeting Sp3, thus indirectly upregulating levels of ABCA1 [96]. Similar results were obtained by Wang and colleagues who demonstrated that SR-IB is targeted not only by miR-223, but also by miR-185 and miR-96 [97].

Intriguingly, miR-223 was shown to be transferred within microvesicles [27, 98] and within HDL and LDL particles [99]. In particular, miR-223 is one of the most abundant miRs in HDL particles [99] and is also present in LDL particles, where one of the most abundant miRs seems to be the inflammation-related miR-155 [26]. From HDL, miR-223 can be transferred to endothelial cells, where miR-223 is not commonly expressed [99, 100], which may at least partly explain the anti-inflammatory properties of HDL [101–105]. Namely, miR-223 targets NOD-like receptor pyrin domain containing 3 (NLRP3) [106] and intercellular adhesion molecule I (ICAM-1) [100] and downregulation of both these molecules suppresses endothelial inflammation and reactivity, thereby preventing leukocyte infiltration and inflammation connected with atherosclerosis [107, 108].

Involvement of miR-223 in Inflammation, Insulin Resistance, and Platelet Activation

Macrophages and platelets were shown to produce microvesicles/exosomes containing miR-223 and miR-223 was again shown to be transferred to various cells, regulating posttranscriptionally gene expression [27, 98, 109]. For example, miR-223 transferred to endothelial cells (HUVECs) increases their apoptosis induced by advanced glycation products [98]. Interestingly, also gap junctions were described to enable miR-223 transfer from macrophages to hepatocellular carcinoma cells, which resulted in inhibition of proliferation [110]. Concerning the potential involvement of miR-223 in the development of insulin resistance, there is evidence proving

that miR-223$^{-/-}$ mice receiving high-fat diet display profound insulin resistance and increased adipose tissue inflammation compared with wild-type animals on high-fat diet [111]. One of the potential miR-223 targets that may be involved in the development of insulin resistance is glucose transporter 4 (GLUT4) as validated in neonatal rat necrosis cytes [112].

Lastly, miR-223 was shown to be involved in platelet function [28] and reactivity to anti-aggregation treatment—in patients with low-response to clopidogrel therapy, platelet miR-223 levels were significantly lower compared to normal-responders [113]. Lower levels of miR-223 were also observed in plasma of low-responders [114]; however, most recently Chyrchel and coworkers presented a study that argues against the notion of low plasma miR-223 as a marker of platelet responsiveness to dual antiplatelet therapy [115]. Further studies are definitely necessary to reveal the true potential of miR-223 in platelets [116–118], since it has already been shown that circulating miR-223 levels negatively correlate with the susceptibility to myocardial infarction [116].

miR-27a/b and Other Essential Transcriptional Hubs

As shown above, miR-33, miR-122, and ,miR-223 can target many mRNAs translated in proteins involved in the regulation of lipid metabolism and they can thus be considered as "transcriptional miR hubs," i.e., important miRs targeting more proteins affecting transcription (and thus function) of lipid metabolism-related proteins. A similar "hub" role was predicted and confirmed for miR-27a/b. In particular, miR-27a targets RXRα, ABCA1 (see below), FAS, both SREBP-1 and SREBP-2, both PPARα (regulator of ABCA1) and PPARγ (regulator of adipocyte differentiation), and last but not least proteins involved in lipoprotein formation: ApoA1, ApoB100, and ApoE3 [119–122]. Similarly to miR-27a, miR-27b was predicted to target 27 various targets involved in lipid metabolism and out of these, PPARγ, angiopoietin-like 3 (ANGPTL3), N-deacetylase/N-sulfotransferase 1 (NDST1), mitochondrial glycerol-3-phosphate acyltransferase (GPAM) and C/EBPα have been already validated [123–128]. Recently, sequential regulation of cholesterol metabolism by miR-27 was reported by Zhang and colleagues—their study elegantly demonstrated that cholesterol efflux (by targeting ABCA1 and apoA1), influx (by targeting LPL and CD36), and esterification (by targeting ACAT1) are affected at one time by miR-27 [129]. Notably, predicted targets of miR-27a and other miRs that are profoundly dysregulated in metabolic syndrome include genes that partake in pathways related to fatty acid and sphingolipid metabolism and are also involved in vascular signaling [3], suggesting thereby a key role for miR-27 in the maintenance of overall metabolic homeostasis.

ABCA1: Target of Multiple miRs

Protein levels of ABCA1, transporter that is crucial for cholesterol loading into ApoA1 and formation of HDL [13], are known not to correlate with its mRNA levels suggesting highly complex posttranscriptional regulation [130]. Besides other factors, due to its remarkably long 3'UTR, ABCA1 was revealed to be targeted by various miRs (Table 5.1), specifically by miR-10b [131], miR-19b [132], miR-26 [133], miR-106a [134], miR-106b [135], miR-128-2 [52], miR-143/145 cluster [136, 137], miR-144 [50, 138], miR-302a [140], miR-613 [33], and miR-758 [141, 142]. Among the miRs mentioned above, also miR-27 [129] and miR-33 [19, 72, 142] target ABCA1. Additional miRs will be certainly validated in the future, since more than 100 miRs have been predicted to bind ABCA1 3'UTR [143]. This probably reflects the different regulation of ABCA1 in particular tissues; studying ABCA1 regulation still represents a challenge since its involvement in insulin resistance/secretion [72, 136] and Alzheimer's disease [135] has also been demonstrated.

Regulation of miRs by miRs: miR-370 and miR-122

The fact that one single miR can target more mRNAs and one mRNA is commonly targeted by several miRs (e.g., as shown above for ABCA1) creates an incredibly complicated network orchestrating lipid metabolism. Even more complexity is added when realizing that each miR can be also directly regulated by another miR. This was described for miR-370 upregulating the expression of miR-122 [29]; using sense and antisense miR-122 and miR-370, the same set of targets was shown to be affected. However, if miR-122 is blocked with its antagomiR, miR-370 effect is blunted [29]. Interestingly, in patients with CAD, miR-122 and miR-370 levels correlate with each other and they were also shown to reflect CAD severity in patients with hyperlipidemia [92]. Both miRs may be involved in the so-called fetal reprogramming: if mice are fed with high-fat diet, levels of both miRs in the liver of their offspring are altered. miRs may thus represent novel mediators involved in transgenerational epigenetic settings of lipid metabolism [144].

Conclusion

MicroRNAs represent important regulators of lipid metabolism working either as important transcriptional hubs (affecting the expression of various proteins at one time), or by fine tuning the regulation of key genes (i.e., more miRs at time controlling the expression of crucial genes) participating in lipid metabolism. Multitarget

Table 5.1 MicroRNAs involved in ABCA1 regulation

miR	Details	References
miR-10b	Gut microbiota produces protocatechuic acid, which was shown to reduce levels of miR-10b in macrophages and thus increase cholesterol efflux exerting anti-atherogenic effect	[131]
miR-19b	Treatment of ApoE−/− mice with miR-19b resulted in decreased HDL (via ABCA1) and increased LDL levels which was shown to increase aortic plaques size	[132]
miR-26	miR-26 overexpression resulted in decreased cholesterol efflux by targeting ABCA1 and ARL7 levels. Moreover, in LDL loaded Hep2G cells, miR-26 levels decreased and ABCA1 levels increased suggesting the role of dietary cholesterol in decreasing miR-26 expression	[133]
miR-106a	miR-106a levels are higher in cisplatin resistant cell line A549 (non small cell lung cancer cell line) and downregulation of ABCA1 by miR-106a represents one of the novel mechanisms of cisplatin resistance	[134]
miR-106b	In Alzheimer disease, ABCA1 is known to affect metabolism of pathogenic amyloid-β; miR-106b was shown to affect ABCA1 levels and thus increase amyloid-β production and decreased its clearance	[135]
miR-128-2	miR-128-2 attenuates cholesterol efflux by targeting ABCA1, ABCG1, and RXRα in HEK293T, HepG2, and MCF7 cells	[52]
miR-143/ miR-145	$Ldlr^{-/-}$ and miR-143/$145^{-/-}$ double knockout mice were fed with high-fat diet. Comparison of atherosclerosis plaque from these animals and $Ldlr^{-/-}$ mice showed that the absence of miR-143/145 resulted in smaller atherosclerotic plaques and decreased macrophage infiltration. This was accompanied by a decrease in circulating cholesterol levels in VLDL and LDL. ABCA1 upregulation was observed in liver and vessels	[136, 137]
	miR-145 levels were shown to be decreased in pancreatic cells after glucose stimulation. This results in an increase in cholesterol efflux and improvement in glucose-stimulated insulin secretion	
miR-144	LXR and FXR agonists increased miR-144 expression, which reduced ABCA1 expression and cholesterol efflux in macrophages and hepatic cells. miR-144 silencing increased HDL	[50, 138, 139]
	miR-144 mimic inhibited cholesterol efflux from macrophages via ABCA1 targeting and it further decreased HDL cholesterol levels and promoted atherosclerosis in $ApoE^{-/-}$ mice. Furthermore, it increased inflammatory cytokines expression (IL-1β, IL6, TNFα) and circulating miR-144 levels correlated with serum CK, CK-MB, LDH, and AST in patients after acute myocardial infarction	
miR-302a	Ldlr−/− mice were treated with antagomiR against miR-302a, which resulted in a decrease in atherosclerotic plaques size and an increase in circulating HDL levels	[140]
miR-613	By targeting PPARγ and ABCA1, miR-613 decreases cholesterol efflux from PPARγ-activated macrophages	[33]

(continued)

Table 5.1 (continued)

miR	Details	References
miR-758	High-fat diet reduced miR-758 levels in peritoneal macrophages and liver followed by increased in ABCA1, which led to increased cholesterol efflux	[141]
	In human atherosclerotic carotid plaques, expression of ABCA1 and ABCG1 was found to be increased, together with an increase in miR-33b and miR-758, which is probably a reason for no changes in the protein levels of mentioned proteins	[142]

ApoE apolipoprotein E, *Ldlr* low density lipoprotein receptor, *HDL* high density lipoprotein, *ARL7* ADP-ribosylation factor-like 7, *HEK293T* human embryonic kidney, *HepG2* liver hepatocellular carcinoma, *MCF7* breast cancer cell line, *ABCG1* ATP binding cassette G1, *RXRα* retinoid X receptor α, *IL* interleukin, *TNF* tumor necrosis factor; *CK* creatin kinase, *CK-MB* creatin kinase-muscle/brain subunits, *LDH* lactate dehydrogenase, *AST* aspartate aminotransferase, *PPARγ* peroxisome proliferator-activated receptor gamma

function of miRs can be definitely exploited as future tool to study pathophysiology of disease and test potential targeted therapies [145]. We can almost say that miRs "show us" how everything is connected together and some connections never thought before may be revealed. Since miRs are highly evolutionary conserved, this can suggest us the way by which the research in the field can continue in order to reveal the true essence of some disorders. This may in the future result in identifying new and enhanced strategies for diagnostics and therapy of lipid metabolism-related disorders.

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References

1. Novak J, Bienertova-Vasku J, Kara T, Novak M. MicroRNAs involved in the lipid metabolism and their possible implications for atherosclerosis development and treatment. Mediators Inflamm. 2014;2014:275867.
2. Santulli G. Effects of low-carbohydrate and low-fat diets. Ann Intern Med. 2015;162:392.
3. Wronska A, Kurkowska-Jastrzebska I, Santulli G. Application of microRNAs in diagnosis and treatment of cardiovascular disease. Acta Physiol (Oxf). 2015;213:60–83.
4. Cheung O, Puri P, Eicken C, Contos MJ, Mirshahi F, Maher JW, et al. Nonalcoholic steatohepatitis is associated with altered hepatic microRNA expression. Hepatology. 2008;48:1810–20.
5. Brown MS, Goldstein JL. The SREBP pathway: regulation of cholesterol metabolism by proteolysis of a membrane-bound transcription factor. Cell. 1997;89:331–40.
6. Horton JD, Goldstein JL, Brown MS. SREBPs: transcriptional mediators of lipid homeostasis. Cold Spring Harb Symp Quant Biol. 2002;67:491–8.
7. Hong C, Tontonoz P. Liver X receptors in lipid metabolism: opportunities for drug discovery. Nat Rev Drug Discov. 2014;13:433–44.

8. Fernandez-Hernando C, Suarez Y, Rayner KJ, Moore KJ. MicroRNAs in lipid metabolism. Curr Opin Lipidol. 2011;22:86–92.
9. Ishigaki Y, Oka Y, Katagiri H. Circulating oxidized LDL: a biomarker and a pathogenic factor. Curr Opin Lipidol. 2009;20:363–9.
10. Rizzo G, Renga B, Mencarelli A, Pellicciari R, Fiorucci S. Role of FXR in regulating bile acid homeostasis and relevance for human diseases. Curr Drug Targets Immune Endocr Metabol Disord. 2005;5:289–303.
11. Lim LP, Lau NC, Garrett-Engele P, Grimson A, Schelter JM, Castle J, et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature. 2005;433:769–73.
12. Santulli G, Iaccarino G, De Luca N, Trimarco B, Condorelli G. Atrial fibrillation and microRNAs. Front Physiol. 2014;5:15.
13. Lv YC, Yin K, Fu YC, Zhang DW, Chen WJ, Tang CK. Posttranscriptional regulation of ATP-binding cassette transporter A1 in lipid metabolism. DNA Cell Biol. 2013;32:348–58.
14. 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.
15. Sardu C, Marfella R, Santulli G, Paolisso G. Functional role of miRNA in cardiac resynchronization therapy. Pharmacogenomics. 2014;15:1159–68.
16. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116: 281–97.
17. 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.
18. Gerin I, Clerbaux LA, Haumont O, Lanthier N, Das AK, Burant CF, et al. Expression of miR-33 from an SREBP2 intron inhibits cholesterol export and fatty acid oxidation. J Biol Chem. 2010;285:33652–61.
19. Goedeke L, Vales-Lara FM, Fenstermaker M, Cirera-Salinas D, Chamorro-Jorganes A, Ramirez CM, et al. A regulatory role for microRNA 33* in controlling lipid metabolism gene expression. Mol Cell Biol. 2013;33:2339–52.
20. Zhang J, Li S, Li L, Li M, Guo C, Yao J, et al. Exosome and exosomal microRNA: trafficking, sorting, and function. Genomics Proteomics Bioinformatics. 2015;13(1):17–24.
21. Kinet V, Halkein J, Dirkx E, Windt LJ. Cardiovascular extracellular microRNAs: emerging diagnostic markers and mechanisms of cell-to-cell RNA communication. Front Genet. 2013; 4:214.
22. Esau C, Davis S, Murray SF, Yu XX, Pandey SK, Pear M, et al. miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab. 2006;3:87–98.
23. Jopling C. Liver-specific microRNA-122: biogenesis and function. RNA Biol. 2012;9:137–42.
24. Li YP, Gottwein JM, Scheel TK, Jensen TB, Bukh J. MicroRNA-122 antagonism against hepatitis C virus genotypes 1–6 and reduced efficacy by host RNA insertion or mutations in the HCV 5' UTR. Proc Natl Acad Sci U S A. 2011;108:4991–6.
25. Tsai WC, Hsu SD, Hsu CS, Lai TC, Chen SJ, Shen R, et al. MicroRNA-122 plays a critical role in liver homeostasis and hepatocarcinogenesis. J Clin Invest. 2012;122:2884–97.
26. 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.
27. Ismail N, Wang Y, Dakhlallah D, Moldovan L, Agarwal K, Batte K, et al. Macrophage microvesicles induce macrophage differentiation and miR-223 transfer. Blood. 2013;121:984–95.
28. Halkein J, De Windt LJ. miR-223: sailing to terra incognita for microRNAs in platelets. Thromb Haemost. 2013;110:1112–3.
29. Iliopoulos D, Drosatos K, Hiyama Y, Goldberg IJ, Zannis VI. MicroRNA-370 controls the expression of microRNA-122 and Cpt1alpha and affects lipid metabolism. J Lipid Res. 2010;51:1513–23.

30. Yang M, Liu W, Pellicane C, Sahyoun C, Joseph BK, Gallo-Ebert C, et al. Identification of miR-185 as a regulator of de novo cholesterol biosynthesis and low density lipoprotein uptake. J Lipid Res. 2014;55:226–38.
31. Zhu DQ, Lou YF, He ZG, Ji M. Nucleotidyl transferase TUT1 inhibits lipogenesis in osteosarcoma cells through regulation of microRNA-24 and microRNA-29a. Tumour Biol. 2014; 35:11829–35.
32. Zhong D, Zhang Y, Zeng YJ, Gao M, Wu GZ, Hu CJ, et al. MicroRNA-613 represses lipogenesis in HepG2 cells by downregulating LXRalpha. Lipids Health Dis. 2013;12:32.
33. Zhao R, Feng J, He G. miR-613 regulates cholesterol efflux by targeting LXRalpha and ABCA1 in PPARgamma activated THP-1 macrophages. Biochem Biophys Res Commun. 2014;448:329–34.
34. Miller AM, Gilchrist DS, Nijjar J, Araldi E, Ramirez CM, Lavery CA, et al. MiR-155 has a protective role in the development of non-alcoholic hepatosteatosis in mice. PLoS One. 2013;8, e72324.
35. Vinod M, Chennamsetty I, Colin S, Belloy L, De Paoli F, Schaider H, et al. miR-206 controls LXRalpha expression and promotes LXR-mediated cholesterol efflux in macrophages. Biochim Biophys Acta. 2014;1841:827–35.
36. Ou Z, Wada T, Gramignoli R, Li S, Strom SC, Huang M, et al. MicroRNA hsa-miR-613 targets the human LXRalpha gene and mediates a feedback loop of LXRalpha autoregulation. Mol Endocrinol. 2011;25:584–96.
37. Walker AK, Yang F, Jiang K, Ji JY, Watts JL, Purushotham A, et al. Conserved role of SIRT1 orthologs in fasting-dependent inhibition of the lipid/cholesterol regulator SREBP. Genes Dev. 2010;24:1403–17.
38. Li X, Zhang S, Blander G, Tse JG, Krieger M, Guarente L. SIRT1 deacetylates and positively regulates the nuclear receptor LXR. Mol Cell. 2007;28:91–106.
39. Yu J, Auwerx J. Protein deacetylation by SIRT1: an emerging key post-translational modification in metabolic regulation. Pharmacol Res. 2010;62:35–41.
40. Ponugoti B, Kim DH, Xiao Z, Smith Z, Miao J, Zang M, et al. SIRT1 deacetylates and inhibits SREBP-1C activity in regulation of hepatic lipid metabolism. J Biol Chem. 2010;285: 33959–70.
41. Xiong H, Pang J, Yang H, Dai M, Liu Y, Ou Y, et al. Activation of miR-34a/SIRT1/p53 signaling contributes to cochlear hair cell apoptosis: implications for age-related hearing loss. Neurobiol Aging. 2015;36(4):1692–701.
42. Fan W, Fang R, Wu X, Liu J, Feng M, Dai G, et al. Shear-sensitive microRNA-34a modulates flow-dependent regulation of endothelial inflammation. J Cell Sci. 2015;128:70–80.
43. Zhang L, Huang D, Wang Q, Shen D, Wang Y, Chen B, et al. MiR-132 inhibits expression of SIRT1 and induces pro-inflammatory processes of vascular endothelial inflammation through blockade of the SREBP-1c metabolic pathway. Cardiovasc Drugs Ther. 2014;28:303–11.
44. Gao J, Wang Y, Zhao X, Chen P, Xie L. MicroRNA-204-5p-mediated regulation of SIRT1 contributes to the delay of epithelial cell cycle traversal in diabetic corneas. Invest Ophthalmol Vis Sci. 2015;56:1493–504.
45. Zhang H, Feng Z, Huang R, Xia Z, Xiang G, Zhang J. MicroRNA-449 suppresses proliferation of hepatoma cell lines through blockade lipid metabolic pathway related to SIRT1. Int J Oncol. 2014;45:2143–52.
46. Duan JH, Fang L. MicroRNA-92 promotes gastric cancer cell proliferation and invasion through targeting FXR. Tumour Biol. 2014;35:11013–9.
47. Zhang Y, Gong W, Dai S, Huang G, Shen X, Gao M, et al. Downregulation of human farnesoid X receptor by miR-421 promotes proliferation and migration of hepatocellular carcinoma cells. Mol Cancer Res. 2012;10:516–22.
48. Lee CG, Kim YW, Kim EH, Meng Z, Huang W, Hwang SJ, et al. Farnesoid X receptor protects hepatocytes from injury by repressing miR-199a-3p, which increases levels of LKB1. Gastroenterology. 2012;142:1206–17. e7.
49. Li J, Zhang Y, Kuruba R, Gao X, Gandhi CR, Xie W, et al. Roles of microRNA-29a in the antifibrotic effect of farnesoid X receptor in hepatic stellate cells. Mol Pharmacol. 2011;80: 191–200.

50. de Aguiar Vallim TQ, Tarling EJ, Kim T, Civelek M, Baldan A, Esau C, et al. MicroRNA-144 regulates hepatic ATP binding cassette transporter A1 and plasma high-density lipoprotein after activation of the nuclear receptor farnesoid X receptor. Circ Res. 2013;112:1602–12.
51. Oda Y, Nakajima M, Tsuneyama K, Takamiya M, Aoki Y, Fukami T, et al. Retinoid X receptor alpha in human liver is regulated by miR-34a. Biochem Pharmacol. 2014;90:179–87.
52. Adlakha YK, Khanna S, Singh R, Singh VP, Agrawal A, Saini N. Pro-apoptotic miRNA-128-2 modulates ABCA1, ABCG1 and RXRalpha expression and cholesterol homeostasis. Cell Death Dis. 2013;4, e780.
53. Hu Z, Shen WJ, Cortez Y, Tang X, Liu LF, Kraemer FB, et al. Hormonal regulation of microRNA expression in steroid producing cells of the ovary, testis and adrenal gland. PLoS One. 2013;8, e78040.
54. Hu Z, Shen WJ, Kraemer FB, Azhar S. MicroRNAs 125a and 455 repress lipoprotein-supported steroidogenesis by targeting scavenger receptor class B type I in steroidogenic cells. Mol Cell Biol. 2012;32:5035–45.
55. Irani S, Hussain MM. Role of microRNA-30c in lipid metabolism, adipogenesis, cardiac remodeling and cancer. Curr Opin Lipidol. 2015;26:139–46.
56. Chen KC, Hsieh IC, Hsi E, Wang YS, Dai CY, Chou WW, et al. Negative feedback regulation between microRNA let-7g and the oxLDL receptor LOX-1. J Cell Sci. 2011;124:4115–24.
57. Loyer X, Mallat Z, Boulanger CM, Tedgui A. MicroRNAs as therapeutic targets in atherosclerosis. Expert Opin Ther Targets. 2015;19:489–96.
58. Rayner KJ, Suarez Y, Davalos A, Parathath S, Fitzgerald ML, Tamehiro N, et al. MiR-33 contributes to the regulation of cholesterol homeostasis. Science. 2010;328:1570–3.
59. Marquart TJ, Allen RM, Ory DS, Baldan A. miR-33 links SREBP-2 induction to repression of sterol transporters. Proc Natl Acad Sci U S A. 2010;107:12228–32.
60. 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;328:1566–9.
61. Allen RM, Marquart TJ, Jesse JJ, Baldan A. Control of very low-density lipoprotein secretion by N-ethylmaleimide-sensitive factor and miR-33. Circ Res. 2014;115:10–22.
62. Marquart TJ, Wu J, Lusis AJ, Baldan A. Anti-miR-33 therapy does not alter the progression of atherosclerosis in low-density lipoprotein receptor-deficient mice. Arterioscler Thromb Vasc Biol. 2013;33:455–8.
63. 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;478:404–7.
64. Rayner KJ, Sheedy FJ, Esau CC, Hussain FN, Temel RE, Parathath S, et al. Antagonism of miR-33 in mice promotes reverse cholesterol transport and regression of atherosclerosis. J Clin Invest. 2011;121:2921–31.
65. Allen RM, Marquart TJ, Albert CJ, Suchy FJ, Wang DQ, Ananthanarayanan M, et al. miR-33 controls the expression of biliary transporters, and mediates statin- and diet-induced hepatotoxicity. EMBO Mol Med. 2012;4:882–95.
66. Horie T, Baba O, Kuwabara Y, Chujo Y, Watanabe S, Kinoshita M, et al. MicroRNA-33 deficiency reduces the progression of atherosclerotic plaque in ApoE−/− mice. J Am Heart Assoc. 2012;1, e003376.
67. Rotllan N, Ramirez CM, Aryal B, Esau CC, Fernandez-Hernando C. Therapeutic silencing of microRNA-33 inhibits the progression of atherosclerosis in Ldlr−/− mice–brief report. Arterioscler Thromb Vasc Biol. 2013;33:1973–7.
68. Fernandez-Hernando C, Moore KJ. MicroRNA modulation of cholesterol homeostasis. Arterioscler Thromb Vasc Biol. 2011;31:2378–82.
69. Davalos A, Goedeke L, Smibert P, Ramirez CM, Warrier NP, Andreo U, et al. miR-33a/b contribute to the regulation of fatty acid metabolism and insulin signaling. Proc Natl Acad Sci U S A. 2011;108:9232–7.
70. Ramirez CM, Goedeke L, Rotllan N, Yoon JH, Cirera-Salinas D, Mattison JA, et al. MicroRNA 33 regulates glucose metabolism. Mol Cell Biol. 2013;33:2891–902.

71. Copps KD, White MF. Regulation of insulin sensitivity by serine/threonine phosphorylation of insulin receptor substrate proteins IRS1 and IRS2. Diabetologia. 2012;55:2565–82.
72. Wijesekara N, Zhang LH, Kang MH, Abraham T, Bhattacharjee A, Warnock GL, et al. miR-33a modulates ABCA1 expression, cholesterol accumulation, and insulin secretion in pancreatic islets. Diabetes. 2012;61:653–8.
73. Chen WJ, Zhang M, Zhao GJ, Fu Y, Zhang DW, Zhu HB, et al. MicroRNA-33 in atherosclerosis etiology and pathophysiology. Atherosclerosis. 2013;227:201–8.
74. Iwakiri Y. A role of miR-33 for cell cycle progression and cell proliferation. Cell Cycle. 2012; 11:1057–8.
75. Herrera-Merchan A, Cerrato C, Luengo G, Dominguez O, Piris MA, Serrano M, et al. miR-33-mediated downregulation of p53 controls hematopoietic stem cell self-renewal. Cell Cycle. 2010;9:3277–85.
76. Chang J, Nicolas E, Marks D, Sander C, Lerro A, Buendia MA, et al. miR-122, a mammalian liver-specific microRNA, is processed from hcr mRNA and may downregulate the high affinity cationic amino acid transporter CAT-1. RNA Biol. 2004;1:106–13.
77. Li ZY, Xi Y, Zhu WN, Zeng C, Zhang ZQ, Guo ZC, et al. Positive regulation of hepatic miR-122 expression by HNF4alpha. J Hepatol. 2011;55:602–11.
78. Gatfield D, Le Martelot G, Vejnar CE, Gerlach D, Schaad O, Fleury-Olela F, et al. Integration of microRNA miR-122 in hepatic circadian gene expression. Genes Dev. 2009;23:1313–26.
79. Katoh T, Sakaguchi Y, Miyauchi K, Suzuki T, Kashiwabara S, Baba T. Selective stabilization of mammalian microRNAs by 3' adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. Genes Dev. 2009;23:433–8.
80. Krutzfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, et al. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005;438:685–9.
81. Elmen J, Lindow M, Silahtaroglu A, Bak M, Christensen M, Lind-Thomsen A, et al. Antagonism of microRNA-122 in mice by systemically administered LNA-antimiR leads to up-regulation of a large set of predicted target mRNAs in the liver. Nucleic Acids Res. 2008; 36:1153–62.
82. Hsu SH, Wang B, Kota J, Yu J, Costinean S, Kutay H, et al. Essential metabolic, anti-inflammatory, and anti-tumorigenic functions of miR-122 in liver. J Clin Invest. 2012;122: 2871–83.
83. Moore KJ, Rayner KJ, Suarez Y, Fernandez-Hernando C. microRNAs and cholesterol metabolism. Trends Endocrinol Metab. 2010;21:699–706.
84. Burchard J, Zhang C, Liu AM, Poon RT, Lee NP, Wong KF, et al. microRNA-122 as a regulator of mitochondrial metabolic gene network in hepatocellular carcinoma. Mol Syst Biol. 2010;6:402.
85. de Jong YP, Jacobson IM. Antisense therapy for hepatitis C virus infection. J Hepatol. 2014;60:227–8.
86. 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.
87. 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.
88. Lanford RE, Hildebrandt-Eriksen ES, Petri A, Persson R, Lindow M, Munk ME, et al. Therapeutic silencing of microRNA-122 in primates with chronic hepatitis C virus infection. Science. 2010;327:198–201.
89. Wang R, Hong J, Cao Y, Shi J, Gu W, Ning G, et al. Elevated circulating microRNA-122 is associated with obesity and insulin resistance in young adults. Eur J Endocrinol. 2015;172:291–300.
90. Pirola CJ, Fernandez Gianotti T, Castano GO, Mallardi P, San Martino J, Mora Gonzalez Lopez Ledesma M, et al. Circulating microRNA signature in non-alcoholic fatty liver disease: from serum non-coding RNAs to liver histology and disease pathogenesis. Gut. 2015;64(5): 800–12.
91. Bala S, Petrasek J, Mundkur S, Catalano D, Levin I, Ward J, et al. Circulating microRNAs in exosomes indicate hepatocyte injury and inflammation in alcoholic, drug-induced, and inflammatory liver diseases. Hepatology. 2012;56:1946–57.

92. Gao W, He HW, Wang ZM, Zhao H, Lian XQ, Wang YS, et al. Plasma levels of lipometabolism-related miR-122 and miR-370 are increased in patients with hyperlipidemia and associated with coronary artery disease. Lipids Health Dis. 2012;11:55.
93. Johnnidis JB, Harris MH, Wheeler RT, Stehling-Sun S, Lam MH, Kirak O, et al. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. Nature. 2008; 451:1125–9.
94. Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C, et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. Cell. 2005;123:819–31.
95. Fukao T, Fukuda Y, Kiga K, Sharif J, Hino K, Enomoto Y, et al. An evolutionarily conserved mechanism for microRNA-223 expression revealed by microRNA gene profiling. Cell. 2007;129:617–31.
96. 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.
97. Wang L, Jia XJ, Jiang HJ, Du Y, Yang F, Si SY, et al. MicroRNAs 185, 96, and 223 repress selective high-density lipoprotein cholesterol uptake through posttranscriptional inhibition. Mol Cell Biol. 2013;33:1956–64.
98. 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.
99. 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.
100. 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.
101. Tanaka N, Ishida T, Nagao M, Mori T, Monguchi T, Sasaki M, et al. Administration of high dose eicosapentaenoic acid enhances anti-inflammatory properties of high-density lipoprotein in Japanese patients with dyslipidemia. Atherosclerosis. 2014;237:577–83.
102. Sang H, Yao S, Zhang L, Li X, Yang N, Zhao J, et al. Walk-run training improves the anti-inflammation properties of high-density lipoprotein in patients with metabolic syndrome. J Clin Endocrinol Metab. 2015;100:870–9.
103. Song GJ, Kim SM, Park KH, Kim J, Choi I, Cho KH. SR-BI mediates high density lipoprotein (HDL)-induced anti-inflammatory effect in macrophages. Biochem Biophys Res Commun. 2015;457:112–8.
104. Van Lenten BJ, Hama SY, de Beer FC, Stafforini DM, McIntyre TM, Prescott SM, et al. Anti-inflammatory HDL becomes pro-inflammatory during the acute phase response. Loss of protective effect of HDL against LDL oxidation in aortic wall cell cocultures. J Clin Invest. 1995;96:2758–67.
105. Gong M, Wilson M, Kelly T, Su W, Dressman J, Kincer J, et al. HDL-associated estradiol stimulates endothelial NO synthase and vasodilation in an SR-BI-dependent manner. J Clin Invest. 2003;111:1579–87.
106. Bauernfeind F, Rieger A, Schildberg FA, Knolle PA, Schmid-Burgk JL, Hornung V. NLRP3 inflammasome activity is negatively controlled by miR-223. J Immunol. 2012;189:4175–81.
107. Weber C, Soehnlein O. ApoE controls the interface linking lipids and inflammation in atherosclerosis. J Clin Invest. 2011;121:3825–7.
108. Harja E, Bu DX, Hudson BI, Chang JS, Shen X, Hallam K, et al. Vascular and inflammatory stresses mediate atherosclerosis via RAGE and its ligands in apoE-/- mice. J Clin Invest. 2008;118:183–94.
109. Laffont B, Corduan A, Ple H, Duchez AC, Cloutier N, Boilard E, et al. Activated platelets can deliver mRNA regulatory Ago2*microRNA complexes to endothelial cells via microparticles. Blood. 2013;122:253–61.
110. Aucher A, Rudnicka D, Davis DM. MicroRNAs transfer from human macrophages to hepato-carcinoma cells and inhibit proliferation. J Immunol. 2013;191:6250–60.

111. Zhuang G, Meng C, Guo X, Cheruku PS, Shi L, Xu H, et al. A novel regulator of macrophage activation: miR-223 in obesity-associated adipose tissue inflammation. Circulation. 2012;125: 2892–903.
112. Lu H, Buchan RJ, Cook SA. MicroRNA-223 regulates Glut4 expression and cardiomyocyte glucose metabolism. Cardiovasc Res. 2010;86:410–20.
113. Shi R, Ge L, Zhou X, Ji WJ, Lu RY, Zhang YY, et al. Decreased platelet miR-223 expression is associated with high on-clopidogrel platelet reactivity. Thromb Res. 2013;131:508–13.
114. Zhang YY, Zhou X, Ji WJ, Shi R, Lu RY, Li JL, et al. Decreased circulating microRNA-223 level predicts high on-treatment platelet reactivity in patients with troponin-negative non-ST elevation acute coronary syndrome. J Thromb Thrombolysis. 2014;38:65–72.
115. Chyrchel B, Toton-Zuranska J, Kruszelnicka O, Chyrchel M, Mielecki W, Kolton-Wroz M, et al. Association of plasma miR-223 and platelet reactivity in patients with coronary artery disease on dual antiplatelet therapy: A preliminary report. Platelets. 2015;26(6):593–7. doi: 10.3109/09537104.2014.974527.
116. Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard JM, et al. Prospective study on circulating microRNAs and risk of myocardial infarction. J Am Coll Cardiol. 2012;60: 290–9.
117. Liverani E, Kilpatrick LE, Kunapuli SP. P2Y12 receptor in cardiovascular disease. J Cardiovasc Dis. 2014;2:203–8.
118. Shameem R, Hamid MS, Randahawa A, Spaccavento C, Garatt K. P2Y12 antagonists: pharmacology, efficacy, and patient consideration. J Cardiovasc Dis. 2014;2:91–100.
119. Kida K, Nakajima M, Mohri T, Oda Y, Takagi S, Fukami T, et al. PPARalpha is regulated by miR-21 and miR-27b in human liver. Pharm Res. 2011;28:2467–76.
120. Shirasaki T, Honda M, Shimakami T, Horii R, Yamashita T, Sakai Y, et al. MicroRNA-27a regulates lipid metabolism and inhibits hepatitis C virus replication in human hepatoma cells. J Virol. 2013;87:5270–86.
121. Ogata M, Tsujita M, Hossain MA, Akita N, Gonzalez FJ, Staels B, et al. On the mechanism for PPAR agonists to enhance ABCA1 gene expression. Atherosclerosis. 2009;205:413–9.
122. Liu Y, Berendsen AD, Jia S, Lotinun S, Baron R, Ferrara N, et al. Intracellular VEGF regulates the balance between osteoblast and adipocyte differentiation. J Clin Invest. 2012;122:3101–13.
123. Lin Q, Gao Z, Alarcon RM, Ye J, Yun Z. A role of miR-27 in the regulation of adipogenesis. FEBS J. 2009;276:2348–58.
124. Vickers KC, Shoucri BM, Levin MG, Wu H, Pearson DS, Osei-Hwedieh D, et al. MicroRNA-27b is a regulatory hub in lipid metabolism and is altered in dyslipidemia. Hepatology. 2013;57:533–42.
125. Santulli G. Angiopoietin-like proteins: a comprehensive look. Front Endocrinol. 2014;5:4.
126. Galhardo M, Sinkkonen L, Berninger P, Lin J, Sauter T, Heinaniemi M. Integrated analysis of transcript-level regulation of metabolism reveals disease-relevant nodes of the human metabolic network. Nucleic Acids Res. 2014;42:1474–96.
127. Wang L, Ye X, Liu Y, Wei W, Wang Z. Aberrant regulation of FBW7 in cancer. Oncotarget. 2014;5:2000–15.
128. Singaravelu R, Chen R, Lyn RK, Jones DM, O'Hara S, Rouleau Y, et al. Hepatitis C virus induced up-regulation of microRNA-27: a novel mechanism for hepatic steatosis. Hepatology. 2014;59:98–108.
129. Zhang M, Wu JF, Chen WJ, Tang SL, Mo ZC, Tang YY, et al. MicroRNA-27a/b regulates cellular cholesterol efflux, influx and esterification/hydrolysis in THP-1 macrophages. Atherosclerosis. 2014;234:54–64.
130. Wellington CL, Walker EK, Suarez A, Kwok A, Bissada N, Singaraja R, et al. ABCA1 mRNA and protein distribution patterns predict multiple different roles and levels of regulation. Lab Invest. 2002;82:273–83.

131. Wang D, Xia M, Yan X, Li D, Wang L, Xu Y, et al. Gut microbiota metabolism of anthocyanin promotes reverse cholesterol transport in mice via repressing miRNA-10b. Circ Res. 2012;111:967–81.
132. Lv YC, Tang YY, Peng J, Zhao GJ, Yang J, Yao F, et al. MicroRNA-19b promotes macrophage cholesterol accumulation and aortic atherosclerosis by targeting ATP-binding cassette transporter A1. Atherosclerosis. 2014;236:215–26.
133. Sun D, Zhang J, Xie J, Wei W, Chen M, Zhao X. MiR-26 controls LXR-dependent cholesterol efflux by targeting ABCA1 and ARL7. FEBS Lett. 2012;586:1472–9.
134. Ma Y, Li X, Cheng S, Wei W, Li Y. MicroRNA-106a confers cisplatin resistance in non-small cell lung cancer A549 cells by targeting adenosine triphosphatase-binding cassette A1. Mol Med Rep. 2015;11:625–32.
135. Kim J, Yoon H, Ramirez CM, Lee SM, Hoe HS, Fernandez-Hernando C. MiR-106b impairs cholesterol efflux and increases Abeta levels by repressing ABCA1 expression. Exp Neurol. 2012;235:476–83.
136. Kang MH, Zhang LH, Wijesekara N, de Haan W, Butland S, Bhattacharjee A, et al. Regulation of ABCA1 protein expression and function in hepatic and pancreatic islet cells by miR-145. Arterioscler Thromb Vasc Biol. 2013;33:2724–32.
137. Sala F, Aranda JF, Rotllan N, Ramirez CM, Aryal B, Elia L, et al. MiR-143/145 deficiency attenuates the progression of atherosclerosis in Ldlr-/-mice. Thromb Haemost. 2014;112:796–802.
138. Ramirez CM, Rotllan N, Vlassov AV, Davalos A, Li M, Goedeke L, et al. Control of cholesterol metabolism and plasma high-density lipoprotein levels by microRNA-144. Circ Res. 2013;112:1592–601.
139. Hu YW, Hu YR, Zhao JY, Li SF, Ma X, Wu SG, et al. An agomir of miR-144-3p accelerates plaque formation through impairing reverse cholesterol transport and promoting pro-inflammatory cytokine production. PLoS One. 2014;9, e94997.
140. Meiler S, Baumer Y, Toulmin E, Seng K, Boisvert WA. MicroRNA 302a is a novel modulator of cholesterol homeostasis and atherosclerosis. Arterioscler Thromb Vasc Biol. 2015;35:323–31.
141. Ramirez CM, Davalos A, Goedeke L, Salerno AG, Warrier N, Cirera-Salinas D, et al. MicroRNA-758 regulates cholesterol efflux through posttranscriptional repression of ATP-binding cassette transporter A1. Arterioscler Thromb Vasc Biol. 2011;31:2707–14.
142. Mandolini C, Santovito D, Marcantonio P, Buttitta F, Bucci M, Ucchino S, et al. Identification of microRNAs 758 and 33b as potential modulators of ABCA1 expression in human atherosclerotic plaques. Nutr Metab Cardiovasc Dis. 2015;25:202–9.
143. Garcia DM, Baek D, Shin C, Bell GW, Grimson A, Bartel DP. Weak seed-pairing stability and high target-site abundance decrease the proficiency of lsy-6 and other microRNAs. Nat Struct Mol Biol. 2011;18:1139–46.
144. Benatti RO, Melo AM, Borges FO, Ignacio-Souza LM, Simino LA, Milanski M, et al. Maternal high-fat diet consumption modulates hepatic lipid metabolism and microRNA-122 (miR-122) and microRNA-370 (miR-370) expression in offspring. Br J Nutr. 2014;111:2112–22.
145. Santulli G, Totary-Jain H. Tailoring mTOR-based therapy: molecular evidence and clinical challenges. Pharmacogenomics. 2013;14:1517–26.

Chapter 6
microRNAs in Pancreatic β-Cell Physiology

Sabire Özcan

Abstract The β-cells within the pancreas are responsible for production and secretion of insulin. Insulin is released from pancreatic β-cells in response to increasing blood glucose levels and acts on insulin-sensitive tissues such as skeletal muscle and liver in order to maintain normal glucose homeostasis. Therefore, defects in pancreatic β-cell function lead to hyperglycemia and diabetes mellitus. A new class of molecules called microRNAs has been recently demonstrated to play a crucial role in regulation of pancreatic β-cell function under normal and pathophysiological conditions. miRNAs have been shown to regulate endocrine pancreas development, insulin biosynthesis, insulin exocytosis, and β-cell expansion. Many of the β-cell enriched miRNAs have multiple functions and regulate pancreas development as well as insulin biosynthesis and exocytosis. Furthermore, several of the β-cell specific miRNAs have been shown to accumulate in the circulation before the onset of diabetes and may serve as potential biomarkers for prediabetes. This chapter will focus on miRNAs that are enriched in pancreatic β-cells and play a critical role in modulation of β-cell physiology and may have clinical significance in the treatment of diabetes.

Keywords miRNA • β-Cell • Islets • Insulin • Diabetes • Insulin secretion • β-Cell failure • Endocrine pancreas • Insulin biosynthesis • Dicer1 • Ago2

Introduction

The β-cells within the pancreas are essential for maintaining normal blood glucose levels by producing and secreting insulin into circulation. Insulin release from pancreatic β-cells is proportional to blood glucose levels. Insulin acts on insulin-sensitive tissues, including muscle, liver, and adipocytes. The initiating event for type 2 diabetes is insulin resistance followed by hyperinsulinemia and β-cell failure.

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Based on the UK Prospective Diabetes Study, which involved over 5000 patients with newly diagnosed type 2 diabetes, it was estimated that at diagnosis of type 2 diabetes, β-cell function might have been already reduced by up to 50 % [1]. Moreover, the reduction in β-cell function appears to start 10–12 years before the onset of type 2 diabetes [2]. Thus, these findings suggest that preserving β-cell function is one of the most important goals in the treatment of type 2 diabetes.

Since the identification of the first β-cell enriched miRNA miR-375 in 2004 [3], many other miRNAs have been identified with important roles in pancreatic β-cell function [4–7]. An updated list of β-cell specific miRNAs is reported in Table 6.1.

β-Cell specific deletion of Dicer1 and Ago2 in mice provided valuable information on the overall function of miRNAs in pancreatic β-cells. Dicer1 is required for cytoplasmic processing of pre-miRNAs to mature miRNAs and homozygous deletion of Dicer1 in mice is embryonic lethal [8, 9]. Deletion of Dicer1 in the developing endocrine pancreas in mice at embryonic day 10.5 resulted in a significant reduction

Table 6.1 List of pancreatic β-cell specific miRNAs

Function	miRNA	Targets	References
Endocrine pancreas development	miR-7	Pax6	[24]
	miR-15a/b	Ngn3	[35]
	miR-124a	FoxA2, Ngn3	[30, 35]
	miR-200c	cMaf, Fog2	[31]
	miR-375	Cadm1	[18]
Insulin biosynthesis	miR-24a	Sox6	[11]
	miR-30a	NeuroD	[40]
	miR-30d	Map4k4	[39, 41]
Insulin exocytosis	miR-7	Snca, Cspa, Cplx1, Pfn2, Wipf2, Phactr1, Zdhhc9	[44]
	miR-9	Onecut-2, Sirt1	[45, 46]
	miR-21	–	[53]
	miR-33a	Abca1	[57]
	miR-124a	Rab27a	[52]
	miR-132	Slc25a20	[61]
	miR-145	Abca1	[56]
	miR-212	Slc25a20	[61]
	miR-375	Mtpn, HuD, Gephyrin, Ywhaz, Aifm1	[3, 17]
β-Cell inflammation	miR-21	Pdcd4	[64]
	miR-29	Mcl1	[66]
β-Cell expansion/replication	miR-7	S6k1, eIF4E, Mknk1, Mknk2, Mapkap1	[68]
	miR-184	Ago2	[16]
	miR-200	Zeb1	[75]

of β-cell mass [10]. These mice died shortly after birth and had abnormal islet structure that was associated with substantial decrease in endocrine progenitors. This provided the first evidence that miRNA maturation by Dicer1 is critical for endocrine pancreas development.

The importance of Dicer1 and mature miRNAs in β-cell function was further confirmed by several independent studies in which Dicer1 was ablated in adult endocrine pancreas [11–13]. These studies found that β-cell specific deletion of Dicer1 after the development of endocrine pancreas leads to the establishment of diabetes due to decreased insulin biosynthesis [11–13], glucose-stimulated insulin secretion [12], and reduced β-cell mass [12, 13]. In summary, the processing of pre-miRNAs to mature miRNAs by Dicer1 is critical for normal development of the endocrine pancreas as well as for production and release of insulin in adult β-cells.

Argonaute 2 or Ago2, a member of the argonaute protein family mediates the interaction of miRNAs with their target mRNAs and is part of the RNA-induced silencing complex (RISC) [14, 15]. In mammalian cells, miRNAs guide the RISC complex to target mRNAs and cause either degradation or translational repression of the target mRNAs. Recent data indicate that Ago2 is required for insulin secretion and β-cell compensatory expansion [16, 17]. Silencing of Ago2 in the mouse insulinoma MIN6 cell line enhanced insulin secretion [17]. Further studies suggested that mice lacking Ago2 are incapable of β-cell mass expansion in response to insulin resistance [16]. This defect was due to increased expression of miR-375 target mRNAs.

Previous studies showed that miR-375 is the most enriched miRNA in pancreatic β-cells and negatively regulates insulin secretion [3]. miR-375 is also important for endocrine pancreas development [18]. Thus, Ago2 appears to be important for directing miR-375 to its target mRNAs in β-cells. In conclusion, the data obtained with Dicer1 and Ago2 knockout mice suggests a critical role for miRNAs in pancreatic β-cell function. This chapter focuses on the role of miRNAs in regulation of endocrine pancreas development, insulin biosynthesis, insulin exocytosis, and β-cell expansion.

Endocrine Pancreas Development and Pancreas Regeneration

The pancreas has exocrine and endocrine functions. The exocrine pancreas consists of acinar and ductal cells and is responsible for the production and release of digestive enzymes into the duodenum. The endocrine system consists of the islets of Langerhans that are important in maintaining glucose homeostasis and make up only 1–2 % of pancreas. The islets consist of cell clusters containing five different types of cells, with the insulin-producing β-cells being the most prominent ones (50–80 %) and glucagon-producing α-cells as the second most abundant cell type [19–21].

Exocrine and endocrine cells originate from the same pool of progenitor cells in the gut endoderm [22, 23]. Pancreas development is morphologically visible around

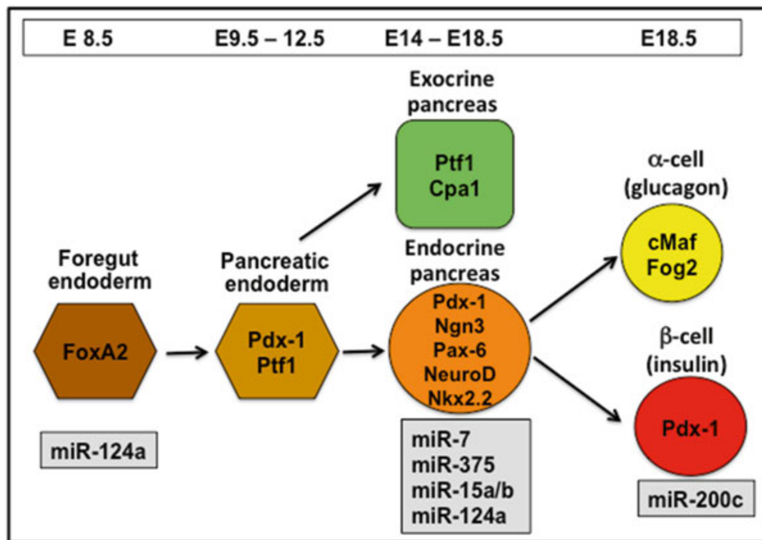

Fig. 6.1 miRNA regulation of endocrine pancreas development. The transcription factors involved in endocrine pancreas development are shown in *bold*. The miRNAs that target the listed transcription factors are displayed on the *bottom*. The approximate embryonic day (*E*) of pancreas development is shown on the *top*

embryonic day E8.5 (Fig. 6.1). Starting with embryonic day E14 the exocrine and endocrine pancreas develop from the pancreatic endoderm. Ngn3 is expressed at embryonic day E14 and marks the precursors of islets cells and the onset of endocrine cell differentiation [22]. From E15 on, the endocrine cell mass expands and is organized into well-shaped islets, expressing glucagon and insulin (Fig. 6.1).

Several miRNAs have been implicated in endocrine pancreas development, including miR-7, miR-15, miR-124a, miR-200, and miR-375. miR-7 has been shown to play a critical role in early endocrine pancreas development by negatively regulating the differentiation of α and β-cells [24]. Initial studies indicated that miR-7 is highly abundant in rodent and human islets [24–27]. Further studies demonstrated the co-localization of miR-7 with insulin and glucagon in the differentiating endocrine pancreas [24, 25]. However, miR-7 was not expressed in acinar or ductal cells. miR-7 levels were also highly upregulated during development of the human fetal pancreas, where miR-7 expression was observed at 9 weeks of gestational age. The expression of miR-7 was highest in endocrine cells around 14 and 18 weeks of gestational age, where the hormone levels start to rise [25, 26].

Kredo-Russo et al. [24] demonstrated that miR-7 acts downstream of Ngn3 and directly suppresses the expression of Pax6 (paired-domain transcription factor 6) in endocrine precursors. Pax6 is important for endocrine pancreas differentiation and specification of hormone producing endocrine cells [28, 29]. Consistent with the idea that miR-7 targets Pax6, overexpression of miR-7 in developing pancreas explants during embryonic day 12.5 caused a reduction in Pax6 as well as insulin

levels [24]. This was further confirmed using a knock-in model where the genomic sequence of miR-7a-1 was inserted into the Rosa26 locus and miR-7 expressed in the pancreatic lineage using Pdx-1-Cre mice. These mice displayed a significant reduction in insulin and glucagon mRNA levels at embryonic day 13.5. However, the expression of exocrine lineage markers Cpa1 and Ptf1a were not altered by overexpression of miR-7 [24]. These findings suggest that miR-7 negatively regulates α- and β-cell differentiation during early endocrine pancreas development by directly targeting Pax6.

Another miRNA that has been implicated in endocrine pancreas development and β-cell differentiation is miR-124a, which is encoded by three different genomic loci (miR-124a1-3) [30]. The expression level of the miR-124a2 isoform is significantly upregulated during endocrine pancreas development, especially at embryonic day E18.5, where β-cell specification occurs. miR-124a has been shown to target the transcription factor FoxA2, which is expressed during endocrine pancreas development and regulates β-cell differentiation [30].

β-Cell specification involves the induction of β-cell specific genes as well as suppression of $\alpha - cell$ specific genes. miR-200c that is highly abundant in pancreatic β-cells regulates β-cell specification by repressing glucagon gene expression [31]. It targets the α-cell specific transcription factors cMaf and Fog2 in β-cells that normally stimulate glucagon gene expression. miR-200c is not expressed in α-cells and forced expression of miR-200c in the α-cell line αTC6 inhibits cMaf and Fog2 levels and thereby reduces glucagon gene expression [31].

miR-375 is one of the most abundant miRNAs in pancreatic β-cells [3]. It is highly expressed in the developing endocrine pancreas and co-localizes with the β-cell transcription factor Pdx-1 (Fig. 6.1). Furthermore, it was shown that miR-375 is essential for islet formation in zebra fish [32]. Similar data were also obtained in mice by deletion of miR-375 [18]. These mice developed hyperglycemia and had decreased number of β-cells, but increased number of α-cells that led to excess production of glucagon. DNA microarray screens carried out in the miR-375 knockout mice revealed that miR-375 targets negative regulators of cell proliferation, including the growth suppressor gene Cadm1 [17, 18]. These data suggest that miR-375 may regulate β-cell differentiation by suppression of non-β-cell specific gene expression.

Ngn3, a marker of endocrine progenitor cells, is required for endocrine pancreas development and mice deleted for Ngn3 lack pancreatic islets [22, 23]. Ngn3 is not expressed in mature islets or during pancreas regeneration. Interestingly, Ngn3 transcript is detected in the regenerating pancreas after partial pancreatectomy, but there is no Ngn3 protein present [33–35]. This suggested that the translation of the Ngn3 transcript might be suppressed by miRNAs during pancreas regeneration. In agreement with this idea, three miRNAs, miR-15a, miR-15b, and miR-124a, were found to target Ngn3 in the regenerating pancreas [35]. All three miRNAs are highly upregulated after partial pancreatectomy. Furthermore, inhibition of miR-15 led to increased expression of Ngn3 and its target genes NeuroD and Nkx2.2 in the regenerating pancreas [35] (Fig. 6.1). Thus, increased expression of miR-15a/b and miR-124a during pancreas regeneration downregulates Ngn3 and thereby initiates the regeneration process.

Insulin Biosynthesis

Insulin biosynthesis in pancreatic β-cells is regulated by changes in blood glucose levels and is mediated by three β-cell specific transcription factors Pdx-1, MafA, and NeuroD (Fig. 6.2) [36]. These transcription factors act synergistically to stimulate insulin gene expression in response to increasing blood glucose levels. In addition to these transcriptional activators, several transcriptional repressors are also involved in suppressing insulin biosynthesis, including the repressors Sox6 and Bhlhe22 (Fig. 6.2). In contrast to humans, rodents have two insulin genes (insulin 1 and 2) and insulin 2 expression and regulation is most similar to that of humans [37]. Several miRNAs, including miR-24, miR-30d, and miR-30a, have been shown to regulate insulin biosynthesis by modulating the expression of β-cell specific transcription factors (Fig. 6.2).

The first evidence that miRNAs are crucial for insulin biosynthesis was provided by ablation of Dicer1 in adult pancreatic β-cells by several groups [12, 13]. These studies utilized the rat insulin promoter RIP-Cre to delete Dicer1 in adult β-cells. Dicer1 deficient animals developed hyperglycemia and diabetes due to decreased insulin mRNA levels and insulin content, while endocrine pancreas development was normal [12, 13]. One group found that these mice had abnormal islet morphology and decreased β-cell mass [13]. Another group demonstrated that insulin 1 and insulin 2 mRNA levels were decreased by 70 % in Dicer1 deficient animals. Such a

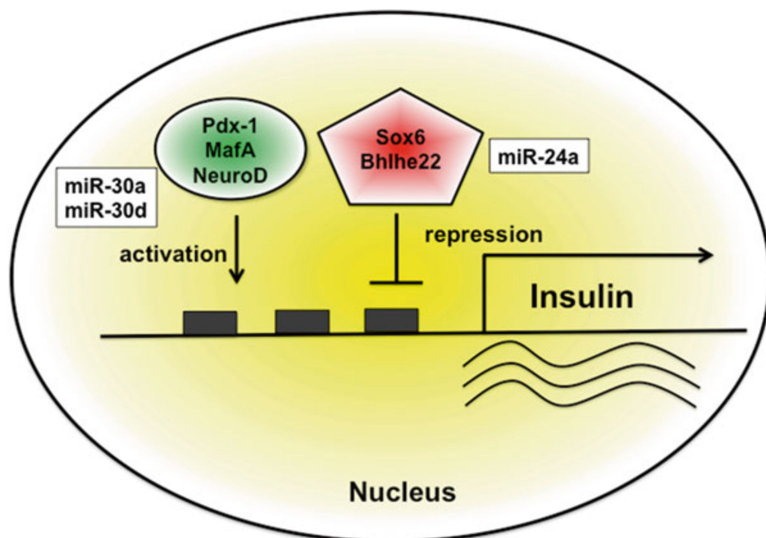

Fig. 6.2 Regulation of insulin gene transcription by miRNAs. The transcription factors Pdx-1, MafA, and NeuroD activate insulin gene transcription in a glucose-dependent manner and are regulated by miR-30a and miR-30d. Sox6 and Bhlhe22 are transcriptional repressors of the insulin gene and are targeted by miR-24a

reduction in insulin levels was associated with increased expression of Sox6 and Bhlhe22, which function as transcriptional repressors of the insulin gene [11] (Fig. 6.2). Further studies with these Dicer1 deficient mice identified four miRNAs, miR-24, miR-26, miR-148, and miR-182, that may be responsible for the observed reduction in insulin biosynthesis [11].

Inhibition of miR-24 in primary islets decreased insulin mRNA levels, but increased the levels of the transcriptional repressors Sox6 [11, 38]. In conclusion, ablation of Dicer1 in adult pancreatic β-cells reduces the levels of miR-24, which leads to increased Sox6 protein levels and repression of insulin gene expression.

miR-30a and miR-30d belong to the miR-30 family of miRNAs, which consist of five members and have been shown to modulate insulin gene transcription by regulating the levels of β-cell transcription factors [39, 40]. miR-30d levels are upregulated in response to hyperglycemia in pancreatic β-cells. Overexpression of miR-30d in insulinoma cell lines and pancreatic islets increased the levels of the insulin gene transcription factor MafA [39]. miR-30d increases MafA and thereby insulin mRNA levels by targeting the TNF-α activated kinase Map4k4 [41]. How suppression of Map4k4 by miR-30d increases MafA and insulin mRNA levels remains to be established.

miR-30a-5p levels are upregulated in pancreatic β-cells during glucotoxic conditions and also in islets of the obese and diabetic db/db animals [40]. Overexpression of miR-30a-5p downregulated insulin biosynthesis and insulin secretion by targeting the β-cell transcription factor β2/NeuroD. Consistent with the idea that miR-30a-5p negatively regulates insulin production and secretion. Injection of anti-miR-30a-5p oligonucleotides into the obese and diabetic db/db animals significantly reduced the non-fasting blood glucose levels [40]. Although mature miR-30a-5p and miR-30d share the same seed sequence and differ only by one nucleotide, they regulate insulin biosynthesis by targeting different genes in pancreatic β-cells.

Insulin Exocytosis

Insulin secretion from pancreatic β-cells is a highly dynamic process that is primarily regulated by blood glucose levels. Glucose-stimulated insulin secretion (GSIS) starts with the uptake of glucose into β-cells by glucose transporter Glut2 in rodents and Glut1 in humans. Glucose is then metabolized in glycolysis to pyruvate, which enters the mitochondria where it is converted to acetyl CoA (Fig. 6.3). The TCA cycle oxidizes acetyl CoA to carbon dioxide and the electrons harvested in the TCA cycle are used in oxidative phosphorylation for ATP production.

The increase in ATP/ADP ratio leads to the closure of ATP-sensitive potassium channels causing the depolarization of the plasma membrane. This depolarization event leads to opening of the voltage-dependent calcium channels and influx of calcium that mediates the fusion of the insulin granules with the plasma membrane leading to release of insulin into blood stream [42, 43] (Fig. 6.3). The miRNAs

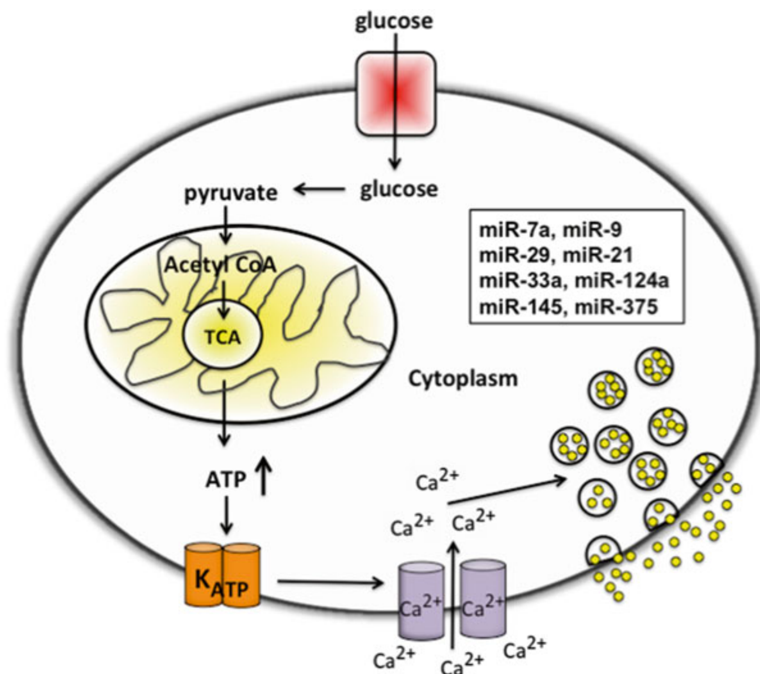

Fig. 6.3 miRNA regulation of insulin exocytosis. Insulin release by glucose involves the uptake and oxidation of glucose, which causes an increase in the ATP/ADP ratio. This leads to closure of the ATP-sensitive potassium channel and depolarization of the plasma membrane, followed by opening of the voltage dependent calcium channel. The influx of calcium triggers the fusion of the insulin granules with the plasma membrane and the release of insulin

miR7a, miR-9, miR-21, miR-29, miR-33a, miR-124a, miR-145, and miR-375 play a significant role in regulation of insulin secretion and are discussed below.

Previous data suggest that miR-7a levels are significantly decreased in human islets of obese and diabetic patients [44]. This decrease was also observed in islets of obese and diabetic mice. Studies on miR-7a2 function in mice demonstrated that miR-7a2 deficient mice display increased insulin secretion. This was due to upregulation of proteins involved in regulation of insulin granule fusion with the plasma membrane in pancreatic β-cells, including Snca, Cspa, Cplx1, Pfn2, Wipf2, Phactr1, and Zdhhc9 [44]. These proteins were shown to be direct targets of miR-7. As expected, overexpression of miR-7a2 in mice resulted in inhibition of insulin secretion and the development of diabetes [44]. These data establish miR-7 as a negative regulator of insulin secretion in pancreatic β-cells.

Another negative regulator of insulin secretion is miR-9, which is highly expressed in brain and pancreatic islets [45]. miR-9 levels are upregulated in response to high glucose in the INS-1E cell line as well as mouse pancreatic islets [46]. Increased levels of miR-9 suppressed insulin secretion in response to glucose

and potassium chloride in the pancreatic β-cell line INS-1E [45]. These findings suggest that miR-9 inhibits insulin secretion at the level of the exocytosis machinery. It was discovered that overexpression of miR-9 increases the levels of granuphilin (Slp4), a Rab3/Rab27 GTPase effector that is associated with the insulin secretory granules [45, 47]. Insulin secretion is significantly increased in granuphilin deficient mice, suggesting a negative role for granuphilin in insulin exocytosis. Granuphilin suppresses insulin exocytosis by regulating the docking of insulin granules to the plasma membrane [48]. Analysis of potential miR-9 targets using the TargetScan database identified the transcription factor Onecut2 [45]. Overexpression of miR-9 reduced Onecut2 levels and increased granuphilin levels. Onecut2 has been shown to bind to the granuphilin promoter and repress the transcription of the granuphilin gene. Silencing of Onecut2 expression increases granuphilin levels and thereby leads to inhibition of GSIS, mimicking the overexpression of miR-9. The conclusion from these data is that miR-9 inhibits insulin secretion by targeting Onecut2 and thereby increases granuphilin levels. Another miR-9 target that may mediate the negative effect of miR-9 on insulin exocytosis was identified as Sirt1, which deacetylates proteins in a NAD-dependent manner [46]. Overexpression of miR-9 has been shown to downregulate Sirt1 protein levels. Sirt1 is a positive regulator of insulin secretion and overexpression of Sirt1 in pancreatic β-cells enhances GSIS by downregulating the levels of the uncoupling protein UCP2 [49, 50]. In summary, miR-9 negatively regulates insulin secretion by targeting Onecut2 and Sirt1 in pancreatic β-cells.

miR-375 was one of the first miRNAs identified from pancreatic β-cells and shown to be a negative regulator of insulin secretion [3]. Initial studies indicated that miR-375 targets myotrophin (Mtpn), which regulates actin depolymerization and insulin granule fusion [3, 51]. This implicated miR-375 in regulation of insulin granule fusion with plasma membrane. Recent studies using the MIN6 insulinoma cells demonstrated that miR-375 is involved in global regulation of GSIS by directly targeting the expression of genes relevant for insulin exocytosis, including Gephyrin, Ywhaz, Aifm1, and Mtpn [17]. This study also discovered that miR-375 was the most enriched miRNA associated with Ago2-ribonucleotide protein complexes. Moreover, it was shown that loss of Ago2 in MIN6 cells enhances insulin release by causing increased expression of miR-375 target genes [17]. In summary, miR-375 and Ago2 have overlapping functions in pancreatic β-cells and inhibit GSIS.

miR-124a was found in a screen for miRNAs that regulate basal insulin secretion. Overexpression of miR-124a in MIN6 cells enhanced basal insulin secretion, but decreased GSIS. Upregulation of miR-124a levels was also associated with increased expression of SNAP25, Rab3A, and Syn1, and decreased levels of Rab27A and Noc2, which are involved in regulation of insulin exocytosis [52]. Further studies revealed that only Rab27A was a direct target of miR-124a. miR-124a has been previously shown to regulate endocrine pancreas development by targeting FoxA2, a transcription factor required for expression of β-cell specific genes [30]. FoxA2 also regulates the expression of K_{ATP} channel subunits Kir6.2 and Sur1, which are critical for insulin exocytosis. Therefore, some of the effects of miR-124a on insulin secretion may be mediated via its target FoxA2.

miR-21 levels are upregulated by cytokines and overexpression of miR-21 in MIN6 β-cells decreased insulin secretion by glucose [53]. The decrease in GSIS by miR-21 was associated with downregulation of VAMP2 and Rab3a levels [53]. VAMP2 is a SNARE protein and Rab3 is a GTPase and both are involved in insulin exocytosis [54, 55]. Treatment of MIN6 β-cells with IL-1β for 24 h also resulted in decreased GSIS and downregulation of VAMP2 and Rab3a levels. Inhibition of miR-21 in MIN6 cells treated with IL-1β prevented the decrease in GSIS and VAMP2, but had no effect on Rab3a levels [53]. VAMP2 does not contain any miR-21 binding sites and, thus, appears to be not a direct target of miR-21. In conclusion, exposure to proinflammatory cytokines inhibits insulin secretion by upregulation of miR-21 levels.

Elevated cholesterol levels in pancreatic β-cells interfere with GSIS. miR-33a and mir-145, two miRNAs involved in regulation of cholesterol levels, have been shown to negatively regulate GSIS. Overexpression of miR-33a and miR-145 in mouse islets increases intracellular cholesterol levels by downregulating the levels of the ATP-binding cassette transporter 1 or Abca1, which mediates cholesterol efflux [56, 57]. Consistent with a role for Abca1 in insulin secretion, mice that lack Abca1 display β-cell dysfunction [58]. Inhibition of miR-33a and miR-145 in pancreatic islets enhances GSIS by increasing the levels of Abca1, which leads to increased cholesterol efflux [56, 57]. In conclusion, increased levels of miR-33a and miR-145 negatively regulate GSIS in pancreatic β-cells by targeting Abca1 transporter and thereby reducing cholesterol efflux.

miR-132 and miR-212 levels have been previously shown to be upregulated in pancreatic islets of obesity-induced diabetic animals [5, 59]. miR-132 and miR-212 are about 200 bp separated from each other and are likely to be generated from a common pri-mRNA [60]. Overexpression of miR-132 and miR-212 enhance GSIS by targeting the carnitine acyl-carnitine translocase (CACT; Slc25a20), which is involved in transport of long-chain acyl-carnitines into the mitochondria for β-oxidation [61]. Consistent with the idea that CACT regulates insulin secretion in pancreatic β-cells, silencing of CACT in INS-1 β-cells resulted in accumulation of fatty acyl-carnitines and increased insulin exocytosis [61, 62]. Furthermore, treatment of INS-1 cells and mouse islets with long-chain fatty acyl-carnitines promoted insulin secretion [61]. Interestingly, the stimulation of insulin release by long-chain fatty acyl-carnitine was independent of their degradation by β-oxidation. It has been well established that chronic exposure to fatty acids inhibits insulin secretion, while acute exposure to fatty acids stimulates insulin release. In conclusion, miR-132 and miR-212 enhance insulin secretion by targeting CACT and thereby increasing the concentration of long-chain fatty acyl-carnitines in the cytoplasm.

β-Cell Inflammation

Type 1 diabetes is caused by an autoimmune destruction of the pancreatic β-cells. Proinflammatory cytokines, including IL-1β, TNF-α, and IFN-γ, play an important role in this process. These proinflammatory cytokines, which are produced by

infiltrating leukocytes and islets cells, contribute to β-cell failure and establishment of diabetes [63]. miR-21 levels have been shown to be upregulated by cytokines in MIN6 cells as well as in pancreatic islets of prediabetic NOD mice that display normal blood glucose levels [53, 64]. These findings suggest that miR-21 plays a critical role in regulation of β-cell apoptosis during type 1 diabetes. In agreement with this idea, miR-21 was shown to suppress β-cell apoptosis by decreasing the levels of the tumor suppressor protein Pdcd4 that induces cell death by activation of Bax family of proteins [64, 65]. Ablation of Pdcd4 in pancreatic β-cells protects them from cell death in NOD and in STZ-treated C57BL/6 mice. Interestingly, miR-21 levels were found to be upregulated by members of the $NF\kappa_B$ family, c-Rel, and p65 [64]. The implications from these data are that the $NF\kappa_B$-miR-21-Pdcd4 axis regulates β-cell death during type 1 diabetes. Similar to miR-21, miR-29 is also upregulated by exposure to proinflammatory cytokines in pancreatic islets of prediabetic NOD mice and in isolated mouse and human islets [66]. Overexpression of miR-29 promoted β-cell apoptosis that was associated with downregulation of the anti-apoptotic protein Mcl1 [66]. In summary, these data suggest that miR-21 and miR-29 play a crucial role in cytokine-induced β-cell dysfunction during prediabetes.

β-Cell Replication/Expansion

Since diabetes is caused by β-cell failure, induction of pancreatic β-cell proliferation is a major goal for treatment of diabetes. However adult pancreatic β-cells have an extremely low replication rate [67]. Detailed understanding of the mechanisms responsible for the low replication rate of human adult β-cells may lead to novel strategies for treatment of diabetes. Recent data suggest that miRNAs are involved in regulation of β-cell replication and expansion.

The mature form of miR-7 is encoded by three different genomic loci in mice and humans. miR-7a has been shown to be the major isoform and miR-7a-2 the major precursor that is expressed in adult pancreatic β-cells [68]. Studies in mouse and human islets indicate that miR-7a is an inhibitor of β-cell proliferation and targets the mTOR-signaling pathway. Inhibition of miR-7a promoted adult β-cell replication in mouse and human islets by activation of mTOR signaling that was blocked by treatment with rapamycin [68]. The mTOR-signaling pathway plays a critical role in regulating metabolism and cell proliferation in response to nutrient availability and induces β-cell replication and expansion [69, 70]. Further studies suggested that miR-7a regulates the mTOR pathway by targeting S6k1, eIF4E, Mknk1, Mknk2, and Mapkap1, which are components of the mTOR-signaling pathway [68]. Thus, miR-7a is a potential target for miRNA therapy to promote adult β-cell replication to replace the β-cells lost in the course of type 2 diabetes.

β-Cells can compensate for insulin resistance by increasing the insulin secretory capacity and β-cell mass [71]. This is especially evident during obesity. miR-184 is the only miRNA that has been implicated to date in β-cell compensatory expansion during insulin resistance [16]. Recent data suggest that miR-184 is silenced in the

pancreatic islets of insulin-resistant mice and humans. The silencing of miR-184 was associated with upregulation of Ago2 levels, which coincided with increased β-cell expansion. Forced expression of miR-184 in ob/ob animals that display obesity-induced diabetes resulted in downregulation of Ago2 levels and prevention of the compensatory β-cell expansion process [16]. Ago2 is part of the RISC complex and is required for miRNA-guided targeting of mRNAs [14]. Loss of Ago2 blocks the compensatory expansion of β-cells in response to insulin resistance by increasing the expression of miR-375 targets, including the growth suppressor Cadm1 [17]. These data suggest that miR-184 negatively regulates β-cell expansion by targeting Ago2.

The thioredoxin-interacting protein (TXNIP) is a cellular redox regulator that is upregulated in pancreatic β-cells during diabetic conditions and has been implicated in causing β-cell death [72, 73]. Lack of TXNIP protects mice against diabetes by increasing β-cell mass and inhibiting β-cell apoptosis [74]. Recent data suggest that the expression of miR-200 family members (miR-200a/b/c, miR-141, and miR-429) are increased by TXNIP in INS-1 β-cells and mouse islets, and overexpression of miR-200 family members promotes β-cell apoptosis [75]. Previous studies in cancer cells implicated miR-200 in inhibition of epithelial–mesenchymal transition (EMT) by targeting the transcriptional repressor Zeb1 and thereby increasing the levels of E-cadherin transmembrane protein [76, 77]. Like in cancer cells, miR-200 has been shown to target Zeb1 also in pancreatic β-cells and increases E-cadherin expression [75]. Increased expression of E-cadherin inhibits EMT and promotes β-cell apoptosis. In conclusion, during diabetic conditions TXNIP levels are increased, which lead to upregulation of miR-200 that then suppresses Zeb1 levels leading to increased E-cadherin expression. Thus, the activation of the TXNIP-miR-200-Zeb1-E-cadherin pathway promotes β-cell apoptosis by inhibition of EMT and β-cell expansion.

Circulating miRNAs as Biomarkers for Diabetes

Circulating miRNAs identified from type 2 diabetes patients have the potential to serve as new biomarkers and therapeutic targets. The levels of several miRNAs are increased in the serum of diabetic patients as well as diabetic animals [78]. miR-375 levels have been shown to be increased in the circulation of STZ-treated and nonobese diabetic NOD mice [79]. Interestingly, miR-375 levels were dramatically increased in the STZ-treated mice before the onset of hyperglycemia. In the NOD mice, miR-375 levels were increased in the circulation 2 weeks prior to diabetes onset [79]. These data indicate that circulating levels of miR-375 may serve as a potential biomarker for diagnosis of prediabetes.

Conclusions

Recent studies have clearly established a role for miRNAs in endocrine pancreas development as well as many aspects of β-cell function, including insulin biosynthesis and insulin exocytosis. However the number of miRNAs with established functions in β-cells is fairly small given the fact that the human gene encodes for over 2500 miRNAs [80]. This suggests that there are many more miRNAs with specific function in pancreatic β-cells that remain to be identified. One limitation in analysis of miRNA function in pancreatic β-cells as well as in other tissues is that most studies focus on a single miRNA. However, the 3′-UTR of most mRNAs contain binding sites for two or more miRNAs, indicating that a single mRNA is likely to be regulated by more than one miRNA. Several of the miRNAs involved in β-cell function have isoforms that are encoded by different genomic loci that result in the same mature miRNA. This is the case for miR-7 and miR-9, which are encoded by three different genomic loci, leading to the same mature miRNA. This raises the question whether pre- or pri-miRNAs may also have a function that is different from that of the mature miRNA.

Another interesting aspect of miRNA function is that the same miRNA may regulate different processes within the β-cell. miRNA-375 has been shown to influence endocrine pancreas development as well as insulin secretion by targeting different mRNAs [3, 18]. Several miRNAs have been demonstrated to be abundant in the circulation of type 2 diabetics and may serve as diagnostic markers for prediabetes. miRNAs are ideal biomarkers for diabetes and other diseases because of their high stability in the circulation, high sensitivity, and tissue specificity. Detailed understanding of how the levels of various miRNAs are regulated during physiological as well as pathophysiological conditions will be crucial in the development of novel strategies to prevent β-cell failure. Moreover the development of new techniques that enable the analysis of genome-wide transcription and of the proteome at the single β-cell level will advance our understanding of metabolic regulation by miRNAs. The field of miRNA-based therapeutics has been emerging and several miRNA candidates are in preclinical development. The therapeutic development of β-cell specific miRNAs will provide powerful strategies in the treatment of diabetes associated with β-cell dysfunction.

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References

1. U.K. prospective diabetes study 16. Overview of 6 years' therapy of type II diabetes: a progressive disease. U.K. Prospective Diabetes Study Group. Diabetes. 1995;44(11):1249–58.
2. Levy J, Atkinson AB, Bell PM, McCance DR, Hadden DR. Beta-cell deterioration determines the onset and rate of progression of secondary dietary failure in type 2 diabetes mellitus: the 10-year follow-up of the Belfast diet study. Diabet Med. 1998;15(4):290–6.

3. 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.
4. Dumortier O, Van Obberghen E. MicroRNAs in pancreas development. Diabetes Obes Metab. 2012;14 Suppl 3:22–8.
5. Nesca V, Guay C, Jacovetti C, Menoud V, Peyot ML, Laybutt DR, et al. Identification of particular groups of microRNAs that positively or negatively impact on beta cell function in obese models of type 2 diabetes. Diabetologia. 2013;56(10):2203–12.
6. Ozcan S. Minireview: microRNA function in pancreatic beta cells. Mol Endocrinol. 2014;28(12):1922–33.
7. Singer RA, Arnes L, Sussel L. Noncoding RNAs in beta cell biology. Curr Opin Endocrinol Diabetes Obes. 2015;22(2):77–85.
8. Harfe BD, McManus MT, Mansfield JH, Hornstein E, Tabin CJ. The RNaseIII enzyme Dicer is required for morphogenesis but not patterning of the vertebrate limb. Proc Natl Acad Sci U S A. 2005;102(31):10898–903.
9. 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.
10. Lynn FC, Skewes-Cox P, Kosaka Y, McManus MT, Harfe BD, German MS. MicroRNA expression is required for pancreatic islet cell genesis in the mouse. Diabetes. 2007;56(12): 2938–45.
11. Melkman-Zehavi T, Oren R, Kredo-Russo S, Shapira T, Mandelbaum AD, Rivkin N, et al. miRNAs control insulin content in pancreatic beta-cells via downregulation of transcriptional repressors. EMBO J. 2011;30(5):835–45.
12. Kalis M, Bolmeson C, Esguerra JL, Gupta S, Edlund A, Tormo-Badia N, et al. Beta-cell specific deletion of Dicer1 leads to defective insulin secretion and diabetes mellitus. PLoS One. 2011;6(12), e29166.
13. Mandelbaum AD, Melkman-Zehavi T, Oren R, Kredo-Russo S, Nir T, Dor Y, et al. Dysregulation of Dicer1 in beta cells impairs islet architecture and glucose metabolism. Exp Diabetes Res. 2012;2012:470302.
14. Meister G. Argonaute proteins: functional insights and emerging roles. Nat Rev Genet. 2013;14(7):447–59.
15. Dueck A, Meister G. Assembly and function of small RNA—argonaute protein complexes. Biol Chem. 2014;395(6):611–29.
16. Tattikota SG, Rathjen T, McAnulty SJ, Wessels HH, Akerman I, van de Bunt M, et al. Argonaute2 mediates compensatory expansion of the pancreatic beta cell. Cell Metab. 2014;19(1):122–34.
17. Tattikota SG, Sury MD, Rathjen T, Wessels HH, Pandey AK, You X, et al. Argonaute2 regulates the pancreatic beta-cell secretome. Mol Cell Proteomics. 2013;12(5):1214–25.
18. Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, et al. miR-375 maintains normal pancreatic alpha- and beta-cell mass. Proc Natl Acad Sci U S A. 2009;106(14): 5813–8.
19. Shih HP, Wang A, Sander M. Pancreas organogenesis: from lineage determination to morphogenesis. Annu Rev Cell Dev Biol. 2013;29:81–105.
20. Murtaugh LC. Pancreas and beta-cell development: from the actual to the possible. Development. 2007;134(3):427–38.
21. Docherty K. Pancreatic stellate cells can form new beta-like cells. Biochem J. 2009;421(2): e1–4.
22. Gu G, Dubauskaite J, Melton DA. Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are distinct from duct progenitors. Development. 2002;129(10):2447–57.
23. Gradwohl G, Dierich A, LeMeur M, Guillemot F. Neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas. Proc Natl Acad Sci U S A. 2000;97(4):1607–11.
24. Kredo-Russo S, Mandelbaum AD, Ness A, Alon I, Lennox KA, Behlke MA, et al. Pancreas-enriched miRNA refines endocrine cell differentiation. Development. 2012;139(16):3021–31.

25. Correa-Medina M, Bravo-Egana V, Rosero S, Ricordi C, Edlund H, Diez J, et al. MicroRNA miR-7 is preferentially expressed in endocrine cells of the developing and adult human pancreas. Gene Expr Patterns. 2009;9(4):193–9.
26. Joglekar MV, Patil D, Joglekar VM, Rao GV, Reddy DN, Mitnala S, et al. The miR-30 family of microRNAs confer epithelial phenotype to human pancreatic cells. Islets. 2009;1(2):137–47.
27. Bravo-Egana V, Rosero S, Molano RD, Pileggi A, Ricordi C, Dominguez-Bendala J, et al. Quantitative differential expression analysis reveals miR-7 as major islet microRNA. Biochem Biophys Res Commun. 2008;366(4):922–6.
28. St-Onge L, Sosa-Pineda B, Chowdhury K, Mansouri A, Gruss P. Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas. Nature. 1997;387(6631):406–9.
29. Sander M, Neubuser A, Kalamaras J, Ee HC, Martin GR, German MS. Genetic analysis reveals that PAX6 is required for normal transcription of pancreatic hormone genes and islet development. Genes Dev. 1997;11(13):1662–73.
30. Baroukh N, Ravier MA, Loder MK, Hill EV, Bounacer A, Scharfmann R, et al. MicroRNA-124a regulates Foxa2 expression and intracellular signaling in pancreatic beta-cell lines. J Biol Chem. 2007;282(27):19575–88.
31. Klein D, Misawa R, Bravo-Egana V, Vargas N, Rosero S, Piroso J, et al. MicroRNA expression in alpha and beta cells of human pancreatic islets. PLoS One. 2013;8(1), e55064.
32. Kloosterman WP, Lagendijk AK, Ketting RF, Moulton JD, Plasterk RH. Targeted inhibition of miRNA maturation with morpholinos reveals a role for miR-375 in pancreatic islet development. PLoS Biol. 2007;5(8), e203.
33. Lee CS, De Leon DD, Kaestner KH, Stoffers DA. Regeneration of pancreatic islets after partial pancreatectomy in mice does not involve the reactivation of neurogenin-3. Diabetes. 2006;55(2):269–72.
34. Joglekar MV, Parekh VS, Hardikar AA. New pancreas from old: microregulators of pancreas regeneration. Trends Endocrinol Metab. 2007;18(10):393–400.
35. Joglekar MV, Parekh VS, Mehta S, Bhonde RR, Hardikar AA. MicroRNA profiling of developing and regenerating pancreas reveal post-transcriptional regulation of neurogenin3. Dev Biol. 2007;311(2):603–12.
36. Andrali SS, Sampley ML, Vanderford NL, Ozcan S. Glucose regulation of insulin gene expression in pancreatic beta-cells. Biochem J. 2008;415(1):1–10.
37. Steiner DF, Chan SJ, Welsh JM, Kwok SC. Structure and evolution of the insulin gene. Annu Rev Genet. 1985;19:463–84.
38. Iguchi H, Ikeda Y, Okamura M, Tanaka T, Urashima Y, Ohguchi H, et al. SOX6 attenuates glucose-stimulated insulin secretion by repressing PDX1 transcriptional activity and is down-regulated in hyperinsulinemic obese mice. J Biol Chem. 2005;280(45):37669–80.
39. Tang X, Muniappan L, Tang G, Ozcan S. Identification of glucose-regulated miRNAs from pancreatic b cells reveals a role for miR-30d in insulin transcription. RNA. 2009;15(2):287–93.
40. Kim JW, You YH, Jung S, Suh-Kim H, Lee IK, Cho JH, et al. miRNA-30a-5p-mediated silencing of Beta2/NeuroD expression is an important initial event of glucotoxicity-induced beta cell dysfunction in rodent models. Diabetologia. 2013;56(4):847–55.
41. Zhao X, Mohan R, Ozcan S, Tang X. MicroRNA-30d induces insulin transcription factor MafA and insulin production by targeting mitogen-activated protein 4 kinase 4 (MAP4K4) in pancreatic beta-cells. J Biol Chem. 2012;287(37):31155–64.
42. Henquin JC. Triggering and amplifying pathways of regulation of insulin secretion by glucose. Diabetes. 2000;49(11):1751–60.
43. Seino S, Shibasaki T, Minami K. Dynamics of insulin secretion and the clinical implications for obesity and diabetes. J Clin Invest. 2011;121(6):2118–25.
44. Latreille M, Hausser J, Stutzer I, Zhang Q, Hastoy B, Gargani S, et al. MicroRNA-7a regulates pancreatic beta cell function. J Clin Invest. 2014;124(6):2722–35.

45. Plaisance V, Abderrahmani A, Perret-Menoud V, Jacquemin P, Lemaigre F, Regazzi R. MicroRNA-9 controls the expression of Granuphilin/Slp4 and the secretory response of insulin-producing cells. J Biol Chem. 2006;281(37):26932–42.
46. Ramachandran D, Roy U, Garg S, Ghosh S, Pathak S, Kolthur-Seetharam U. Sirt1 and mir-9 expression is regulated during glucose-stimulated insulin secretion in pancreatic beta-islets. FEBS J. 2011;278(7):1167–74.
47. Fukuda M. Rab27 and its effectors in secretory granule exocytosis: a novel docking machinery composed of a Rab27.effector complex. Biochem Soc Trans. 2006;34(Pt 5):691–5.
48. Gomi H, Mizutani S, Kasai K, Itohara S, Izumi T. Granuphilin molecularly docks insulin granules to the fusion machinery. J Cell Biol. 2005;171(1):99–109.
49. Moynihan KA, Grimm AA, Plueger MM, Bernal-Mizrachi E, Ford E, Cras-Meneur C, et al. Increased dosage of mammalian Sir2 in pancreatic beta cells enhances glucose-stimulated insulin secretion in mice. Cell Metab. 2005;2(2):105–17.
50. Bordone L, Motta MC, Picard F, Robinson A, Jhala US, Apfeld J, et al. Sirt1 regulates insulin secretion by repressing UCP2 in pancreatic beta cells. PLoS Biol. 2006;4(2), e31.
51. Poy MN, Spranger M, Stoffel M. MicroRNAs and the regulation of glucose and lipid metabolism. Diabetes Obes Metab. 2007;9 Suppl 2:67–73.
52. Lovis P, Gattesco S, Regazzi R. Regulation of the expression of components of the exocytotic machinery of insulin-secreting cells by microRNAs. Biol Chem. 2008;389(3):305–12.
53. Roggli E, Britan A, Gattesco S, Lin-Marq N, Abderrahmani A, Meda P, et al. Involvement of microRNAs in the cytotoxic effects exerted by proinflammatory cytokines on pancreatic beta-cells. Diabetes. 2010;59(4):978–86.
54. Regazzi R, Sadoul K, Meda P, Kelly RB, Halban PA, Wollheim CB. Mutational analysis of VAMP domains implicated in Ca2+-induced insulin exocytosis. EMBO J. 1996;15(24):6951–9.
55. Yaekura K, Julyan R, Wicksteed BL, Hays LB, Alarcon C, Sommers S, et al. Insulin secretory deficiency and glucose intolerance in Rab3A null mice. J Biol Chem. 2003;278(11):9715–21.
56. Kang MH, Zhang LH, Wijesekara N, de Haan W, Butland S, Bhattacharjee A, et al. Regulation of ABCA1 protein expression and function in hepatic and pancreatic islet cells by miR-145. Arterioscler Thromb Vasc Biol. 2013;33(12):2724–32.
57. Wijesekara N, Zhang LH, Kang MH, Abraham T, Bhattacharjee A, Warnock GL, et al. miR-33a modulates ABCA1 expression, cholesterol accumulation, and insulin secretion in pancreatic islets. Diabetes. 2012;61(3):653–8.
58. Brunham LR, Kruit JK, Pape TD, Timmins JM, Reuwer AQ, Vasanji Z, et al. Beta-cell ABCA1 influences insulin secretion, glucose homeostasis and response to thiazolidinedione treatment. Nat Med. 2007;13(3):340–7.
59. Zhao E, Keller MP, Rabaglia ME, Oler AT, Stapleton DS, Schueler KL, et al. Obesity and genetics regulate microRNAs in islets, liver, and adipose of diabetic mice. Mamm Genome. 2009;20(8):476–85.
60. Vo N, Klein ME, Varlamova O, Keller DM, Yamamoto T, Goodman RH, 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.
61. Soni MS, Rabaglia ME, Bhatnagar S, Shang J, Ilkayeva O, Mynatt R, et al. Downregulation of carnitine acyl-carnitine translocase by miRNAs 132 and 212 amplifies glucose-stimulated insulin secretion. Diabetes. 2014;63(11):3805–14.
62. Prentki M, Matschinsky FM, Madiraju SR. Metabolic signaling in fuel-induced insulin secretion. Cell Metab. 2013;18(2):162–85.
63. Eizirik DL, Colli ML, Ortis F. The role of inflammation in insulitis and beta-cell loss in type 1 diabetes. Nat Rev Endocrinol. 2009;5(4):219–26.
64. Ruan Q, Wang T, Kameswaran V, Wei Q, Johnson DS, Matschinsky F, et al. The microRNA-21-PDCD4 axis prevents type 1 diabetes by blocking pancreatic beta cell death. Proc Natl Acad Sci U S A. 2011;108(29):12030–5.

65. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem. 2008;283(2):1026–33.
66. Roggli E, Gattesco S, Caille D, Briet C, Boitard C, Meda P, et al. Changes in microRNA expression contribute to pancreatic beta-cell dysfunction in prediabetic NOD mice. Diabetes. 2012;61(7):1742–51.
67. Bernal-Mizrachi E, Kulkarni RN, Scott DK, Mauvais-Jarvis F, Stewart AF, Garcia-Ocana A. Human beta-cell proliferation and intracellular signaling part 2: still driving in the dark without a road map. Diabetes. 2014;63(3):819–31.
68. Wang Y, Liu J, Liu C, Naji A, Stoffers DA. MicroRNA-7 regulates the mTOR pathway and proliferation in adult pancreatic beta-cells. Diabetes. 2013;62(3):887–95.
69. Zoncu R, Efeyan A, Sabatini DM. mTOR: from growth signal integration to cancer, diabetes and ageing. Nat Rev Mol Cell Biol. 2011;12(1):21–35.
70. Hamada S, Hara K, Hamada T, Yasuda H, Moriyama H, Nakayama R, et al. Upregulation of the mammalian target of rapamycin complex 1 pathway by Ras homolog enriched in brain in pancreatic beta-cells leads to increased beta-cell mass and prevention of hyperglycemia. Diabetes. 2009;58(6):1321–32.
71. Araujo TG, Oliveira AG, Saad MJ. Insulin-resistance-associated compensatory mechanisms of pancreatic Beta cells: a current opinion. Front Endocrinol. 2013;4:146.
72. Minn AH, Hafele C, Shalev A. Thioredoxin-interacting protein is stimulated by glucose through a carbohydrate response element and induces beta-cell apoptosis. Endocrinology. 2005;146(5):2397–405.
73. Chen J, Saxena G, Mungrue IN, Lusis AJ, Shalev A. Thioredoxin-interacting protein: a critical link between glucose toxicity and beta-cell apoptosis. Diabetes. 2008;57(4):938–44.
74. Chen J, Hui ST, Couto FM, Mungrue IN, Davis DB, Attie AD, et al. Thioredoxin-interacting protein deficiency induces Akt/Bcl-xL signaling and pancreatic beta-cell mass and protects against diabetes. FASEB J. 2008;22(10):3581–94.
75. Filios SR, Xu G, Chen J, Hong K, Jing G, Shalev A. MicroRNA-200 is induced by thioredoxin-interacting protein and regulates Zeb1 protein signaling and beta cell apoptosis. J Biol Chem. 2014;289(52):36275–83.
76. Bracken CP, Gregory PA, Kolesnikoff N, Bert AG, Wang J, Shannon MF, et al. A double-negative feedback loop between ZEB1-SIP1 and the microRNA-200 family regulates epithelial-mesenchymal transition. Cancer Res. 2008;68(19):7846–54.
77. 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.
78. Guay C, Regazzi R. Role of islet microRNAs in diabetes: which model for which question? Diabetologia. 2015;58(3):456–63.
79. Erener S, Mojibian M, Fox JK, Denroche HC, Kieffer TJ. Circulating miR-375 as a biomarker of beta-cell death and diabetes in mice. Endocrinology. 2013;154(2):603–8.
80. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 2014;42(Database issue):D68–73.

Chapter 7
microRNA and Cardiac Regeneration

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Abstract Heart diseases are a very common health problem in developed as well as developing countries. In particular, ischemic heart disease and heart failure represent a plague for the patients and for the society. Loss of cardiac tissue after myocardial infarction or dysfunctioning tissue in nonischemic cardiomyopathies may result in cardiac failure. Despite great advancements in the treatment of these diseases, there is a substantial unmet need for novel therapies, ideally addressing repair and regeneration of the damaged or lost myocardium. Along this line, cardiac cell based therapies have gained substantial attention. Three main approaches are currently under investigation: stem cell therapy with either embryonic or adult stem cells; generation of patient-specific induced pluripotent stem cells; stimulation of endogenous regeneration trough direct reprogramming of fibroblasts into cardiomyocytes, activation of resident cardiac stem cells or induction of native resident cardiomyocytes to reenter the cell cycle. All these strategies need to be optimized since their efficiency is low.

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It has recently become clear that cardiac signaling and transcriptional pathways are intimately intertwined with microRNA molecules which act as modulators of cardiac development, function, and disease. Moreover, miRNA also regulates stem cell differentiation. Here we describe how miRNA may circumvent hurdles that hamper the field of cardiac regeneration and stem cell therapy, and how miRNA may result as the most suitable solution for the damaged heart.

Keywords microRNA • Acute myocardial infarction • Cell therapy • Cardiovascular disease • Stromal cells

Introduction

Cardiovascular diseases (CVD) remain the leading cause of death worldwide. Among CVD, postischemic heart failure is one of the most severe and debilitating, with a prevalence on the rise. Approximately 80 % of all cardiovascular-related deaths occur in low- and middle-income countries and at a younger age in comparison with high-income countries. Heart failure is a clinical syndrome that occurs when the working capacity of the myocardium decreases, for example as a complication of an acute myocardial infarction (AMI), and the heart becomes unable to pump blood in sufficient quantity to meet the metabolic needs of the organism. AMI is caused by the abrupt closure of a coronary artery due primarily to thrombus formation. The most effective therapy for AMI is represented by timely revascularization of the related artery. This is achieved with either thrombolytic agents, percutaneous coronary intervention, or bypass surgery. If not promptly reperfused, the ischemic heart can undergo massive loss of contractile tissue, replaced in time by scar tissue. The resulting compensatory mechanisms cause an increase in blood volume, increased filling pressures in the heart chambers, increased heart rate and heart mass. However, despite these compensatory mechanisms, the ability of the heart to contract and to relax progressively decreases, resulting in the negative remodeling of the left ventricle (LV) and a worsening of the clinical picture and finally heart failure ensues [1, 2]. Currently, there are drugs able to reduce the severity of this pathological remodeling, but no therapeutic approach or surgery is able to restore the contractility of the infarcted heart. The result is an increasing number of patients with a poor quality of life due to recurrent hospitalizations and the large number of drugs they must assume. The economic impact in regards to loss of productive years of life and the costs that the healthcare system must bear is substantial. Heart transplantation represents the only definitive solution. With current surgical techniques and the refinement of immunosuppressive therapies, the survival of transplanted patients at 5 years after surgery is higher than 70 % [3, 4]. Unfortunately, due to the low availability of transplantable hearts, only a very limited number of patients receive this treatment. For all these reasons, it is essential to find innovative therapeutic approaches able to prevent LV postischemic remodeling or, even better, approaches able to replace scarred or noncompetent cardiac tissue.

Although the mammalian heart has conventionally been viewed as a postmitotic organ, cardiac cells have recently been shown to display some regenerative potential, good enough to maintain the physiologic homeostasis of the heart but insufficient to regenerate relevant cardiac lesions [5]. Cardiac regeneration in adulthood is observed in lower vertebrates such as certain amphibians and fish, which are able to regenerate cardiac muscle after injury or genetic ablation of cardiomyocytes (CMC) [6–10]. Lineage tracing experiments have revealed that differentiated CMC are the major source of the newly generating cardiac muscle. In response to injury, preexisting mature CMC undergo a process of sarcomeric disassembly, dedifferentiation, reentry into the cell cycle and proliferation to replenish the lost myocardium [11, 12]. This process is associated with minimal fibrosis. In addition, it has recently been demonstrated that early during the postnatal period, mice also exhibit a comparable regenerative capacity [13]. This regenerative plasticity is strictly limited to the first week after birth when CMC have not terminally exited the cell cycle. Once CMC have completed terminal differentiation, following injury the myocardium undergoes a reparative process mostly associated with fibrosis and compensatory cardiac hypertrophy [13]. These findings mean that mammals retain a quiescent regenerative capacity, which could potentially be reactivated in the adult heart.

Moreover, the demonstration that it is possible to mobilize and activate endogenous progenitor cells in diseased heart or to introduce exogenous stem cells able to regenerate cardiac tissue, has paved the way to revolutionary strategies in myocardial repair. Reports that embryonic stem cells (ESC) and adult stem cells (ASC) can differentiate into CMC, vascular smooth muscle cells (VSMC), and endothelial cells (EC) have stimulated studies investigating the use of stem cells to treat cardiovascular disease. During the past decade, several studies have demonstrated and suggested that the potential for cardiac regeneration may still be present in the organ, albeit silenced. Therefore, insights into the determinant roles that tiny molecular switches play in cardiac cell proliferation and differentiation are of great relevance, not only to complement our current understanding of heart biology, but also to open new paths for the development of innovative strategies to treat cardiac-related pathologies.

Strategies to Regenerate Cardiac Tissue

Three different approaches are currently pursued to form new CMC and achieve myocardial regeneration: (1) differentiation of ESC or ASC into CMC; (2) full reprogramming of fibroblasts into induced pluripotent stem cells (iPS) and subsequent cardiac differentiation; and (3) stimulation of endogenous regeneration trough direct reprogramming of fibroblasts into CMC, activation of cardiac stem cells (CSC) or induction of the native resident CMC to reenter the cell cycle.

The rational for the first two methods is that the newly generated CMC can be then transplanted into damaged hearts and replace scarred or non-functional tissue. Regarding the use of ESC or ESC-derived CMC, the most compelling issue is ethical

because of the source from which ESC are isolated. People are divided on the question of whether the promise of considerable therapeutic breakthroughs can be weighed against the ethical problem connected with the destruction of early human life [14]. Nonetheless, embryo destruction is not the only problem we face in the ESC research field. For example, the developmental potential of ESC is another challenging problem. In the public debate on the use of early human embryos, the inner cell mass of a blastocyst, the usual starting point for the production of ESC, is very often considered just a cell cluster, namely the morula. Embryologists, however, are still divided on the question of whether a morula or the inner cell mass constitute a homogeneous population of cells with equal developmental information or whether there is any cryptic spatial information embedded in this cluster. The question remains as to whether the ensuing ESC have properties that force us to regard them as cells that are nevertheless so close to early embryos that they deserve special protection with regard to potentiality and exceptional treatment on ethical questions. However, in many countries this ethical dilemma is bypassed and ESC can be used after obtaining written informed consent from the pregnant woman who decides to donate the fertilized egg.

ESC are pluripotent and can differentiate into several specialized cell types, including CMC. The most recognized method to induce cardiac differentiation consists in growing the ESC in suspension, thus allowing the generation of embryoid bodies (EB). The CMC derived from ESC are now well characterized [15, 16]. Their phenotype is mostly immature, so new techniques able to induce more effective differentiation are desirable.

Mostly to avoid ethical issues, investigators started to test ASC for regeneration purposes. Different types of ASC from mice, rats, pigs, and humans have been administered in experimental models of permanent coronary ligation and ischemia/reperfusion injury (I/R). A great variety of readouts have been employed to evaluate the effects of ASC transplantation into injured hearts, and all the analyses have documented an overall reduction in infarct size, less severe ventricular remodeling, and improved vascularization [17, 18]. Furthermore, ASC administration improved ventricular function in most cases. Much of the research in cardiovascular regenerative therapies has been conducted using bone marrow-derived mesenchymal stem cells (BM-MSC). In particular, it has been demonstrated that the administration of BM-MSC can rescue damaged hearts and improve cardiac function in animal models of AMI and improve vasculogenesis in chronic ischemia models [19].

Unlike ESC, BM-MSC are multipotent and not pluripotent, and can be propagated in culture only for a limited number of passages. Despite these limitations, BM-MSC seem to be a good population for regenerative cell therapy, primarily because they are not limited by ethical or legal restrictions. Furthermore, BM-MSC can be easily isolated and expanded ex vivo from a tissue biopsy or from peripheral blood; thus, they can be used for autologous transplantation and immunosuppressive therapy is not required. Unfortunately, the efficiency with which BM-MSC differentiate into CMC is extremely low and the beneficial effects documented are mostly mediated by paracrine mechanisms [20–22]. Another important ASC type for cardiac regeneration is represented by CSC, tissue-specific progenitors harbored within the adult mammalian heart [23–26]. The isolation of CSC has raised

harsh discussion in the scientific community since it contradicted the old concept according to which the adult heart was considered a post-mitotic organ without any regenerative capability. However, extensive evidence from genetic studies has established that CSC are able to generate new adult CMC, for example lost by injury, throughout adult life [27]. Moreover, it is a new but accepted concept that CMC death and CMC renewal occur and are physiological events participating to cardiac homeostasis and in which the CSC play a pivotal role [24]. These findings have opened new perspectives and made possible to hypothesize new approaches for the treatment of cardiac disease by considering the heart an organ with regenerative potential like the liver and skin. CSC were first characterized as c-kit positive cells and CD45 negative cells: c-kit is the receptor of the stem cell factor and CD45 is a common hematopoietic marker. Recently, also other different membrane epitopes, such as Sca-1 and Flk-1, were identified as characterizing CSC markers. Also transcription factors, for example, NKX2.5 and GATA4 have been identified in certain sub-types of CSC and then used to identify and characterize CSC in the embryonic and adult life [28]. These cells display very attractive therapeutic potential, since they are clonogenic, self-renewing, and both in vitro and in vivo are able to generate the three main cell components of the myocardium: CMC, VSMC, and EC [23, 24, 26]. When CMC are cultured in suspension they form cardiospheres, similar to the same structures which ESC generate during their cardiac differentiation, namely EB [29]. Cardiospheres consist of a mixture of CSC and differentiating progenitors. Cardiosphere cells stain positive for the endothelial marker, von Willebrand factor, and also for the cardiac differentiation markers, myosin heavy chain and cardiac troponin I (cTnI), demonstrating their ability to differentiate into distinctive lineages with proper stimulus. As for ESC, a method to improve the differentiation capacity of ASC is mandatory in order to achieve clinically relevant cardiac regeneration [30–32].

The biggest discovery in the last 15 years in the stem cell field was reported by Takahashi and his colleague Yamanaka: starting from dermal fibroblasts they were able to create a cell type exhibiting the morphology and growth properties of ESC and expressing pluripotency markers, namely iPS cells. With this approach they overcame the ethical problems related to the isolation of ESC from the inner cell mass of the blastocysts because they obtained the iPS cells from fibroblasts, fully differentiated cells, which are easily isolated with a minimally invasive biopsy. Nuclear reprogramming with ectopic stemness factors has provided the opportunity to generate autologous patient-derived iPS from adult somatic cells [33, 34]. The ability of both mouse and human iPS to differentiate into functional CMC has been well demonstrated and the ability of these cells to engraft in the infarcted heart has been assayed by intramyocardial injection of functionally undifferentiated iPS. It has been shown that iPS can engraft and improve contractile function of infarcted myocardium while attenuating adverse remodeling in immunocompetent mice. Only in immunocompetent mice, was the environment after cardiac transplantation permissive for differentiation, whereas in immunodeficient mice, tumor development was observed, which highlights the importance of immune surveillance to prevent tumor growth [35–37]. Furthermore, iPS become immunologically relevant while they differentiate, with upregulation of histocompatibility antigens. This

aspect would narrow the range of applications to the autologous setting [38]. Thus, several important hurdles have to be resolved before clinical translation of iPS-based therapies can become reality, including safety issues to prevent tumor growth that may require determination of the appropriate differentiation of iPS-derived cells and removal of residual undifferentiated cells before transplantation [36, 39]. In addition, up-scaling to achieve effective iPS-based cell generation will be required. Also in the case of iPS-derived CMC, the efficiency of cardiac differentiation is limited and would need a boost in order to achieve fully mature CMC.

More recently, it has been described how to directly convert fibroblasts into another cell type by introducing a combination of transcription factors into these cells. The direct reprogramming approach is particularly attractive because it bypasses the integration step of the CMC into the damaged heart tissue. In 2010, Vierbuchen and colleagues succeeded in making neuronal-like or induced neuronal cells by introducing three genes, encoding transcription factors necessary for neuronal differentiation, into mouse fibroblasts [40]. This was the first successful report of direct reprogramming without the need of a middle step with iPS cells, using organ-specific transcription factors. Following this study, it has been speculated that mouse neonatal cardiac fibroblasts may be converted into CMC-like cells through the introduction of genes encoding cardiac-specific transcription factors such as GATA4, MEF2C and TBX5 [41]. In the future, this strategy may provide a safer and novel alternative to cell transplant. Particularly, direct reprogramming technology would represent an ideal treatment for post-AMI damage by turning the fibrotic tissue of the scar into new cardiac tissue. However, in order for direct reprogramming to be used in clinical applications, the efficiency of this method must be optimized and a nontoxic/non-integrating inducer identified and developed. Currently, the reprogramming efficiency of fibroblasts into mature CMC is variable and low. Although there are several reports on the direct reprogramming of human cardiac fibroblasts into CMC, further studies are required for optimization [42].

miRNA: Little Molecules, Big Effects

The myocardial regeneration process involves different types of molecules such as cytokines, chemokines, growth factors, nuclear factors, and miRNA. In recent years, all of these molecules have been studied and miRNA resulted to be the most attractive and promising agents. miRNA are a class of 21–25 nucleotide noncoding RNA, which share common biogenesis and effector pathways. miRNA can either be derived from genes or introns during splicing. Those derived from genes are initially transcribed in the nucleus, by RNA polymerases (II and III), into primary transcripts called pri-miRNA, which are processed into pre-miRNA by a microprocessor complex consisting of the Drosha enzyme and RNA-binding protein, DiGeorge critical region 8 (DGCR8) [43, 44]. The initial pri-miRNA precursors have long hairpin structures with a terminal loop and flanking segments. The flanking segments are essential for the binding of DGCR8 to the pri-miRNA genes [45].

miRNA derived from introns are called "mirtrons" and enter the miRNA biogenesis pathway bypassing the Drosha-mediated cleavage [46]. The pre-miRNA are then transported through a nuclear pore into the cytoplasm by an exportin-5 transporter protein, where they are further processed into a double-stranded nucleotide intermediate by Dicer, an RNAse III enzyme. The intermediates are further processed into mature miRNA by Dicer [47]. These mature miRNA unwind within the complex, resulting in a single miRNA strand, which is finally incorporated, along with Argonaute (Arg) proteins, into an RNA-induced silencing (RISC) complex. The miRNA strand guides the RISC complex to conserved recognition sites on target mRNA, where either translation is directly repressed by the miRNA or the target mRNA is degraded. This largely depends on the complementary match of the miRNA to the target mRNA, which is mediated by the associated Arg proteins. Typically, more than one binding site is present on the 3′ untranslated region (uTR) of the target, thus numerous miRNA can bind cooperatively to a 3′ uTR to provide stronger repression [48]. However, the mechanisms of translation repression are only partially understood. In addition to translation repression, mammal miRNA are thought to induce target degradation, similar to small interfering RNA (siRNA) [49]. Moreover, some miRNA might have motifs that redirect them to the nucleus where they can mediate transcriptional, rather than the more usual posttranscriptional, gene silencing [50, 51]. Finally, new data indicate that miRNA might also stimulate translation: these different findings have sparked some controversy, and more work will need to be done to elucidate the mechanisms by which miRNA regulate gene expression [52–54]. miRNA are known to exist in a variety of different terminally differentiated cells, but also inside stem cells, where they act as molecular regulators of gene expression. The physiological roles of miRNA are only partially known and need to be better characterized. However, the involvement of different miRNA in heart embryogenesis, heart disease, and differentiation of stem cells into CMC has already been described. Here, we attempt to summarize the most relevant discoveries reported so far on the role of miRNA in cardiac regeneration.

Role of miRNA in Heart Embryogenesis

The heart is the first organ to function during vertebrate embryogenesis. Heart development is a complex process involving the integration of multiple cell lineages. Abnormalities in heart formation may lead to congenital heart disease, the most common human birth defect, and abnormalities in the function of the adult heart result in a spectrum of fatal disorders, including arrhythmias, cardiomyopathies, heart failure, and sudden death [55].

At the molecular level, the developmental process involves several transcription factors, transcriptional co-activators and repressors, their corresponding enhancer and promoter elements and chromatin-modifying enzymes, generating a complex gene network that coordinates heart development. The discovery of the regulatory

role of noncoding RNAs, and miRNA in particular, has added a new layer of complexity to our understanding of cardiovascular development. Their primary role seems to be the “fine-tuning” of gene expression to control development and tissue homeostasis [56]. The critical role of miRNA during cardiac development was discovered by studying loss-of-function mutations of the miRNA-processing enzymes Dicer and Drosha/DGCR8. Albeit global deletion of miRNA-processing enzymes causes lethality during early gestation [57], conditional knockout of these enzymes in specific cell lineages has highlighted their essential roles during heart development. Conditional deletion of Dicer using Cre recombinase under the control of the endogenous NKX2.5 regulatory region (NKX2.5–Cre) abolished the processing of pre-miRNA into their mature form in early cardiac progenitors at embryonic day 8.5 [58]. Early deletion of Dicer in cardiac progenitors led to embryonic lethality resulting from cardiac failure at embryonic day 12.5 [58]. The ventricular myocardium of NKX2.5–Cre-knockout mice was poorly developed and the heart exhibited signs of pericardial edema. miRNA are also necessary for cardiac outflow tract alignment and chamber septation [59]. The development of the outflow tract involves the coordinated regulation of cell proliferation, differentiation, migration, and apoptosis of multiple cell types, including those derived from the neural crest. These cells are required for proper development of craniofacial structures, as well as development of the great arteries and outflow tract septation [60]. Selective deletion of Dicer from the neural crest lineage causes severe defects in craniofacial and cardiovascular structures, such as the ventricular septal defect, double outlet right ventricle, and interrupted aortic arch.

Among the miRNA involved in heart development,miRNA1 is the most abundant in the mammalian heart, accounting for almost 40 % of all miRNA in the adult murine heart [61]. In vertebrates, members of the miRNA1 (miRNA1-1, miRNA1-2, miRNA206) and miRNA133 (miRNA133a-1, miRNA133a-2, miRNA133b) families cluster and are generated from common bicistronic transcripts. The expression of miRNA1 and miRNA133 is cardiac and skeletal muscle specific. Gain and loss of function experiments have established critical roles for the miRNA1 and miRNA133 families during heart development. Gene deletion experiments on miRNA1 have highlighted the role that it plays in the process of muscle differentiation [62]. Overexpression of miRNA1 under the control of the isoform β of the myosin heavy chain (βMHC) promoter disrupted embryonic mouse heart development and was associated with thin-walled ventricles, heart failure, and lethality at embryonic day 13.5 [58]. Overexpression of this miRNA causes a reduction in the number of proliferating CMC without affecting the number of apoptotic cells. Genetic deletion of miRNA1-2 provided the first demonstration of an essential role for an individual miRNA during cardiogenesis. Surprisingly, disruption of just one member of this miRNA1 family resulted in a range of abnormalities, including cell cycle dysregulation, heart malformations, and postnatal electrophysiological defects. Heterozygous miRNA1-2-null mice survived to reproduce, but 50 % of miRNA1-2 homozygous null mice died between embryonic day 15.5 and birth from ventricular septal defects and cardiac dysfunction. These defects can occur from dysregulation of a multitude of events during cardiogenesis, and it is likely that miRNA1-2 regulates numerous genes during this process. Homozygous deletion of miRNA1-2 in mice causes

lethality, with incomplete penetrance, between embryonic day 15.5 and birth due to interventricular septal defects [63]. Consistent with previous gain-of-function experiments, which proposed a role for miRNA1 as a negative regulator of CMC proliferation via translational repression of the heart and neural crest derivatives expressed protein 2 (HAND2), miRNA1-2-knockout mice have an increased number of mitotic CMC and higher expression levels of HAND2 protein [63]. miRNA133a double mutant hearts are characterized by increased CMC proliferation and apoptosis, profound sarcomere disarray, and cardiac fibrosis.

There are two major MHC genes ,expressed in the heart, αMHC (MYH6) and βMHC (MYH7). The ratio of αMHC to βMHC affects both the contractility of cardiac sarcomeres and cardiac energy consumption since they have distinct rates of converting ATP to mechanical work. In rodent hearts, the slower isoform βMHC is specifically expressed in embryonic and fetal stages while the faster isoform αMHC is expressed predominantly in the adult heart. One of the most interesting and surprising discoveries in recent years has been the finding that these muscle-specific myosin genes are under the control of a family of intronic miRNA, known as myomiRNA [64]. The myomiRNA consist of miRNA208a, miRNA208b, and miRNA499, which are embedded within the introns of Myh6, Myh7, and Myh7b, respectively. miRNA208a and miRNA208b have identical "seed" sequences and miRNA499 is very similar to the miRNA208 family members, with six overlapping nucleotides in the "seed" region, suggesting that myomiRNA probably share common target genes and functions. These observations also imply that there has been strong selective pressure to maintain these miRNA within myosin gene regulatory networks throughout vertebrate evolution [65]. Genetic loss-of-function studies in mice have revealed that miRNA208a is not essential for cardiac development and mutants for this miRNA are viable, have normal cardiac contractile function under baseline conditions and do not display any overt signs of cardiac pathology up to 20 weeks of age [66]. However, a deficiency in this miRNA may lead to a decline in cardiac function with age (up to 6 months), due to abnormalities in sarcomere structure [66]. Although these results suggest that miRNA208a is dispensable for embryonic cardiac development, another study has revealed striking cardiac conduction abnormalities in 4 month old miRNA208a mutant mice [67]. In contrast, acute administration of anti-miRNA208a in adult mice was not sufficient to induce any obvious electrophysiological abnormalities, suggesting that, although miRNA208a may play an important role in the development of the cardiac conduction system, its function is not required for maintenance of electrical conduction in adulthood [68]. Despite the relatively subtle effects of miRNA208a loss-of-function on cardiac development, miRNA208a is indispensable for mediating some aspects of the cardiac stress response during pressure overload or hypothyroidism. One of the verified targets of miRNA208a is thyroid hormone receptor-associated protein 1 (THRAP1), which is a key component of the thyroid hormone signal pathway. Deletion of miRNA208a resulted in an increase in THRAP1 expression and enhanced thyroid hormone receptor mediated suppression of MYH7 [64]. Thus, the miRNA208a/THRAP connection may explain why miRNA208a and thyroid hormone are involved in cardiac hypertrophy. Consistent with these findings, miRNA208a overexpression in transgenic mice is sufficient to induce βMHC/MYH7 upregulation prior to the onset of any overt signs of cardiac hypertrophy

and/or pathology [66]. Conversely, developmental loss of miRNA208a does not appear to have any effect on βMHC protein levels in the embryonic and neonatal heart, suggesting that distinct regulatory hierarchies control the expression of βMHC/MYH7 during embryogenesis and under conditions of cardiac stress in adulthood [66, 69]. Complete loss-of-function of miRNA208a, miRNA208b, and miRNA499 will be required to overcome potential issues of functional redundancy and to fully assess the functions of the myomiRNA network during embryonic heart development. Using an elegant series of genetic deletions and rescue experiments, van Rooij et al. demonstrated that re-expression of miRNA499 in the adult heart is sufficient to reactivate MYH7, MYH7b, and miRNA208b expression, as well as prevent aberrant upregulation of fast skeletal muscle troponin isoform, in hypothyroid miRNA208a-mutant mice [69]. These results indicate that miRNA499 is a major downstream effector of the actions of miRNA208a in the heart and define a positive-feedback loop, whereby miRNA208a regulates the expression of MYH7b and its intronic miRNA499 which, in turn, regulates βMHC. The miRNA17-92 cluster, also known as oncomiRNA1, consists of five miRNA (miRNA17, miRNA18a, miR19a, miRNA19b-1, and miRNA92-1), belonging to four miRNA families that are generated from a common pri-miRNA transcript. Despite the well-known involvement of the miRNA17-92 cluster in tumorigenesis, recent mouse genetic studies reveal that this miRNA cluster is essential for cardiac development and function. Ventura et al. generated null mice for the miRNA17-92 cluster as well as its paralogs miRNA106b-25 and miRNA106a-363 [70]. Complete deletion of the miRNA17-92 cluster resulted in smaller embryos and perinatal lethality. Mutant animals displayed thin ventricle walls, ventricular septal defects, and lung hypoplasia. While deletion of the other two paralogs individually did not affect heart development or survival, compound loss of either miRNA106b-25 or miRNA106a-363 with miRNA17-92 led to embryonic lethality with higher penetration than the miRNA17-92 deletion alone. Together, these studies established the primary effect of the miRNA17-92 cluster in cardiac development in a dose-dependent manner with the other two paralogs [70].

Heart development requires precise coordination of cell fate determination, pattern formation, and cell movement [71]. In these highly conserved events, transcriptional networks accurately control specific gene expression in a spatial and/or temporal manner [72]. It is not surprising that recent investigations revealed that miRNA regulate cardiac patterning. Morton and coworkers found that miRNA138 is expressed in specific domains of the zebrafish heart and is required to establish appropriate chamber-specific gene expression patterns [73]. Disruption of miRNA138 by morpholino and antagomir in zebrafish embryos led to expansion of the atrio-ventricular canal (AVC)-restricted genes to the ventricular chamber. As a result, ventricular CMC failed to fully mature. These observations suggest that miRNA138 regulates cardiac patterning during heart development by restricting AVC gene expression.

Further evidence for a role of miRNA during vertebrate cardiac morphogenesis and patterning comes from recent gain- and loss-of-function studies of miRNA218 in zebrafish. The miRNA218 family consists of three highly conserved members including miRNA218a-1, miRNA218a-2 and miRNA218b. In vertebrates, miRNA218a-1 and miRNA218a-2 are intronically encoded in the slit2 and slit3 genes,

whereas miRNA218b is intergenic. Knock-down of miRNA218 using two different morpholinos in one- to two-cell zebrafish embryos results in cardiac morphological defects, impaired migration of heart field progenitors to the midline during heart tube formation and pericardial edema at 48 h postfertilization, but does not cause any severe vascular defects [74]. Furthermore, a sub-phenotypic dose of roundabout homolog 1 (Robo1) morpholino significantly rescued the miRNA218 morphant phenotype, suggesting that miRNA218 regulates heart field migration and heart tube formation through modulation of Robo1 dosage [74].

In summary, it is clear that several miRNA and miRNA families are involved in cardiac development. It is also clear that a more in-depth knowledge of the mechanisms of action through which these miRNA regulate cardiac development should lead to future therapies modulating specific miRNA also in the adult heart to achieve cardiac regeneration.

miRNA and Differentiation of Stem Cells into Cardiomyocytes

Mostly by adopting data obtained from embryogenesis studies, investigators started to test the hypothesis of using miRNA to induce cardiac regeneration (Fig. 7.1). From a therapeutic point of view, understanding the role of miRNA in stem cell differentiation toward the cardiac lineage is of great relevance [75]. Since the differentiation of ESC into CMC is considered the gold standard assay, when studying cardiac regeneration, the role of miRNA has been tested mostly using this experimental model.

Several intriguing observations have been reported. For instance, it has been shown that miRNA145 regulates the expression of target genes which play a pivotal role in maintaining the pluripotency of ESC: OCT4, SOX2, and KFL4 [78]. ESC maintain their pluripotency when these three genes are expressed in a specific manner and with a certain balance. However, an upregulation or downregulation of either one of these genes can trigger ESC differentiation toward specialized cell types. For example, an increase in OCT4 protein converts ESC into primitive endoderm and mesoderm, whereas repression of OCT4 results in trophectoderm [76]. An increase in SOX2 protein triggers ESC differentiation into neuroectoderm, mesoderm, and trophectoderm [77]. miRNA145 in ESC are able to repress the expression of OCT4, SOX2, and KLF4, providing modulation of the three genes' levels when their copy numbers are in excess [78]. This is only one example of the miRNA action on ESC and it supports the notion that miRNA molecules are strong regulators and counterbalance the strength of the ESC transcription circuitry.

A role for miRNA1, miRNA133, miRNA499, and miRNA208 in the differentiation of ESC into CMC has been reported [79, 80]. The role of miRNA1 and miRNA133 seems controversial. Reduced levels of miRNA1 and miRNA133 have been observed in mouse ESC following artificial induction of myocardial differentiation using trichostatin A, a histone deacetylase inhibitor [79]. Moreover, overexpression of miRNA1 and miRNA133 reduced the expression of the specific

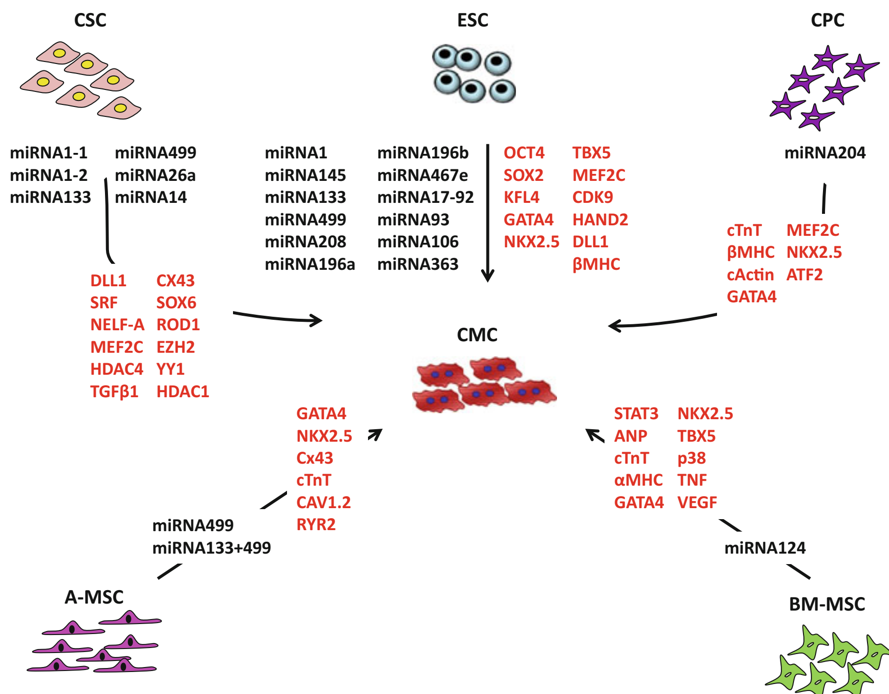

Fig. 7.1 miRNAs and stem cell cardiac differentiation

cardiac gene NKX2.5 in ESC. Overexpression of miRNA1 also seems to inhibit the translation of cyclin-dependent kinase-9 (CDK9), which is known to activate cardiac-specific transcription factor GATA4 [79]. miRNA1 also targets the transcription factor that plays a major role in the formation of ventricular CMC [58]. This evidence suggests that both miRNA1 and miRNA133 tend to inhibit cardiac differentiation of ESC. However, other studies demonstrate that the role of these two miRNA is probably bi-modal and likely depends by their expression level during the differentiation process. For instance, it has been shown that miRNA1 can repress the notch ligand delta-like 1 (DLL1), which in turn promotes the expression of cardiac genes and suppresses the expression of noncardiac genes [81]. Studying miRNA profiling of human ESC-derived CMC, Wilson and collaborators were able to show that several miRNA, including miRNA1, miRNA208, and miRNA133, significantly changed expression during differentiation [82]. Other miRNA also showed cardiac-specific expression; among them, miRNA499 was strongly associated with cardiac differentiation and appeared to share many predicted targets with miRNA208. Overexpression of miRNA499 caused upregulation of the cardiac transcription factor MEF2C and co-expression of miRNA499 with miRNA1 resulted in the upregulation of cardiac myosin heavy-chain genes in EB [82]. On the contrary, inhibition of miRNA499 action blocked cardiac differentiation, demonstrating that miRNA499 controls the cardiac commitment of ESC.

Besides miRNA1, miRNA133, miRNA499 and miRNA208, other miRNA are involved in ESC cardiac differentiation. For example, miRNA196a, miRNA196b, and miRNA467e are expressed at significantly lower levels in beating areas compared with non-beating areas, suggesting that the expression of these miRNA may be inversely correlated with ESC differentiation [83].

The miRNA17-92 cluster has also been associated with cardiac differentiation of ESC [84]. This cluster operates via repression of a number of pro-apoptotic proteins, including Bim, which contains 3′-uTR-binding sites for miRNA19, miRNA25, and miRNA92. Moreover, the Bim gene sequence also targets other miRNA sequences, such as miRNA93, miRNA106, and miRNA363, but the role of these miRNA during ESC differentiation into CMC needs to be clarified. The same miRNA implicated in the regulation of ESC differentiation into CMC have also been reported to play a role in ASC cardiac transformation. Mixed results have been reported for the roles of miRNA1 and miRNA133 in regulating the function of CSC and also of cardiac progenitors cells (CPC) [58, 81]. For example, miRNA1 increased differentiation of CSC by repressing the translation of DLL1, a transcription factor that promotes the expression of cardiac mesoderm genes and suppresses the expression of non-mesoderm genes [81, 85]. Conversely, miRNA133 seems to inhibit this process thus repressing the differentiation of CSC. Although the molecular nature of this repression is unclear, the negative elongation factor-A (NELF-A), which is known to promote cardiogenesis, has been identified as a target for miRNA133 [86]. It is also known that miRNA1 indirectly upregulates MEF2C, an essential muscle-related transcription factor, by targeting the transcriptional co-repressor histone deacetylase 4 (HDAC4), thereby promoting myogenesis.

The cardiac-specific miRNA499 seems to strongly promote differentiation of ASC. Indeed, the overexpression of miRNA499 in human CPC indirectly promoted cardiac differentiation by targeting sex determining region Y-box 6 (SOX6) and regulator of differentiation 1 (ROD1) in vitro [80, 87]. In confirmation of the key role of miRNA499, when its activity was blocked using specific oligonucleotides the differentiation of CSC was prevented [80].

Other less common miRNA, such as miRNA204, miRNA124, miRNA669a, miRNA669q, miRNA23a, and miRNA23b, play a role in promoting the differentiation of CPC [88, 89]. For instance, miRNA204 is moderately expressed in adult CMC but its level results increased in differentiating CPC [88]. When studying the differentiation of CSC, miRNA204 inhibition significantly downregulated the expression of sarcomeric proteins, including cardiac troponin T (cTnT), βMHC, and cActin, which are structural cardiac proteins found in mature and functional CMC while miRNA204 mimics significantly upregulated βMHC and early cardiac transcription factor expression, including MEF2C, GATA4, and NKX2.5 [88]. miRNA124 seems to control CMC differentiation of BM-MSC by targeting STAT3 signaling, a pathway which plays an essential role in self-renewal, trans-differentiation, and paracrine actions of ASC [90, 91]. miRNA124 is also able to regulate the differentiation of BM-MSC into CMC affecting the expression of the atrial natriuretic peptide (ANP), cTnT, αMHC and GATA4, NKX2.5, and TBX5 genes [43]. Recent evidence supports the translational potential of miRNA-mediated cardiac

differentiation of stem cells for heart disease. Transplantation of ESC overexpressing miRNA1 in the border zone of infarcted mouse hearts seems to repair the myocardium from ischemic injury in vivo [92, 93]. miRNA1 inhibited expression of phosphatase and tensin homolog (PTEN) in ESC, triggered the phosphoinositide3-kinase/Akt signaling pathway, improved cardiac function, enhanced CMC differentiation, and attenuated apoptotic cell death [92, 93]. Similarly, overexpression of miRNA499 in c-kit$^+$ CSC improved CMC differentiation and promoted restoration of myocardial mass following injection into the border zone of infarcted rat hearts and resulted in the improvement of myocardial function [87]. Thus, ectopic expression of miRNA in CSC and CPC can alter the differentiation potential of engrafted cells in vivo and could be used to improve the efficacy of stem cell therapies for heart disease.

Synergic Effect of Different miRNA in Cardiac Differentiation

Very recently, our group investigated whether the concomitant overexpression of different miRNA can synergically improve the differentiation of cardiac progenitors and ASC into CMC [94]. To test this hypothesis, we first used P19 cells, a well-known and well-established cell line, to study cardiac differentiation. Our results demonstrated that miRNA499 is a powerful activator of cardiac differentiation. However, when co-expressed with miRNA133 the results were significantly and strikingly superior compared with the overexpression of miRNA499 alone. In particular, miRNA499 plus miRNA133 almost doubled the number of beating EB compared with miRNA499 alone. Most importantly, by simultaneously overexpressing miRNA499 and miRNA133 the number of P19 cells expressing cTnI was 30-fold greater compared with the standard differentiation protocol. In addition, the expression of genes encoding for cardiac-specific transcription factors, such as GATA4 and NKX2.5, and cardiac-specific proteins, such as connexin43 (Cx43) and cTnT, was enhanced in cells treated with miRNA499 plus miRNA133. Gene expression analysis documented that our protocol results in the production of both atrial and ventricular myocytes, as testified by the overexpression of MLC2A and IRX4, two well-known differentiation markers. Western blot and immunocytochemistry analyses confirmed that cardiac proteins are indeed expressed at higher levels when P19 cells are co-transfected with miRNA499 plus miRNA133. Importantly, functional electrophysiological characterization of contracting EB reinforced evidence that miRNA499 and miRNA133 synergistically induce cardiac differentiation. To verify whether miRNA499 and miRNA133 exert their effects also on other cell types, more suitable for clinical use, we tested our protocol on fetal MSC derived from the amniotic membrane of human placenta (A-MSC). Gene and protein expression analyses showed that miRNA499 and miRNA133 are able to induce the differentiation of A-MSC into cells expressing typical cardiac markers such as NKX2.5, GATA4, cTnT, Cx43, the ryanodine receptor 2 (RYR2), and CAV1.2. It was impossible to document the same results using a different combination of

miRNA, confirming that only the couple miRNA499/miRNA133 triggers the differentiation of A-MSC toward a cardiac-like phenotype. These results suggest that the machinery regulated by miRNA is far more complex than expected and that the optimization of a differentiation protocol involving the modulation of miRNA will require more in-depth studies. However, they also confirm the notion that by overexpressing, probably in a time specific manner, certain miRNA will help produce more mature CMC, and more efficiently compared with methods used so far.

Direct Reprogramming with miRNA to Regenerate Cardiac Tissue

The discovery of iPS in 2006 by Takahashi and Yamanaka [33] inspired a new approach that aims to generate specific cell types without the need to transition through a stem cell state by introducing combinations of lineage-specific factors, called direct reprogramming. Recent studies demonstrated that direct reprogramming has the potential to yield a diverse range of cell types including CMC and EC [41, 95]. These findings indicate that cell fate plasticity is much wider than previously anticipated, and that direct reprogramming may offer a new system to study the mechanisms underlying cell fate decisions during development, which is currently a major focus of investigation in basic biology and translational medicine.

The first direct reprogramming was attempted by modulation of transcription factors. Srivastava's group demonstrated that a cocktail of three cardiac-specific transcription factors, GATA4, MEF2C, and TBX5, referred with the acronym GMT, was sufficient to reprogram mouse cardiac fibroblasts (CF) into induced CMC in vitro [96]. Subsequently, Srivastava and Olson laboratories independently reported that endogenous CF could be directly reprogrammed into CMC to improve the cardiac function of infarcted mouse hearts by gene delivery of GMT with or without HAND2 [97, 98].

Other studies showed that human fibroblasts could be also reprogrammed into CMC with various combinations of cardiac transcription factors in the presence or absence of miRNA, even though the efficiency was lower compared with that observed in mice [99, 100]. A very interesting finding comes from the Dzau's laboratory, showing that it is possible to achieve direct reprogramming just by using miRNA [101]. Testing different miRNA with a combinatorial approach, Dzau and collaborators showed that miRNA1 and the combination of miRNA1, miRNA133a, and miRNA208a induced the expression of early markers of commitment to the CMC lineage. Even though miRNA1 alone was found to be sufficient to drive cardiac gene expression, the efficiency was higher in combination with miRNA133a and miRNA208a. Additional studies showed that the addition of miRNA499 further augmented the efficiency of cardiac reprogramming. The authors named this combination "miRNA combo" (miRNA1, miRNA133a, miRNA208a, and miRNA499) and showed that a single transient transfection with this "miRNA combo" was sufficient to induce fibroblasts to express cardiac markers, such as MEF2C and αMHC. Full maturation of the reprogrammed cells was observed only after prolonged culture. Approximately

4 weeks after transfection, organized sarcomeres, contraction, and spontaneous calcium transients were documented. Most importantly, it has been shown that the administration of "miRNA" combo into mouse infarcted hearts converted resident cardiac fibroblast into induced CMC and improved cardiac function. By lineage tracing of non-myocytes it was documented that 12 % of CMC at the infarct border zone were newly generated. The induced CMC generated by in vivo reprogramming expressed typical cardiac markers, sarcomeric organization, excitation–contraction coupling, and action potentials characteristic of mature ventricular CMC. Serial echocardiographic examinations revealed that there was a time-delayed and progressive improvement in ventricular function, beginning 1–2 months post-surgery and was enhanced at 3 months, which was similar to the period reported for reprogramming with transcription factors. For clinical applications, the development of a non-viral delivery method, such as synthesized miRNA mimics, it is certainly an attractive therapeutic option, as they do not integrate into host chromosomes. Together, these results suggest that the abundant pool of endogenous CF could be a cell source for new CMC by direct reprogramming and that this new technology could improve cardiac function and reduce scar size after AMI. Moreover, the in vivo induced CMC are more mature than those in vitro, suggesting that the in vivo environment may improve the quality of cardiac reprogramming [102].

Giacca's group suggested that it is possible to experimentally drive adult mammalian CMC toward a proliferative state and use this approach to regenerate infracted mouse hearts [103]. In their elegant study, in vivo proliferation of mature CMC was achieved by manipulation of miRNA expression. With a high-throughput analysis and high-content microscopy screening, the proliferation ability of neonatal CMC were analyzed by using more than 800 miRNA mimics. The study focused on finding miRNA able to enhance the expression of proliferative markers such as Ki67 and the incorporation of DNA analogs, such as 5-ethynyl-deoxyuridine, as indicative of DNA synthesis. The authors found 204 miRNA that induce the proliferation of rat and mouse CMC more than twofold. Among these 204 miRNA, two were identified as the most efficient inducers of CMC proliferation: miRNA199a-3p and miRNA590-3p. The delivery of these two promising miRNA with adeno-associated virus (AAV) in infracted mouse hearts was sufficient to achieve a high increase in the number of proliferating CMC, a reduction of infarct size and a significant improvement of cardiac function after myocardial infarction. This elegant study demonstrated how endogenous cardiac regeneration can be achieved through activation of native CMC proliferation and expansion.

miRNA-Based Therapeutics for Myocardial Regeneration

The results summarized in this chapter highlight the pivotal role that miRNA play in cardiac development, reprogramming, and stem cell differentiation. It appears reasonable to hypothesize that targeting specific miRNA might be a rational strategy for cardiac regenerative therapy. In particular, miRNA-based therapy can be

used to promote CMC proliferation, to directly reprogram fibroblasts into CMC, as well as driving the differentiation of ESC, iPS or ASC into CMC. Obviously there are still several issues that need to be addressed before miRNA can be taken into the clinic. The most obvious is defining which combination of miRNA may result the most efficient in determining cardiac regeneration. So far, different results obtained in different species do not allow to reach any definitive conclusion. Moreover, besides the miRNA already explored, there might be others equally important or even more important for cardiac differentiation. New technologies and additional studies will shortly shed light on this issue.

Another crucial question is how these miRNA will be delivered into the patient. Retro-, lentiviruses, and AAV are all suitable delivery systems each with their own specific advantages and disadvantages [104]. The AAV approach is probably the most suitable since they do not integrate into the host genome. Major efforts have been made to design and deliver pharmacologically active synthetic miRNA, or miRNA mimics/mimetics. The first strategy developed for systemic delivery of miRNA consisted in synthesizing chemically modified miRNA to prevent their degradation by nucleases in the blood circulation. The disadvantage of this strategy is the possible rapid renal and hepatic clearance, resulting in short miRNA half-life. A second delivery strategy is focused on nanoparticle formulation for passive diffusion into target tissues based on enhanced permeability and retention effects. Although miRNA formulated in nanoparticles can enhance favorable tissue distribution compared with naked miRNA, the degree of enhancement is often not sufficient. A third-generation delivery strategy has recently emerged which adds surface modifications to the nanoparticles, allowing specific binding to the target cells, facilitating the internalization of nanoparticles into the target cells through receptor-mediated endocytosis [105]. Non-viral carriers such as polymer or lipid-based delivery vehicles have been developed and have delivery profiles. Even though non-viral delivery systems usually show lower transfection efficiency and shorter duration of target gene expression compared with viral vectors, recent studies successfully demonstrated that they can achieve clinically relevant efficiency upon specific modifications [83, 106]. Non-viral miRNA carriers use inorganic nanoparticles made of gold, carbon and biocompatible silica, non-immunogenic and nontoxic materials [107]. Also polyethylene glycol (PEG) has been modified with gold and has exhibited high miRNA loading capacity and low toxicity with efficient endosomal release [105]. Another miRNA delivery method that has been widely studied is the polymer-based delivery. The most common polymers used for this application are poly-lactic-coglycolic acid (PLGA) and polyethylenimine PEI. PLGA, which has been studied for many years and well characterized by its safe, biocompatible, and biodegradable nature, is used for making nanoparticles with high production efficiency and stable mechanical properties. PLGA-based nanoparticles are capable of sustained release in the cytosol through endo-lysosomal escape owing to reversal of the surface charge after cellular internalization [108].

miRNA therapeutics for heart repair do not necessarily or exclusively rely on the overexpression of certain miRNA but may also implicate the downregulation of miRNA. One way to block miRNA action is through antagomiR, which are miRNA

modified with cholesterol or phosphorothioate moieties to increase their stability in vivo. AntagomiR have been used, for example, to reduce the expression of miRNA that mediate pathological response after myocardial injury [109]. Most importantly, antagomiR may also prove to be useful for cardiac reprogramming. It is well known that there are significant barriers to reprogramming. Cell type-specific miRNA prevent translation of lineage-inappropriate mRNA. Targeting these cell type-specific miRNA with antagomiR will likely enhance cardiac reprogramming. CMC proliferation, differentiation, and reprogramming involve the coordinated action of many proteins acting in multiple pathways.

Conclusion

miRNA have become a source of great excitement as regulators of cardiac differentiation because they provide new insights into heart repair and can be therapeutically targeted. However, much remains to be investigated, specifically the precise mechanisms of miRNA action in cardiac differentiation and direct reprogramming and whether such modest regulators can, indeed, be modulated in settings of chronic disease and in a tissue-specific manner. Certainly we need to avoid a tendency to oversimplify the mechanistic basis of miRNA functions in the context of single downstream targets, but this is clearly not their primary mode of action. Thus, we need a deeper understanding as well as system biology approaches to fully explain miRNA activity under conditions of homeostasis and disease. Despite these uncertainties and given the pace of this field, it seems likely that some of the many miRNA therapeutics under study will soon enter the clinical arena in the setting of heart failure and AMI with the ultimate goal of achieving myocardial regeneration and restoration of cardiac function.

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References

1. Braunwald E, Antman EM. Evidence-based coronary care. Ann Intern Med. 1997;126: 551–3.
2. Braunwald E. Shattuck lecture—cardiovascular medicine at the turn of the millennium: triumphs, concerns, and opportunities. N Engl J Med. 1997;337:1360–9.
3. Costanzo MR. The role of histoincompatibility in cardiac allograft vasculopathy. J Heart Lung Transplant. 1995;14:S180–4.
4. Costanzo MR, Augustine S, Bourge R, et al. Selection and treatment of candidates for heart transplantation. A statement for health professionals from the Committee on Heart Failure

and Cardiac Transplantation of the Council on Clinical Cardiology, American Heart Association. Circulation. 1995;92:3593–612.
5. Ounzain S, Crippa S, Pedrazzini T. Small and long non-coding RNAs in cardiac homeostasis and regeneration. Biochim Biophys Acta. 1833;2013:923–33.
6. Oberpriller JO, Oberpriller JC. Response of the adult newt ventricle to injury. J Exp Zool. 1974;187:249–53.
7. Poss KD, Wilson LG, Keating MT. Heart regeneration in zebrafish. Science. 2002;298: 2188–90.
8. Chablais F, Jazwinska A. The regenerative capacity of the zebrafish heart is dependent on TGFbeta signaling. Development. 2012;139:1921–30.
9. Gonzalez-Rosa JM, Martin V, Peralta M, Torres M, Mercader N. Extensive scar formation and regression during heart regeneration after cryoinjury in zebrafish. Development. 2011;138:1663–74.
10. Wang J, Panakova D, Kikuchi K, et al. The regenerative capacity of zebrafish reverses cardiac failure caused by genetic cardiomyocyte depletion. Development. 2011;138:3421–30.
11. Jopling C, Sleep E, Raya M, Marti M, Raya A, Izpisua Belmonte JC. Zebrafish heart regeneration occurs by cardiomyocyte dedifferentiation and proliferation. Nature. 2010;464: 606–9.
12. Kikuchi K, Holdway JE, Werdich AA, et al. Primary contribution to zebrafish heart regeneration by gata4(+) cardiomyocytes. Nature. 2010;464:601–5.
13. Porrello ER, Mahmoud AI, Simpson E, et al. Transient regenerative potential of the neonatal mouse heart. Science. 2011;331:1078–80.
14. Holm S. The blastocyst transfer method cannot rescue human embryonic stem cell research. Am J Bioeth. 2005;5:20–1; discussion W10-3.
15. Kehat I, Kenyagin-Karsenti D, Snir M, et al. Human embryonic stem cells can differentiate into myocytes with structural and functional properties of cardiomyocytes. J Clin Invest. 2001;108:407–14.
16. Arbel G, Caspi O, Huber I, Gepstein A, Weiler-Sagie M, Gepstein L. Methods for human embryonic stem cells derived cardiomyocytes cultivation, genetic manipulation, and transplantation. Methods Mol Biol. 2010;660:85–95.
17. Melo LG, Pachori AS, Kong D, et al. Molecular and cell-based therapies for protection, rescue, and repair of ischemic myocardium: reasons for cautious optimism. Circulation. 2004;109:2386–93.
18. Laflamme MA, Murry CE. Regenerating the heart. Nat Biotechnol. 2005;23:845–56.
19. Quevedo HC, Hatzistergos KE, Oskouei BN, et al. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. Proc Natl Acad Sci U S A. 2009;106:14022–7.
20. Gnecchi M, Zhang Z, Ni A, Dzau VJ. Paracrine mechanisms in adult stem cell signaling and therapy. Circ Res. 2008;103:1204–19.
21. Gnecchi M, He H, Liang OD, et al. Paracrine action accounts for marked protection of ischemic heart by akt-modified mesenchymal stem cells. Nat Med. 2005;11:367–8.
22. Gnecchi M, Danieli P, Cervio E. Mesenchymal stem cell therapy for heart disease. Vascul Pharmacol. 2012;57:48–55.
23. Beltrami AP, Barlucchi L, Torella D, et al. Adult cardiac stem cells are multipotent and support myocardial regeneration. Cell. 2003;114:763–76.
24. Nadal-Ginard B, Kajstura J, Leri A, Anversa P. Myocyte death, growth, and regeneration in cardiac hypertrophy and failure. Circ Res. 2003;92:139–50.
25. Ellison GM, Torella D, Dellegrottaglie S, et al. Endogenous cardiac stem cell activation by insulin-like growth factor-1/hepatocyte growth factor intracoronary injection fosters survival and regeneration of the infarcted pig heart. J Am Coll Cardiol. 2011;58:977–86.
26. Torella D, Ellison GM, Karakikes I, Nadal-Ginard B. Resident cardiac stem cells. Cell Mol Life Sci. 2007;64:661–73.
27. Hsieh PC, Segers VF, Davis ME, et al. Evidence from a genetic fate-mapping study that stem cells refresh adult mammalian cardiomyocytes after injury. Nat Med. 2007;13:970–4.

28. Ellison GM, Galuppo V, Vicinanza C, et al. Cardiac stem and progenitor cell identification: different markers for the same cell? Front Biosci (Schol Ed). 2010;2:641–52.
29. Messina E, De Angelis L, Frati G, et al. Isolation and expansion of adult cardiac stem cells from human and murine heart. Circ Res. 2004;95:911–21.
30. Kong D, Melo LG, Mangi AA, et al. Enhanced inhibition of neointimal hyperplasia by genetically engineered endothelial progenitor cells. Circulation. 2004;109:1769–75.
31. Herzog EL, Chai L, Krause DS. Plasticity of marrow-derived stem cells. Blood. 2003;102:3483–93.
32. Tosh D, Slack JM. How cells change their phenotype. Nat Rev Mol Cell Biol. 2002;3: 187–94.
33. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. Cell. 2006;126:663–76.
34. Gurdon JB, Melton DA. Nuclear reprogramming in cells. Science. 2008;322:1811–5.
35. Mauritz C, Schwanke K, Reppel M, et al. Generation of functional murine cardiac myocytes from induced pluripotent stem cells. Circulation. 2008;118:507–17.
36. Nelson TJ, Martinez-Fernandez A, Terzic A. Induced pluripotent stem cells: developmental biology to regenerative medicine. Nat Rev Cardiol. 2010;7:700–10.
37. Zhang J, Wilson GF, Soerens AG, et al. Functional cardiomyocytes derived from human induced pluripotent stem cells. Circ Res. 2009;104:e30–41.
38. Swijnenburg RJ, Govaert JA, van der Bogt KE, et al. Timing of bone marrow cell delivery has minimal effects on cell viability and cardiac recovery after myocardial infarction. Circ Cardiovasc Imaging. 2010;3:77–85.
39. Nelson TJ, Martinez-Fernandez A, Yamada S, Ikeda Y, Perez-Terzic C, Terzic A. Induced pluripotent stem cells: advances to applications. Stem Cells Cloning. 2010;3:29–37.
40. Vierbuchen T, Ostermeier A, Pang ZP, Kokubu Y, Sudhof TC, Wernig M. Direct conversion of fibroblasts to functional neurons by defined factors. Nature. 2010;463:1035–41.
41. Ieda M, Fu JD, Delgado-Olguin P, et al. Direct reprogramming of fibroblasts into functional cardiomyocytes by defined factors. Cell. 2010;142:375–86.
42. Fu JD, Stone NR, Liu L, et al. Direct reprogramming of human fibroblasts toward a cardiomyocyte-like state. Stem Cell Reports. 2013;1:235–47.
43. Cai B, Li J, Wang J, et al. microRNA-124 regulates cardiomyocyte differentiation of bone marrow-derived mesenchymal stem cells via targeting STAT3 signaling. Stem Cells. 2012;30:1746–55.
44. Lee Y, Kim M, Han J, et al. MicroRNA genes are transcribed by RNA polymerase II. EMBO J. 2004;23:4051–60.
45. Han J, Lee Y, Yeom KH, et al. Molecular basis for the recognition of primary microRNAs by the drosha-DGCR8 complex. Cell. 2006;125:887–901.
46. Ruby JG, Jan CH, Bartel DP. Intronic microRNA precursors that bypass Drosha processing. Nature. 2007;448:83–6.
47. Gangaraju VK, Lin H. MicroRNAs: key regulators of stem cells. Nat Rev Mol Cell Biol. 2009;10:116–25.
48. Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. PLoS Biol. 2005;3, e85.
49. Bartel B. MicroRNAs directing siRNA biogenesis. Nat Struct Mol Biol. 2005;12:569–71.
50. Hwang HW, Wentzel EA, Mendell JT. A hexanucleotide element directs microRNA nuclear import. Science. 2007;315:97–100.
51. Kim DH, Saetrom P, Snove Jr O, Rossi JJ. MicroRNA-directed transcriptional gene silencing in mammalian cells. Proc Natl Acad Sci U S A. 2008;105:16230–5.
52. Vasudevan S, Tong Y, Steitz JA. Switching from repression to activation: microRNAs can up-regulate translation. Science. 2007;318:1931–4.
53. Kozak M. Faulty old ideas about translational regulation paved the way for current confusion about how microRNAs function. Gene. 2008;423:108–15.
54. Eulalio A, Huntzinger E, Izaurralde E. Getting to the root of miRNA-mediated gene silencing. Cell. 2008;132:9–14.

55. Olson EN, Schneider MD. Sizing up the heart: development redux in disease. Genes Dev. 2003;17:1937–56.
56. Liu N, Olson EN. MicroRNA regulatory networks in cardiovascular development. Dev Cell. 2010;18:510–25.
57. 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;102:12135–40.
58. Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. Nature. 2005;436:214–20.
59. Saxena A, Tabin CJ. miRNA-processing enzyme dicer is necessary for cardiac outflow tract alignment and chamber septation. Proc Natl Acad Sci U S A. 2010;107:87–91.
60. Knecht AK, Bronner-Fraser M. Induction of the neural crest: a multigene process. Nat Rev Genet. 2002;3:453–61.
61. Rao PK, Toyama Y, Chiang HR, et al. Loss of cardiac microRNA-mediated regulation leads to dilated cardiomyopathy and heart failure. Circ Res. 2009;105:585–94.
62. Kwon C, Han Z, Olson EN, Srivastava D. MicroRNA1 influences cardiac differentiation in drosophila and regulates notch signaling. Proc Natl Acad Sci U S A. 2005;102:18986–91.
63. Zhao Y, Ransom JF, Li A, et al. Dysregulation of cardiogenesis, cardiac conduction, and cell cycle in mice lacking miRNA-1–2. Cell. 2007;129:303–17.
64. Chen J, Wang DZ. microRNAs in cardiovascular development. J Mol Cell Cardiol. 2012;52:949–57.
65. Porrello ER. microRNAs in cardiac development and regeneration. Clin Sci (Lond). 2013;125:151–66.
66. 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.
67. Callis TE, Pandya K, Seok HY, et al. MicroRNA-208a is a regulator of cardiac hypertrophy and conduction in mice. J Clin Invest. 2009;119:2772–86.
68. Montgomery RL, Hullinger TG, Semus HM, et al. Therapeutic inhibition of miR-208a improves cardiac function and survival during heart failure. Circulation. 2011;124:1537–47.
69. van Rooij E, Quiat D, Johnson BA, et al. A family of microRNAs encoded by myosin genes governs myosin expression and muscle performance. Dev Cell. 2009;17:662–73.
70. Ventura A, Young AG, Winslow MM, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell. 2008;132:875–86.
71. Yelon D. Cardiac patterning and morphogenesis in zebrafish. Dev Dyn. 2001;222:552–63.
72. Srivastava D. Making or breaking the heart: from lineage determination to morphogenesis. Cell. 2006;126:1037–48.
73. Morton SU, Scherz PJ, Cordes KR, Ivey KN, Stainier DY, Srivastava D. microRNA-138 modulates cardiac patterning during embryonic development. Proc Natl Acad Sci U S A. 2008;105:17830–5.
74. Fish JE, Wythe JD, Xiao T, et al. A Slit/miR-218/Robo regulatory loop is required during heart tube formation in zebrafish. Development. 2011;138:1409–19.
75. Small EM, Olson EN. Pervasive roles of microRNAs in cardiovascular biology. Nature. 2011;469:336–42.
76. Niwa H. Self-renewal and differentiation of ES cells. Hum Cell. 2000;13:161–75.
77. Kopp JL, Ormsbee BD, Desler M, Rizzino A. Small increases in the level of Sox2 trigger the differentiation of mouse embryonic stem cells. Stem Cells. 2008;26:903–11.
78. Xu N, Papagiannakopoulos T, Pan G, Thomson JA, Kosik KS. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell. 2009;137:647–58.
79. Takaya T, Ono K, Kawamura T, et al. MicroRNA-1 and MicroRNA-133 in spontaneous myocardial differentiation of mouse embryonic stem cells. Circ J. 2009;73:1492–7.
80. Sluijter JP, van Mil A, van Vliet P, et al. MicroRNA-1 and -499 regulate differentiation and proliferation in human-derived cardiomyocyte progenitor cells. Arterioscler Thromb Vasc Biol. 2010;30:859–68.

81. Ivey KN, Muth A, Arnold J, et al. MicroRNA regulation of cell lineages in mouse and human embryonic stem cells. Cell Stem Cell. 2008;2:219–29.
82. Wilson KD, Hu S, Venkatasubrahmanyam S, et al. Dynamic microRNA expression programs during cardiac differentiation of human embryonic stem cells: role for miR-499. Circ Cardiovasc Genet. 2010;3:426–35.
83. Chen F, Chen ZY, Yang HT. Expression profile of microRNAs in the cardiomyocytes derived from mouse embryonic stem cells. Sheng Li Xue Bao. 2014;66:702–8.
84. Porrello ER, Mahmoud AI, Simpson E, et al. Regulation of neonatal and adult mammalian heart regeneration by the miR-15 family. Proc Natl Acad Sci U S A. 2013;110:187–92.
85. Chen JF, Mandel EM, Thomson JM, et al. The role of microRNA-1 and microRNA-133 in skeletal muscle proliferation and differentiation. Nat Genet. 2006;38:228–33.
86. Care A, Catalucci D, Felicetti F, et al. MicroRNA-133 controls cardiac hypertrophy. Nat Med. 2007;13:613–8.
87. Hosoda T, Zheng H, Cabral-da-Silva M, et al. Human cardiac stem cell differentiation is regulated by a mircrine mechanism. Circulation. 2011;123:1287–96.
88. Xiao J, Zhu X, He B, et al. MiR-204 regulates cardiomyocyte autophagy induced by ischemia-reperfusion through LC3-II. J Biomed Sci. 2011;18:35.
89. Crippa S, Cassano M, Messina G, et al. miR669a and miR669q prevent skeletal muscle differentiation in postnatal cardiac progenitors. J Cell Biol. 2011;193:1197–212.
90. Arminan A, Gandia C, Bartual M, et al. Cardiac differentiation is driven by NKX2.5 and GATA4 nuclear translocation in tissue-specific mesenchymal stem cells. Stem Cells Dev. 2009;18:907–18.
91. Wang F, Gui YH. Progress in studies on microRNAs regulation of cardiac development. Zhonghua Er Ke Za Zhi. 2010;48:791–4.
92. Glass C, Singla DK. MicroRNA-1 transfected embryonic stem cells enhance cardiac myocyte differentiation and inhibit apoptosis by modulating the PTEN/Akt pathway in the infarcted heart. Am J Physiol Heart Circ Physiol. 2011;301:H2038–49.
93. Glass C, Singla DK. ES cells overexpressing microRNA-1 attenuate apoptosis in the injured myocardium. Mol Cell Biochem. 2011;357:135–41.
94. Pisano F, Altomare C, Cervio E, et al. Combination of miRNA499 and miRNA133 exerts a synergic effect on cardiac differentiation. Stem Cells. 2015;33:1187–99.
95. Han JK, Chang SH, Cho HJ, et al. Direct conversion of adult skin fibroblasts to endothelial cells by defined factors. Circulation. 2014;130:1168–78.
96. Qian L, Berry EC, Fu JD, Ieda M, Srivastava D. Reprogramming of mouse fibroblasts into cardiomyocyte-like cells in vitro. Nat Protoc. 2013;8:1204–15.
97. Qian L, Huang Y, Spencer CI, et al. In vivo reprogramming of murine cardiac fibroblasts into induced cardiomyocytes. Nature. 2012;485:593–8.
98. Song K, Nam YJ, Luo X, et al. Heart repair by reprogramming non-myocytes with cardiac transcription factors. Nature. 2012;485:599–604.
99. Nam YJ, Song K, Luo X, et al. Reprogramming of human fibroblasts toward a cardiac fate. Proc Natl Acad Sci U S A. 2013;110:5588–93.
100. Wada R, Muraoka N, Inagawa K, et al. Induction of human cardiomyocyte-like cells from fibroblasts by defined factors. Proc Natl Acad Sci U S A. 2013;110:12667–72.
101. Jayawardena TM, Egemnazarov B, Finch EA, et al. MicroRNA-mediated in vitro and in vivo direct reprogramming of cardiac fibroblasts to cardiomyocytes. Circ Res. 2012;110: 1465–73.
102. Sadahiro T, Yamanaka S, Ieda M. Direct cardiac reprogramming: progress and challenges in basic biology and clinical applications. Circ Res. 2015;116:1378–91.
103. Eulalio A, Mano M, Dal Ferro M, et al. Functional screening identifies miRNAs inducing cardiac regeneration. Nature. 2012;492:376–81.
104. Huang J, Zhang Z, Guo J, et al. Genetic modification of mesenchymal stem cells overexpressing CCR1 increases cell viability, migration, engraftment, and capillary density in the injured myocardium. Circ Res. 2010;106:1753–62.

105. Tivnan A, Orr WS, Gubala V, et al. Inhibition of neuroblastoma tumor growth by targeted delivery of microRNA-34a using anti-disialoganglioside GD2 coated nanoparticles. PLoS One. 2012;7, e38129.
106. Ibrahim AG, Cheng K, Marban E. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. Stem Cell Reports. 2014;2:606–19.
107. Ghosh R, Singh LC, Shohet JM, Gunaratne PH. A gold nanoparticle platform for the delivery of functional microRNAs into cancer cells. Biomaterials. 2013;34:807–16.
108. Vasir JK, Labhasetwar V. Biodegradable nanoparticles for cytosolic delivery of therapeutics. Adv Drug Deliv Rev. 2007;59:718–28.
109. Thum T, Gross C, Fiedler J, et al. MicroRNA-21 contributes to myocardial disease by stimulating MAP kinase signalling in fibroblasts. Nature. 2008;456:980–4.

Chapter 8
microRNAs and Endometrial Pathophysiology

Henry H. Chill, Uri P. Dior, Liron Kogan, and Ariel Revel

Abstract Embryo implantation requires a reciprocal interaction between the blastocyst and endometrium and is associated with complex regulatory mechanisms. Since their discovery, microRNAs became prominent candidates providing missing links for many biological pathways. In recent years, microRNAs were implicated as one of the important players in regulation of various biological and physiological endometrial related processes. This chapter aims to present recent knowledge pertaining to the diverse aspects of microRNAs in the embryo–endometrial relationship. We will focus on the role of microRNAs in decidualization and their part in natural and stimulated cycles. Next, we will present recent studies deliberating the role of microRNAs in recurrent pregnancy loss and in the important phenomenon of recurrent implantation failure. Lastly, demonstrating an important aspect of embryo implantation and invasion, we will outline few microRNA related shared pathways of implantation and carcinogenesis.

Keywords Endometrium • Decidualization • Implantation • Gynecology • Angiogenesis • Immunomodulation • Pregnancy

Introduction

Implantation of an embryo in the endometrium requires a dialog between the embryo and a receptive endometrium [1]. Decidualization is a process in which the endometrium undergoes morphological and biological changes enabling trophoblast invasion and pregnancy [2]. Decidualization and implantation are regulated complexly by a wide range of signaling molecules and transcriptional factors [3–5].

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Lately there is emerging evidence for the role of miRNA in the regulation of these processes. The aim of this chapter is to summarize and highlight the role of miRNA in embryo implantation.

miRNAs and Decidualization

Decidualization, characterized by morphological and biochemical transformation of endometrial stromal fibroblast into differentiated decidual cells is critical for embryo implantation, placental formation, and pregnancy establishment [1]. From a clinical point of view, decidualization is the postovulatory process in which the endometrium prepares itself for the upcoming pregnancy. Histologically, the main endometrial alterations are extensive angiogenesis and vascular remodeling, secretory transformation of the uterine glands and influx of specialized uterine natural killer cells [2]. Flawed decidualization has been linked to severe pregnancy complications including preeclampsia [6] and placenta accrete [7].

The decidualization process is dependent mainly on progesterone signaling pathways and is regulated by various molecular and genetic pathways. Yet, until recently, little has been known about posttranscriptional regulation of decidual transformation of the post-ovulatory endometrium. Qian et al. were one of the first to describe the role of miRNA in the regulation of decidualization [8]. By microarray analysis they have revealed 49 miRNA genes differentially expressed between induced and non-induced endometrial stromal cells. They have further shown that hsa-miR-222 has a key role in the differentiation of endometrial stromal cells.

The study of Estella et al. aimed to profile the miRNAs expression throughout human endometrial stromal decidualization [9]. Possibly due to different molecular methods, they have found different miRNAs than Qian et al. Three miRNAs families, miR-181, miR-183, and miR-200, were found to be downregulated and 26 miRNAs were upregulated during the decidualization process. These miRNAs target various transcriptional factors, growth factors, extracellular matrix enzymes, and interleukins involved in pathways related to the regulation of actin filaments, a key process required for the correct differentiation of endometrial stromal cells. They have also found that the RNAse Dicer has a minor effect on endometrial stromal cells during decidualization.

miRNAs are differentially expressed during the physiological phases of the menstrual cycle. One study found that miRNAs that show increased transcript abundance during the mid-secretory phase are related to downregulation of the expression of cell cycle genes, thereby suppressing cell cycle progression and cell proliferation in human secretory-phase endometrial epithelium [10]. Figure 8.1 demonstrates miRNAs associated with various biologic aspects of embryo implantation. As is shown, specific miRNA are known to play important roles in decidualization, angiogenesis, immunomodulation, adhesion, and invasion. In summary, recent discoveries have shown that miRNA have an important role in the decidualization process though there is still much to be discovered regarding the complex pathways through which this modulation takes place.

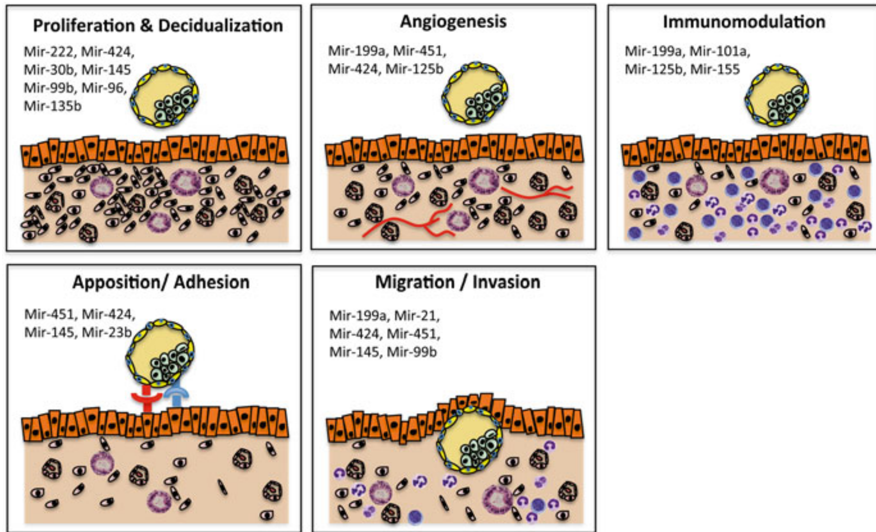

Fig. 8.1 miRNAs associated with embryo implantation

Expression of miRNA in Natural and Stimulated Cycles

In the past few decades there has been a constant rise in the use of artificial reproductive technology (ART) as a means of achieving higher pregnancy rates. Questions have been raised regarding biological differences on the molecular level between natural and stimulated cycles. These questions transcend to the realm of miRNA and recently have been addressed by research targeted towards this topic.

Through microarray analysis, Zhao et al. sought to find whether endometrial miRNA expression is affected by different luteal phase support protocols in IVF cycles [11]. They have found that luteal support protocol has a profound influence on miRNA profile following controlled ovarian stimulation. Specifically, progesterone supplementation was associated with a significant increase in miRNA expression. Those findings are in accordance with previous reports identifying differential expression patterns of miRNA between natural and stimulated cycles.

By deep sequencing techniques, Sha et al. investigated miRNA expression profiles of human endometrium 2 and 7 days after the LH surge in natural cycles as compared to those on 4 and 7 days after administration of hCG in stimulated cycles during IVF treatment [12]. They have demonstrated a few interesting findings. First, within the natural cycles, miRNAs targeting genes associated with cell cycle, transport, cell adhesion, cell death, and metabolism were differentially expressed between days 2 and 7 after the LH surge. Second, 22 miRNAs were significantly dysregulated on the seventh day after hCG injection, as compared to the seventh day after the LH surge in natural cycles. Third, miRNA expression profile on day hCG+4 was similar to that on day LH+7, suggesting that ovarian stimulation may alter the window of endometrial receptivity.

Elevated serum progesterone (P) on the day of human chorionic gonadotrophin (hCG) injection has been reported to occur in 20–40 % of in vitro fertilization (IVF) and embryo transfer (ET) cycles [13]. Clinical studies have demonstrated decreased endometrial receptivity in women with high serum P concentrations [14, 15]. In an attempt to explain the lower pregnancy rates in stimulated cycles in which progesterone levels are elevated, Li et al. sampled endometrial tissue 6 days after oocyte retrieval. Through array analysis they have found that four miRNAs (mir-451, mir-424, mir-125b, and mir-30b) were differentially regulated in normal and high progesterone level tissues [16]. Hence, ovarian steroids may have an impact on miRNA expression and regulation in the uterus. Those findings may implicate that miRNAs are potential biomarkers for human endometrial receptivity and may be used to optimize IVF treatment protocol and subsequent pregnancy rate.

miRNA and Recurrent Pregnancy Loss

Approximately 20 % of all clinical pregnancies result in miscarriages [17], most of which occur during the first trimester. Cytogenetic analyses had shown that 50 % or more of them are related to chromosomal abnormalities [18]. Extensive research effort is dedicated to understanding of the pathophysiology of euploid miscarriages.

Recent studies have shown that more sensitive genetic techniques, such as array comparative genomic hybridization (array-CGH), can identify genome imbalances in miscarriages [19]. The presence of miRNAs on both sides of the maternal–fetal interface appears to be important for implantation and subsequent pregnancy [20]. Viaggi et al. performed an array-CGH analysis of 40 fetal tissue samples derived from first trimester spontaneous abortions [21] and identified 45 copy number variants (CNVs). CNVs refer to extensive genomic structural variation, ranging in size from kilobases to megabases. They can be inherited or sporadic and may have phenotypic effects [22]. Out of the 45 CNVs identified by Viaggi et al., 14 were classified as unique and 5 miRNAs were contained within these CNVs.

Through PCR analysis, Joo Jeon et al. have shown that chromosomally normal spontaneous aborted fetuses had significantly different frequencies of the miR-196a2CC, miR-146aCC/miR-196a2CC, and miR-149TT/miR-196a2CC genotypes compared with control subjects [23]. The targets of those miRNAs are genes involved in apoptosis [24], homocysteine regulation [25], implantation [26], cell growth [27], and inflammation [28].

miRNA-mediated posttranscriptional regulation may be required for normal embryogenesis [29, 30] and embryonic germ cell development [31]. For example, mice studies had shown that mir-290 through mir-295 that plays important roles in embryonic development results in partially penetrant embryonic lethality and germ cell defects when deficient [32]. Similarly, out of eight potential miRNAs, miRNA-450a-3p was found to represses cell proliferation and regulates embryo development by regulating Bub1. Bub1 is a critical component of the spindle assembly checkpoint [33]. The authors therefore speculated that blockade of miR-450a-3p

may be explored as a novel therapeutic strategy for preventing spontaneous miscarriages. In a recent study, miRNA 17 and 19b and their main target PTEN mRNA, were found to be significantly down- and upregulated, respectively, in early pregnancy loss [34]. Also this study highlights the potential clinical implications of miRNAs in pregnancy loss.

Recurrent pregnancy loss (RPL), defined by two or more failed clinical pregnancies [35], is a major fertility concern and is experienced by approximately 5 % of women trying to conceive [36]. A putative diagnosis will be made in approximately 50 % of patients with RPL, acknowledging that our understanding of this field is significantly limited [37]. Polymorphisms in specific miRNAs related with RPL implicate a possible genetic predisposition to RPL [38, 39].

Hu et al. described one nucleotide mutation in pri-miR-125a which related to RPL. By scanning pri-miR-125a coding region in 389 Chinese women with RPL it was indicated that an A>G mutation reduced mature miR-125a expression and led to less efficient inhibition of specific target genes [40]. Further evidence associates regulation of human leukocyte antigen (HLA)-G by mir-133a to RPL, by reducing its translation [40]. HLA-G is expressed in the placenta throughout gestation [41]. Because of its unique expression pattern at the fetal–maternal interface, it has been postulated that HLA-G plays a critical role in maternal immune tolerance to the fetus [42]. Alteration in its expression, due to overexpression of mir-133a may be related to RPL, raising the possibility that miR-133a may be another target for treatment of RPL.

Natural killer (NK) cells, an important component of the innate immune system, form the largest population of leucocytes in the endometrium [43] and are adjacent to trophoblast cells in early pregnancy. Many studies have associated a high density of uterine NK cells with RPL [44]. Liu et al. revealed that miRNA-155 overexpression enhanced and miRNA-155 antagonist impaired the NK cell-mediated killing activity, indicating that miRNA-155 plays an important role in the control of NK cell cytotoxicity [45]. These findings may have clinical relevance for targeting miR-155 in RPL.

miRNA and Recurrent Implantation Failure

Implantation failure, particularly the unexplained repeated implantation failure (RIF), is still the unsolved and principal problem to affect the outcome of assisted reproductive technology [46]. In a previous study Revel et al. identified 13 miRNAs, differentially expressed in RIF endometrial samples, which putatively regulate the expression of 3800 genes. It was found that ten miRNAs were overexpressed (including miR 145, 23b, and 99a) and three were underexpressed. Consistent with the miRNA-predicted targets, mRNA levels of N-cadherin, H2AFX, netrin-4 and secreted frizzled-related protein-4, belonging to the cell adhesion molecules, Wnt signaling and cell cycle pathways were lower in RIF-IVF patients [47]. According to these findings, RIF-associated miRNAs could be exploited as new candidates for diagnosis and treatment of embryo implantation failures.

Kang et al. assessed the role of miR-145 and its target IGF1R in early implantation. This was done by using Ishikawa endometrial cells in which miR-145 overexpression and IGF1R knockdown were achieved. The effects of altered miR-145 and/or IGF1R were studied by following the attachment of mouse embryos or IGF-I coated beads to endometrial cells. miR-145 overexpression or specific reduction of IGF1R impaired attachment in both cases. According to this study, not only is miR-145 a regulator of IGF1R expression but there seems to be evidence pointing towards IGF1R playing a role in embryo implantation [47].

In another study Chu et al. showed that there is downregulation of the miR-181 family in mouse uterus on day 4 of pregnancy with levels of miR-181a and miR-181b rising back to normal on day 6 and 7. Later mice were given repeated administrations of nanoparticles packed with either miR-181a or miR-181b mimics on day 2 and 3 of pregnancy. When inspected on day 4 of pregnancy mouse uterus were indeed enriched with miR-181a and miR-181b. In mice exposed to these miRNAs the number of implanted embryos was significantly lower than the control mice showing that miR-181a and miR-181b may inhibit embryo implantation [47].

It has been recognized that leukemia inhibitory factor (LIF) plays a crucial role in embryo implantation. In mice LIF is known to be upregulated by different factors some of which are known, while others remain obscure. Estrogen has been shown to be one of the factors to upregulate LIF [51–53]. In LIF knockout mice, administration of exogenous LIF on day 4 of pregnancy restored embryo implantation. Chu et al. continued their study by generating miR-181a and miR-181b transgenic mice which did indeed express high levels of miR-181a/b in uterus during pregnancy compared to control mice [47]. These transgenic mice showed a decreased pregnancy rate and decreased litter size compared with control mice as well as lower LIF expression in uteri on day 4 of pregnancy. To assess the connection between miR-181a/b and LIF, the latter was administered to miR-181a/b transgenic mice on day 4 of pregnancy. LIF injection greatly improved implantation in theses mice implying that miR-181a/b inhibit embryo implantation through LIF.

Finally, it has been shown that expression of miR181 is controlled by the transcriptional factor Emx2. It is known that Emx2 is downregulated by estrogen [54] and it can be hypothesized that high levels of estrogen downregulating Emx2 mediate the downregulation of miR-181 increasing levels of LIF to allow embryo implantation.

The Emx2-miR-181-LIF axis, depicted in Fig. 8.2, seems to play a major role in this process. These discoveries leave room for future research towards finding more pathways through which microRNA may influence embryo implantation.

miRNA Related Shared Pathways of Implantation and Cancer

The embryo implantation process is complex, requiring reciprocal interactions between the developing blastocyst and the receptive uterus. This process involves a host of endocrine, paracrine, autocrine, and juxtacrine modulators [48].

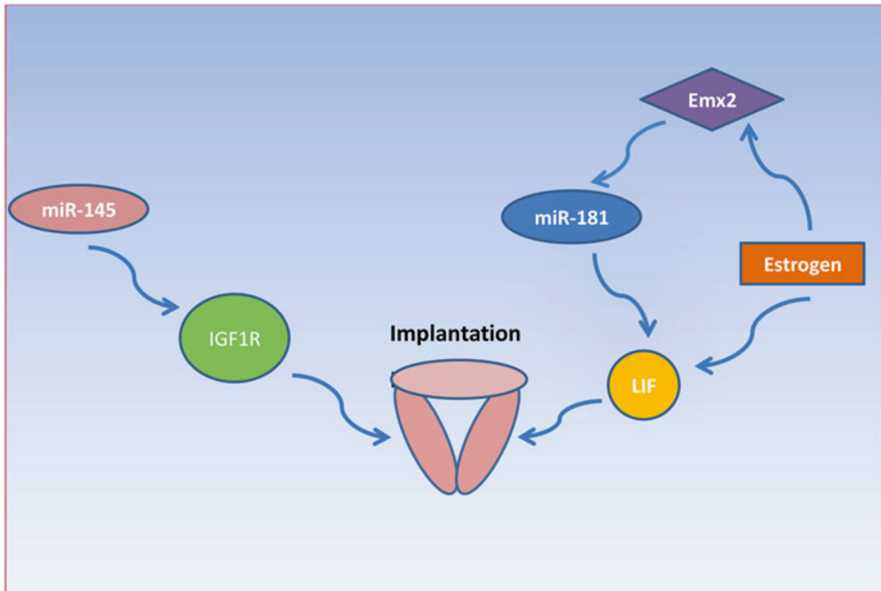

Fig. 8.2 Shown is the Emx2-miR-181-LIF (leukemia inhibitory factor) axis. miR-145 and IGF1R (insulin-like growth factor 1 receptor) give another example of miRNA playing an important role in the implantation process

"Endometrial receptivity" refers to the ability of the uterus to accept and accommodate a nascent embryo, resulting in a successful pregnancy. Reduced endometrial receptivity in stimulated IVF cycles may result in low implantation rate. Aggressive behaviors of trophoblast during embryo implantation resemble those of malignant tumor cells. Shared miRNAs and target genes may thus be potentially involved in endometrial receptivity and in various tumorigenesis models.

Cox-2 has a critical role in implantation [49] as well as in precancerous hyperplastic endometrium. Chakrabarty et al. have demonstrated that mmu-miR-101a and mmu-miR-199a are expressed in the mouse uterus during implantation coincident with expression of cyclooxygenase-2 (cox-2). Cox-2 gene expression regulation plays a role also in endometrial cancer pathogenesis. In a mouse model of endometrial cancer, levels of two miRNAs, miR-199a and miR-101a, targeting Cox-2 expression, were downregulated in uterine cancer [50]. Other studies have shown that human endometrial cancer and endometria with hyperplasia express elevated Cox-2 [51–53].

miR-199a plays a role in regulation of cell adhesion, migration, and invasion in various tissues including endometrial stromal cells. These are key biological processes in embryo implantation [54–57] and in cancer. A functional analysis revealed that ectopic expression of miR-199a suppresses tumor angiogenesis in ovarian cancer tissues, in vitro and in vivo, indicating an anti-angiogenic function [58]

(see Chap. 8 of the volume "microRNA: Cancer" for a detailed discussion of miRNA and ovarian cancer). Overexpression of miR-199a was shown to down-regulate HIF-1a protein expression and VEGF mRNA levels, suggesting that miR-199a suppresses tumor angiogenesis by decreasing the expression of HIF-1a and VEGF [58].

mir-21 is also involved in regulation of implantation and cancer. Previous studies have demonstrated a differential expression of miRNAs in different endometrial locations. Eight miRNAs were found to be upregulated in implantation sites, as compared to inter-implantation sites [59]. Among these, mir-21 was highly expressed in the subluminal stromal cells at implantation sites on day 5 of pregnancy [59]. mir-21, however, has been found to be associated also with endometriosis, leiomyoma and cervical and endometrial cancer [60, 61]. Recent studies confirmed that miR-21 is overexpressed in endometrioid endometrial cancer (EEC) tissues compared with their adjacent matched non-tumor endometrium [62, 63]. PTEN, a tumor suppressor gene, has been validated as a target gene of miR-21 in a variety of malignancies [64, 65].

Overexpression of miR-21 modulated EEC cell proliferation through the downregulation of phosphatase and tensin homolog deleted from chromosome-10 (PTEN) [62]. These correlations between altered miR-21 expression and malignancy-related cellular processes, including proliferation, migration, and apoptosis, have been well demonstrated [60, 66, 67]. Similarly, PTEN plays a role in embryo implantation. Laguë et al. demonstrated novel roles of PTEN in the mammalian uterus and its requirement for proper trophoblast invasion and decidual regression [68].

As previously mentioned, mir-451 and mir-424 are differentially expressed under different hormonal stimulation [16]. Early and recent studies have shown that mir-424 and mir-451 play a critical role in tumor genesis and progression [69, 70]. Another shared pathway of implantation and cancer, regulated by those miRNAs, can be demonstrated by the function of two essential proteins in the pathogenesis of implantation and cancer: Osteopontin and Vascular endothelial growth factor (VEGF).

Spp1 is the gene encoding for Osteopontin protein and the Ang gene encodes the VEGF protein. Both of those genes are regulated by miR-451 and mir-424 [16]. Osteopontin is a glycoprotein that mediates cellular adhesion and migration during embryo implantation and is regulated mainly by progesterone. Its maximal expression in endometrial epithelial cells has been observed during the window of implantation [71]. In tumorigenesis, Osteopontin signaling could result in the activation of anti-apoptosis and pro-survival pathways. This is enabled via PI3-KAkt and NF-κB signaling molecules, angiogenesis modulation via VEGF induction and extracellular matrix (ECM) degradation via Matrix Metallo-Proteinases (MMPs) [72]. All of which are fundamental pathways in embryo implantation. VEGF is a key regulator of embryo implantation [73] and cancer [74]. Importantly, the Osteopontin protein was shown to be expressed more predominantly and VEGF expression was less intense in women with high versus normal serum progesterone group in stimulated cycles [16].

In a study mentioned earlier of miRNA expression analysis in IVF patients experiencing recurrent implantation failure (RIF), mRNA levels of N-cadherin, H2AFX,

netrin-4 and secreted frizzled-related protein-4, belonging to the cell adhesion molecules, Wnt signaling and cell cycle pathways were lower in RIF-IVF patients in correlation with the miRNA-predicted targets, including mir-145 and mir-23b [47]. Several studies have validated the downregulation of miR-145 in ovarian cancer [75–77].

In a recent study, Wu et al. found that overexpression of miR-145 led to a reduction of cell growth and invasion abilities, and overexpression was also found to induce cell apoptosis in ovarian cancer tissue [78]. This study suggests that miR-145 modulates ovarian cancer growth and invasion by suppressing p70S6K1 and MUC1 genes, functioning as a tumor suppressor [78]. miR-23b was identified as a potent modulator of cytoskeletal dynamics via co-repression of a set of cytoskeleton specific genes, affecting motile and invasive properties necessary for breast cancer cells to metastasize [79]. miR-23b represents a central effector of cytoskeletal remodeling. It increases cell–cell interactions, modulates focal adhesion and reduces cell motility and invasion by directly regulating several genes involved in these processes [80].

Conclusion

Rising evidence implicate a vital role for miRNAs in various biological events involving the endometrium. Here, we reviewed the role of miRNAs in the complex process of decidualization. We further have demonstrated that the decidualization process may have distinct biological pathways in natural and stimulated cycles, mediated in part by miRNAs. These observations have instigated many researchers to study the role of miRNAs in the still enigmatic RPL entity. Indeed, the findings relating to miRNAs in this field are important and may lead to further understanding of this biological phenomenon. Lastly, we have outlined common miRNAs relating to embryo implantation and malignancies, implicating common pathways for these biological events.

Indeed, the rapid progress in understanding the role of miRNAs in the endometrium may open a window to deepening of our knowledge and for potential treatments in fertility practice.

References

1. Paria BC, Reese J, Das SK, Dey SK. Deciphering the cross-talk of implantation: advances and challenges. Science. 2002;296(5576):2185–8.
2. Gellersen B, Brosens IA, Brosens JJ. Decidualization of the human endometrium: mechanisms, functions, and clinical perspectives. Semin Reprod Med. 2007;25(6):445–53.
3. Tierney EP, Tulac S, Huang ST, Giudice LC. Activation of the protein kinase A pathway in human endometrial stromal cells reveals sequential categorical gene regulation. Physiol Genomics. 2003;16(1):47–66.

4. Ueno Y, Yagasaki S. Toxicological approaches to the metabolites of Fusaria. X. Accelerating effect of zearalenone on RNA and protein syntheses in the uterus of ovariectomized mice. Jpn J Exp Med. 1975;45(3):199–205.
5. Popovici RM, Kao LC, Giudice LC. Discovery of new inducible genes in in vitro decidualized human endometrial stromal cells using microarray technology. Endocrinology. 2000;141(9): 3510–3.
6. Sibai B, Dekker G, Kupferminc M. Pre-eclampsia. Lancet. 2005;365(9461):785–99.
7. Jauniaux E, Jurkovic D. Placenta accreta: pathogenesis of a 20th century iatrogenic uterine disease. Placenta. 2012;33(4):244–51.
8. Qian K, Hu L, Chen H, Li H, Liu N, Li Y, et al. Hsa-miR-222 is involved in differentiation of endometrial stromal cells in vitro. Endocrinology. 2009;150(10):4734–43.
9. Estella C, Herrer I, Moreno-Moya JM, Quinonero A, Martinez S, Pellicer A, et al. miRNA signature and Dicer requirement during human endometrial stromal decidualization in vitro. PLoS One. 2012;7(7):e41080.
10. Kuokkanen S, Chen B, Ojalvo L, Benard L, Santoro N, Pollard JW. Genomic profiling of microRNAs and messenger RNAs reveals hormonal regulation in microRNA expression in human endometrium. Biol Reprod. 2010;82(4):791–801.
11. Zhao Y, Zacur H, Cheadle C, Ning N, Fan J, Vlahos NF. Effect of luteal-phase support on endometrial microRNA expression following controlled ovarian stimulation. Reprod Biol Endocrinol. 2012;10:72.
12. Sha AG, Liu JL, Jiang XM, Ren JZ, Ma CH, Lei W, et al. Genome-wide identification of micro-ribonucleic acids associated with human endometrial receptivity in natural and stimulated cycles by deep sequencing. Fertil Steril. 2011;96(1):150–5 e5.
13. Ubaldi F, Camus M, Smitz J, Bennink HC, Van Steirteghem A, Devroey P. Premature luteinization in in vitro fertilization cycles using gonadotropin-releasing hormone agonist (GnRH-a) and recombinant follicle-stimulating hormone (FSH) and GnRH-a and urinary FSH. Fertil Steril. 1996;66(2):275–80.
14. Shulman A, Ghetler Y, Beyth Y, Ben-Nun I. The significance of an early (premature) rise of plasma progesterone in in vitro fertilization cycles induced by a “long protocol” of gonadotropin releasing hormone analogue and human menopausal gonadotropins. J Assist Reprod Genet. 1996;13(3):207–11.
15. Li R, Qiao J, Wang L, Zhen X, Lu Y. Serum progesterone concentration on day of HCG administration and IVF outcome. Reprod Biomed Online. 2008;16(5):627–31.
16. Li R, Qiao J, Wang L, Li L, Zhen X, Liu P, et al. MicroRNA array and microarray evaluation of endometrial receptivity in patients with high serum progesterone levels on the day of hCG administration. Reprod Biol Endocrinol. 2011;9:29.
17. Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, et al. Incidence of early loss of pregnancy. N Engl J Med. 1988;319(4):189–94.
18. Guerneri S, Bettio D, Simoni G, Brambati B, Lanzani A, Fraccaro M. Prevalence and distribution of chromosome abnormalities in a sample of first trimester internal abortions. Hum Reprod. 1987;2(8):735–9.
19. Schaeffer AJ, Chung J, Heretis K, Wong A, Ledbetter DH, Lese MC. Comparative genomic hybridization-array analysis enhances the detection of aneuploidies and submicroscopic imbalances in spontaneous miscarriages. Am J Hum Genet. 2004;74(6):1168–74.
20. Wessels JM, Edwards AK, Khalaj K, Kridli RT, Bidarimath M, Tayade C. The microRNAome of pregnancy: deciphering miRNA networks at the maternal-fetal interface. PLoS One. 2013; 8(11), e72264.
21. Viaggi CD, Cavani S, Malacarne M, Floriddia F, Zerega G, Baldo C, et al. First-trimester euploid miscarriages analysed by array-CGH. J Appl Genet. 2013;54(3):353–9.
22. Stankiewicz P, Lupski JR. Structural variation in the human genome and its role in disease. Annu Rev Med. 2010;61:437–55.
23. Jeon YJ, Kim SY, Rah H, Choi DH, Cha SH, Yoon TK, et al. Association of the miR-146aC>G, miR-149T>C, miR-196a2T>C, and miR-499A>G polymorphisms with risk of spontaneously aborted fetuses. Am J Reprod Immunol. 2012;68(5):408–17.

24. Fluhr H, Wenig H, Spratte J, Heidrich S, Ehrhardt J, Zygmunt M. Non-apoptotic Fas-induced regulation of cytokines in undifferentiated and decidualized human endometrial stromal cells depends on caspase-activity. Mol Hum Reprod. 2011;17(2):127–34.
25. Kim SY, Park SY, Choi JW, Kim do J, Lee SY, Lim JH, et al. Association between MTHFR 1298A>C polymorphism and spontaneous abortion with fetal chromosomal aneuploidy. Am J Reprod Immunol. 2011;66(4):252–8.
26. Vitiello D, Kodaman PH, Taylor HS. HOX genes in implantation. Semin Reprod Med. 2007; 25(6):431–6.
27. Murakami A, Ishida S, Thurlow J, Revest JM, Dickson C. SOX6 binds CtBP2 to repress transcription from the Fgf-3 promoter. Nucleic Acids Res. 2001;29(16):3347–55.
28. Luthra R, Singh RR, Luthra MG, Li YX, Hannah C, Romans AM, et al. MicroRNA-196a targets annexin A1: a microRNA-mediated mechanism of annexin A1 downregulation in cancers. Oncogene. 2008;27(52):6667–78.
29. 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.
30. 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.
31. 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.
32. Medeiros LA, Dennis LM, Gill ME, Houbaviy H, Markoulaki S, Fu D, et al. Mir-290-295 deficiency in mice results in partially penetrant embryonic lethality and germ cell defects. Proc Natl Acad Sci U S A. 2011;108(34):14163–8.
33. Luo M, Weng Y, Tang J, Hu M, Liu Q, Jiang F, et al. MicroRNA-450a-3p represses cell proliferation and regulates embryo development by regulating Bub1 expression in mouse. PLoS One. 2012;7(10), e47914.
34. Ventura W, Koide K, Hori K, Yotsumoto J, Sekizawa A, Saito H, et al. Placental expression of microRNA-17 and -19b is down-regulated in early pregnancy loss. Eur J Obstet Gynecol Reprod Biol. 2013;169(1):28–32.
35. Practice Committee of the American Society for Reproductive Medicine. Evaluation and treatment of recurrent pregnancy loss: a committee opinion. Fertil Steril. 2012;98(5):1103–11.
36. Stirrat GM. Recurrent miscarriage. Lancet. 1990;336(8716):673–5.
37. Jaslow CR, Carney JL, Kutteh WH. Diagnostic factors identified in 1020 women with two versus three or more recurrent pregnancy losses. Fertil Steril. 2010;93(4):1234–43.
38. Hu Y, Liu CM, Qi L, He TZ, Shi-Guo L, Hao CJ, et al. Two common SNPs in pri-miR-125a alter the mature miRNA expression and associate with recurrent pregnancy loss in a Han-Chinese population. RNA Biol. 2011;8(5):861–72.
39. Jeon YJ, Choi YS, Rah H, Kim SY, Choi DH, Cha SH, et al. Association study of microRNA polymorphisms with risk of idiopathic recurrent spontaneous abortion in Korean women. Gene. 2012;494(2):168–73.
40. Wang X, Li B, Wang J, Lei J, Liu C, Ma Y, et al. Evidence that miR-133a causes recurrent spontaneous abortion by reducing HLA-G expression. Reprod Biomed Online. 2012;25(4): 415–24.
41. Goldman-Wohl DS, Ariel I, Greenfield C, Hanoch J, Yagel S. HLA-G expression in extravillous trophoblasts is an intrinsic property of cell differentiation: a lesson learned from ectopic pregnancies. Mol Hum Reprod. 2000;6(6):535–40.
42. Christiansen OB. Reproductive immunology. Mol Immunol. 2013;55(1):8–15.
43. Bulmer JN, Morrison L, Longfellow M, Ritson A, Pace D. Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies. Hum Reprod. 1991;6(6):791–8.
44. Tuckerman E, Laird SM, Prakash A, Li TC. Prognostic value of the measurement of uterine natural killer cells in the endometrium of women with recurrent miscarriage. Hum Reprod. 2007;22(8):2208–13.
45. Liu X, Wang Y, Sun Q, Yan J, Huang J, Zhu S, et al. Identification of microRNA transcriptome involved in human natural killer cell activation. Immunol Lett. 2012;143(2):208–17.

46. Coughlan C, Yuan X, Nafee T, Yan J, Mariee N, Li TC. The clinical characteristics of women with recurrent implantation failure. J Obstet Gynaecol. 2013;33(5):494–8.
47. Revel A, Achache H, Stevens J, Smith Y, Reich R. MicroRNAs are associated with human embryo implantation defects. Hum Reprod. 2011;26(10):2830–40.
48. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. Nat Rev Genet. 2006;7(3):185–99.
49. Chakrabarty A, Tranguch S, Daikoku T, Jensen K, Furneaux H, Dey SK. MicroRNA regulation of cyclooxygenase-2 during embryo implantation. Proc Natl Acad Sci U S A. 2007;104(38): 15144–9.
50. Daikoku T, Hirota Y, Tranguch S, Joshi AR, DeMayo FJ, Lydon JP, et al. Conditional loss of uterine Pten unfailingly and rapidly induces endometrial cancer in mice. Cancer Res. 2008; 68(14):5619–27.
51. Hasegawa K, Ohashi Y, Ishikawa K, Yasue A, Kato R, Achiwa Y, et al. Expression of cyclooxygenase-2 in uterine endometrial cancer and anti-tumor effects of a selective COX-2 inhibitor. Int J Oncol. 2005;26(5):1419–28.
52. Nasir A, Boulware D, Kaiser HE, Lancaster JM, Coppola D, Smith PV, et al. Cyclooxygenase-2 (COX-2) expression in human endometrial carcinoma and precursor lesions and its possible use in cancer chemoprevention and therapy. In Vivo. 2007;21(1):35–43.
53. Tong BJ, Tan J, Tajeda L, Das SK, Chapman JA, DuBois RN, et al. Heightened expression of cyclooxygenase-2 and peroxisome proliferator-activated receptor-delta in human endometrial adenocarcinoma. Neoplasia. 2000;2(6):483–90.
54. Shen Q, Cicinnati VR, Zhang X, Iacob S, Weber F, Sotiropoulos GC, et al. Role of microRNA-199a-5p and discoidin domain receptor 1 in human hepatocellular carcinoma invasion. Mol Cancer. 2010;9:227.
55. Song G, Zeng H, Li J, Xiao L, He Y, Tang Y, et al. miR-199a regulates the tumor suppressor mitogen-activated protein kinase kinase kinase 11 in gastric cancer. Biol Pharm Bull. 2010; 33(11):1822–7.
56. Cheung HH, Davis AJ, Lee TL, Pang AL, Nagrani S, Rennert OM, et al. Methylation of an intronic region regulates miR-199a in testicular tumor malignancy. Oncogene. 2011;30(31): 3404–15.
57. Dai L, Gu L, Di W. MiR-199a attenuates endometrial stromal cell invasiveness through suppression of the IKKbeta/NF-kappaB pathway and reduced interleukin-8 expression. Mol Hum Reprod. 2012;18(3):136–45.
58. He J, Jing Y, Li W, Qian X, Xu Q, Li FS, et al. Roles and mechanism of miR-199a and miR-125b in tumor angiogenesis. PLoS One. 2013;8(2), e56647.
59. Hu SJ, Ren G, Liu JL, Zhao ZA, Yu YS, Su RW, et al. MicroRNA expression and regulation in mouse uterus during embryo implantation. J Biol Chem. 2008;283(34):23473–84.
60. Pan Q, Luo X, Chegini N. microRNA 21: response to hormonal therapies and regulatory function in leiomyoma, transformed leiomyoma and leiomyosarcoma cells. Mol Hum Reprod. 2010; 16(3):215–27.
61. Ramon LA, Braza-Boils A, Gilabert-Estelles J, Gilabert J, Espana F, Chirivella M, et al. microRNAs expression in endometriosis and their relation to angiogenic factors. Hum Reprod. 2011;26(5):1082–90.
62. Qin X, Yan L, Zhao X, Li C, Fu Y. microRNA-21 overexpression contributes to cell proliferation by targeting PTEN in endometrioid endometrial cancer. Oncol Lett. 2012;4(6):1290–6.
63. Torres A, Torres K, Paszkowski T, Radej S, Staskiewicz GJ, Ceccaroni M, et al. Highly increased maspin expression corresponds with up-regulation of miR-21 in endometrial cancer: a preliminary report. Int J Gynecol Cancer. 2011;21(1):8–14.
64. Lee YS, Dutta A. MicroRNAs in cancer. Annu Rev Pathol. 2009;4:199–227.
65. Frankel LB, Christoffersen NR, Jacobsen A, Lindow M, Krogh A, Lund AH. Programmed cell death 4 (PDCD4) is an important functional target of the microRNA miR-21 in breast cancer cells. J Biol Chem. 2008;283(2):1026–33.

66. Meng F, Henson R, Lang M, Wehbe H, Maheshwari S, Mendell JT, et al. Involvement of human micro-RNA in growth and response to chemotherapy in human cholangiocarcinoma cell lines. Gastroenterology. 2006;130(7):2113–29.
67. Zhu S, Wu H, Wu F, Nie D, Sheng S, Mo YY. MicroRNA-21 targets tumor suppressor genes in invasion and metastasis. Cell Res. 2008;18(3):350–9.
68. Lague MN, Detmar J, Paquet M, Boyer A, Richards JS, Adamson SL, et al. Decidual PTEN expression is required for trophoblast invasion in the mouse. Am J Physiol Endocrinol Metab. 2010;299(6):E936–46.
69. Pan X, Wang R, Wang ZX. The potential role of miR-451 in cancer diagnosis, prognosis, and therapy. Mol Cancer Ther. 2013;12(7):1153–62.
70. Oneyama C, Kito Y, Asai R, Ikeda J, Yoshida T, Okuzaki D, et al. MiR-424/503-mediated rictor upregulation promotes tumor progression. PLoS One. 2013;8(11), e80300.
71. Talbi S, Hamilton AE, Vo KC, Tulac S, Overgaard MT, Dosiou C, et al. Molecular phenotyping of human endometrium distinguishes menstrual cycle phases and underlying biological processes in normo-ovulatory women. Endocrinology. 2006;147(3):1097–121.
72. Cao DX, Li ZJ, Jiang XO, Lum YL, Khin E, Lee NP, et al. Osteopontin as potential biomarker and therapeutic target in gastric and liver cancers. World J Gastroenterol. 2012;18(30):3923–30.
73. Hannan NJ, Paiva P, Meehan KL, Rombauts LJ, Gardner DK, Salamonsen LA. Analysis of fertility-related soluble mediators in human uterine fluid identifies VEGF as a key regulator of embryo implantation. Endocrinology. 2011;152(12):4948–56.
74. Kieran MW, Kalluri R, Cho YJ. The VEGF pathway in cancer and disease: responses, resistance, and the path forward. Cold Spring Harb Perspect Med. 2012;2(12):a006593.
75. Chung YW, Bae HS, Song JY, Lee JK, Lee NW, Kim T, et al. Detection of microRNA as novel biomarkers of epithelial ovarian cancer from the serum of ovarian cancer patient. Int J Gynecol Cancer. 2013;23(4):673–9.
76. Miles GD, Seiler M, Rodriguez L, Rajagopal G, Bhanot G. Identifying microRNA/mRNA dysregulations in ovarian cancer. BMC Res Notes. 2012;5:164.
77. Nam EJ, Yoon H, Kim SW, Kim H, Kim YT, Kim JH, et al. MicroRNA expression profiles in serous ovarian carcinoma. Clin Cancer Res. 2008;14(9):2690–5.
78. Wu H, Xiao Z, Wang K, Liu W, Hao Q. MiR-145 is downregulated in human ovarian cancer and modulates cell growth and invasion by targeting p70S6K1 and MUC1. Biochem Biophys Res Commun. 2013;441(4):693–700.
79. Pellegrino L, Stebbing J, Braga VM, Frampton AE, Jacob J, Buluwela L, et al. miR-23b regulates cytoskeletal remodeling, motility and metastasis by directly targeting multiple transcripts. Nucleic Acids Res. 2013;41(10):5400–12.
80. Pellegrino L, Krell J, Roca-Alonso L, Stebbing J, Castellano L. MicroRNA-23b regulates cellular architecture and impairs motogenic and invasive phenotypes during cancer progression. Bioarchitecture. 2013;3(4):119–24.

Chapter 9
microRNA and NF-kappa B

Ye Yuan, Lingying Tong, and Shiyong Wu

Abstract Nuclear Factor kappa B (NF-κB) plays important roles in regulation of countless cellular functions, including cell cycle and apoptosis. As a versatile transcription factor, NF-κB is a target of a large amount of miRNAs. Abnormal NF-κB activity is frequently associated with an abnormal level of miRNAs, which is found to play critical roles in disease progression including cancer. While the expression and activity of NF-κB can be directly or indirectly up-regulated or downregulated by various miRNAs, NF-κB can also regulate the expression of many miRNAs. Intriguingly, reciprocal regulation between miRNAs and NF-κB, which exists in the form of positive and negative feedback loops, is often observed in various cancers. In this chapter, the mechanisms and roles of miRNA-regulated NF-κB and NF-κB-regulated miRNAs in a variety of cancers will be discussed. The potential therapeutic use of miRNAs that are up- and down-stream of NF-κB signaling pathways as targets for cancer treatment will also be accessed.

Keywords NF-κB • Cancer • Therapeutics • Apoptosis • Cell cycle • Carcinogens • Angiogenesis

Introduction

NF-κB has been shown to be tightly related to cancer development and progression. It is a transcription factor that regulates the expression of many genes involved in developmental process, the immune system, and inflammatory response, as well as cellular growth and apoptosis [1–4]. The NF-κB family is composed of five family

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members: p65 (RelA), RelB, c-Rel, NF-κB1 (p50 and its precursor p105), and NF-κB2 (p52 and its precursor p100) [5]. The NF-κB activity is constitutively high in many cancer cells [3, 6]. Under most circumstances, the increase of NF-κB activity leads to the activation of antiapoptosis pathways, as well as an increase of cell proliferation and cancer progression but not limited to invasion, metastasis, and angiogenesis [7–9].

The role of miRNAs in cancers has also been intensively studied in recent years. It has been shown that in many cancer cells the miRNA expression profile has been changed compared to that of a normal cell. In the meanwhile, the deregulation of miRNAs has also been proven to be related to cell apoptosis and proliferation [10]. Therefore, deregulation of miRNAs is potentially related to cancer development and progression. In this chapter, we will discuss the interaction of NF-κB and miRNA in various cancers, starting with the miRNA-regulated NF-κB activity, followed by NF-κB-regulated miRNAs expression, as well as reciprocal regulation.

Positive Regulation of NF-κB by miRNAs

miRs-30e*, 124, 125a/b, 182, and 520h up-regulate NF-κB indirectly by suppressing NF-κB inhibitors (Table 9.1). miR-30e* expression level is increased in advanced glioma and also negatively correlates with favorable prognosis in patients [11]. miR-30e* targets IκBα mRNA to enhance NF-κB activation and nuclear translocation, which is essential for miR-30e*-induced invasiveness in a variety of glioma cell lines. Given that a negative correlation between MMP-9 and IκBα has been identified, miR-30e* is suggested to promote cancer cell invasion via MMP-9 through NF-κB activation.

Of note, miR-30e, the sibling of miR-30e*, has no influence on NF-κB signaling or invasive potential, though miR-30e could possibly cooperate with miR-30e* in glioma carcinogenesis because both of them are induced by carcinogen hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) [25, 26].

Table 9.1 Effect of miRNA on NF-κB

miRNA	Effect	Targets	Cancer type	Ref.
miR-30e*	↑	IκBα	Glioma	[11]
miR-124	↑	iASPP	Colon	[12]
miR-125a/b	↑	TNFAIP3	Lymphoma	[13]
miR-182	↑	CYLD	Glioma	[14]
miR-520h	↑	PP2A/C	Breast	[15]
miR-31	↓	NIK	Breast; leukemia	[16, 17]
miR-146	↓	IRAK1, TRAF6	Breast; pancreatic; gastric	[18–21]
miR-520b	↓	HBXIP	Breast	[22]
miR-520c	↓	RELA (p65)	Breast	[23]
miR-520e	↓	NIK	HCC	[24]

Interestingly, in a subset of glioma where miR-30e* expression level is low, miR-182 becomes a candidate to activate NF-κB signaling. An upstream inhibitor of NF-κB, CYLD (cylindromatosis) has been identified as a target of miR-182. Functionally, this K63-specific deubiquitinase suppresses ubiquitination of TRAF2, TRAF6, and NEMO, leading to a reduction in IKK activation [14, 27]. The effect of miR-182 in promoting the tumor growth and angiogenesis is abrogated by blocking IκBα, which indicates the essential role of miR-182-induced NF-κB signaling in glioma progression.

miR-124 expression is reduced in colon cancer cells. Overexpression of miR-124 in SW480 and HT29 cell lines decreases the cancer cell growth and colony formation via targeting an inhibitor of apoptosis-stimulating protein of p53 (iASPP) [12]. Reducing iASPP expression increases NF-κB (p65) expression indicating miR-124 up-regulates p65 at least partially due to suppressed iASPP. Given that iASPP regulates multiple pathways and differential roles of NF-κB in regulation of cell death, further study is still needed to understand the correlation between miR-124 and NF-κB activities [28, 29].

miR-125a/b expression has positive correlation with NF-κB and negative correlation of A20 (also known as TNFAIP3) in diffuse large B-cell lymphoma (DLBCL) biopsies [13]. High levels of miR-125a/b are involved in maintaining NF-κB activation in DLBCL cells through targeting A20, which dismantles IKK complex by interfering with the interaction between E3 ligases and E2 ubiquitin conjugation [30].

Protein phosphatase 2A catalytic subunit (PP2A/C) is an NF-κB inhibitor due to its role in suppressing phosphorylation of IκB [31]. miR-520h, unlike miR-520b/c/e, has been shown to activate NF-κB by targeting 3′ UTR of PP2A/C, leading to up-regulation of Twist [15]. miR-520h-induced NF-κB activation is followed by enhanced invasiveness of breast cancer cells. Adenovirus type 5 E1A (E1A) could reduce invasiveness through inhibiting miR-520h and its downstream NF-κB signaling. Anti-miR520h also diminishes metastatic potential of breast cancer cells.

Negative Regulation of NF-κB by miRNAs

miRs-31, 520b/c/e, and 146a/b downregulate NF-κB indirectly by suppressing NF-κB activators (Table 9.1). miR-31, which was drastically repressed in adult T cell leukemia (ATL) and breast cancer cells, has been found to be an NF-κB inhibitor [16, 17]. In ATL cells, by suppressing expression of NF-κB-inducing kinase (NIK), overexpression of miR-31 reduces phosphorylation of both IKKα and IκB. Inhibition of NF-κB is followed by corresponding growth inhibition and cell apoptosis [17]. In addition to NIK, protein kinase C ε (PKCε) is also a direct target of miR-31 as reported in MDA-MB-231 breast cancer cells [16]. PKCε inhibitor Go6976 has been shown to have antitumor activity through repressing NF-κB signaling [32]. Similarly, overexpression of miR-31 downregulates PKCε protein but not mRNA, which is accompanied by diminished NF-κB nuclear translocation and

reduced p65 phosphorylation. The miR-31-reduced NF-κB activity is correlated with increased cell apoptosis and sensitization of chemo- and radio-therapies. However, overexpression of miR-31 also shows a cytotoxic effect on immortalized normal breast MCF-10A cells. Thus, optimizing the dosage of miR-31 for therapeutic purposes is still a potential challenge.

miR-146a/b inhibits NF-κB activity by downregulating IL-1 receptor-associated kinase 1 (IRAK1) and TNF receptor-associated factor 6 (TRAF6) during immune response to endotoxin [33]. TRAF6 and IRAK1 activate NF-κB through recruiting transforming growth factor β-activated kinase (TAK1), which phosphorylates IKK complex [34]. Overexpression of miR-146a/b in MDA-MB-231 cells results in downregulation of IRAK1 and TRAF6 as well as a decrease in NF-κB DNA binding activity [18], with a corresponding inhibition of NF-κB downstream genes including inflammatory cytokines (IL-8 and IL-6) and MMP-9. Reduced invasiveness suggests therapeutic potential of miR-146 in breast cancer cells. Suppression of NF-κB via miR-146-TRAF6 cascade has also been reported in NK/T cell lymphoma (NKTL). Besides miR-146a-induced inhibition of cancer cell proliferation and improved sensitivity to chemotherapy, a positive correlation between expression of miR-146a and NKTL patients' prognosis has been identified [35]. The role of miR-146a in suppressing NF-κB has been further confirmed by experiments in vivo. miR-146a knockout mice develop progressive myeloproliferation, which shows higher NF-κB activity in their spleen and bone marrow. Myeloproliferation is alleviated after deleting p50 subunit of NF-κB, preventing the formation of myeloid sarcoma [36]. Similarly, miR-146a is shown to target IRAK1 to inhibit NF-κB activity in pancreatic cancer cells [21]. Moreover, treatment of 3,3′-diinodolylmethane (DIM) or isoflavone is found to induce expression of miR-146a, which suppresses tumor cell metastatic potential through downregulating NF-κB, metastasis-associated 1 family, member 2 (MTA-2) and epidermal growth factor receptor (EGFR). Suppressed NF-κB by miR-146a is also associated with downregulated EGFR. In addition to IRAK1 and TRAF6, miR-146a in gastric cancer has been shown to directly target caspase recruitment domain-containing protein 10 (CARD10) and COP9 constitutive photomorphogenic homolog subunit 8 (COPS8), which activates NF-κB through G protein-coupled receptor (GPCR) pathways [20]. Although miR-146a is a potent NF-κB inhibitor and shows antitumor effects in many cancer types, up-regulation of miR-146a has been observed in both mouse models of gastric cancer and human gastric adenocarcinoma cells. In addition, miR-146a expression might be positively controlled by NF-κB [33]. More investigation is still needed for the underlying mechanism.

Several miR-520/373 family members have been shown to suppress cancers at least partially by inhibition of NF-κB via different pathways. miR-520b has been reported to negatively regulate NF-κB by repressing hepatitis B X-interacting protein (HBXIP) which is highly expressed in breast cancer cells as well as metastatic lymph nodes [22]. HBXIP is shown to promote IκB phosphorylation to activate NF-κB, and the resultant IL-8 secretion is believed to be an essential contributor to cell migration. miR-520b reduces IL-8 levels not only by targeting HBXIP-mediated NF-κB activation but also by targeting 3′ UTR of IL-8 directly, leading to a

decreased migration. Mimics of miR-520c and miR-373-inhibited RELA (p65) after TNFα stimulation, suppress IL-6/IL-8 expression and reduce invasiveness of MDA-MB-231 cells [23]. Although the reduction of cancer invasiveness by the mimics has been attributed to inhibition of transforming growth factor-β (TGFβ), the inhibition of RELA should also be examined considering the role of NF-κB-mediated IL-6/IL-8 expression in promoting cancer metastasis. miR-520e is one of the most downregulated miRNAs in hepatocellular carcinoma, where DNA methylation might play a role [24, 37]. NIK, an NF-κB activator, has been identified as the direct target of miR-520e, suggesting that miR-520e inhibits NF-κB activity by suppressing NIK [38]. Of interest, miR-520e-mediated NF-κB inhibition is observed only in hepatocellular carcinoma cells but not in corresponding normal cells. In addition, miR-520e does not show cytotoxic effects on normal tissue while it inhibits tumor growth in vivo [24]. This effect might be contributed by the low basal level of miR-520e expression in hepatocellular carcinoma cells, suggesting its potential advantage for therapeutics.

Positive and Negative Regulation of miRNA Expression by NF-κB

Comparing the numbers of miRNAs that can regulate NF-κB activity, relatively fewer numbers of miRNAs are regulated by NF-κB without a reciprocal regulation. NF-κB activation increases the expression of miRNAs-16 and 224 by direct promoter binding and reduces the expression of miRNAs-199 and 214 (Table 9.2). miR-16 and miR-21 play roles in nicotine-mediated tumor progression [39]. Nicotine treatment up-regulates both miR-16 and miR-21 expression in gastric adenocarcinoma cells. Moreover, activation of NF-κB increases expression of both the miRNAs through direct binding to their promoter regions. Of interest, NF-κB inhibitor or NF-κB-targeting siRNA abolishes the nicotine-induced expression of miR-16 and miR-21, suggesting NF-κB activation is involved in the process. Thus, nicotine-induced cancer growth might be through prostaglandin E receptor-mediated NF-κB activation, and consequently increases expression of miR-16 and miR-21. miR-224 is highly expressed in HCC cells and contributes to cell invasion and migration [37, 41]. Three conservative NF-κB binding sites in the promoter region of miR-224 have been identified. NF-κB activators such as TNFα induce miR-224

Table 9.2 Effect of NF-κB on miRNA

miRNA	Effect	Cancer type	Ref.
miR-16	↑	Gastric	[39]
miR-224	↑	HCC	[37]
miR-199	↓	HCC	[40]
miR-214	↓	HCC	[40]

expression while the IKK inhibitor BMS reduces miR-224 level, which confirms that NF-κB up-regulates miR-224.

The expression of miR-199/214 in HCC is suppressed and suppression of miR-199/miR-214 is alleviated when NF-κB is inhibited by pyrrolidine dithiocarbamate (PDTC) indicating their expression is negatively regulated by NF-κB [40]. miR-199 and miR-214 have been shown to inhibit X-box binding protein 1 (XBP-1), which activates unfolded protein response of the cell. Considering that UPR-induced NF-κB also suppresses miR-199 and miR-214, reciprocal regulation between NF-κB and miR-199 and miR-214 might exist but no direct evidence has been shown.

Positive Regulation of NF-κB Activity with Positive Feedback Loop for miRNA Expression

miRs-21, 155, 181b-1, 301a, and 1290 up-regulate NF-κB with a positive feedback loop (Table 9.3). Besides contributing to cancer progression induced by nicotine as described in detail in the Volume "microRNA: Cancer", miR-21 has been reported to work with miR-181b-1 as one part of a positive feedback loop that mediates transformation of immortalized normal mammary epithelial cells [42]. In ER-Src cell model, tamoxifen treatment can activate Src, initiate transformation, and induce signal transducer and activator of transcription 3 (STAT3). STAT3 up-regulates both miR-21 and miR-181b-1, which lead to NF-κB activation through different pathways.

miR-21 activates NF-κB through inhibiting phosphatase and tensin homolog (PTEN) and then AKT; and miR-181b-1 activate NF-κB through suppressing cylindromatosis (CYLD). Meanwhile, NF-κB can positively regulate IL-6 via Lin28B and Let-7 [58], which is able to activate STAT3. In this regard, miR-21 and miR-181b-1 work in a loop to maintain NF-κB activation, linking inflammation to carcinogenesis.

Table 9.3 Reciprocal effects of miRNA and NF-κB

miRNA	Effect of miR on NF-κB	Targets	Effect of NF-κB on miR	Cancer type	Ref.
miR-21	↑	PTEN	↑	Gastric; breast; colon	[39, 42, 43]
miR-155	↑	PPP2CA	↑	Leukemia; HCC; colon	[43–46]
miR-181b-1	↑	CYLD	↑	Breast; colon	[42]
miR-301a	↑	Gax	↑	HCC; pancreatic	[47–49]
miR-1290	↑	KIF13B, NKRF	↑	Colon	[50]
miR-200c	↓	ZEB1	↓	Breast	[51]
miR-448	↓	SATB1	↓	Breast	[52]
miR-9	↓	NF-κB1 (p105)	↑	Ovarian; lung; gastric; melanoma; HCC	[53–57]

Physiological effects of miR-21 and miR-181b-1 are not limited to mammary cells because both miRNAs also enhance the clonogenic ability of different cancer cells [42]. In breast cancer cells, the high expression level of miR-21 has been associated with increased metastatic potential and poor prognosis [59, 60]. In addition to an STAT3-dependent manner, further evidence has shown that DNA-damaging reagents inducing NF-κB activation could up-regulate miR-21 by binding to its promoter region directly [61]. Suppressed PTEN and PDCD4 by miR-21 contribute to metastatic potential and resistance to apoptosis.

miR-155 is commonly overexpressed in lymphoma, where it is associated with enhanced NF-κB activity [44, 62, 63]. Both miR-155 and the precursor of miR-155 (B-cell integration cluster or BIC) have been induced by infection of Epstein-Barr virus (EBV) or overexpression of LMP1, the chief transforming protein originated from the virus [64, 65]. The EBV-induced elevation of miR-155 is blunted by the NF-κB inhibitor indicating the involvement of by NF-κB for this process [45]. Besides EBV, B-cell receptor also triggers miR-155 expression while nondegradable IκB suppresses this induction, suggesting that NF-κB activation is involved [66]. It is of note that transfection of precursor of miR-155 did not necessarily result in higher levels of miR-155, which indicates that some other critical factors play a role in regulating miR-155 processing. In addition to the association with cancer formation, miR-155 also contributes to adrenaline-induced chemoresistance and cell growth in HT29 cells [46]. NF-κB is activated by catecholamine, and then leads to up-regulation of miR-155. Inhibition of miR-155 abolishes increased proliferation and resistance to cisplatin of the cells. Actually, downregulation of serine/threonine-protein phosphatase 2A catalytic subunit alpha isoform (PPP2CA) is observed after adrenaline treatment, suggesting that a possible positive feedback also exists in response to catecholamine-mediated tumor progression.

Both miR-21 and miR-155 are also expressed in a higher level in colon cancer specimens than normal tissues [43]. The expression of miR-21 and miR-155 can be induced in non-transformed colonic epithelial cells by neurotensin (NT) treatment [43], which promotes production of inflammatory cytokines and colon cancer cell growth by binding neurotensin-1 receptor (NTR1) [67, 68]. Direct binding of NF-κB on promoters of miR-21 and miR-155 is necessary for their up-regulation after NT treatment. miR-21 inhibits PTEN while miR-155 suppresses PPP2CA to activate Akt, which in turn enhances NF-κB activity. Targeting this loop by knockdown of miR-155 and miR-21 significantly inhibits NT-induced cancer invasiveness and progression.

miR-301a is up-regulated in hepatoma and pancreatic adenocarcinoma cells [47–49]. miRNA-301a induces the expression of NF-κB by indirectly targeting upstream NF-κB suppressor's homeobox gene Gax [47] or NF-κB-repressing factor (NKRF) [49]. The expressions of NF-κB are inhibited in cells overexpressing Gax, which agrees with a previous report indicating that Gax targets NF-κB in endothelial cells [69]. While miR-301a induces NF-κB expression, activated NF-κB can transcriptionally activate miR-301a expression. The function of miRNA-301a as an NF-κB activator partially explains its role in promoting cancer growth and metastases. Targeting miR-301a expression in PANC-1 cells inhibits tumor growth in a mouse xenografts model [49].

miR-1290 has been shown to be elevated in 19 out of 25 colon cancer specimens and plays a role in suppressing cytokinesis by targeting kinesin family member 13B (KIF13B) at the last stage in cell division, leading to a nuclear fusion in cancer cells [50]. miR-1290 also activates Akt and NF-κB by targeting NF-κB repressing factor (NKRF). The miR-1290 stimulated NF-κB activation leads to the induction of a series of genes including Wnt, c-Myc, and cyclin D1. Meanwhile, activated Wnt and NF-κB also facilitates transcription of miR-1290. The regulation of cytokinesis and Wnt signaling by miR-1290 shows its potential role in cancer cell reprogramming and development of cell stemness [70–72].

Negative Regulation of NF-κB Activity with Positive and Negative Feedback Loops for miRNA Expression

miRs-9 and 143 downregulate NF-κB with a positive feedback loop (Table 9.3). miR-9 has been shown to be involved in regulating cell proliferation and progression of gastric, ovarian, cervical, and skin cancers [55, 73–75]. miR-9 has been found to target 3′ UTR of NF-κB1 (p105) directly to inhibit ovarian cancer cell proliferation and colony formation [55]. Similar cancer growth inhibition is also seen in gastric adenocarcinoma which expresses low level of miR-9 [54]. Both cell proliferation in vitro and xenograft tumor growth in vivo decrease after overexpression of miR-9. Besides decreased mRNA level of NF-κB1, expression of p105 and p50 protein is reduced as well. Overexpression of NF-κB1 with miR-9 together restores cancer cell growth, indicating the therapeutic effect is due to NF-κB1 inhibition by miR-9. miR-9-inhibited NF-κB1 also plays a role in regulation of melanoma metastasis. Overexpression of miR-9 suppresses cytoskeleton proteins as well as matrix metallopeptidases (MMP)-2/9 via inhibiting NF-κB1-Snail 1 signaling pathway, leading to a decrease in motility and growth of melanoma cells [75, 76]. In view of important roles of NF-κB1 in DNA damage repair, the effect of miRNAs-induced NF-κB1 suppression on sensitivity in response to ionizing radiation (IR) also drew researchers' attention [56]. A time-dependent decrease of miR-9 expression is observed after IR while overexpression of miR-9 increases sensitivity of cancer cells to radiation. miR-9 does not only possess the ability to directly target 3′ UTR of NF-κB1, but also serves as a downstream gene of NF-κB. It has been shown that NF-κB could activate pri-miR-9-1 gene to up-regulate miR-9, which suppresses CD166 [57], a key regulator of cancer cell migration and growth [77]. In hepatocellular carcinoma cells, serum depletion up-regulates both mRNA and protein level of CD166 within 24 h, though its protein level drops after 48 h. Meanwhile, serum depletion also promotes p50/p65 translocation. Given that NF-κB activates miR-9 through pri-miR-9-1 in a delayed manner, decrease of CD166 after 48 h could be partially due to miR-9 activation. In addition, suppression of CD166 is believed to be the major cause of miR-9-promoted cell migration during serum depletion.

miR-143 suppresses NF-κB expression and increases the sensitivity of colon cancer cells to 5-fluorouracil [78]. The direct association between NF-κB inhibition and miR-143 in regulation of apoptosis is not clear because miR-143 also inhibits the expression of ERK5 and Bcl-2. In addition, the miR-143 level increases sharply in the HBV-HCC-bearing mice and HBV-coated HBV X protein (HBx) transiently transfected HCC cells. The expression of miR-143, both in vitro and in vivo, can be induced by HBx via activation of NF-κB pathway [79–82]. Through suppressing fibronectin protein FNDC3B, miR-143 promotes metastasis of HCC cells while leaving cell viability intact, which suggests the involvement of HBV/NF-κB/miR-143 signaling cascade in regulation of cancer metastasis. Although the underlying mechanism for regulation of NF-κB by miR-143 is still unknown, it appears that miR-143 could be either an upstream or downstream gene of NF-κB.

miR-200c and miR-448 downregulate NF-κB with a negative feedback loop. miR-200c negatively regulates NF-κB (p65) activation by activating protein tyrosine phosphatase Z1 (PTPRZ1) to dephosphorylate IκBα [51]. Both NF-κB and MEK/ERK are involved in the early stage of inflammatory signaling after stimulation with monocyte chemotactic protein-1 (MCP-1). The resultant increase of IL-6 further inhibits miR-200c to activate p65 as well as JNK2, which then in turn up-regulates IL-6 through inducing heat shock factor protein 1 (HSF1). The maintenance of this IL-6-mediated inflammatory signaling loop drives transformation of breast epithelial cells [51].

A negative feedback loop of miR-448 and NF-κB plays a critical role in chemotherapy-induced epithelial–mesenchymal transition (EMT) [52]. Special AT-rich sequence-binding protein-1 (SATB1), which activates NF-κB through an AR/EGFR/PI3K pathway, is a target of miR-448. Moreover, suppressing miR-448 could reinstate NF-κB activity, suggesting that miR-448 is an NF-κB inhibitor. Nevertheless, NF-κB has also been shown to negatively regulate miR-448 through binding the promoter region of HRT2C which hosts miR-448. Adriamycin-induced mesenchymal phenotype in breast cancer cells is mediated by miR-448 inhibition because anti-miR-448 can promote EMT. Since Twist-1, one of the key regulators of EMT [83], is controlled by EGFR signaling, miR-448-reduced chemotherapy-induced EMT of breast cancer could be partially contributed by suppressing Twist-1.

Conclusion

NF-κB-targeting miRNAs, which either activate or inhibit NF-κB activity, play a pivotal role in regulation of cancer progression and resistance to treatment. Instead of targeting NF-κB, miRNAs frequently influence NF-κB through suppressing NF-κB inhibitors or activators, which extend the signaling network. NF-κB also incorporates some miRNAs into its downstream signaling by showing the competency of up-regulating or downregulating miRNAs. Moreover, reciprocal regulation between miRNAs and NF-κB has been associated with not only tumor progression but also aberrant inflammatory status, which contributes to early stage of carcinogenesis.

Given that miRNA deregulation correlates closely with aberrant NF-κB activation, targeting miRNAs may be a promising strategy to interfere with pathological constitutive NF-κB activation. Although many obstacles still have to be overcome, with the development of drug delivery technologies and deepened knowledge of sophisticated regulation between NF-κB and miRNAs, therapeutic potential of NF-κB-targeting miRNAs could be realized to treat diseases like cancers.

References

1. Baldwin Jr AS. Series introduction: the transcription factor NF-kappaB and human disease. J Clin Invest. 2001;107(1):3–6. PubMed Pubmed Central PMCID: 198555. Epub 2001/01/03. eng.
2. Baldwin AS. Control of oncogenesis and cancer therapy resistance by the transcription factor NF-kappaB. J Clin Invest. 2001;107(3):241–6. PubMed Pubmed Central PMCID: 199203. Epub 2001/02/13. eng.
3. Karin M, Cao Y, Greten FR, Li ZW. NF-kappaB in cancer: from innocent bystander to major culprit. Nat Rev Cancer. 2002;2(4):301–10. PubMed Epub 2002/05/11. eng.
4. Karin M, Lin A. NF-kappaB at the crossroads of life and death. Nat Immunol. 2002;3(3): 221–7. PubMed Epub 2002/03/05. eng.
5. Grimm S, Baeuerle PA. The inducible transcription factor NF-kappa B: structure-function relationship of its protein subunits. Biochem J. 1993;290(Pt 2):297–308. PubMed Pubmed Central PMCID: 1132272.
6. Dhawan P, Singh AB, Ellis DL, Richmond A. Constitutive activation of Akt/protein kinase B in melanoma leads to up-regulation of nuclear factor-kappaB and tumor progression. Cancer Res. 2002;62(24):7335–42. PubMed Epub 2002/12/25. eng.
7. Karin M. NF-kappaB as a critical link between inflammation and cancer. Cold Spring Harb Perspect Biol. 2009;1(5):a000141. PubMed Pubmed Central PMCID: 2773649.
8. Karin M, Greten FR. NF-kappaB: linking inflammation and immunity to cancer development and progression. Nat Rev Immunol. 2005;5(10):749–59. PubMed.
9. Karin M. NF-kappaB and cancer: mechanisms and targets. Mol Carcinog. 2006;45(6):355–61. PubMed.
10. Sassen S, Miska EA, Caldas C. MicroRNA: implications for cancer. Virchows Arch. 2008;452(1):1–10. PubMed Pubmed Central PMCID: 2151131.
11. Jiang L, Lin C, Song L, Wu J, Chen B, Ying Z, et al. MicroRNA-30e* promotes human glioma cell invasiveness in an orthotopic xenotransplantation model by disrupting the NF-kappaB/IkappaBalpha negative feedback loop. J Clin Invest. 2012;122(1):33–47. PubMed Pubmed Central PMCID: 3248293. Epub 2011/12/14. eng.
12. Liu K, Zhao H, Yao H, Lei S, Lei Z, Li T, et al. MicroRNA-124 regulates the proliferation of colorectal cancer cells by targeting iASPP. BioMed Res Int. 2013;2013:867537. PubMed Pubmed Central PMCID: 3652105. Epub 2013/05/22. eng.
13. Kim SW, Ramasamy K, Bouamar H, Lin AP, Jiang D, Aguiar RC. MicroRNAs miR-125a and miR-125b constitutively activate the NF-kappaB pathway by targeting the tumor necrosis factor alpha-induced protein 3 (TNFAIP3, A20). Proc Natl Acad Sci U S A. 2012;109(20):7865–70. PubMed Pubmed Central PMCID: 3356650. Epub 2012/05/03. eng.
14. Song L, Liu L, Wu Z, Li Y, Ying Z, Lin C, et al. TGF-beta induces miR-182 to sustain NF-kappaB activation in glioma subsets. J Clin Invest. 2012;122(10):3563–78. PubMed Pubmed Central PMCID: 3589141. Epub 2012/09/26. eng.
15. Su JL, Chen PB, Chen YH, Chen SC, Chang YW, Jan YH, et al. Downregulation of microRNA miR-520h by E1A contributes to anticancer activity. Cancer Res. 2010;70(12):5096–108. PubMed Pubmed Central PMCID: 2891368. Epub 2010/05/27. eng.

16. Korner C, Keklikoglou I, Bender C, Worner A, Munstermann E, Wiemann S. MicroRNA-31 sensitizes human breast cells to apoptosis by direct targeting of protein kinase C epsilon (PKCepsilon). J Biol Chem. 2013;288(12):8750–61. PubMed Pubmed Central PMCID: 3605692. Epub 2013/02/01. eng.
17. Yamagishi M, Nakano K, Miyake A, Yamochi T, Kagami Y, Tsutsumi A, et al. Polycomb-mediated loss of miR-31 activates NIK-dependent NF-kappaB pathway in adult T cell leukemia and other cancers. Cancer Cell. 2012;21(1):121–35. PubMed Epub 2012/01/24. eng.
18. Bhaumik D, Scott GK, Schokrpur S, Patil CK, Campisi J, Benz CC. Expression of microRNA-146 suppresses NF-kappaB activity with reduction of metastatic potential in breast cancer cells. Oncogene. 2008;27(42):5643–7. PubMed Pubmed Central PMCID: 2811234. Epub 2008/05/28. eng.
19. Li X, Xu B, Moran MS, Zhao Y, Su P, Haffty BG, et al. 53BP1 functions as a tumor suppressor in breast cancer via the inhibition of NF-kappaB through miR-146a. Carcinogenesis. 2012;33(12):2593–600. PubMed Epub 2012/10/03. eng.
20. 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. PubMed Pubmed Central PMCID: 3515505. Epub 2012/09/21. eng.
21. Li Y, Vandenboom 2nd TG, Wang Z, Kong D, Ali S, Philip PA, et al. miR-146a suppresses invasion of pancreatic cancer cells. Cancer Res. 2010;70(4):1486–95. PubMed Pubmed Central PMCID: 2978025. Epub 2010/02/04. eng.
22. Hu N, Zhang J, Cui W, Kong G, Zhang S, Yue L, et al. miR-520b regulates migration of breast cancer cells by targeting hepatitis B X-interacting protein and interleukin-8. J Biol Chem. 2011;286(15):13714–22. PubMed Pubmed Central PMCID: 3075715. Epub 2011/02/24. eng.
23. Keklikoglou I, Koerner C, Schmidt C, Zhang JD, Heckmann D, Shavinskaya A, et al. MicroRNA-520/373 family functions as a tumor suppressor in estrogen receptor negative breast cancer by targeting NF-kappaB and TGF-beta signaling pathways. Oncogene. 2012;31(37):4150–63. PubMed Epub 2011/12/14. eng.
24. Zhang S, Shan C, Kong G, Du Y, Ye L, Zhang X. MicroRNA-520e suppresses growth of hepatoma cells by targeting the NF-kappaB-inducing kinase (NIK). Oncogene. 2012;31(31):3607–20. PubMed Epub 2011/11/23. eng.
25. Ro S, Park C, Young D, Sanders KM, Yan W. Tissue-dependent paired expression of miRNAs. Nucleic Acids Res. 2007;35(17):5944–53. PubMed Pubmed Central PMCID: 2034466.
26. Zhang B, Pan X. RDX induces aberrant expression of microRNAs in mouse brain and liver. Environ Health Perspect. 2009;117(2):231–40. PubMed Pubmed Central PMCID: 2649225.
27. Kovalenko A, Chable-Bessia C, Cantarella G, Israel A, Wallach D, Courtois G. The tumour suppressor CYLD negatively regulates NF-kappaB signalling by deubiquitination. Nature. 2003;424(6950):801–5. PubMed.
28. Li G, Wang R, Gao J, Deng K, Wei J, Wei Y. RNA interference-mediated silencing of iASPP induces cell proliferation inhibition and G0/G1 cell cycle arrest in U251 human glioblastoma cells. Mol Cell Biochem. 2011;350(1-2):193–200. PubMed.
29. Lin B, Williams-Skipp C, Tao Y, Schleicher MS, Cano LL, Duke RC, et al. NF-kappaB functions as both a proapoptotic and antiapoptotic regulatory factor within a single cell type. Cell Death Differ. 1999;6(6):570–82. PubMed.
30. Vereecke L, Beyaert R, van Loo G. The ubiquitin-editing enzyme A20 (TNFAIP3) is a central regulator of immunopathology. Trends Immunol. 2009;30(8):383–91. PubMed.
31. Palkowitsch L, Leidner J, Ghosh S, Marienfeld RB. Phosphorylation of serine 68 in the IkappaB kinase (IKK)-binding domain of NEMO interferes with the structure of the IKK complex and tumor necrosis factor-alpha-induced NF-kappaB activity. J Biol Chem. 2008;283(1):76–86. PubMed.
32. Biswas DK, Martin KJ, McAlister C, Cruz AP, Graner E, Dai SC, et al. Apoptosis caused by chemotherapeutic inhibition of nuclear factor-kappaB activation. Cancer Res. 2003;63(2):290–5. PubMed.

33. 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(33):12481–6. PubMed Pubmed Central PMCID: 1567904.
34. Akira S, Takeda K. Toll-like receptor signalling. Nat Rev Immunol. 2004;4(7):499–511. PubMed.
35. Paik JH, Jang JY, Jeon YK, Kim WY, Kim TM, Heo DS, et al. MicroRNA-146a downregulates NFkappaB activity via targeting TRAF6 and functions as a tumor suppressor having strong prognostic implications in NK/T cell lymphoma. Clin Cancer Res. 2011;17(14):4761–71. PubMed Epub 2011/05/26. eng.
36. Zhao JL, Rao DS, Boldin MP, Taganov KD, O'Connell RM, Baltimore D. NF-kappaB dysregulation in microRNA-146a-deficient mice drives the development of myeloid malignancies. Proc Natl Acad Sci U S A. 2011;108(22):9184–9. PubMed Pubmed Central PMCID: 3107319. Epub 2011/05/18. eng.
37. Li Q, Wang G, Shan JL, Yang ZX, Wang HZ, Feng J, et al. MicroRNA-224 is upregulated in HepG2 cells and involved in cellular migration and invasion. J Gastroenterol Hepatol. 2010;25(1):164–71. PubMed.
38. Cui W, Zhang Y, Hu N, Shan C, Zhang S, Zhang W, et al. miRNA-520b and miR-520e sensitize breast cancer cells to complement attack via directly targeting 3′UTR of CD46. Cancer Biol Ther. 2010;10(3):232–41. PubMed.
39. Shin VY, Jin H, Ng EK, Cheng AS, Chong WW, Wong CY, et al. NF-kappaB targets miR-16 and miR-21 in gastric cancer: involvement of prostaglandin E receptors. Carcinogenesis. 2011;32(2):240–5. PubMed Epub 2010/11/18. eng.
40. Duan Q, Wang X, Gong W, Ni L, Chen C, He X, et al. ER stress negatively modulates the expression of the miR-199a/214 cluster to regulates tumor survival and progression in human hepatocellular cancer. PLoS One. 2012;7(2):e31518. PubMed Pubmed Central PMCID: 3281082. Epub 2012/02/24. eng.
41. Scisciani C, Vossio S, Guerrieri F, Schinzari V, De Iaco R, D'Onorio de Meo P, et al. Transcriptional regulation of miR-224 upregulated in human HCCs by NFkappaB inflammatory pathways. J Hepatol. 2012;56(4):855–61. PubMed Epub 2011/12/20. eng.
42. 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(4):493–506. PubMed Pubmed Central PMCID: 2929389. Epub 2010/08/28. eng.
43. Bakirtzi K, Hatziapostolou M, Karagiannides I, Polytarchou C, Jaeger S, Iliopoulos D, et al. Neurotensin signaling activates microRNAs-21 and -155 and Akt, promotes tumor growth in mice, and is increased in human colon tumors. Gastroenterology. 2011;141(5):1749–61 e1. PubMed Epub 2011/08/03. eng.
44. Kluiver J, Poppema S, de Jong D, Blokzijl T, Harms G, Jacobs S, et al. BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. J Pathol. 2005;207(2):243–9. PubMed.
45. Rahadiani N, Takakuwa T, Tresnasari K, Morii E, Aozasa K. Latent membrane protein-1 of Epstein-Barr virus induces the expression of B-cell integration cluster, a precursor form of microRNA-155, in B lymphoma cell lines. Biochem Biophys Res Commun. 2008;377(2):579–83. PubMed Epub 2008/10/18. eng.
46. Pu J, Bai D, Yang X, Lu X, Xu L, Lu J. Adrenaline promotes cell proliferation and increases chemoresistance in colon cancer HT29 cells through induction of miR-155. Biochem Biophys Res Commun. 2012;428(2):210–5. PubMed Epub 2012/10/06. eng.
47. Zhou P, Jiang W, Wu L, Chang R, Wu K, Wang Z. miR-301a is a candidate oncogene that targets the homeobox gene Gax in human hepatocellular carcinoma. Dig Dis Sci. 2012;57(5):1171–80. PubMed Epub 2012/03/01. eng.
48. Lee EJ, Gusev Y, Jiang J, Nuovo GJ, Lerner MR, Frankel WL, et al. Expression profiling identifies microRNA signature in pancreatic cancer. Int J Cancer. 2007;120(5):1046–54. PubMed Pubmed Central PMCID: 2680248.

49. Lu Z, Li Y, Takwi A, Li B, Zhang J, Conklin DJ, et al. miR-301a as an NF-kappaB activator in pancreatic cancer cells. EMBO J. 2011;30(1):57–67. PubMed Pubmed Central PMCID: 3020116. Epub 2010/11/30. eng.
50. Wu J, Ji X, Zhu L, Jiang Q, Wen Z, Xu S, et al. Up-regulation of microRNA-1290 impairs cytokinesis and affects the reprogramming of colon cancer cells. Cancer Lett. 2013;329(2):155–63. PubMed Epub 2012/11/13. eng.
51. Rokavec M, Wu W, Luo JL. IL6-mediated suppression of miR-200c directs constitutive activation of inflammatory signaling circuit driving transformation and tumorigenesis. Mol Cell. 2012;45(6):777–89. PubMed Pubmed Central PMCID: 3319241. Epub 2012/03/01. eng.
52. Li QQ, Chen ZQ, Cao XX, Xu JD, Xu JW, Chen YY, et al. Involvement of NF-kappaB/miR-448 regulatory feedback loop in chemotherapy-induced epithelial-mesenchymal transition of breast cancer cells. Cell Death Differ. 2011;18(1):16–25. PubMed Pubmed Central PMCID: 3131865. Epub 2010/08/28. eng.
53. Arora H, Qureshi R, Jin S, Park AK, Park WY. miR-9 and let-7g enhance the sensitivity to ionizing radiation by suppression of NFkappaB1. Exp Mol Med. 2011;43(5):298–304. PubMed Pubmed Central PMCID: 3104252. Epub 2011/04/06. eng.
54. Wan HY, Guo LM, Liu T, Liu M, Li X, Tang H. Regulation of the transcription factor NF-kappaB1 by microRNA-9 in human gastric adenocarcinoma. Mol Cancer. 2010;9:16. PubMed Pubmed Central PMCID: 2835654. Epub 2010/01/28. eng.
55. Guo LM, Pu Y, Han Z, Liu T, Li YX, Liu M, et al. MicroRNA-9 inhibits ovarian cancer cell growth through regulation of NF-kappaB1. FEBS J. 2009;276(19):5537–46. PubMed Epub 2009/08/26. eng.
56. Janssens S, Tinel A, Lippens S, Tschopp J. PIDD mediates NF-kappaB activation in response to DNA damage. Cell. 2005;123(6):1079–92. PubMed.
57. Wang J, Gu Z, Ni P, Qiao Y, Chen C, Liu X, et al. NF-kappaB P50/P65 hetero-dimer mediates differential regulation of CD166/ALCAM expression via interaction with microRNA-9 after serum deprivation, providing evidence for a novel negative auto-regulatory loop. Nucleic Acids Res. 2011;39(15):6440–55. PubMed Pubmed Central PMCID: 3159468. Epub 2011/05/17. eng.
58. Iliopoulos D, Hirsch HA, Struhl K. An epigenetic switch involving NF-kappaB, Lin28, Let-7 MicroRNA, and IL6 links inflammation to cell transformation. Cell. 2009;139(4):693–706. PubMed Pubmed Central PMCID: 2783826. Epub 2009/11/03. eng.
59. Farazi TA, Horlings HM, Ten Hoeve JJ, Mihailovic A, Halfwerk H, Morozov P, et al. MicroRNA sequence and expression analysis in breast tumors by deep sequencing. Cancer Res. 2011;71(13):4443–53. PubMed Pubmed Central PMCID: 3129492.
60. Yan LX, Huang XF, Shao Q, Huang MY, Deng L, Wu QL, et al. MicroRNA miR-21 overexpression in human breast cancer is associated with advanced clinical stage, lymph node metastasis and patient poor prognosis. RNA. 2008;14(11):2348–60. PubMed Pubmed Central PMCID: 2578865.
61. Niu J, Shi Y, Tan G, Yang CH, Fan M, Pfeffer LM, et al. DNA damage induces NF-kappaB-dependent microRNA-21 up-regulation and promotes breast cancer cell invasion. J Biol Chem. 2012;287(26):21783–95. PubMed Pubmed Central PMCID: 3381141. Epub 2012/05/02. eng.
62. Barbarotto E, Schmittgen TD, Calin GA. MicroRNAs and cancer: profile, profile, profile. Int J Cancer. 2008;122(5):969–77. PubMed.
63. Rai D, Karanti S, Jung I, Dahia PL, Aguiar RC. Coordinated expression of microRNA-155 and predicted target genes in diffuse large B-cell lymphoma. Cancer Genet Cytogenet. 2008;181(1):8–15. PubMed Pubmed Central PMCID: 2276854. Epub 2008/02/12. eng.
64. Epstein MA, Achong BG, Barr YM. Virus particles in cultured lymphoblasts from Burkitt's lymphoma. Lancet. 1964;1(7335):702–3. PubMed.
65. Weiss LM, Movahed LA, Warnke RA, Sklar J. Detection of Epstein-Barr viral genomes in Reed-Sternberg cells of Hodgkin's disease. N Engl J Med. 1989;320(8):502–6. PubMed.
66. Kluiver J, van den Berg A, de Jong D, Blokzijl T, Harms G, Bouwman E, et al. Regulation of pri-microRNA BIC transcription and processing in Burkitt lymphoma. Oncogene. 2007;26(26):3769–76. PubMed Epub 2006/12/19. eng.

67. Koon HW, Kim YS, Xu H, Kumar A, Zhao D, Karagiannides I, et al. Neurotensin induces IL-6 secretion in mouse preadipocytes and adipose tissues during 2,4,6,-trinitrobenzensulphonic acid-induced colitis. Proc Natl Acad Sci U S A. 2009;106(21):8766–71. PubMed Pubmed Central PMCID: 2688970.
68. Maoret JJ, Anini Y, Rouyer-Fessard C, Gully D, Laburthe M. Neurotensin and a non-peptide neurotensin receptor antagonist control human colon cancer cell growth in cell culture and in cells xenografted into nude mice. Int J Cancer. 1999;80(3):448–54. PubMed.
69. Patel S, Leal AD, Gorski DH. The homeobox gene Gax inhibits angiogenesis through inhibition of nuclear factor-kappaB-dependent endothelial cell gene expression. Cancer Res. 2005;65(4):1414–24. PubMed.
70. Powell AE, Anderson EC, Davies PS, Silk AD, Pelz C, Impey S, et al. Fusion between Intestinal epithelial cells and macrophages in a cancer context results in nuclear reprogramming. Cancer Res. 2011;71(4):1497–505. PubMed Pubmed Central PMCID: 3079548.
71. Luscan A, Shackleford G, Masliah-Planchon J, Laurendeau I, Ortonne N, Varin J, et al. The activation of the WNT signalling pathway is a hallmark in neurofibromatosis type 1 tumorigenesis. Clin Cancer Res. 2013;20(2):358–71. PubMed.
72. Luis TC, Naber BA, Roozen PP, Brugman MH, de Haas EF, Ghazvini M, et al. Canonical wnt signaling regulates hematopoiesis in a dosage-dependent fashion. Cell Stem Cell. 2011;9(4):345–56. PubMed.
73. Hu X, Schwarz JK, Lewis Jr JS, Huettner PC, Rader JS, Deasy JO, et al. A microRNA expression signature for cervical cancer prognosis. Cancer Res. 2010;70(4):1441–8. PubMed Pubmed Central PMCID: 2844247.
74. Tsai KW, Liao YL, Wu CW, Hu LY, Li SC, Chan WC, et al. Aberrant hypermethylation of miR-9 genes in gastric cancer. Epigenetics. 2011;6(10):1189–97. PubMed Pubmed Central PMCID: 3225840.
75. Liu N, Sun Q, Chen J, Li J, Zeng Y, Zhai S, et al. MicroRNA-9 suppresses uveal melanoma cell migration and invasion through the NF-kappaB1 pathway. Oncol Rep. 2012;28(3):961–8. PubMed Epub 2012/07/25. eng.
76. Liu S, Kumar SM, Lu H, Liu A, Yang R, Pushparajan A, et al. MicroRNA-9 up-regulates E-cadherin through inhibition of NF-kappaB1-Snail1 pathway in melanoma. J Pathol. 2012;226(1):61–72. PubMed Epub 2011/12/02. eng.
77. Ohneda O, Ohneda K, Arai F, Lee J, Miyamoto T, Fukushima Y, et al. ALCAM (CD166): its role in hematopoietic and endothelial development. Blood. 2001;98(7):2134–42. PubMed.
78. Borralho PM, Kren BT, Castro RE, da Silva IB, Steer CJ, Rodrigues CM. MicroRNA-143 reduces viability and increases sensitivity to 5-fluorouracil in HCT116 human colorectal cancer cells. FEBS J. 2009;276(22):6689–700. PubMed Epub 2009/10/22. eng.
79. Kim SY, Kim JC, Kim JK, Kim HJ, Lee HM, Choi MS, et al. Hepatitis B virus X protein enhances NFkappaB activity through cooperating with VBP1. BMB Rep. 2008;41(2):158–63. PubMed.
80. Su F, Schneider RJ. Hepatitis B virus HBx protein activates transcription factor NF-kappaB by acting on multiple cytoplasmic inhibitors of rel-related proteins. J Virol. 1996;70(7):4558–66. PubMed Pubmed Central PMCID: 190392.
81. Yun C, Um HR, Jin YH, Wang JH, Lee MO, Park S, et al. NF-kappaB activation by hepatitis B virus X (HBx) protein shifts the cellular fate toward survival. Cancer Lett. 2002;184(1): 97–104. PubMed.
82. Zhang X, Liu S, Hu T, He Y, Sun S. Up-regulated microRNA-143 transcribed by nuclear factor kappa B enhances hepatocarcinoma metastasis by repressing fibronectin expression. Hepatology. 2009;50(2):490–9. PubMed Epub 2009/05/28. eng.
83. Yang J, Mani SA, Donaher JL, Ramaswamy S, Itzykson RA, Come C, et al. Twist, a master regulator of morphogenesis, plays an essential role in tumor metastasis. Cell. 2004;117(7):927–39. PubMed.

Chapter 10
microRNAs: Key Players in Hematopoiesis

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Abbreviations

CLP	Common lymphoid progenitor
CMP	Common myeloid progenitor
CTCL	Cutaneous T-cell lymphoma
DLBCL	Diffuse large B-cell lymphoma
GMP	Granulocyte-monocyte progenitor
HL	Hodgkin lymphoma
HSC	Hematopoietic stem cell
HSPC	Hematopoietic stem and progenitor cells
LT-HSCs	Long-term reconstituting HSCs
MALT	Mucosa-associated lymphoid tissue lymphoma
MCL	Mantle cell lymphoma
MEP	Megakaryocyte-erythrocyte progenitor
MM	Multiple myeloma
ST-HSCs	Short-term reconstituting HSCs
SzS	Sézary syndrome
Tregs	T regulatory cells

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Introduction

microRNAs (miRNAs) are one of the most studied noncoding RNA molecules found in animals, plants, and DNA viruses. They regulate gene expression at post-transcriptional level by RNA silencing or destabilizing target transcripts. Mature miRNAs are small, about 20–25 nucleotides in length, generated from pre-miRNA that underwent the slicing mechanism by Dicer [1]. These pre-miRNAs are in turn generated from the pri-miRNAs that undergo splicing mechanism by Drosha in nucleolus [2, 3], which then transported to the cytoplasm with the help of Ran-GTP and karyopherin exportin complexes [4–6]. Mature miRNA in the presence of Argonaute family proteins binds to the target mRNA in the RNA-induced silencing complex (RISC) [7]. This leads to gene silencing either by the degradation of the mRNA or translation repression (Fig. 10.1).

Recent studies have evidenced an increased role of miRNA in the regulation of normal hematopoiesis, the formation of blood components from hematopoietic stem cells (HSC). The differentiation of HSC into different lineages involves complex network of transcription factors, growth factors, and cytokines, signaling path-

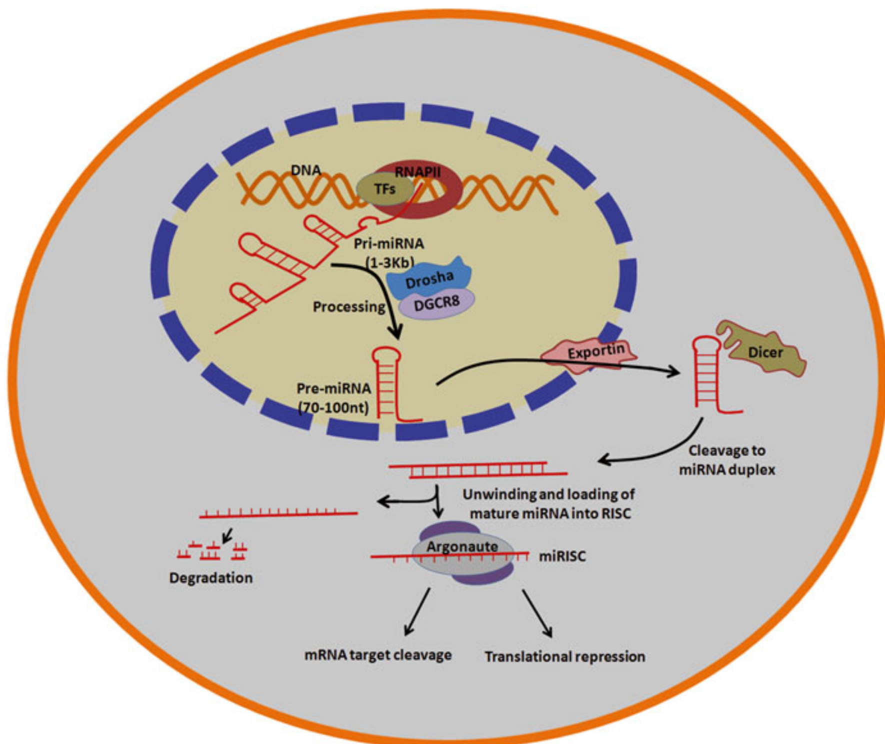

Fig. 10.1 Biogenesis pathway of miRNA, RISC, and silencing mechanism

ways, and interconnected molecular networks that requires proper regulation. Even though several transcription factors have been found to be regulating the process of hematopoiesis, evidence to establish the role of miRNAs in hematopoiesis has been improved significantly in recent years (Table 10.1). Here, we focus on the role of miRNA in hematopoiesis and hematological disorders.

Hematopoiesis

Hematopoiesis is the formation of blood cells from HSC. Hematopoiesis is highly regulated and operates throughout the life of an organism. During embryonic development, the first wave of blood cell production (termed "primitive hematopoiesis") comes from yolk sac. The primitive wave of hematopoiesis is transient and rapidly replaced by the adult type hematopoiesis (termed "definitive hematopoiesis") in fetal liver, which finally form niche in bone marrow [8, 9]. The hematopoietic stem and progenitor cells (HSPC) reside primarily in the bone marrow during adult life, though several studies have shown their presence in the blood and lymph during homeostasis and stress [10–13].

During hematopoiesis, HSC responds to several extracellular signals and differentiate into different cells of the myeloid and lymphoid lineages (Fig. 10.2). Overall, the entire hematopoietic system is organized as a tree where the developmental potential is restricted in each branch point: each step is tightly regulated by signals from microenvironmental and macroenvironment, specific transcription factors, signaling molecules, as well as miRNAs [14].

Hematopoietic Differentiation

Several reports have shown the key roles of miRNAs in normal hematopoiesis [15, 17]. After the first groundbreaking discovery of miRNA Lin 4 which downregulates Lin14 transcript, several studies have shown significant role of miRNA in hematopoietic system markedly increased to more than 500 as listed in miRBase (www.mirbase.org) [18]. The role of miR-125a is known when deletion studies of Dicer in HSCs led to enhanced apoptosis and reduction in hematopoietic ability [19]. Studies have shown that several miRNAs which are expressed at early progenitor stage of CD34+ HSCs of bone marrow or peripheral blood compartments are similar. Functional validation studies have shown that miR-17, miR-24, miR-146, miR-155, miR-128, and miR-181 prevent the differentiation of early stage progenitor cells. And few miRNAs such as miR-16, miR-103, and miR-107 act later on, and miR-221, miR-222, and miR-223 regulate the terminal stages of hematopoietic development [20].

Table 10.1 miRNAs role in hematopoiesis

miRNA	Expression	Targets	Function	Regulated by	References
Granulopoiesis and Monopoiesis					
miR-223	Up G	NFI-A/E2F1 MEF2C	Inhibits granulocytic proliferation and activity	CEBPα, NFI-A, E2F1	Fazi et al. [89], Johnnidis et al. [91], Pullikan et al. [179], Garzon et al. [180]
miR-21 miR-196a	Down GMP	Unknown	Regulation between CMP-GMP transition	GFI-1	Velu et al. [93]
miR-424	Up Mo	NFI-A	Induces Mo differentiation and proliferation	PU.1	Rosa et al. [95], Forrest et al. [96]
miR-17-92	Down Mo	AML1	Inhibits Monopoiesis in vitro but no effects in vivo	AML1	Fontana et al. [94], Ventura et al. [191]
Megakaryopoiesis					
miR-130a	Down	MAFB	Unknown	Unknown	Garzon et al. [58]
miR-10a	Down	HOXA1	Unknown	Unknown	Garzon et al. [58]
miR-150	Up	c-Myb	Drives MK differentiation	Unknown	Lu et al. [56], Barroga et al. [59]
miR-155	Down	Ets-1/Meis1	Inhibits MK differentiation and proliferation	AP-1, JNK	Kanellopoulou et al. [181], O'Connell et al. [182], Rodriguez et al. [183], Romania et al. [62]
miR-146a	Down	CXCR4	Inhibits MK differentiation and proliferation	PLZF	Navarro et al. [63]
miR-125b-2	Down	DICER1/St18	Increase MK proliferation	Unknown	Klusmann et al. [184]
Erythropoiesis					
miR-221 miR-222	Down	c-KIT	Inhibits erythroid proliferation and perinatal Hb switching	Unknown	Felli et al. [76], Bruchova et al. [78], Zhan et al. [80], Gabbianelli et al. [77]

(continued)

Table 10.1 (continued)

miRNA	Expression	Targets	Function	Regulated by	References
miR-451 miR-144	Up	RAB14, 14-3-3Zeta	Regulates oxidative stress, positive regulation of terminal erythroid differentiation and in zebrafish hemoglobin synthesis (miR-144)	GATA-1	Bruchova et al. [78], Zhan et al. [80], Rasmussen et al. [185], Kim et al. [85], patrick et al. [83], Yu et al. [84]
miR-24	Bi-phasic	hALK4	Modulates negatively erythropoiesis	Unknown	Wang et al. [86]
miR-155	Down	PU-1/ETS-1 CEBPb/SHIP1	Inhibits erythropoiesis	AP-1, JNK	Escobar et al. [22], Ghisi et al. [23], Wu et al. [30], Lu et al. [31], Bruchova et al. [78], Masaki et al. [186], O'Connell et al. [182], Rodriguez et al. [183]
miR-223	Down	LMO2	Inhibits erythropoiesis	CEBPa, NF1A, E2F1	Felli et al. [72]
Lymphopoiesis					
miR-150	Down prog.	c-Myb	Inhibits the transition from pro-B to pre-B cells	Unknown	Xiao et al. [187], Zhou et al. [188]
	Up mature B/T cells		Block DN3 to DN4 T-cell transition		
miR-181a	Up prog. and in DP T cells Down in mature B/T cells	BCL2/CD69 TCR/DUSP5 DUSP6/SHP2 PTPN22	Increases CD19+ cells/Modulate late thymic T-cell development, TCR sensitivities and strength	Unknown	Neilson et al. [189], Li et al. [190]
miR-17-92	Up in B/T precursors Down in mature B/T cells	Bim PTEN	Modulates positively the transition from pro-B to pre-B cells	c-Myc, E2F1	Ventura et al. [191]

(continued)

Table 10.1 (continued)

miRNA	Expression	Targets	Function	Regulated by	References
miR-155	Up in Tregs and in activated T/B cells	SOCS1 SHIP-1 CEBPb AID	Enhanced Tregs proliferation Maintain competitive Treg fitness Regulates GC-B cells responses, innate immunity, T-cell dependent antibody responses, negative regulator of somatic hypermutation	Foxp3	Rodriguez et al. [183], Thai et al. [192], Vigorito et al. [193], Stahl et al. [194], Zheng et al. [195], Teng et al. [196], Dorsett et al. [197]

MK megakaryopoiesis, *Mo* monopoiesis, *GC* germinal center, *DN3* double negative stage 3, *DN4* double negative stage 4 *Prog* progenitors

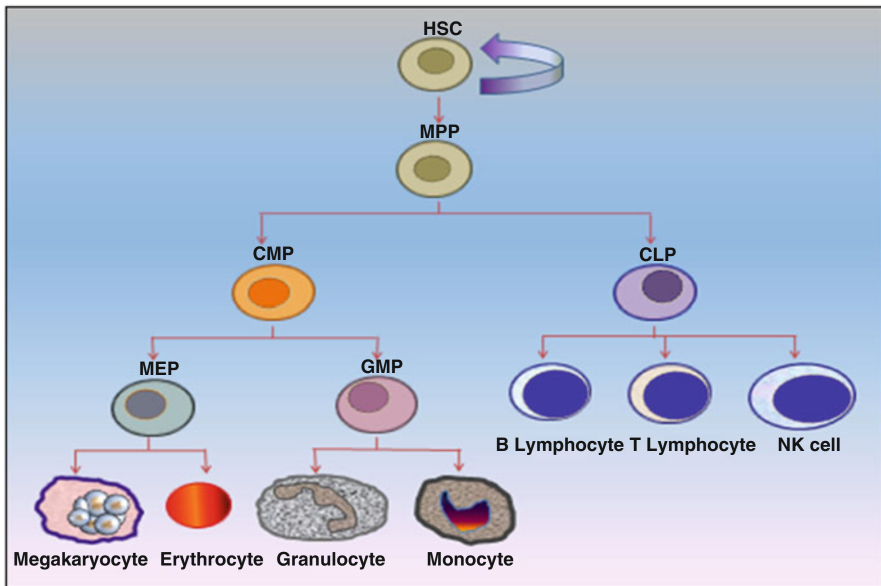

Fig. 10.2 A schematic representation of hematopoietic development depicting the specific points in differentiation

Lymphopoiesis

Lymphopoiesis refers to a developmental process involving distinct stages of commitment and differentiation, finally resulting in the generation of immune cells that mediate adaptive immunity, namely—T cells, B cells, and NK cells. These cells develop from a common lymphoid progenitor which is developed from the pluripotent HSC. The process ends up in the generation of a vast and highly diverse repertoire of T, B, and NK cell which efficiently fights against pathogenic organisms. Evidences suggest that these short noncoding RNA extends their influence in almost every aspect of lymphocyte development.

Expression pattern of miRNA in HSCs have been studied extensively. miR-125b is a highly expressed class of miRNA in HSCs and its expression decreases in committed progenitors. Mature peripheral blood cells derived from the miR-125b-overexpressing HSC increase early B-progenitor cells in the spleen and induce the expansion and enrichment of the lymphoid-balanced and lymphoid-biased HSC subset through an antiapoptotic mechanism, reducing the mRNA expression levels of two proapoptotic targets, Bmf and KLF13, which can have drastic effect on the phenotype of an organism [21]. miR-155-deficient CD4+ T cells in vitro and in vivo have shown defective Th17 cell cytokine and Jarid2 is found to be more expressed which is required to recruit Polycomb Repressive Complex 2 to chromatin in Th 17 and T regulatory (Treg) cells [22].

T Cell Development and Differentiation

T cells are immune cells that are the critical controllers of adaptive immune response in organisms. T cells develop from the common lymphoid progenitor cells through several critically regulated developmental and differentiation steps.

T lymphocyte has several subtypes that are classified on the basis of certain surface marker expression, type of cytokines they release, and maturation status. Cell surface markers such as CD4, CD8, and CD3 define the developmental stages of a particular T lymphocyte in the thymus. On the basis of expression of these markers T cells are grouped as double negative (DN), double positive (DP), and single positive (SP) CD4+ or CD8+ cells. Mature T cells that get released from the thymus forms another class of T cells—naïve T cells, effector T cells, and memory cells T cells each with corresponding cell surface components.

The miRNA miR-155 can be induced by signals that activate T cells, B cells, and antigen-presenting cells, and regulate immune response of these cells such as the differentiation of T regulatory (Treg) cells and CD4+ T helper (Th) cells. They are extensively studied class of miRNA as it influences several immune cells. miR-181 family member, miR-181a, has a profound role in T cell biology as it is evident from distinct studies carried out by several independent research groups.

miRNA profiling study of three different maturation stages of T lymphocytes, DP (CD4+ CD8+), SP CD4 (CD4+ CD8−), and SP CD8 (CD4− CD8+), has identified the role of miRNAs in regulation of T cell maturation. miR-150 was highly expressed in this population of cells during maturation and that its target was identified to be NOTCH3, a key player in T cell development. The studies have identified an indirect relation between miR-150 and NOTCH-3p by upregulation of miRNA 150 during T cell maturation [23]. They are highly expressed in double negative (DN) and double positive (DP) population and downregulated to undetectable levels in mature T lymphocytes [24].

CD4 and CD8 T Cell Biology

CD4 T cells are lymphoid derived immune cells which plays a central role in adaptive immunity against)pathogens. These cells not)only help B cells to make antigen specific antibodies but also involved in recruitment of neutrophils, eosinophils, and basophils to the site of activation, enhancing microbicidal activity of macrophages and synthesis of their own cytokines and chemokines.

Majority of the studies conducted to identify the expression pattern of miRNAs reported that their expression is higher in the primitive forms when compared to the differentiated form. This can be mainly attributed to the genetic switch from idealness of a progenitor cell or the hematopoietic stem cell itself to a more metabolically active developed cell. The number of miRNAs involved in different stages of T cell development has been listed in Table 10.2. For instance, miR-125b belonging to miR-125 family was reported to be highly expressed in HSCs but as it progresses into a more differentiated progenitors, the expression level gradually decreased [21]. AHR or aryl hydrocarbon receptor is often involved in the differentiation process of TH 17 cells. Study has shown TH17 activated AHR upregulates miR-132/212 clusters but not Treg cells and has also shown AHR activated TH17 differentiation was not happened in miR-132/212 cluster deficient cells. Mice deficient in the miR-132/212 cluster have shown more resistance to the development of experimental autoimmune encephalomyelitis [25].

T cells express mir-21 expression and activated αCD3/CD28 upregulates mir-21 expression. Inhibition studies of mir-21 functions in activated naive and memory T-cells have shown the regulatory role of this miRNA in different aspects of T-cell biology. Inhibition of miR-21 function in activated memory T-cells led to apoptosis, whereas in activated naive T-cells led to induction of CCR7 protein expression, hence proving multiple roles for single miRNA in two subsets of CD4+ T cells [26]. As a result of T cell specific Dicer ablation resulted in increased level of effector CD4 T cells committing to Th1 (T-helper 1) responses [27] with reduction in the development to T regulatory cells [28]. miRNA also plays a regulatory role in maintaining T cell numbers. This was reported in a study conducted by Boldin et al. The study reported miR-146a as a negative regulator of inflammation. Mice lacking miR-146 were found to be more responsive to bacterial challenges and produced more pro-inflammatory cytokines [29].

Table 10.2 miRNAs in the different stages of T cell development

miRNAs	Target	Function	References
miR-146	*STAT1*	Regulation of the Treg suppressor function through the targeting of *STAT1* promotion of differentiation into Th1 cells rather than Th2 cells	Neilson et al. [189], Monticelli et al. [198], Lu et al. [199]
miR-17-92	*CREB1* *PTEN* Bim	Regulation of effector and memory CD8+ T-cell differentiation	Wu et al. [34]
miR-150	*NOTCH3*	Controls of T-cell differentiation	Zhou et al. [188]
		Regulation of differentiation into the memory or effector phenotypes of T cells	Almanza et al. [200]
		Helps in differentiation from DP into CD4+ and CD8+ T-cells	Neilson et al. [189], Ghisi et al. [23], Bellavia et al. [201], Indraccolo et al. [202]
		Regulation of the intrathymic pre-T-cell receptor selection of T-cells	Bellavia et al. [201], Campese et al. [203]
miR-155	*SOCS1*	Regulation of differentiation into the memory or effector phenotypes of T cells	Almanza et al. [200]
		Controls of T-cell differentiation: to favor Th1 responses partially by modulating cytokine production	Rodriguez, et al. [183], Thai et al. [192]
		Control of proliferation and homeostasis of Treg cells by stabilizing the signal of *FOXP3* through the targeting of *SOCS1*	Lu et al. [31]
miR-181a	CD69 *BCL2* TCR-α (DUSP5, DSP6, SHP2, PTPN22)	Regulation of positive selection by governing the homeostasis of CD4/CD8 lymphocytes and modulation of T-cell sensitivity by increasing TCR signaling to peptide antigens through the downregulation of multiple phosphatases	Chen et al. [18], Fabbri et al. [204], Neilson et al. [189], Liu et al. [205], Li et al. [206], Li et al. [190]
		Regulation of iNKT cell development through the modulation of TCR signaling threshold resulting the increase responsiveness of DP thymocytes to TCR signals	Ziętara et al. [207]

CD8 T cells are critical mediators of adaptive immune response in an organism against several pathogens. Microbe entry enables these cells to undergo clonal expansion, resulting in production of numerous clones of antigen specific CD8 T cells. Wu et al. made a comparative study of miRNA expression profiles of naïve, effectors, and memory CD8+ T cells and showed that only few miRNAs are dominantly expressed in CD8+ T cells and these miRNAs expression is dynamically regulated during differentiation process. Interestingly, a significant decrease in

miRNA expression level is seen in effector T cells, as antigen stimulation of naïve T cells results in upregulation of several genes [30].

T Regulatory Cell Biology

T regulatory cells are essential components of the adaptive immune response as they are responsible for the)maintenance of immune self-tolerance.)These CD24+ CD4+ cells are characterized with expression of a transcription factor Foxp3. Most of the Foxp3 T regulatory cells are produced as a functionally mature T cell which can effectively bring about immune suppression. Like in all T cell subsets, miRNA has a regulatory role in development of these regulatory T cells also. miRNA deletion can result in certain deleterious effect; one such effect is caused when miR-146a Knockout Tregs are generated.

Foxp3 is the most crucial factor reported in the establishment of regulatory T cell lineage. miR-155 is an extensively studied microRNA in hematopoiesis, mainly in lymphopoiesis. miR-155 regulates SOCS1 (suppressor of cytokine signaling 1), a molecule which is essential for the immune suppressive function regulatory T cells. T reg cells lacking miR-155 exhibited poor expansion in response to IL2. This is due to inhibition in IL-2 signaling by SOCS-1 [31]. These cells are characterized by poorly functional Foxp3+ Tregs in large numbers in the periphery. miR-17-92 cluster is another miRNA class studied to have an impact on T regulatory cell development. It was found that miR-17-92 was required for the accumulation of antigen specific T reg after activation and also for differentiation into effector T cells [32]. These miRNAs also promote T cell survival and Th1 responses, regulate CD8 effectors vs. memory differentiation, and inhibit TGF-β-induced in vitro differentiation into induced Tregs [33]. miR-31 and miR-21 were also addressed to regulate Foxp3 expression in T reg cells, former one inducing a negative effect as it directly binds to the 3′ UTR of Foxp3 mRNA whilst the latter being a positive regulator [34]. miR-155, a multifunctional miRNA, is found to have its profound influence on hematopoiesis, inflammation, and immunity [35]. The role of miR-155 is well studied in immune modulation and in the developmental process of T and B cells.

B Cell Development

The pluripotent hematopoietic progenitor) cell differentiates to form common lymphoid progenitors, which in turn give rise to the B-cell lineage. The stages of B-cell development are early pro-B cell, late pro-B cell, large pre-B cell, small pre-B cell, and mature B cell.

Expression profiling of miRNA in B cell subsets in human and mouse have identified distinct miRNAs and the results has great clinical significance which enabled for an in-depth study in exploring the world of miRNA in B lymphopoiesis and

lymphomagenesis. The studies also indicated that miR-155 has distinct expression profiles and plays a key role in hematopoietic lineage differentiation, inflammation, immunity, cardiovascular diseases, and cancer [36]. Another study has shown that the role of miR-181 expression in lineage negative hematopoietic progenitor cells in mouse doubled the cells belonging to B lymphoid lineage without any significant effect on T lymphoid lineage [37], suggesting it to be a positive regulator of B lymphopoiesis. The same study reported miRNA to be upregulated in B lymphoid and myeloid lineages miR-142 [18].

Dicer ablation conducted by Koralov et al. resulted in inhibition of transition of B cell lineage committed cells from pro-B cell state to pre-B cell state. It was found that miRs related to miR-17-92 cluster were detected in pro-B cells. Bim being the target gene, it is concluded that miR-17-92 cluster is involved in B cell progenitor survival [38]. Overexpression of miR-34a led to prevention of B cell development at the pro-B-cell-to-pre-B-cell transition, leading to a reduction in number of mature B cells. Co-transduction of Foxp1 lacking its 3′ UTR with miR-34a rescued B cell maturation whereas inhibition of Foxp1 causes B cell developmental phenotype through miR-34a activation [39].

Natural Killer Cells

Natural Killer (NK) cells are)large granular lymphocytes which could produce inflammatory cytokines. Bezman et al. have shown the effect of deletion of Dicer and Dgcr8 which resulted in defective NK cell differentiation and functioning suggest that Dicer and a double-stranded RNA binding protein Dgcr8 have a key role in NK cell activation, survival, and function during infection in mouse mounted by cytomegalovirus. The study also reported that an increase in the mRNA level of Bcl-2, an anti-apoptotic factor, was found to be downregulated in cell deficient in Dicer and Dgcr8 and also exhibited an increased rate of apoptosis in peripheral NK cells thereby proving a role for these enzymes in cell survival [40]. Some of the miRNAs studied so far to influence NK cell development include miR-150, miR-583, miR-223, miR-155, miR-181, and miR-15/16.

Lack of miR-150 inhibits the terminal maturation of cells and also decreases the number of total peripheral NK cells. As mentioned above, expression level of this miRNA is low when compared to the differentiated cells and hence suggesting them to have less chance of miR-150 mediated regulation of its development. An ectopic expression of miR-150 showed increase in number of hyperfunctional natural killer cells in miR-150 transgenic mice with no difference in the rate of apoptosis between normal and miR-150 transgenic NK cells [41]. miR-223 is also known to be involved in the developmental regulation of NK cells through granzyme B, a direct activator of proapoptotic molecule Bid thereby initiating caspase activation [42]. Recently, Yun et al. showed that miR-583 had an inhibitory effect on NK cell differentiation [43]. miR-181 mediates NK cell development to a minor extent by regulating NLK (nemo-like kinase), an inhibitor of Notch signaling. Notch signaling is involved in NK cells during the early stages of development [44].

miRNA in Myelopoiesis

Myelopoiesis is a complex process of hematopoietic CMP development that gives rise to all myeloid lineages; these committed cells further differentiate to give rise to erythrocytes, megakaryocytes, and granulocytes as well as macrophages [45]. Till the past decade, the integrated effects of cytokines regulated signaling pathways and intrinsic TFs were known to control myelopoiesis [46, 47]. However, a number of recent investigations have also evaluated the role of miRNAs in myeloid development and function. Hox9A, a transcription factor, was reported to have a role in normal myeloid differentiation [48] and lymphoid lineage commitment from MPP [49] Loss of function of Hox9A results in contractions in myeloid and lymphoid progenitors and loss of peripheral leukocytes [48], whereas overexpression in mouse bone marrow cells results in AML [214]. miR-126 regulates the expression of Hox9A by binding to the homeobox; miR-126 and hox9A are parallely downregulated during progenitor cell differentiation [50]. Hence, miR-126 appears to control HSCs differentiation and lineage commitment process by regulating Hox9A expression. miR-9 was reported as a critical regulator in cell fate decision in hematopoietic development, its ectopic expression in BM cultures accelerates terminal myelopoiesis, and its inhibition with a miRNA sponge blocks myelopoiesis; miR-9 was also found to enhance myelopoiesis but inhibits lymphopoiesis in mice model [51]. miR-9 inhibits expression of FoxO1 and FoxO3 by targeting 3′ UTR region of the murine *FoxO1* and *FoxO3* genes and FoxO3 inhibits myeloid differentiation induced by miR-9 [51]. miR29a, an important miRNA in hematopoietic development, is highly expressed in HSCs and LSCs and downregulated in more committed progenitor cells; ectopic expression of miR-29a induces CMP and GMP self-renewal, biased myeloid differentiation, and also induces the development of myeloproliferative disorder [52] (Fig. 10.3).

miRNAs in Megakaryocytopoiesis

Traditionally, the mechanistic studies on megakaryocytopoiesis have focused primarily on different cytokines [53] and transcription factors [54, 55]. Over the past many years, numerous laboratories have established the essential role of miRNAs in megakaryocytopoiesis [56–58]. Garzon et al. [58] suggest a regulatory role of miRNAs in megakaryocytopoiesis by targeting different megakaryocytic transcription factors. One study reports the expression profile of 435 miRNAs; among them 13 miRNAs were upregulated and 81 were downregulated during murine MKs differentiation [57]. miR-150 is an important miRNA which plays a role in megakaryocyte-erythrocyte-progenitors (MEPs) fate decision; it favors the commitment of MEP into MKs lineage rather than erythroid lineage [56]. miR-150 is moderately expressed in MEP, and exhibits increased expression in cells undergoing megakaryocytopoiesis whereas progressively downregulated during the erythropoiesis

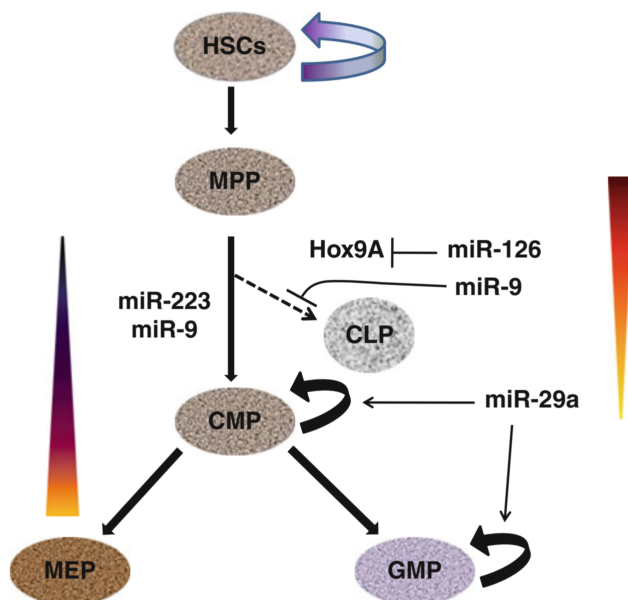

Fig. 10.3 Schematic representation of the role of miRNAs in CMP differentiation

[56]. Thrombopoietin (TPO) increases the expression of miR-150, which in turn knocked down the expression of c-Myb via its 3′-UTR [59], previous data show that the low levels of c-Myb favors megakaryocytopoiesis [60]. miR-28 is a TPO receptor (MPL) targeting miRNA detected in platelet fraction isolated from myeloproliferative neoplasm patient, the overexpansion of miR-28 downregulates differentiation of CD34+ cells into Mks by targeting TPO signaling [61]. miR-155 is highly expressed in CD34+ cells but the expression is dramatically reduced during the CD34+ cells differentiation into Mks, the decline expression of miR-155 is required for proliferation and differentiation of Mks at progenitor and precursor level, the sustained expression of miR-155 inhibits the Mks differentiation by regulating the targets Meis-1 and Ets-1 [62]. miR-146a was reported as a modulator of megakaryocytopoiesis, the expression of miR-146a dramatically changes when HSCs differentiate into MKs [57, 63, 64]. Opalinska et al. [57] reported that the miR-146a expression level increases when the murine and human derived HSCs induced to differentiate into MKs, but the forced expression of miR-146a had no effects on megakaryocytopoiesis and platelet activation. Labbaye et al. [63] found decreased levels of miR-146 when the human cord stem cells were induced to differentiate into MKs, and decrease in cell ploidy was detected due to forced expression of miR-146a. During megakaryocytopoiesis the level of miR-146a is reduced by PLZF transcription factor; the PLZF binds the promoter region of miR-146a and inhibits its expression, whereas miR146a targets the CXCR4 mRNA and inhibits its translation [63]. Starczynowski et al. [64] found the results of miR-146a expression was similar

to Labbaye et al. [63], but the effect of forced expression on megakaryocytopoiesis was not observed, similar to Opalinska et al. [57]. miR-146a negatively regulates megakaryocytopoiesis by targeting TRAF6, the downregulation of miR-146a increases the activity of TRAF6 which in turn increases the IL-6 level [64]. miR-146b, a member of miR-146 family, is upregulated in progenitor cells undergoing erythroid or megakaryocytic differentiation, and PDGFRA, a negative regulator of erythroid and megakaryocytic differentiation, is a direct target of miR-146b; miR-146b could affect the expression of GATA-1 via downregulating PDGFRA/JNK/JUN/HEY1 pathway, whereas the transcription of miR-146b is positively regulated by GATA-1 [65]. miR-34a was found to be upregulated during megakaryocytic differentiation of K562 cells, but not during erythrocyte differentiation [66] and miR-34a promotes differentiation of bi-potent K562 cells into MKs by targeting c-Myb, CDK4, CDK6 [66], and MEK1 [67]. miR-34a is also upregulated during TPO induced differentiation of CD34+ cells, and its enforced expression increases the number of MKs [66]. Thus, miR34a is illustrated to enhance megakaryocytopoiesis. Recent reports show the downregulation of miR-130a during the CD34+ cells differentiation into MKs targets the MAFB transcription factor, which is upregulated during megakaryocytopoiesis [58]. Thus, miR-130a downregulation may help to MEPs fate decision by upregulating MAFB, which inhibits erythroid differentiation [68]. Several other miRNAs in megakaryocytopoiesis have been identified, but there is no much information related to their activity. miR-27a was found to be upregulated during MKs differentiation by RUNX1, miR27a targets RUNX1 and modulates the steady-state level of RUNX1 during MKs differentiation [69]. In this way, miR-27a and RUNX1 maintain a regulatory feedback loop. miR-10a is downregulated during MKs differentiation and was found to target HOXA1 [58, 70]. Thus, miR-10a appears to inhibit MKs differentiation. miR-181 is upregulated during initial stage of MKs differentiation and after that it is progressively downregulated, miR-181 was found to interrupt the Lin28-Let7 feedback circuit by downregulating the Lin28 in early stage of MKs differentiation [71]. Thus miR-181 appears to promote megakaryocytopoiesis by indirectly promoting the expression of Let-7, but not promoting the erythroid differentiation [71]. Hence, it works as a molecular switch during MEPs progression toward MKs differentiation. miR-223 expression increases during MKs differentiation and it downregulates LMO2 expression by directly targeting LMO2 mRNA, whereas the miR-223 is downregulated during erythrocyte differentiation and forced expression shows suppressive effects on erythroid differentiation [72, 73]. Recent study evidenced the role of miR-125b in regulation of cell proliferation and survival in neonatal megakaryocytes [16] (Fig. 10.4). Represents the summary of study on miRNAs in megakaryocytopoiesis.

miRNAs in Erythropoiesis

Erythropoiesis is the process which produces red blood cells (erythrocytes). Erythropoiesis occurs in bone marrow (in humans) and stimulated by erythropoietin, which is secreted by kidneys in response of decreased oxygen levels in

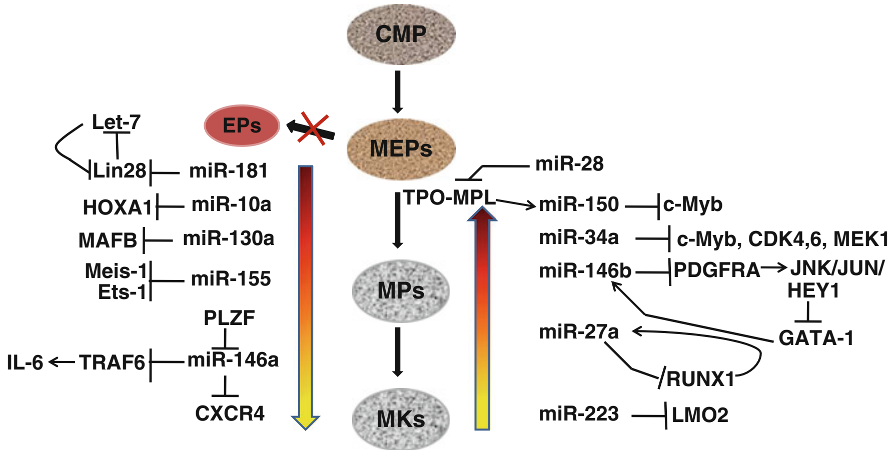

Fig. 10.4 Schematic representation of the role of miRNAs in megakaryocytopoiesis

circulation. The role of certain miRNAs in erythropoiesis has also been identified in last 6–7 year research work. c-KIT, a receptor of SCF, has important role in early erythropoiesis to regulate survival and proliferation of erythroblasts [74, 75], and its expression is blocked in late erythropoiesis by GATA1 and other mechanisms [75]. Felli et al. [76] first reported that miR-221/222's expression is downregulated during differentiation of CD34+ cells into erythroid precursors, miR-221 and 222 directly targets c-KIT 3′UTR and the expression of both miRs is inversely related to that of c-KIT. Gabbianelli et al. [77] reported that the perinatal HbF switching is controlled by c-KIT. Thus miR-221/222 appears to block HbF switching and downregulates erythroblasts proliferation. One in vitro culture study on peripheral blood CD34+ cells revealed progressive downregulation of miR-221, miR-222, miR-150, and miR-155; upregulation of miR-451 and miR-16 at late stage of erythrocyte differentiation; and biphasic regulation of miR-339 and miR-378 during erythroid differentiation [78]. miR-150 is downregulated during erythropoiesis and directly targets c-Myb which regulates MEP fate decision towards erythrocyte or megakaryocytes [56]. Vegiopoulos et al. [79] reported that c-Myb is promoting MEP commitment toward erythropoiesis and progression of cells from their early to late stage of differentiation. Accumulating data from many erythroid miRNA profiling studies proved that miR-451 is upregulated after erythroid differentiation [78, 80, 81]. The expression of miR-451 is erythroid specific and it transcribed as a bicistronic transcript with miR-144 by GATA-1 transcription factor [82, 83]. miR-451 was found to protect erythroid cells from oxidative stress via inhibiting 14-3-3ζ, which inhibits nuclear accumulation of FoxO3 transcription factor, a positive regulator of erythroid antioxidant genes [84]. Patrick et al. [83] demonstrate that repression of 14-3-3ζ by miR-451 enhances erythroid differentiation. Recently, Kim et al. [85] identified RAB14 as a novel physiological inhibitor of human erythropoiesis, which is a direct target of both miR144 and miR451; the expression of miR-144 and miR-451 is inversely proportional to that of RAB14. The forced expression

of RAB14 shows the phenocopy of the effects of miR144 and/or miR451 depletion and knockdown expression of RAB14 protects cells from miR-144/451 depletion mediated inhibition of erythropoiesis, the knockdown expression of RAB14 increases the cell number and β-Hb expression during erythropoiesis [85]. miR-233 is downregulated during erythroid differentiation and the expression of its target LMO2 is upregulated, LMO2 is required for erythroid differentiation [72, 73]. Thus miR-223 is an inhibitor of erythroid differentiation. miR-24 regulates the erythrocytic differentiation by controlling the expression of ALK4 receptor by directly targeting 3′UTR region of AKL4 mRNA, miR-24 blocks the expression of activin-mediated accumulation of Hb and decreases the CFU and BFU of CD34+ cells. The expression of miR-24 is downregulated during erythropoiesis [86]. The c-Myb is an important factor for lineage commitment, proliferation, and differentiation. Zhao et al. [87] suggested a negative autoregulatory feedback mechanism between c-Myb and miR-15a. c-Myb binds promoter region of miR-15a and promotes its expression whereas miR-15a downregulates the c-Myb expression by blocking its translation; the forced expression of miR-15a in bone marrow mononuclear cells blocked the erythroid transition from BFU to CFU in vitro [87]. miR-320 was found to inhibit erythroid differentiation by targeting SMAR1 mRNA, which in turn downregulates miR-222 expression by binding promoter region of miR-221/222 [88]. miR-320 is downregulated during erythroid differentiation [80] resulting in upregulation of SMAR1, which downregulates miR-222 expression [88]. miR-222 inhibits erythropoiesis by targeting c-KIT [76]. Thus miR-320 regulates erythropoiesis through SMAR1 and miR-221/222. (Fig. 10.5) represents the summary of the regulatory role of miRNAs in erythropoiesis.

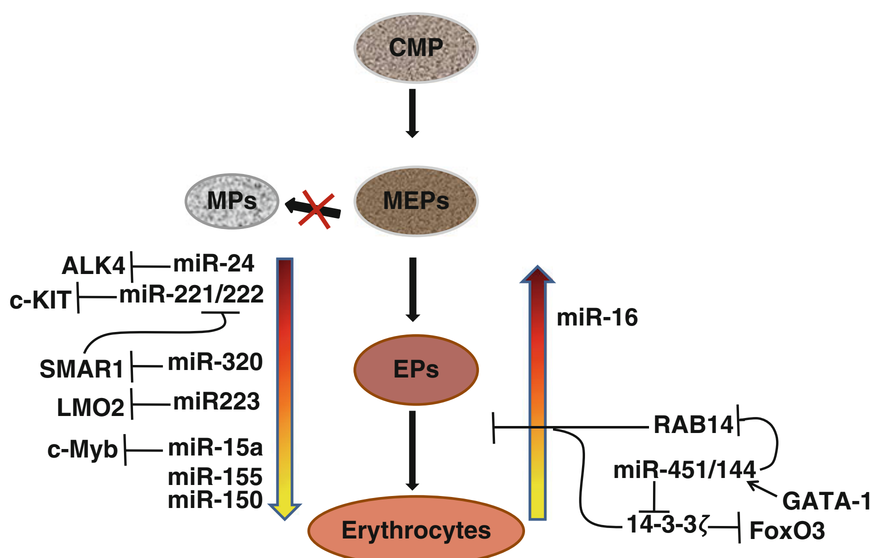

Fig. 10.5 Schematic representation of the role of miRNAs in erythropoiesis

miRNA in Granulopoiesis and Monopoiesis

Transcription factors are well known to play important role in GMP fate decision; these regulate the commitment of GMP into granulocyte or monocyte. An increasing body of data implicates the importance of various miRNAs in regulatory circuit of these transcription factors. miRNAs regulate these transcription factors activity by downregulating their translation, many TFs also regulates the expression of miRNAs. miR-223 with two other TFs, NFI-A and C/EBPα, was reported to be involved in a regulatory circuit of retinoic acid (RA)-induced differentiation of granulocytes; both NFI-A and C/EBPα compete for binding to the promoter region of *miR-223* gene [89]. NFI-A downregulates promoter activity of *miR-223*, whereas C/EBPα promotes promoter activity of *miR-223* and increases transcription of miR-223 which represses NFI-A translation [89]. Zardo et al. [90] reported in human CD34+ cells that NFI-A expression is also downregulated at transcriptional level by coordinated epigenetic events triggered via recruitment of polycomb (PcG)-RNAi complexes and endogenous miR-223. AML1/ETO, a fusion protein expressed in AML cells, was found to inhibit granulopoiesis by recruiting chromatin remodeling enzymes (HDAC and DNMT) on *pre-miR-223* gene, in turn these enzymes downregulates *pre-miR-223* gene expression via chromatin remodeling [89]. Above reports show that miR-223 upregulates granulocyte differentiation by transcriptional and posttranscriptional targeting of NFI-A gene expression. Johnnidis et al. [91] reported the increased expression of miR-223 during granulocytic differentiation while it decreases during monocytic and erythrocytic differentiation and it was found as the fine-tuner of granulocyte differentiation and function in mice model. GFI-1 is a transcriptional repressor protein which has role in normal granulopoiesis, the GFI-1 deficiency in mice and human has been found to exhibit an arrest in myeloid differentiation [9, 92]. It is reported that GFI-1 binds to the promoter of miR-21 and miR-196b and downregulates their transcription; GFI-1 upregulation in mice GMP cells is associated with dramatic reduction of both miR-21 and miR-196b [93]. miR-21 overexpression in Lin− murine BM cells was found to increase the monocytic colonies and its antagomiR showed apposite effects, whereas ectopic expression of miR-196b was observed with significant decrease in granulocytic colonies [93]. However, coexpression of both miRNAs completely blocked the G-CSF induced granulopoiesis and lead to the accumulation of immature granulocyte and monocyte precursors [93]. Thus, miR-21 appears to act as a promonopoietic factor and miR-196b as an antigranulopoietic factor and both miRNAs with GFI-1 control the fate decision of GMP to granulocytes. Fontana et al. [94] investigated the role of miR-17-5p, 20a, and 106a cluster in monocytic differentiation and maturation of human CD34+ cells, this cluster is downregulated during monocytic differentiation. AML-1, a direct target of this cluster, is unblocked and upregulated during monocytic differentiation, thereby promoting monocytic differentiation and maturation; AML-1 also acts as a suppresser of these three miRNAs by directly targeting their promoter region [94]. Rosa et al. [95] reported that human monocyte/macrophase differentiation is regulated by an interconnected circuit of PU.1, miR-424 and NFI-A; PU.1 activates transcription of miR-424, which in turn induces

monocyte/macrophase differentiation by translational repression of NFI-A, which is an inhibitor of monocytic differentiation. Other reports also found the miR-424 upregulation during monocytic differentiation of leukemia cell lines [96, 97]. Bousquet et al. [98] identified that miR-125 is upregulated in MDS and AML with t(2;11)(p21;q23); miR-125b interferes with primary human CD34+ cell differentiation, and also inhibits granulocytic and monocytic differentiation in HL60 and NB4 leukemic cell lines. Ooi et al. [21] reported that miR-125b expression level is high in mouse HSC, and its expression decreases progressively during lymphoid and myeloid progenitor commitment; the miR-125b expression also decreases during CMP differentiation into GMP. Surdziel et al. [99] reported that the miR-125b affects myelopoiesis in various ways and blocks the G-CSF induced differentiation of granulocytes. Shen et al. [97] reported that miR-424 decreases the miR-125b expression by direct targeting to CDX2 (caudal type homeobox 2) and promotes monocytic differentiation. CDX2 TF was reported as a transcriptional regulator of miR-125b, which binds promoter region of miR-125b and increase its expression [100]. (Fig. 10.6) represents the regulatory role of miRNAs in granulocyte and monocyte differentiation.

Hematological Disorders

Hematological malignancies account for 9.5 % and lymphomas are more common than leukemia. Hematological malignancies are malignant neoplasms which affect blood, lymph, bone marrow and lymphatic system. Leukemias are classified into

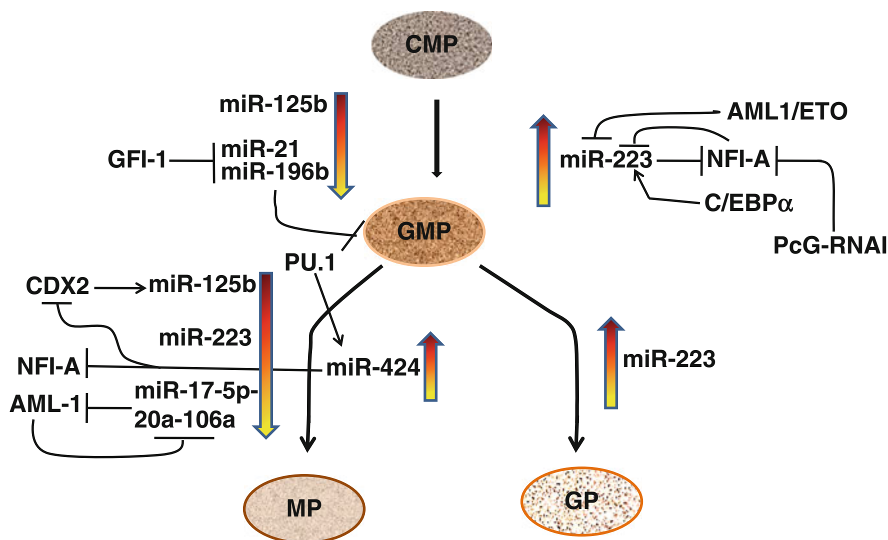

Fig. 10.6 Schematic representation of the role of miRNAs in granulopoiesis and monopoiesis

acute myelogenous leukemia (AML), chronic myelogenous leukemia (CML), acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute monocytic leukemia (AMOL), and so forth. Lymphocytic leukemia and myeloma arise from lymphoid lineage cells, whereas CML, AML, and myelodysplastic syndromes arise from myeloid lineage cells. Lymphomas are classified into Hodgkin's lymphomas and non-Hodgkin's lymphomas. Recent expression profiles of miRNAs studies have evidenced their involvement in the development of hematological malignancies.

Acute Myeloid Leukemia

AML is the most common type of leukemia in adults and is a rapidly growing malignant neoplasm in which immature WBCs are found in blood and bone marrow. Recent study indicated that miR-126/126* is upregulated in this malignancy and induces inhibition of apoptosis and also increases cell viability and proliferation. This study found that tumor suppressor protein PLK2 (polo-like kinase2) which is involved in the cell cycle checkpoint was targeted by miR-126 [101]. miR-125b and miR-99a which are present on chromosome 21 are shown to involve in co-regulation of vincristine resistance in childhood acute megakaryoblastic leukemia [102]. miR-223 regulates granulopoiesis by inhibiting the cell cycle regulator protein E2F1. But in AML E2F1 is a transcriptional inhibitor of miR-223 by binding to the miR-223 promoter [215]. The study has show that mir-223 suppression is not caused by nucleotide sequence change or hypermethylation of DNA, its suppression in AML is caused by impaired miR-223 upstream factors [103]. miRNA-221 inhibits the CDK inhibitor p27 thereby enhancing cell proliferation in AML [104] and overexpression of miRNA 221 has also been associated with several solid tumors. The role of Hypoxia-inducible factor 1 (HIF-1) has been observed in miRNA signaling network by regulating the miR-20a and miR-17 which can inhibit the p21 [105]. Tumor suppressor let-7b miRNA expression was found to be downregulated in AML [104]. Recent studies have shown involvement of several other miRNAs in AML pathogenesis which has been listed in Table 10.3.

Chronic Myelogenous Leukemia

Uncontrolled growth of granulocytes (eosinophils, basophils, and neutrophils) has been observed in chronic myelogenous leukemia or chronic granulocytic leukemia. This leukemia is well characterized by presence of fused BCR-ABL protein present in Philadelphia chromosome. The translocation of BCR gene between chromosome 9 and 22t (9; 22) (q34; q11) [106] leads to fusion protein BCR-ABL. This protein inhibits DNA repair and cause genome instability, which in turn leads to accumulation of genetic abnormalities [106]. Overexpression studies of miR-29b have shown that it decreased the cell growth and colony formation by inhibiting ABL1 and

BCR/ABL1 [107]. The overexpression of miR-138 also inhibits BCR/ABL1 and CCND3 by binding to the coding and 3′UTR regions [108]. The list of miRNAs which are involved in CML pathogenesis is given in Table 10.4.

Multiple Myeloma

Recent studies have evidenced that deregulated miRNAs have associated with myeloma genesis and most of the miRNAs are upregulated in plasma cell of myeloma compared to normal condition [109] and further studies have shown deregulated miRNAs could be used for diagnostic purpose [109]. Ninety-five miRNAs have shown more expression in an analysis of 464 miRNAs in multiple myeloma (MM) condition when compared to healthy individuals in a comparative study of miRNA expression profiles of 52 MM patients and healthy persons [110]. miRNA-15a expression was downregulated in MM cells which could be a tumor regulator [111]. In another study miRNA-15a and miRNA-16 expression

Table 10.3 miRNAs involved in AML

miRNA	Targets	Function	References
miR-126*	ADAT2,FOF1, LMO7	Upregulation leads to chromosomal translocations and inhibits apoptosis	Li et al. [101]
miR-126	TOM1, CKMT2, ZNF131, RGS3	Targets tumor suppressor PLK2	Li et al. [101]
miR-223	ANKH, SCN1A, SCN3A, CBFB, CDH11, NEBL, RILPL1, CENPN	C/EBPα regulates its expression in turn it inhibits E2F1 in AML cells	Eyholzer et al. [103]
miR-221	HIPK1, RAB18, DNM3, ZNF547,	Oncogenic miRNA, it inhibits the CDK inhibitor p27	Cammarata et al. [104]
miR-124a		Target the C/EBPα, silenced and block differentiation gives leukemia phenotype	Hackanson et al. [208]
miR-20a and miR-17	POLQ, KLF12, STK38, CENTD1, NUP35, GNB5, CTSK	Inhibits the p21	He et al. [105]
miR-29b	SCML2, C1orf96, COL3A1, COL7A1, COL11A1	Tumor suppressor in AML and reduce tumorigenicity	Garzon et al. [216]
miR-29b	CD93, HBP1, SNX21, GNS, HMGCR, HNF4G, DNMT3B	Reduced expression of DNA methyltransferases	Garzon et al. [216]
miR-196a and miR-196b	FOS, GATA6, HOXB6, HOXC8, ZNF24, CCDC47	They target ERG expression	Coskun et al. [217]
miR-193b	MMP19, ARMC1, ARPC5	Downregulated in AML and it targets c-Kit	Gao et al. [218]

Table 10.4 miRNAs involved in CML

miRNA	Targets	Function	References
miR-17-92	IRF9, RAB10, TXNIP, TET2	Downregulated in imatinib treated CML cells	Venturini et al. [219]
miR-21	TXPAN2, LUM, SUZ12, MSH2, PDZD2	Antisense inhibition leads to inhibition of migration and cell growth and induces apoptosis	Hu et al. [220]
miR-203	RTKN2, AAK1, MYST4 CD109, IL21, PLD2	Methylated in AML, CML, ALL, CLL. Inhibit the expression of BCR-ABL	Chim et al. [221]
miR-451	TSC1, ACADSB, GRSF1, MAML1, GDI1, NAMPT	Associated with Bcr-Abl	Lopotová et al. [222]
miR-29b	HAS3, SNX24, CD93, SCML2, COL7A1, ZNF396, HMGCR, ICOS	Inhibits ABL1 and BCR/ABL1 there by inhibiting cell growth and colony formation	Li et al. [107]
miR-138	KLF12, H3F3B, MYO5C, NXN, NEBL, PDPN, STK38	Represses BCR/ABL1 and CCND3, increases by GATA1	Xu et al. [108]
miR-212	APAF1, EP300, EDNRA, CFL2, NOS1, SOX4, SOX11	Increases the ABCG2 expression	Turrini et al. [223]

levels were elevated in the plasma cells (PCs) of diagnosed MM patients [112]. miRNA-29b has been reported to downregulate Mcl-1 and to induce apoptosis of myeloma cells [113]. miRNAs-720, 1308, and 1246 and miRNA-193b-365 cluster were found to be upregulated in PCs of myeloma patients than healthy controls [114, 115].

Diffuse Large B-Cell Lymphoma

Recent studies have shown the deregulation of miRNAs in DLBCL. miR-155 has been shown to overexpress in this lymphoma and play key role in progression of pathogenesis [116–118]. The study showed transgenic mice which carry miR-*155* develops high grade B-cell lymphoma after 6 months [119]. Another study showed pre-B cells transformation into malignant when SHIP1 and C/EBPβ were targeted [120–123]. Another study evidenced of showing regression of lymphoma when the inducible promoter of miR-155 was repressed [124]. The clinical importance of miRNA was shown to reduce lymphomas when *miR-155* antagomirs was delivered in the form of nanoparticles into explants of tumors. The growth inhibitory effects of TGF-β1 and BMP2/4 in DLBCL cells were suppressed via SMAD5 when *miR-155* was expressed in in vitro [125]. It was well-studied in in vitro and in vivo xenotransplant models that onco-mir-155 targets SHIP1 to promot TNFα-dependent growth of DLBCL [121], and was also shown to be involved in regulation of

PI3K–AKT pathway by targeting negative regulator PIK3R1 in DLBCL [126]. Differential expression of SHIP1 was observed in two molecular subtypes of DLBCL, activated B-cell like, and germinal center B-cell like DLBCL [127]. CD10 is a prognostic marker for DLBCL, the expression of CD10 is higher in GC subtype whereas the CD10 expression is low or absent in ABC subtype of DLBCL; the ABC type cells show lower prognosis after treatment and higher activity of NF-κB which targets mir-155 and increases expression of this onco-miR which results in down regulation of PU1 and lower expression of CD10 [128]. This study is correlated with previous studies as miR155 expression levels are different between ABC- and GC-type DLBCL. miR-34a is well-studied miRNA which can play a role as tumor suppressor that is linked with p53 network in tumors [129]. Interestingly p53 activates *miR-34a* expression and in turn miR-34a induces p53 via SIRT1 inhibition [130]. The role of tumor suppressor has shown to be involved when treatment of ABC-type DLBCL cell line (U2932) with *miR-34a* lead to regression of tumor growth through suppression of Foxp1 [131].

Lymphoid malignancies and increased tumor aggressiveness was associated with upregulation of *miR-17-92* along with MYC which could be mediated by MYC/*miR-17-92*/E2F circuit [132, 133]. MYC upregulates the miR-17-92 cluster which targets E2F1, while conversely pro-proliferative E2F3 regulates the miR-17-92 cluster [134]. The miR-19 increases the Akt–mTOR pathway through suppression of PTEN and leads to cell proliferation in Eμ-myc model and shown to be a key component of the miR-17-92 cluster [135].

Mantle Cell Lymphoma

Deregulated miRNAs have been demonstrated in pathogenesis of mantle cell lymphoma (MCL) [136–139]. Studies evidence that overexpression of Cyclin D1 in MCL is due to loss of miRNA target sites in the 3′UTR of CCND1 for miR-15/16 and members of the miR-17-92 cluster [140, 141]. MCL is well characterized by higher expression levels of MYC and miR-17-92 cluster, continuous PI3K/AKT pathway, and resistance to apoptosis [66, 139, 142]. In addition to PTEN and BIM, PHLPP2 was shown to be an important regulator of the PI3K/Akt pathway and miR-17-92 cluster [143]. The tumor growth was decreased when silenced the miR-17-92 expression in MCL xenotransplant model lead to suppression of PI3K/Akt pathway. Finally, downregulation of miR-29 was shown to activate CDK4/CDK6, and serves as a potential prognostic marker for this malignancy [136].

Hodgkin Lymphoma

The involvement of miRNAs in Hodgkin lymphoma (HL) progression was demonstrated after miRNA profiling studies in HL cell lines [144–146]. Aberrant miRNAs which are associated with cell proliferation, apoptosis, and the p53 pathway are

identified in HL cell lines using ribonucleoprotein chromatin immunoprecipitation (RIP-ChIP) approach [147]. Upregulated miR-135a in HL cells lead to enhancement of apoptosis and decreases cell proliferation via Bcl-xL inhibition by targeting JAK2 [138]. miRNA-9 is overexpressed in HL and several other cancer. miR-9 targets dicer1 and HuR in HL, the inhibition of mir-9 leads to derepression of HuR and DICER, which results in a decrease in cytokine production by HL cells; the lower levels of cytokines inpair the attraction of normal inflammatory cells by HL cells and also result in decreased tumour growth [148]. Further studies showed that decrease in tumor growth of a xenotransplant model when miR-9 antagomir delivered in tumor. In addition silencing of let-7 and miR-9 in HL cell lines inhibits PC differentiation with decreased expression of PRDM1/BLIMP1 [149]

Other B-Cell Lymphomas

Recent studies demonstrated involvement of miRNA deregulation in mucosa-associated lymphoid tissue (MALT) lymphoma. Methylation of miR-203 leads to transformation of gastritis to MALT lymphoma and provide the clues that ABL1 as a potential target for treatment of this lymphoma. 27-miRNA expression distinguishes gastric DLBCL from MALT lymphoma [150]. Differential expression of miRNAs were shown to differentiate gastritis to MALT lymphoma, i.e., five miRNAs (miR-150, miR-550, miR-124a, miR-518b, and miR-539) were present in gastritis but absent in MALT lymphoma [151]. Another miR-223 expression levels were shown to increase E2A expression in gastric MALT lymphoma [152]. Involvement of miRNAs in B-cell lymphomas now has been studied but yet to be studied in rarer neoplasms such as B-cell primary cutaneous lymphoma, plasmablastic lymphoma, and B-cell prolymphocytic leukemia (B-PLL).

T-Cell Lymphomas

The role of miRNAs in T-cell lymphoma is less studied when compared to B-cell lymphomas. Few studies have been reported on involvement of miRNA in T-cell lymphoma. Recent study showed that more than 100 miRNA are aberrantly expressed in the cutaneous T-cell lymphoma (CTCL), Sézary syndrome (SzS) [153]. QRT-PCR analysis in SzS has shown to miR-223 as a diagnostic marker in clinical study. In addition these studies also showed the role of miR-342 in the pathogenesis of SzS through its targeting of RANKL which was associated with the protection of SzS cells from apoptosis [154].

Downregulation of miR-150 is shown to involve in NK/T-cell lymphomagenesis [155]. This miR-150 regulates NK cells via Myb inhibition and other T-cell subsets through NOTCH3 targeting [23, 155]. miR-150 enhances apoptosis and decreases cell proliferation through DKC1 and AKT2 and causes downregulation of BIM, p53, and phosphorylated AKT expression levels when this miR was introduced into

NK/T cell lymphoma. Overexpression studies of miR-122 in CTCL induced AKT phosphorylation coupled with a decreased sensitivity to chemotherapy-induced apoptosis as well as inhibition of p53. Another study has shown that overexpression of miR-21 and miR-155 lead to enhancement of PI3K–Akt pathway activity [122] whereas CTCL enhances AKT phosphorylation which couple with a decreased sensitivity to chemotherapy-induced apoptosis as well as inhibition of p53 [156]. Further studies have shown the increased apoptosis after miR-21 silencing in SzS cells, in this way miR-21 might represent a therapeutic target for the treatment of SzS [157].

Polymorphisms in miRNA-Binding Sites in Hematological Malignancies

Computational analysis have predicted of hundreds of single nucleotide polymorphisms (SNPs) located within miRNA-binding sites and experimentally validated. Recent studies have evidenced that there is strong negative selection on SNPs in miRNA binding sites (miRSNPs) when compared to the entire 3′UTR sequence [158–160] and have shown functional significance of those sites. Several reports have evidenced the involvement of miRSNPs with cancer and other diseases [161, 162]. First report of association of miRSNPs in hematological malignancies was reported in 2012 [163]. Bioinformatics analysis has shown that 111 putative miRSNPs are associated with 137 leukemia-associated genes.

Bioinformatics analysis of SNPs in the 3′UTRs of 137 leukemia-associated genes revealed 111 putative miRSNPs. 10 miRSNPs have been observed in patients of childhood acute lymphoblastic leukemia (ALL), adult CML and AML, and healthy controls. This study concluded that polymorphic genotypes of ETV6_rs1573613 and TLX1_rs2742038 have been observed and this association would enhance the risk of disease. Carriers of the variant allele of PML_rs9479 were at lower risk of ALL and AML [164]. The involvement of SNPs in polymorphisms in miRNA binding sites of hematological disorders should be validated in in vivo and the mechanisms by which the miRSNPs regulates hematological disorders.

Key Modulators in Therapeutics

Recent studies have evidenced involvement of miRNAs as key regulators of signaling cascades that regulate cell fate and the function of normal and pathological lymphoid progenitor/precursor cells (Table 10.5). Several studies about miRNA expression profiles of lymphomas could provide the key roles of miRNAs in development of lymphoma pathogenesis. Latest studies have shown miRNA as promising agent in the role of diagnosis, prognosis, and treatment but also become the prospects for novel therapeutic strategies.

Table 10.5 miRNAs, lymphoma-associated or treatment response correlated

Disease	miRNA	UP or DR	Response	References
DLBCL vs. FL	miR-150, miR-17-5p, miR-145, miR-328 vs. miR-9/9*, miR-301, miR-338 and miR-213		Differentially expressed miRNAs in DLBCL and FL identify signatures respectively in DLBCL and FL	Roehle et al. [209]
DLBCL/FL/RNL	mir-330, mir-17-5p, mir-106a and mir-210		Correctly identifies 98 % of DLBCL, FL and RLN	Roehle et al. [209]
FL-t vs. FL-nt	miR-223, miR-217, miR-222, miR-221, let-7i, miR-7b	UP	Differentially expressed ($p<0.05$) between FL-t and FL-nt, accurately (89 %) predict transformation of FL cases	Lawrie et al. [145]
ABC-DLBCL vs. GC-DLBCL	miR-155, miR-21 and miR-221	UP	More highly expressed in ABC- than GC-subtypes, distinguish between ABC- and GC-DLBCL cases ($p<0.05$). miR-21 expression is an independent prognostic indicator in de novo DLBCL ($p<0.05$)	Lawrie et al. [117]
FL	miR-9, miR-301, miR-213, miR-9*, miR-330, miR-106a, miR-338, miR-155, miR-210	UP	Significantly higher expression in FL than RLN	Roehle et al. [209]

(continued)

Table 10.5 (continued)

Disease	miRNA	UP or DR	Response	References
DLBCL-dn vs. DLBCL-t	miR-27a, miR-19b, miR-25, miR-18a, miR-636, miR-92, miR-621, miR-526c, miR-766, miR-299-5p, miR-380-3p, miR-129, miR-588	UP	More highly expressed ($p<0.05$) in DLBCL *de novo* than DLBCL-t cases, correctly predict transformation >85 %	Lawrie et al. [145]
DLBCL	miR-210, miR-155, miR-106a, miR-17-5p	UP	Significantly higher expression in DLBCL than RLN	Roehle et al. [209]
	miR-150, miR-145, miR-328, miR-139, miR-95, miR-99a, miR-10a, miR-149, miR-320, miR-151, let-7e (miR-17-3, miR-595, miR-663)	DR/lost	Significantly lower expression in DLBCL than RLN miR-17-3, miR-595, miR-663 most significantly lost in DLBCL	Di Lisio et al. [137]
HL	miR-17-92 cluster members, miR-16, miR-21, miR-24, and miR-155.	UP	The HL-specific miRNAs upregulated	Gibcus et al. [146]
	miR-150	DR	Only miR-150 is significantly downregulated in HL compared with NHL	
cHL vs. HL EBV+	miR-96, miR-128a, miR-128b	DR	Selectively downregulated in HL lymph nodes of EBV+ HL patients	Navarro, et al. [210]

(continued)

Table 10.5 (continued)

Disease	miRNA	UP or DR	Response	References
cHL prognosis	miR-135a		Expression of miR-135a in HL lymph nodes correlates with clinical outcome. Patients with low miR-135a expression had a higher probability of relapse and a shorter disease-free survival	Navarro et al. [138]
	miR-21, miR-30e, miR-30d and miR-92b		To identify two different risk groups for 5-year FFS	Sánchez-Espiridión et al. [211]
B-CLL poor prognosis	miR-29			
	miR-15a, miR-195, miR-221, miR-23b, miR-155, miR-24-1, miR-146, miR-16-1, miR-16-2	UP	Significant relationship between the expression of 9 miRNAs and the time from diagnosis to beginning of chemotherapy	Calin et al. [212]
CLL vs. B-NHL	miR-182, miR-199a*(5p), let-7 family, miR-424, miR-10a, miR-7, mir-126, miR-218, MiR-197, miR-595, miR-483	DR/UP	miR-197 the most highly expressed miRNA, miR-595 and miR-483 also upregulated	Di Lisio et al. [137]
BL vs. B-NHL	miR-17-3p, miR-18a, miR-19a, miR-19b, miR-92	UP	Up/Downregulated in BL vs. NHL	Di Lisio et al. [137]
	let-7 family, miR-29 (a, b, c), miR-155, miR-146a	DR		

(continued)

Table 10.5 (continued)

Disease	miRNA	UP or DR	Response	References
DLBCL prognosis	miR-637, miR-608 and miR-302		Poor prognosis	Lawrie et al. [145]
	miR-330, miR-30e, miR-425, miR-27a, miR-24, miR-23a, miR-199b, miR-199a* and miR-100		Better outcome	Lawrie et al. [145]
	miR-21, miR-127, miR-34a, miR-195, let-7 g, miR-19a, miR-27a		Correlate with EFS and OS	Roehle et al. [209]
DLBCL drug sensitivity	miR-181a, miR-222 and miR-18a		Independent prognostic indicators of survival in R-CHOP treated DLBCL	Alencar et al. [213]

The role of miRNAs in development and pathogenesis is shown to be evidenced increasingly in vitro and in vivo that ectopic expression or silencing of specific miRNAs lead to alter signaling of malignant cell survival. Recent studies have demonstrated that onco-miR silencing by miRNA mimics suppresses the tumour growth. First cancer-targeted miRNA drug MRX34, a liposomal formulation loaded with tumor suppressor miR-34a mimic, entered a Phase I clinical trial in patients with primary liver cancer or those with liver metastasis from other cancers in April 2013 [165].

miRNAs are generally classified as oncomirs or tumor suppressor genes based on their function, i.e., oncomirs or tumor suppressor genes provide approach for therapeutics development. Silencing or suppression of tumor-inducing miRNAs and replacement of downregulated miRNA by re-introducing miRNAs with tumor suppressor function can be used as therapeutic agents.

Anti-miRNAs have been synthesized to silence or block a target miRNA that acts as an oncogene. Cholesterol-conjugated “antagomirs,” polylysine-conjugated peptide nucleic acids (PNAs), and locked nucleic acid oligonucleotides are chemically modified anti-miRNAs that have demonstrated effectiveness in in vivo treatment [166–168]. Anti-miRNAs have been used against miR-155, miR-17-92 cluster, miR-9, and let7 which are overexpressed in many lymphomas.

miR-17-92 cluster overexpression demonstrates to significantly enhance resistance to radiotherapy in human MCL, and become potential molecular target for

improving the effectiveness of conventional treatments. Another evidence that antagomir 17-5p abolish the growth of therapy-resistant neuroblastoma cells which express the miR-17-92 cluster at an elevated level [169, 170].

Even though antisense oligonucleotides have been shown to silence many oncomiRs such as miR-155, miR-17-92, and miR-21, yet the use of anti-miRNAs as drug in clinical practice is still a long way and a number of obstacles have to be overcome before implementation of these therapeutic strategies in human diseases. LNA-modified anti-miRNAs could enhance the specificity and reduce effective doses of anti-miRNA molecules [46]. The incorporation of endonucleolytic DNAzyme motif in LNA-based anti-miRNAs and siRNAs can enhance the catalytic efficiency of these anti-miRNAs and siRNAs [171]. Several parameters such as stability, safety, and successful delivery of therapeutic anti-miRNAs to the appropriate tissue and into the cells need to be evaluated [172].

Babar et al. [173] have developed technology based on nanoparticles coated with a cell-penetrating peptide encapsulating antisense peptide nucleic acids to systemically deliver anti-miR-155 (antagomir) in a B-lymphoma mouse model. In this study, overexpression of miR-155 was induced in the lymphoid tissues of a deficient miR-155 mouse model, causing disseminated lymphoma characterized by clonal transplantable pre-B-cells. In this inducible system, nanoparticle-based therapy targeting miR-155 resulted in the ability to reduce lymphoma growth. In particular, nanoparticles injected in situ or systemically produced a rapid regression of lymphadenopathy, partially due to apoptosis of the malignant B-lymphocytes.

Another approach is exogenous administration of short double-stranded miRNA mimics or endogenous miRNAs into diseased tissues to regulate miRNAs in order to regain physiological levels of downregulated miRNAs in systematic approach or tissue specific approach. In several malignancies, including lymphomas and leukemia, frequent aberration of miR-34 gene expression is demonstrated which has tumor suppressor function. So developments of miR-34a mimic formulations are become promising drug in treatment of solid tumors.

Craig et al. have shown the relevance of miR-34 deregulation in lymphomagenesis by inoculating ABC-type cell line (U2932) with low expression of miR-34a into humanized immunodeficient NOD/SCID/IL2RG−/− mice [131]. The tumor growth was decreased by 76 % in this mouse with U2932 lymphoma and also showed apoptosis of malignant B-lymphocytes when intravenous injections of a neutral lipid emulsion (NLE)/miR-34a formulation in this mouse [131]. In addition, miR-34a along with cytotoxic chemotherapeutic agents enables chemosensitivity mainly through modulation of p53 in many cancers and hematological malignancies [173, 174]. Recent studies evidenced the involvement of restoration of tumor suppressor effects of miRNA using epigenetic drugs 5-aza-2′-deoxycytidine and zeburaline that change the DNA methylation status. Epigenetic modifications such as hypermethylation could downregulate the miR-34a in hematological malignancies NK/T-cell lymphoma. The down-regulated expression of miR-34 showed re-expression when pharmacological reversal of epigenetic silencing by epigenetic inhibitors was employed in lymphoma [175]. Epigenetic downregulation of miR-124 in acute lymphoblastic leukemia (ALL) showed poor prognosis in ALL patients

due to a high relapse and mortality rate [176]. Recent study showed that 5-aza-2-deoxycytidine and 4-phenylbutyric acid (PBA) drugs activate the expression of miR-127 and decrease the proto-oncogene BCL6 in Ramos lymphoma cell line and other cancer cell lines [177]. The downregulation of proto-oncogene BCL6 suppresses PRDM1 which is implicated in DLBCL [178]. The histone modification restores the function of miR-127 and shown clinical importance to treat cancer.

Conclusion

Studies have evidenced the role of miRNA in regulation of all stages of hematopoiesis and differentiation. The mode of action of several miRNAs is not yet understood due to its pleiotrophic nature of function, even though several miRNAs have been known. Increasing evidences showed the role of miRNA based interventions in treatment of hematological disorders could provide a powerful therapeutic approach. Several substantial obstacles such as potential off-target effects, efficacy, and safety are needed to overcome before entering into clinical trials. Hematological malignancies from different origin have distinct miRNA profile which help to distinguish different hematological entities and could be useful for diagnosis, and evaluation for therapeutic potential. Recent decade has shown tremendous progress in research to understand the role of miRNAs in developmental hematopoiesis and molecular mechanism of hematopoietic malignancies. This chapter updates the miRNAs become crucial regulators in diversity of regulatory pathways and also involve in the regulation of positive and negative regulatory feedback loops. Moreover this chapter also illustrates that involvement of aberrant expression of miRNA in several hematological disorders in association with transcriptional repressors or activators.

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References

1. Lee YS, Nakahara K, Pham JW, Kim K, He Z, Sontheimer EJ, Carthew RW. Distinct roles for Drosophila Dicer-1 and Dicer-2 in the siRNA/miRNA silencing pathways. Cell. 2004;117(1):69–81.
2. Lee Y, Ahn C, Han J, Choi H, Kim J, Yim J, Lee J, Provost P, Rådmark O, Kim S, Kim VN. The nuclear RNase III Drosha initiates microRNA processing. Nature. 2003;425(6956):415–9.
3. Landthaler M, Yalcin A, Tuschl T. The human DiGeorge syndrome critical region gene 8 and its D. melanogaster homolog are required for miRNA biogenesis. Curr Biol. 2004;14(23):2162–7.
4. Yi R, Qin Y, Macara IG, Cullen BR. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev. 2003;17(24):3011–6.

5. Moore MS, Blobel G. The GTP-binding protein Ran/TC4 is required for protein import into the nucleus. Nature. 1993;365(6447):661–3.
6. Lund E, Güttinger S, Calado A, Dahlberg JE, Kutay U. Nuclear export of microRNA precursors. Science. 2004;303(5654):95–8.
7. Bartel DP. MicroRNAs: target recognition and regulatory functions. Cell. 2009;136(2):215–33.
8. Baron MH, Isern J, Fraser ST. The embryonic origins of erythropoiesis in mammals. Blood. 2012;119(21):4828–37.
9. Orkin SH, Zon LI. Hematopoiesis: an evolving paradigm for stem cell biology. Cell. 2008;132(4):631–44.
10. Massberg S, Schaerli P, Knezevic-Maramica I, et al. Immunosurveillance by hematopoietic progenitor cells trafficking through blood, lymph, and peripheral tissues. Cell. 2007; 131(5):994–1008.
11. Wright DE, Wagers AJ, Pathak Gulati A, Johnson FL, Weissman IL. Physiological migration of hematopoietic stem and progenitor cells. Science. 2001;294(5548):1933–6.
12. Kim MH, Granick JL, Kwok C, et al. Neutrophil survival and c-kit+-progenitor proliferation in Staphylococcus aureus-infected skin wounds promote resolution. Blood. 2011; 117(12):3343–52.
13. Si Y, Tsou CL, Croft K, Charo IF. CCR2 mediates hematopoietic stem and progenitor cell trafficking to sites of inflammation in mice. J Clin Invest. 2010;120(4):1192–203.
14. Wang LD, Wagers AJ. Dynamic niches in the origination and differentiation of haematopoietic stem cells. Nat Rev Mol Cell Biol. 2011;12(10):643–55.
15. Liao R, Sun J, Zhang L, Lou G, Chen M, Zhou D, Chen Z, Zhang S. MicroRNAs play a role in the development of human hematopoietic stem cells. J Cell Biochem. 2008;104(3):805.
16. Kandi R, Undi R, Gutti RK. MiR-125b regulates cell proliferation and survival in neonatal megakaryocytes. Ann Hematol. 2014;93(6):1065–6.
17. Undi RB, Kandi R, Gutti RK. MicroRNAs as haematopoiesis regulators. Adv Hematol. 2013;2013:695754.
18. Chen CZ, Li L, Lodish HF, Bartel DP. MicroRNAs modulate hematopoietic lineage differentiation. Science. 2004;2004(303):83–6.
19. Guo S, Lu J, Schlanger R, Zhang H, Wang JY, Fox MC, et al. MicroRNA miR-125a controls hematopoietic stem cell number. Proc Natl Acad Sci U S A. 2010;107(32):14229–34.
20. Georgantas III RW, Hildreth R, Morisot S, Alder J, Liu CG, Heimfeld S, et al. CD34+ hematopoietic stem-progenitor cell microRNA expression and function: a circuit diagram of differentiation control. Proc Natl Acad Sci U S A. 2007;104(8):2750–5.
21. Ooi AGL, et al. MicroRNA-125b expands hematopoietic stem cells and enriches for the lymphoid-balanced and lymphoid-biased subsets. Proc Natl Acad Sci. 2010; 107(50):21505–10.
22. Escobar TM, et al. miR-155 activates cytokine gene expression in Th17 cells by regulating the DNA-binding protein Jarid2 to relieve polycomb-mediated repression. Immunity. 2014;40(6):865–79.
23. Ghisi M, Corradin A, Basso K, Frasson C, Serafin V, Mukherjee S, et al. Modulation of microRNA expression in human T-cell development: targeting of NOTCH3 by miR-150. Blood. 2011;117(26):7053–62.
24. Felli MP, et al. Expression pattern of Notch1, 2 and 3 and Jagged1 and 2 in lymphoid and stromal thymus components: distinct ligand–receptor interactions in intrathymic T cell development. Int Immunol. 1999;11(7):1017–25.
25. Nakahama T, et al. Aryl hydrocarbon receptor-mediated induction of the microRNA-132/212 cluster promotes interleukin-17–producing T-helper cell differentiation. Proc Natl Acad Sci. 2013;110(29):11964–9.
26. Smigielska-Czepiel K, et al. Dual role of miR-21 in CD4+ T-cells: activation-induced miR-21 supports survival of memory T-cells and regulates CCR7 expression in naive T-cells. PLoS One. 2013;8(10), e76217.
27. Muljo SA, et al. Aberrant T cell differentiation in the absence of Dicer. J Exp Med. 2005;202(2):261–9.

28. Cobb BS, et al. A role for Dicer in immune regulation. J Exp Med. 2006;203(11):2519–27.
29. Boldin MP, et al. miR-146a is a significant brake on autoimmunity, myeloproliferation, and cancer in mice. J Exp Med. 2011;208(6):1189–201.
30. Wu H, Neilson JR, Kumar P, Manocha M, Shankar P, Sharp PA, Manjunath N. miRNA profiling of naive, effector and memory CD8 T cells. PLoS One. 2007;2(10):e1020.
31. Lu LF, Thai TH, Calado DP, Chaudhry A, Kubo M, Tanaka K, Loeb GB, Lee H, Yoshimura A, Rajewsky K, Rudensky AY. Foxp3-dependent microRNA155 confers competitive fitness to regulatory T cells by targeting SOCS1 protein. Immunity. 2009;30(1):80–91.
32. de Kouchkovsky D, et al. microRNA-17–92 regulates IL-10 production by regulatory T cells and control of experimental autoimmune encephalomyelitis. J Immunol. 2013;191(4):1594–605.
33. Jiang S, Li C, Olive V, Lykken E, Feng F, Sevilla J, Wan Y, He L, Li QJ. Molecular dissection of the miR-17-92 cluster's critical dual roles in promoting Th1 responses and preventing inducible Treg differentiation. Blood. 2011;118:5487–97.
34. Wu T, Wieland A, Araki K, Davis CW, Ye L, Hale JS, Ahmed R. Temporal expression of microRNA cluster miR-17-92 regulates effector and memory CD8+T-cell differentiation. Proc Natl Acad Sci U S A. 2012;109:9965–70.
35. Rouas R, et al. Human natural Treg microRNA signature: role of microRNA-31 and microRNA-21 in FOXP3 expression. Eur J Immunol. 2009;39(6):1608–18.
36. Faraoni I, et al. miR-155 gene: a typical multifunctional microRNA. Biochim Biophys Acta. 2009;1792(6):497–505.
37. Basso K, et al. Identification of the human mature B cell miRNome. Immunity. 2009;30(5):744–52.
38. Koralov SB, et al. Dicer ablation affects antibody diversity and cell survival in the B lymphocyte lineage. Cell. 2008;132(5):860–74.
39. Rao DS, et al. MicroRNA-34a perturbs B lymphocyte development by repressing the forkhead box transcription factor Foxp1. Immunity. 2010;33(1):48–59.
40. Bezman NA, et al. Distinct requirements of microRNAs in NK cell activation, survival, and function. J Immunol. 2010;185(7):3835–46.
41. Bezman NA, et al. miR-150 regulates the development of NK and iNKT cells. J Exp Med. 2011;208(13):2717–31.
42. Sullivan RP, et al. MicroRNA-deficient NK cells exhibit decreased survival but enhanced function. J Immunol. 2012;188(7):3019–30.
43. Yun S, Lee SU, Kim JM, Lee HJ, Song HY, Kim YK, Jung H, Park YJ, Yoon SR, Oh SR, Kim TD, Choi I. Integrated mRNA-microRNA profiling of human NK cell differentiation identifies MiR-583 as a negative regulator of IL2Rγ expression. PLoS One. 2014;9(10), e108913.
44. Cichocki F, et al. Cutting edge: microRNA-181 promotes human NK cell development by regulating Notch signaling. J Immunol. 2011;187(12):6171–5.
45. Akashi K, Traver D, Miyamoto T, Weissman IL. A clonogenic common myeloid progenitor that gives rise to all myeloid lineages. Nature. 2000;404(6774):193–7.
46. Zhu J, Emerson SG. Hematopoietic cytokines, transcription factors and lineage commitment. Oncogene. 2002;21(21):3295–313.
47. Friedman AD. Transcriptional regulation of myelopoiesis. Int J Hematol. 2002;75:466–72.
48. Lawrence HJ, Helgason CD, Sauvageau G, Fong S, Izon DJ, Humphries RK, Largman C. Mice bearing a targeted interruption of the homeobox gene HOXA9 have defects in myeloid, erythroid, and lymphoid hematopoiesis. Blood. 1997;89:1922–30.
49. Gwin KA, Shapiro MB, Dolence JJ, Huang ZL, Medina KL. Hoxa9 and Flt3 signaling synergistically regulate an early checkpoint in lymphopoiesis. J Immunol. 2013;191(2):745–54.
50. Shen WF, Hu YL, Uttarwar L, Passegue E, Largman C. MicroRNA-126 regulates HOXA9 by binding to the homeobox. Mol Cell Biol. 2008;28(14):4609–19.
51. Senyuk V, Zhang Y, Liu Y, Ming M, Premanand K, Zhou L, Chen P, Chen J, Rowley JD, Nucifora G, Qian Z. Critical role of miR-9 in myelopoiesis and EVI1-induced leukemogenesis. Proc Natl Acad Sci U S A. 2013;110(14):5594–9.

52. Han YC, Park CY, Bhagat G, Zhang J, Wang Y, Fan JB, Liu M, Zou Y, Weissman IL, Gu H. MicroRNA-29a induces aberrant self-renewal capacity in hematopoietic progenitors, biased myeloid development, and acute myeloid leukemia. J Exp Med. 2010;207(3):475–89.
53. Peeters K, Stassen JM, Collen D, Van Geet C, Freson K. Emerging treatments for thrombocytopenia: increasing platelet production. Drug Discov Today. 2008;13(17–18):798–806.
54. Chang AN, et al. GATA-factor dependence of the multitype zinc-finger protein FOG-1 for its essential role in megakaryopoiesis. Proc Natl Acad Sci U S A. 2002;99(14):9237–42.
55. Szalai G, et al. Molecular mechanisms of megakaryopoiesis. Cell Mol Life Sci. 2006;63(21):2460–76.
56. Lu J, Guo S, Ebert BL, et al. MicroRNA-mediated control of cell fate in megakaryocyte-erythrocyte progenitors. Dev Cell. 2008;14(6):843–53.
57. Opalinska JB, Bersenev A, Zhang Z, et al. MicroRNA expression in maturing murine megakaryocytes. Blood. 2010;116(23):e128–38.
58. Garzon R, Pichiorri F, Palumbo T, et al. MicroRNA fingerprints during human megakaryocytopoiesis. Proc Natl Acad Sci U S A. 2006;103(13):5078–83.
59. Barroga CF, Pham H, Kaushansky K. Thrombopoietin regulates c-myb expression by modulating microRNA (miR)150 expression. Exp Hematol. 2008;36(12):1585–92.
60. Emambokus N, Vegiopoulos A, Harman B, Jenkinson E, Anderson G, Frampton J. Progression through key stages of haemopoiesis is dependent on distinct threshold levels of c-Myb. EMBO J. 2003;22(17):4478–88.
61. Girardot M, Pecquet C, Boukour S, et al. miR-28 is a thrombopoietin receptor targeting MicroRNA detected in a fraction of myeloproliferative neoplasm patient platelets. Blood. 2010;116(3):437–45.
62. Romania P, Lulli V, Pelosi E, et al. MicroRNA 155 modulates megakaryopoiesis at progenitor and precursor level by targeting Ets-1 and Meis1 transcription factors. Br J Haematol. 2008;143(4):570–80.
63. Labbaye C, Spinello I, Quaranta MT, et al. A three-step pathway comprising PLZF/miR-146a/CXCR4 controls megakaryopoiesis. Nat Cell Biol. 2008;10(7):788–801.
64. Starczynowski DT, Kuchenbauer F, Wegrzyn J, et al. MicroRNA-146a disrupts hematopoietic differentiation and survival. Exp Hematol. 2011;39(2):167–78.
65. Zhai P-F, Wang F, Rui S, Lin H-S, Jiang C-L, Yang G-H, Yu J, Zhang J-W. The regulatory roles of MicroRNA-146b-5p and its target platelet-derived growth factor receptor α (PDGFRA) in erythropoiesis and megakaryocytopoiesis. J Biol Chem. 2014;289:22600–13. doi:10.1074/jbc.M114.547380.
66. Navarro F, Gutman D, Meire E, et al. miR-34a contributes to megakaryocytic differentiation of K562 cells independently of p53. Blood. 2009;114(10):2181–92.
67. Ichimura A, Ruike Y, Terasawa K, Shimizu K, Tsujimoto G. MicroRNA-34a inhibits cell proliferation by repressing mitogen-activated protein kinase kinase 1 during megakaryocytic differentiation of K562 cells. Mol Pharmacol. 2010;77(6):1016–24.
68. Sieweke MH, Tekotte H, Frampton J, Graf T. MafB represses erythroid genes and differentiation through direct interaction with c-Ets-1. Leukemia. 1997;3:486–8.
69. Ben-Ami O, Pencovich N, Lotem J, Levanon D, Groner Y. A regulatory interplay between miR-27a and Runx1 during megakaryopoiesis. Proc Natl Acad Sci U S A. 2009;106(1):238–43.
70. Zarif MN, Soleimani M, Abolghasemi H, Amirizade N, Arefian E, Rahimian A. Megakaryocytic differentiation of CD133+ hematopoietic stem cells by down-regulation of microRNA-10a. Hematology. 2013;18(2):93–100.
71. Li X, Zhang J, Gao L, et al. miR-181 mediates cell differentiation by interrupting the Lin28 and let-7 feedback circuit. Cell Death Differ. 2012;19(3):378–86.
72. Felli N, Pedini F, Romania P, Biffoni M, Morsilli O, Castelli G, Santoro S, Chicarella S, Sorrentino A, Peschle C, Marziali G. MicroRNA 223-dependent expression of LMO2 regulates normal erythropoiesis. Haematologica. 2009;94(4):479–86.
73. Yuan JY, Wang F, Yu J, Yang GH, Liu XL, Zhang JW. MicroRNA-223 reversibly regulates erythroid and megakaryocytic differentiation of K562 cells. J Cell Mol Med. 2009;13(11–12):4551–9.

74. Nocka K, Majumder S, Chabot B, Ray P, Cervone M, Bernstein A, Besmer P. Expression of c-kit gene products in known cellular targets of W mutations in normal and W mutant mice: evidence for an impaired c-kit kinase in mutant mice. Genes Dev. 1989;3:816–26.
75. Munugalavadla V, Dore LC, Tan BL, Hong L, Vishnu M, Weiss MJ, Kapur R. Repression of c-kit and its downstream substrates by GATA-1 inhibits cell proliferation during erythroid maturation. Mol Cell Biol. 2005;25(15):6747–59.
76. Felli N, Fontanan L, Pelosi E, et al. 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.
77. Gabbianelli M, Testa U, Morsilli O, et al. Mechanism of human Hb switching: a possible role of the kit receptor/miR 221–222 complex. Haematologica. 2010;95:1253–60.
78. Bruchova H, Yoon D, Agarwal AM, et al. Regulated expression of microRNAs in normal and polycythemia vera erythropoiesis. Exp Hematol. 2007;35:1657–67.
79. Vegiopoulos A, García P, Emambokus N, Frampton J. Coordination of erythropoiesis by the transcription factor c-Myb. Blood. 2006;107(12):4703–10.
80. Zhan M, Miller CP, Papayannopoulou T, et al. MicroRNA expression dynamics during murine and human erythroid differentiation. Exp Hematol. 2007;35:1015–25.
81. Pase L, Layton JE, Kloosterman WP, et al. miR-451 regulates zebrafish erythroid maturation in vivo via its target gata2. Blood. 2009;113:1794–804.
82. Dore LC, Amigo JD, Dos Santos CO, et al. A GATA-1-regulated microRNA locus essential for erythropoiesis. Proc Natl Acad Sci U S A. 2008;105:3333–8.
83. Patrick DM, Zhang CC, Tao Y, et al. Defective erythroid differentiation in miR-451 mutant mice mediated by 14-3-3zeta. Genes Dev. 2010;24(15):1614–9.
84. Yu D, dos Santos CO, Zhao G, Jiang J, Amigo JD, Khandros E, Dore LC, Yao Y, D'Souza J, Zhang Z, Ghaffari S, Choi J, Friend S, Tong W, Orange JS, Paw BH, Weiss MJ. miR-451 protects against erythroid oxidant stress by repressing 14-3-3zeta. Genes Dev. 2010;24:1620–33.
85. Kim M, Tan YS, Cheng WC, Kingsbury TJ, Heimfeld S, Civin CI. MIR144 and MIR451 regulate human erythropoiesis via RAB14. Br J Haematol. 2015;168(4):583–97. doi:10.1111/bjh.13164.
86. Wang Q, Huang Z, Xue H, et al. MicroRNA miR-24 inhibits erythropoiesis by targeting activin type I receptor ALK4. Blood. 2008;111:588–95.
87. Zhao H, Kalota A, Jin S, Gewirtz AM. The c-myb proto-oncogene and micro-RNA-15a comprise an active autoregulatory feedback loop in human hematopoietic cells. Blood. 2009;113:505–16.
88. Mittal SP, Mathai J, Kulkarni AP, Pal JK, Chattopadhyay S. MiR-320a regulates erythroid differentiation through MAR binding protein SMAR1. Int J Biochem Cell Biol. 2013;45(11):2519–29.
89. Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C, Bozzoni I. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. Cell. 2005;123(5):819–31.
90. Zardo G, Ciolfi A, Vian L, Starnes LM, Billi M, Racanicchi S, Maresca C, Fazi F, Travaglini L, Noguera N, Mancini M, Nanni M, Cimino G, Lo-Coco F, Grignani F, Nervi C. Polycombs and microRNA-223 regulate human granulopoiesis by transcriptional control of target gene expression. Blood. 2012;119(17):4034–46.
91. Johnnidis JB, Harris MH, Wheeler RT, Stehling-Sun S, Lam MH, Kirak O, Brummelkamp TR, Fleming MD, Camargo FD. Regulation of progenitor cell proliferation and granulocyte function by microRNA-223. Nature. 2008;451(7182):1125–9.
92. Person RE, Li FQ, Duan Z, Benson KF, Wechsler J, Papadaki HA, Eliopoulos G, Kaufman C, Bertolone SJ, Nakamoto B, Papayannopoulou T, Grimes HL, Horwitz M. Mutations in proto-oncogene GFI1 cause human neutropenia and target ELA2. Nat Genet. 2003;34(3):308–12.
93. Velu CS, Baktula AM, Grimes HL. Gfi1 regulates miR-21 and miR-196b to control myelopoiesis. Blood. 2009;113(19):4720–8.

94. Fontana L, Pelosi E, Greco P, et al. MicroRNAs 17–5p-20a-106a control monocytopoiesis through AML1 targeting and M-CSF receptor upregulation. Nat Cell Biol. 2007;9:775–87.
95. Rosa A, Ballarino M, Sorrentino A, et al. The interplay between the master transcription factor PU. 1 and miR-424 regulates human monocyte/macrophage differentiation. Proc Natl Acad Sci U S A. 2007;104:19849–54.
96. Forrest ARR, Kanamori-Katayama M, Tomaru Y, et al. Induction of micro-RNAs, mir-155, mir-222, mir-424 and mir-503, promotes monocytic differentiation through combinatorial regulation. Leukemia. 2010;24:460–6.
97. Shen X, Tang J, Hu J, Guo L, Xing Y, Xi T. MiR-424 regulates monocytic differentiation of human leukemia U937 cells by directly targeting CDX2. Biotechnol Lett. 2013;35(11):1799–806.
98. Bousquet M, Quelen C, Rosati R, et al. Myeloid cell differentiation arrest by miR-125b-1 in myelodysplastic syndrome and acute myeloid leukemia with the t(2;11) (p21;q23) translocation. J Exp Med. 2008;205:2499–506.
99. Surdziel E, Cabanski M, Dallmann I, Lyszkiewicz M, Krueger A, Ganser A, Scherr M, Eder M. Enforced expression of miR-125b affects myelopoiesis by targeting multiple signaling pathways. Blood. 2011;117(16):4338–48.
100. Lin KY, Zhang XJ, Feng DD, Zhang H, Zeng CW, Han BW, Zhou AD, Qu LH, Xu L, Chen YQ. miR-125b, a target of CDX2, regulates cell differentiation through repression of the core binding factor in hematopoietic malignancies. J Biol Chem. 2011;286:38253–63.
101. Li Z, Lu J, Sun M, et al. Distinct MicroRNA expression profiles in acute myeloid leukemia with common translocations. Proc Natl Acad Sci U S A. 2008;105(40):15535–40.
102. Kandi R, Gutti U, Saladi RGV, Gutti RK. MiR-125b and miR-99a encoded on chromosome 21 co-regulate vincristine resistance in childhood acute megakaryoblastic leukemia. Hematol Oncol Stem Cell Ther. 2014;8(2):95–7. doi:10.1016/j.hemonc.2014.11.008. pii: S1658-3876(14)00110-1.
103. Eyholzer M, Schmid S, Schardt JA, Haefliger S, Mueller BU, Pabst T. Complexity of miR-223 regulation by CEBPA in human AML. Leuk Res. 2010;34(5):672–6.
104. Cammarata G, Augugliaro L, Salemi D, et al. Differential expression of specific MicroRNA and their targets in acute myeloid leukemia. Am J Hematol. 2010;85(5):331–9.
105. He M, Wang QY, Yin QQ, et al. HIF-1alpha downregulates miR-17/20a directly targeting p21 and STAT3: a role in myeloid leukemic cell differentiation. Cell Death Differ. 2013;20(3):408–18.
106. Faderl S, Talpaz M, Estrov Z, Kantarjian HM. Chronic myelogenous leukemia: biology and therapy. Ann Intern Med. 1999;131(3):207–19.
107. Li Y, Wang H, Tao K, et al. miR-29b suppresses CML cell proliferation and induces apoptosis via regulation of BCR/ABL1 protein. Exp Cell Res. 2013;319(8):1094–101.
108. Xu C, Fu H, Gao L, et al. BCR-ABL/GATA1/miR-138 mini circuitry contributes to the leukemogenesis of chronic myeloid leukemia. Oncogene. 2014;33(1):44–54.
109. Corthals SL, Sun SM, Kuiper R, et al. MicroRNA signatures characterize multiple myeloma patients. Leukemia. 2011;25(11):1784–9.
110. Zhou Y, Chen L, Barlogie B, et al. High-risk myeloma is associated with global elevation of miRNAs and overexpression of EIF2C2/AGO2. Proc Natl Acad Sci U S A. 2010;107(17):7904–9.
111. Roccaro AM, Sacco A, Thompson B, Leleu X, Azab AK, Azab F, Runnels J, Jia X, Ngo HT, Melhem MR, Lin CP, Ribatti D, Rollins BJ, Witzig TE, Anderson KC, Ghobrial IM. MicroRNAs 15a and 16 regulate tumor proliferation in multiple myeloma. Blood. 2009;113(26):6669–80. doi:10.1182/blood-2009-01-198408.
112. Corthals SL, Jongen-Lavrencic M, de Knegt Y, et al. Micro-RNA-15a and Micro-RNA-16 expression and chromosome 13 deletions in multiple myeloma. Leuk Res. 2010;34(5):677–81.
113. Zhang Y-K, Wang H, Leng Y, et al. Overexpression of MicroRNA-29b induces apoptosis of multiple myeloma cells through down regulating Mcl-1. Biochem Biophys Res Commun. 2011;414(1):233–9.

114. Unno K, Zhou Y, Zimmerman T, Platanias LC, Wickrema A. Identification of a novel MicroRNA cluster miR-193b-365 in multiple myeloma. Leuk Lymphoma. 2009;50(11):1865–71.
115. Jones CI, Zabolotskaya MV, King AJ, et al. Identification of circulating MicroRNAs as diagnostic biomarkers for use in multiple myeloma. Br J Cancer. 2012;107(12):1987–96.
116. Kluiver J, Poppema S, de Jong D, Blokzijl T, Harms G, Jacobs S, et al. BIC and miR-155 are highly expressed in Hodgkin, primary mediastinal and diffuse large B cell lymphomas. J Pathol. 2005;207:243–9.
117. Lawrie CH, Soneji S, Marafioti T, Cooper CD, Palazzo S, Paterson JC, et al. MicroRNA expression distinguishes between germinal center B cell-like and activated B cell-like subtypes of diffuse large B cell lymphoma. Int J Cancer. 2007;121:1156–61.
118. Eis PS, Tam W, Sun L, Chadburn A, Li Z, Gomez MF, et al. Accumulation of miR-155 and BIC RNA in human B cell lymphomas. Proc Natl Acad Sci U S A. 2005;102:3627–32.
119. Costinean S, Zanesi N, Pekarsky Y, Tili E, Volinia S, Heerema N, et al. Pre-B cell proliferation and lymphoblastic leukemia/high-grade lymphoma in E(mu)-miR155 transgenic mice. Proc Natl Acad Sci U S A. 2006;103:7024–9.
120. 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.
121. Pedersen IM, Otero D, Kao E, Miletic AV, Hother C, Ralfkiaer E, et al. Onco-miR-155 targets SHIP1 to promote TNFalpha-dependent growth of B cell lymphomas. EMBO Mol Med. 2009;1:288–95.
122. Yamanaka Y, Tagawa H, Takahashi N, Watanabe A, Guo YM, Iwamoto K, et al. Aberrant overexpression of microRNAs activate AKT signaling via down-regulation of tumor suppressors in natural killer-cell lymphoma/leukemia. Blood. 2009;114:3265–75.
123. Costinean S, Sandhu SK, Pedersen IM, Tili E, Trotta R, Perrotti D, et al. Src homology 2 domain-containing inositol-5-phosphatase and CCAAT enhancer-binding protein beta are targeted by miR-155 in B cells of Emicro-MiR-155 transgenic mice. Blood. 2009;114:1374–82.
124. Garofalo M, Di Leva G, Romano G, Nuovo G, Suh SS, Ngankeu A, et al. miR-221&222 regulate TRAIL resistance and enhance tumorigenicity through PTEN and TIMP3 downregulation. Cancer Cell. 2009;16:498–509.
125. Rai D, Kim SW, McKeller MR, Dahia PL, Aguiar RC. Targeting of SMAD5 links microRNA-155 to the TGF-beta pathway and lymphomagenesis. Proc Natl Acad Sci U S A. 2010;107:3111–6.
126. Huang X, Shen Y, Liu M, Bi C, Jiang C, Iqbal J, et al. Quantitative proteomics reveals. that miR-155 regulates the PI3K–AKT pathway in diffuse large B-cell lymphoma. Am J Pathol. 2012;181:26–33.
127. Alizadeh AA, Eisen MB, Davis RE, Ma C, Lossos IS, Rosenwald A, et al. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature. 2000;403:503–11.
128. Thompson RC, Herscovitch M, Zhao I, Ford TJ, Gilmore TD. NF-kappaB down-regulates expression of the B-lymphoma marker CD10 through a miR-155/PU.1 pathway. J Biol Chem. 2011;286:1675–82.
129. He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, et al. A microRNA component of the p53 tumour suppressor network. Nature. 2007;447:1130–4.
130. Yamakuchi M, Lowenstein CJ. MiR-34, SIRT1 and p53: the feedback loop. Cell Cycle. 2009;8:712–5.
131. Craig VJ, Tzankov A, Flori M, Schmid CA, Bader AG, Muller A. Systemic microRNA-34a delivery induces apoptosis and abrogates growth of diffuse large B-cell lymphoma in vivo. Leukemia. 2012;26:2421–4.
132. 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;435:828–33.
133. O'Donnell KA, Wentzel EA, Zeller KI, Dang CV, Mendell JT. c-Myc-regulated microRNAs modulate E2F1 expression. Nature. 2005;435:839–43.

134. Woods K, Thomson JM, Hammond SM. Direct regulation of an oncogenic micro-RNA cluster by E2F transcription factors. J Biol Chem. 2007;282:2130–4.
135. Olive V, Bennett MJ, Walker JC, Ma C, Jiang I, Cordon-Cardo C, et al. miR-19 is a key oncogenic component of mir-17-92. Genes Dev. 2009;23:2839–49.
136. Zhao JJ, Lin J, Lwin T, Yang H, Guo J, Kong W, et al. microRNA expression profile and identification of miR-29 as a prognostic marker and pathogenetic factor by targeting CDK6 in mantle cell lymphoma. Blood. 2010;115:2630–9.
137. Di Lisio L, Sánchez-Beato M, Gómez-López G, Rodríguez ME, Montes-Moreno S, Mollejo M, et al. MicroRNA signatures in B-cell lymphomas. Blood Cancer J. 2012; 2(2): e57.
138. Navarro A, Bea S, Fernandez V, Prieto M, Salaverria I, Jares P, et al. MicroRNA expression, chromosomal alterations, and immunoglobulin variable heavy chain hypermutations in Mantle cell lymphomas. Cancer Res. 2009;69:7071–8.
139. Iqbal J, Shen Y, Liu Y, Fu K, Jaffe ES, Liu C, et al. Genome-wide miRNA profiling of mantle cell lymphoma reveals a distinct subgroup with poor prognosis. Blood. 2012;119:4939–48.
140. Chen RW, Bemis LT, Amato CM, Myint H, Tran H, Birks DK, et al. Truncation in CCND1 mRNA alters miR-16-1 regulation in mantle cell lymphoma. Blood. 2008;112:822–9.
141. Deshpande A, Pastore A, Deshpande AJ, Zimmermann Y, Hutter G, Weinkauf M, et al. 3′UTR mediated regulation of the cyclin D1 proto-oncogene. Cell Cycle. 2009;8:3584–92.
142. Chaudhuri AA, So AY, Mehta A, Minisandram A, Sinha N, Jonsson VD, et al. Oncomir miR-125b regulates hematopoiesis by targeting the gene Lin28A. Proc Natl Acad Sci U S A. 2012;109:4233–8.
143. Rao E, Jiang C, Ji M, Huang X, Iqbal J, Lenz G, et al. The miRNA-17 approximately 92 cluster mediates chemoresistance and enhances tumor growth in mantle cell lymphoma via PI3K/AKT pathway activation. Leukemia. 2012;26:1064–72.
144. 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;129:1401–14.
145. Lawrie CH, Chi J, Taylor S, Tramonti D, Ballabio E, Palazzo S, et al. Expression of microRNAs in diffuse large B cell lymphoma is associated with immunophenotype, survival and transformation from follicular lymphoma. J Cell Mol Med. 2009;13:1248–1260.
146. Gibcus JH, Tan LP, Harms G, Schakel RN, de Jong D, Blokzijl T, et al. Hodgkin lymphoma cell lines are characterized by a specific miRNA expression profile. Neoplasia. 2009;11:167–76.
147. Tan LP, Seinen E, Duns G, de Jong D, Sibon OC, Poppema S, et al. A high throughput experimental approach to identify miRNA targets in human cells. Nucleic Acids Res. 2009;37(20), e137.
148. Leucci E, Zriwil A, Gregersen LH, Jensen KT, Obad S, Bellan C, et al. Inhibition of miR-9 de-represses HuR and DICER1 and impairs Hodgkin lymphoma tumour outgrowth in vivo. Oncogene. 2012;31:5081–9.
149. Nie K, Gomez M, Landgraf P, Garcia JF, Liu Y, Tan LH, et al. MicroRNA-mediated downregulation of PRDM1/Blimp-1 in Hodgkin/Reed-Sternberg cells: a potential pathogenetic lesion in Hodgkin lymphomas. Am J Pathol. 2008;173:242–52.
150. Craig VJ, Cogliatti SB, Imig J, Renner C, Neuenschwander S, Rehrauer H, et al. Myc-mediated repression of microRNA-34a promotes high-grade transformation of B-cell lymphoma by dysregulation of FoxP1. Blood. 2011;117:6227–36.
151. Thorns C, Kuba J, Bernard V, Senft A, Szymczak S, Feller AC, et al. Deregulation of a distinct set of microRNAs is associated with transformation of gastritis into MALT lymphoma. Virchows Arch. 2012;460:371–7.
152. Liu TY, Chen SU, Kuo SH, Cheng AL, Lin CW. E2A-positive gastric MALT lymphoma has weaker plasmacytoid infiltrates and stronger expression of the memory B-cell-associated miR-223: possible correlation with stage and treatment response. Mod Pathol. 2010;23:1507–17.
153. Ballabio E, Mitchell T, van Kester MS, Taylor S, Dunlop HM, Chi J, et al. MicroRNA expression in Sezary syndrome: identification, function, and diagnostic potential. Blood. 2010;116:1105–13.

154. Narducci MG, Arcelli D, Picchio MC, Lazzeri C, Pagani E, Sampogna F, et al. MicroRNA profiling reveals that miR-21, miR486 and miR-214 are upregulated and involved in cell survival in Sezary syndrome. Cell Death Dis. 2011;2, e151.
155. Watanabe A, Tagawa H, Yamashita J, Teshima K, Nara M, Iwamoto K, et al. The role of microRNA-150 as a tumor suppressor in malignant lymphoma. Leukemia. 2011;25:1324–34.
156. Manfe V, Biskup E, Rosbjerg A, Kamstrup M, Skov AG, Lerche CM, et al. miR-122 regulates p53/Akt signalling and the chemotherapy-induced apoptosis in cutaneous T-cell lymphoma. PLoS One. 2012;7:e29541.
157. van der Fits L, van Kester MS, Qin Y, Out-Luiting JJ, Smit F, Zoutman WH, et al. MicroRNA-21 expression in CD4 T cells is regulated by STAT3 and is pathologically involved in Sezary syndrome. J Invest Dermatol. 2011;131:762–8.
158. Chen K, Rajewsky N. Natural selection on human microRNA binding sites inferred from SNP data. Nat Genet. 2006;38:1452–6.
159. Yu Z, Li Z, Jolicoeur N, Zhang L, Fortin Y, Wang E, Wu M, Shen SH. Aberrant allele frequencies of the SNPs located in microRNA target sites are potentially associated with human cancers. Nucleic Acids Res. 2007;35:4535–41.
160. Saunders MA, Liang H, Li WH. Human polymorphism at microRNAs and microRNA target sites. Proc Natl Acad Sci U S A. 2007;104:3300–5.
161. Salzman DW, Weidhaas JB. SNPing cancer in the bud: microRNA and microRNA target site polymorphisms as diagnostic and prognostic biomarkers in cancer. Pharmacol Ther. 2013;137:55–63.
162. Mishra PJ, Bertino JR. MicroRNA polymorphisms: the future of pharmacogenomics, molecular epidemiology and individualized medicine. Pharmacogenomics. 2009;10:399–416.
163. de Larrea CF, Navarro A, Tejero R, Tovar N, Díaz T, Cibeira MT, Rosiñol L, Ferrer G, Rovira M, Rozman M, Monzó M, Bladé J. Impact of MiRSNPs on survival and progression in patients with multiple myeloma undergoing autologous stem cell transplantation. Clin Cancer Res. 2012;18:3697–704.
164. Dzikiewicz-Krawczyk A, Macieja A, Mały E, Januszkiewicz-Lewandowska D, Mosor M, Fichna M, Strauss E, Nowak J. Polymorphisms in microRNA target sites modulate risk of lymphoblastic and myeloid leukemias and affect microRNA binding. J Hematol Oncol. 2014;7:43.
165. Bouchie A. First microRNA mimic enters clinic. Nat Biotechnol. 2013;7:577.
166. Fabani MM, Abreu-Goodger C, Williams D, Lyons PA, Torres AG, Smith KG, Enright AJ, Gait MJ, Vigorito E. Efficient inhibition of miR-155 function in vivo by peptide nucleic acids. Nucleic Acids Res. 2010;38:4466–75.
167. Krützfeldt J, Rajewsky N, Braich R, Rajeev KG, Tuschl T, Manoharan M, Stoffel M. Silencing of microRNAs in vivo with 'antagomirs'. Nature. 2005;438(7068):685–9.
168. Lennox KA, Behlke MA. Chemical modification and design of anti-miRNA oligonucleotides. Gene Ther. 2011;18(12):1111–20.
169. Chang TC, Yu D, Lee YS, Wentzel EA, Arking DE, West KM, Dang CV, Thomas-Tikhonenko A, Mendell JT. Widespread microRNA repression by Myc contributes to tumorigenesis. Nat Genet. 2008;40(1):43–50.
170. Fontana L, Fiori ME, Albini S, Cifaldi L, Giovinazzi S, Forloni M, Boldrini R, Donfrancesco A, Federici V, Giacomini P, Peschle C, Fruci D. Antagomir-17-5p abolishes the growth of therapy-resistant neuroblastoma through p21 and Bim. PLoS One. 2008;3, e2236.
171. Karnati HK, Yalagala RS, Undi R, Pasupuleti SR, Gutti RK. Therapeutic potential of siRNA and DNAzymes in cancer. Tumour Biol. 2014;35(10):9505–21.
172. Pereira DM, Rodrigues PM, Borralho PM, Rodrigues CM. Delivering the promise of miRNA cancer therapeutics. Drug Discov Today. 2013;18:282–9.
173. Babar IA, Cheng CJ, Booth CJ, Liang X, Weidhaas JB, Saltzman WM. Nanoparticle-based therapy in an in vivo microRNA-155 (miR-155)-dependent mouse model of lymphoma. Proc Natl Acad Sci U S A. 2012;109:1695–704.

174. Zhao J, Kelnar K, Bader AG. In-depth analysis shows synergy between erlotinib and miR-34a. PLoS One. 2014;9, e89105.
175. Roman-Gomez J, Agirre X, Jiménez-Velasco A, Arqueros V, Vilas-Zornoza A, Rodriguez-Otero P, Martin-Subero I, Garate L, Cordeu L, San José-Eneriz E, Martin V, Castillejo JA, Bandrés E, Calasanz MJ, Siebert R, Heiniger A, Torres A, Prosper F. Epigenetic regulation of microRNAs in acute lympho-blastic leukemia. J Clin Oncol. 2009;27:1316–22.
176. Agirre X, Vilas-Zornoza A, Jiménez-Velasco A, Martin-Subero JI, Cordeu L, Gárate L, San José-Eneriz E, Abizanda G, Rodríguez-Otero P, Fortes P, Rifón J, Bandrés E, Calasanz MJ, Martín V, Heiniger A, Torres A, Siebert R, Román-Gomez J, Prósper F. Epigenetic silencing of the tumor suppressor microRNA Hsa-miR-124a regulates CDK6 expression and confers a poor prognosis in acute lymphoblastic leukemia. Cancer Res. 2009;69:4443–53.
177. Saito Y, Liang G, Egger G, Friedman JM, Chuang JC, Coetzee GA, Jones PA. Specific activation of micro-RNA-127 with down-regulation of the proto-oncogene BCL6 by chromatin-modifying drugs in human cancer cells. Cancer Cell. 2006;9:435–43.
178. Parekh S, Polo JM, Shaknovich R, Juszczynski P, Lev P, Ranuncolo SM, Melnick A. BCL6 programs lymphoma cells for survival and differentiation through distinct biochemical mechanisms. Blood. 2007;110:2067–74.
179. Pullikan JA, Dengler V, Peramangalam PS, et al. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. Blood. 2010;115:1768–78.
180. Garzon R, Pichiorri F, Palumbo T, et al. MicroRNA gene expression during retinoic acid-induced differentiation of human acute promyelocytic leukemia. Oncogene. 2007;26:4148–57.
181. Kanellopoulou C, Muljo SA, Kung AL, et al. Dicer-deficient mouse embryonic stem cells are defective in differentiation and centromeric silencing. Genes Dev. 2005;19:489–501.
182. O'Connell RM, Rao DS, Chaudhuri AA, Boldin MP, Taganov KD, Nicoll J, Paquette RL, Baltimore D. Sustained expression of microRNA-155 in hematopoietic stem cells causes a myeloproliferative disorder. J Exp Med. 2008;205(3):585–94.
183. Rodriguez A, Vigorito E, Clare S, et al. Requirement of bic/microRNA-155 for normal immune function. Science. 2007;316:608–11.
184. Klusmann JH, Li Z, Böhmer K, et al. MiR-125b-2 is a potential oncomiR on human chromosome 21 in megakaryoblastic leukemia. Genes Dev. 2010;24:478–90.
185. Rasmussen KD, Simmini S, Abreu-Goodger C, et al. The miR-144/451 locus is required for erythroid homeostasis. J Exp Med. 2010;207:1351–8.
186. Masaki S, Ohtsuka R, Abe Y, et al. Expression patterns of microRNAs 155 and 451 during normal human erythropoiesis. Biochem Biophys Res Commun. 2007;364:509–14.
187. Xiao C, Calado DP, Galler G, et al. MiR-150 controls B cell differentiation by targeting the transcription factor c-Myb. Cell. 2007;131:146–59.
188. Zhou B, Wang S, Mayr C, et al. MiR-150, a microRNA expressed in mature B and T cells, blocks early B cell development when expressed prematurely. Proc Natl Acad Sci U S A. 2007;104:7080–5.
189. Neilson JR, Zheng GX, Burge CB, et al. Dynamic regulation of miRNA expression in ordered stages of cellular development. Genes Dev. 2007;21:578–89.
190. Li QJ, Chau J, Ebert PJ, et al. MiR-181a is an intrinsic modulator of T cell sensitivity and selection. Cell. 2007;129:147–61.
191. Ventura A, Young AG, Winslow MM, et al. Targeted deletion reveals essential and overlapping functions of the miR-17 through 92 family of miRNA clusters. Cell. 2008;132:875–86.
192. Thai TH, Calado DP, Casola S, et al. Regulation of the germinal center response by microRNA-155. Science. 2007;316:604–8.
193. Vigorito E, Perks KL, Abreu-Goodger C, et al. MicroRNA-155 regulates the generation of immunoglobulin class-switched plasma cells. Immunity. 2007;27:847–59.
194. Stahl HF, Fauti T, Ullrich N, et al. MiR-155 inhibition sensitizes CD4+ Th cells for TREG mediated suppression. PLoS One. 2009;4, e7158.

195. Zheng Y, Josefowicz SZ, Kas A, et al. Genome-wide analysis of Foxp3 target genes in developing and mature regulatory T cells. Nature. 2007;445:936–40.
196. Teng G, Hakimpour P, Landgraf P, et al. MicroRNA-155 is a negative regulator of activation-induced cytidine deaminase. Immunity. 2008;28:621–9.
197. Dorsett Y, McBride KM, Jankovic M, et al. MicroRNA-155 suppresses activation-induced cytidine deaminase-mediated Myc-Igh translocation. Immunity. 2008;28:630–8.
198. Monticelli S, Ansel KM, Xiao C, Socci ND, Krichevsky AM, Thai TH, Rajewsky N, Marks DS, Sander C, Rajewsky K, et al. MicroRNA profiling of the murine hematopoietic system. Genome Biol. 2005;6:R71.
199. Lu LF, Boldin MP, Chaudhry A, Lin LL, Taganov KD, Hanada T, Yoshimura A, Baltimore D, Rudensky AY. Function of miR-146a in controlling Treg cell-mediated regulation of Th1 responses. Cell. 2010;142:914–29.
200. Almanza G, Fernandez A, Volinia S, Cortez-Gonzalez X, Croce CM, Zanetti M. Selected microRNAs define cell fate determination of murine central memory CD8 T-cells. PLoS One. 2010;5, e11243.
201. Bellavia D, Campese AF, Vacca A, Gulino A, Screpanti I. Notch3, another Notch in T-cell development. Semin Immunol. 2003;15:107–12.
202. Indraccolo S, Minuzzo S, Masiero M, Pusceddu I, Persano L, Moserle L, Reboldi A, Favaro E, Mecarozzi M, di Mario G, et al. Cross-talk between tumor and endothelial cells involving the Notch3-Dll4 interaction marks escape from tumor dormancy. Cancer Res. 2009;69:1314–23.
203. Campese AF, Bellavia D, Gulino A, Screpanti I. Notch signaling at the crossroads of T-cell development and leukemogenesis. Semin Cell Dev Biol. 2003;14(2):151–7.
204. Fabbri M, Croce CM, Calin GA. MicroRNAs in the ontogeny of leukemias and lymphomas. Leuk Lymphoma. 2009;50:160–70.
205. Liu J, Wu CP, Lu BF, Jiang JT. Mechanism of T cell regulation by microRNAs. Cancer Biol Med. 2013;10:131–7.
206. Li L, Zhang J, Diao W, Wang D, Wei Y, Zhang C, Zen K. MicroRNA-155 and MicroRNA-21 promote the expansion of functional myeloid-derived suppressor cells. J Immunol. 2014;192:1034–43.
207. Ziętara N, Łyszkiewicz M, Witzlau K, Naumann R, Hurwitz R, Langemeier J, Bohne J, Sandrock I, Ballmaier M, Weiss S, et al. Critical role for miR-181a/b-1 in agonist selection of invariant natural killer T cells. Proc Natl Acad Sci U S A. 2013;110:7407–12.
208. Hackanson B, Bennett KL, Brena RM, Jiang J, Claus R, Chen SS, Blagitko-Dorfs N, Maharry K, Whitman SP, Schmittgen TD, Lübbert M, Marcucci G, Bloomfield CD, Plass C. Epigenetic modification of CCAAT/enhancer binding protein α expression in acute myeloid leukemia. Cancer Res. 2008;68:3142–51.
209. Roehle A, Hoefig KP, Repsilber D, Thorns C, Ziepert M, Wesche KO, et al. MicroRNA signatures characterize diffuse large B-cell lymphomas and follicular lymphomas. Br J Haematol. 2008; 142(5):732–44.
210. Navarro A, Gaya A, Martinez A, Urbano-Ispizua A, Pons A, Balagué O, et al. MicroRNA expression profiling in classic Hodgkin lymphoma. Blood. 2008; 111(5):2825–32.
211. Sánchez-Espiridión B, Martín-Moreno AM, Montalbán C, Figueroa V, Vega F, Younes A, er al. MicroRNA signatures and treatment response in patients with advanced classical Hodgkin lymphoma. Br J Haematol. 2013;162(3):336–47.
212. Calin GA, Ferracin M, Cimmino A, Di Leva G, Shimizu M, Wojcik SE, et al. A MicroRNA signature associated with prognosis and progression in chronic lymphocytic leukemia. N Engl J Med. 2005;353(17):1793–801.
213. Alencar AJ, Malumbres R, Kozloski GA, Advani R, Talreja N, Chinichian S, et al. MicroRNAs are independent predictors of outcome in diffuse large B-cell lymphoma patients treated with R-CHOP. Clin Cancer Res. 2011;17(12):4125–35.
214. Thorsteinsdottir U, Mamo A, Kroon E, Jerome L, Bijl J, Lawrence HJ, et al. Overexpression of the myeloid leukemia-associated Hoxa9 gene in bone marrow cells induces stem cell expansion. Blood. 2002;99(1):121–9.

215. Pulikkan JA, Dengler V, Peramangalam PS, Peer Zada AA, Müller-Tidow C, Bohlander SK, Tenen DG, Behre G. Cell-cycle regulator E2F1 and microRNA-223 comprise an autoregulatory negative feedback loop in acute myeloid leukemia. Blood. 2010; 115(9): 1768–1778.
216. Garzon R, Liu S, Fabbri M, Liu Z, Heaphy CE, Callegari E, et al. MicroRNA-29b induces global DNA hypomethylation and tumor suppressor gene reexpression in acute myeloid leukemia by targeting directly DNMT3A and 3B and indirectly DNMT1. Blood. 2009;113(25):6411–8.
217. Coskun E, von der Heide EK, Schlee C, Kühnl A, Gökbuget N, Hoelzer D, Hofmann WK, Thiel E, Baldus CD. The role of microRNA-196a and microRNA-196b as ERG regulators in acute myeloid leukemia and acute T-lymphoblastic leukemia. Leuk Res. 2011;35(2):208–13.
218. Gao XN, Lin J, Gao L, Li YH, Wang LL, Yu L. MicroRNA-193b regulates c-Kit proto-oncogene and represses cell proliferation in acute myeloid leukemia. Leuk Res. 2011;35(9):1226–32.
219. Venturini L, Battmer K, Castoldi M, Schultheis B, Hochhaus A, Muckenthaler MU, Ganser A, Eder M, Scherr M. Expression of the miR-17-92 polycistron in chronic myeloid leukemia (CML) CD34+ cells. Blood. 2007;109(10):4399–405.
220. Hu H, Li Y, Gu J, Zhu X, Dong D, Yao J, Lin C, Fei J. Antisense oligonucleotide against miR-21 inhibits migration and induces apoptosis in leukemic K562 cells. Leuk Lymphoma. 2010;51(4):694–701.
221. Chim CS, Wong KY, Leung CY, Chung LP, Hui PK, Chan SY, Yu L. Epigenetic inactivation of the hsa-miR-203 in haematological malignancies. J Cell Mol Med. 2011;15(12):2760–7.
222. Lopotová T, Záčková M, Klamová H, Moravcová J. MicroRNA-451 in chronic myeloid leukemia: miR-451-BCR-ABL regulatory loop? Leuk Res. 2011 Jul;35(7):974–7.
223. Turrini E, Haenisch S, Laechelt S, Diewock T, Bruhn O, Cascorbi I. MicroRNA profiling in K-562 cells under imatinib treatment: influence of miR-212 and miR-328 on ABCG2 expression. Pharmacogenet Genomics. 2012 Mar;22(3):198–205.

Chapter 11
Regulatory Roles of miRNAs in Aging

Xurde M. Caravia and Carlos López-Otín

Abstract Aging is a biological process characterized by the progressive deterioration of physiological functions that occurs through the accumulation of macromolecular and cellular damage. This phenomenon impairs tissue function and is a risk factor for many disorders including cardiovascular disease, neurodegenerative disorders, and cancer. A recent study has enumerated nine cellular and molecular hallmarks that represent common denominators of aging and together determine the aging phenotype, highlighting the concept of aging plasticity. Among the multiple molecular mechanisms which may contribute to aging modulation, microRNAs (miRNAs) are raising enormous interest due to their ability to affect all the "Hallmarks of Aging." In this chapter, we will focus on the description of the diverse functional roles of geromiRs, the large and growing subgroup of miRNAs implicated in aging. We will also address the molecular mechanisms underlying miRNA function in aging and discuss potential strategies for managing aging and extending longevity based on geromiR modulation.

Keywords Aging • geromiRs • Life-span • Longevity • Senescence

Abbreviations

DDR DNA damage response
IGF-1 Insulin-like growth factor 1

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Introduction

Inevitably, all of us will experience a progressive deterioration of our body's fitness due to the biological process known as aging. This dramatic phenomenon has long interested scientists, and with the extraordinary life-span increase of human populations over the last century, there is a growing interest for understanding this condition at the molecular level [1]. Aging is characterized by a progressive loss of physiological integrity that occurs through the accumulation of macromolecular and cellular damage, leading to impaired fitness and increased vulnerability to death. This deterioration is the primary risk factor for major human pathologies, including cancer, diabetes, cardiovascular disorders, and neurodegenerative diseases. One of the main advances in aging research was the discovery of aging plasticity, which implies that this process can be modulated by genetic, nutritional, and pharmacological factors. The first evidence of aging plasticity came from the discovery that caloric restriction—underfeeding without malnutrition—extended life-span in many model organisms [2]. Likewise, other external perturbations, such as temperature or oxygen levels, were found to influence life-span in several organisms. A recent study [3] has enumerated nine cellular and molecular hallmarks that represent common denominators of aging and together determine the aging phenotype. These "hallmarks of aging" have been divided into three categories: primary hallmarks, antagonistic hallmarks, and integrative hallmarks. Primary hallmarks include genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis. These are the main culprits of the molecular damage associated with aging and, therefore, they are all unequivocally negative hallmarks. The second category involves the compensatory or antagonistic responses to this damage, and includes three hallmarks: deregulated nutrient-sensing, mitochondrial dysfunction, and cellular senescence. Antagonistic hallmarks can be positive or negative depending on their intensity. At low levels they mediate beneficial effects, but at high levels are deleterious. Finally, stem cell exhaustion and altered intercellular communication fall into the category of integrative hallmarks that are the end result of the previous two groups and are ultimately responsible for the functional decline associated with aging [3].

The discovery of miRNAs in 1993 [4] has considerably changed the classical view of gene expression regulation, revealing a new group of molecules that can contribute to the complex process of aging. As addressed in other chapters of this book, miRNAs participate in virtually all biological processes within the cell as well as in numerous pathological conditions [5, 6]. Accordingly, it is tempting to speculate that aging-related miRNAs that we have previously termed geromiRs [7] are able to widely repress target genes driving the abovementioned hallmarks. In this chapter, we will summarize the significant changes in miRNA expression during aging in invertebrate and mammalian model organisms. In addition, we will present the regulatory roles of miRNAs in relation to the cellular and molecular hallmarks of aging and will discuss their experimental manipulation in order to improve healthspan and life-span (Fig. 11.1).

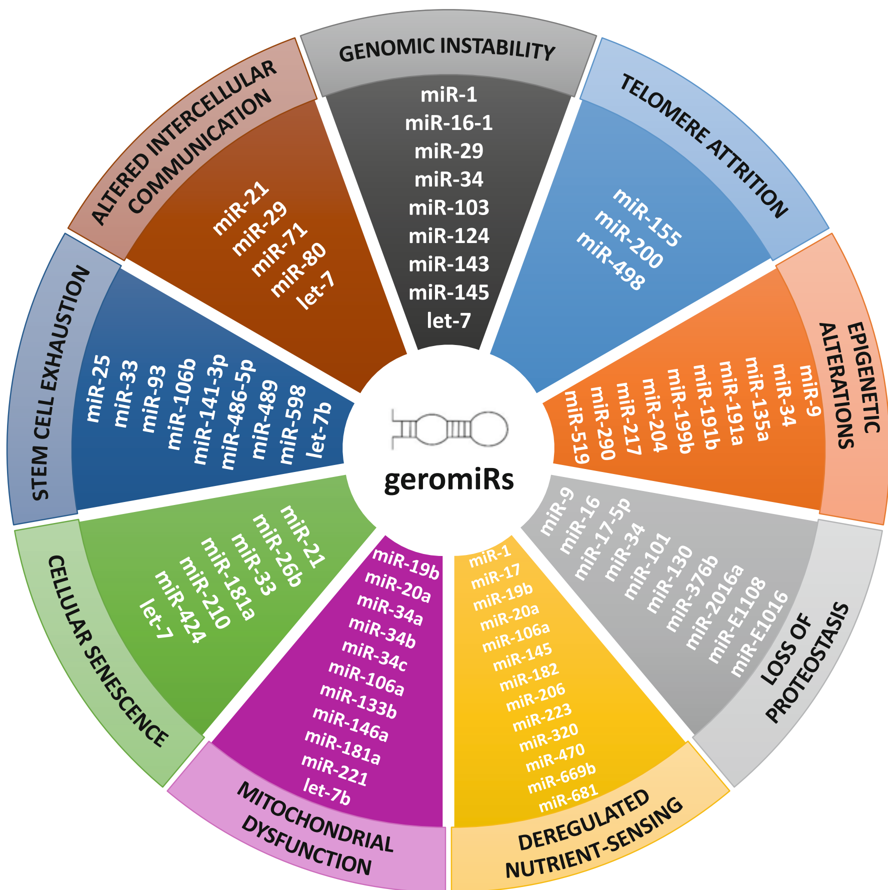

Fig. 11.1 Micro-managing the hallmarks of aging. Schematic representation of miRNAs that regulate the nine hallmarks: genomic instability, telomere attrition, epigenetic regulation, loss of proteostasis, deregulated nutrient-sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication

MicroRNA Expression Profiles in Aging

Several miRNAs appear to play a key role in aging. First, numerous works have reported significant age-related changes in miRNA expression during animal life-span. Additionally, a growing number of miRNAs have been demonstrated to influence most of the well-known longevity and senescence pathways. In this section, we will address the effect of aging on miRNA profiles in humans and animal models.

The first landmark study that identified a geromiR was carried out by Boehm and Slack, who found that *lin-4* miRNA loss-of-function *Caenorhabditis elegans* mutants had a shortened longevity compared to wild-type animals, while overexpression of this miRNA extended organism life-span [8]. To date, we know

that a large number of *C. elegans* miRNAs display changes in their expression during adulthood. Thus, the adult-specific disruption of argonaute-like gene-1 (*alg-1*), which is necessary for miRNA maturation and function, results in abnormal longevity, suggesting that miRNAs are essential for normal aging [9]. Further studies characterized the abundance of miRNAs expressed in individual worms at different ages and noticed that expression variability increased with age. One of these miRNAs, miR-71, is required for normal longevity. Accordingly, transgenic worms overexpressing mir-71 were found to be long-lived [10].

Similar to works on *C. elegans*, studies on *Drosophila melanogaster* have also provided evidence for the contribution of miRNAs to the aging process. For example, flies harboring a hypomorphic mutation in *loquacious* (*loqs*), a key component of the miRNA machinery, develop normally but show late-onset brain degeneration and a reduced life-span, thus implicating miRNA functions in age-associated pathologies [11]. A detailed analysis of brain miRNAs in this organism has also revealed that the *loqs* mutation causes an accelerated aging phenotype that is characterized by progressive neurodegeneration, increased stress sensitivity, and locomotion alterations. A global analysis of miRNA expression levels singled out miR-34 as the most likely mediator of this phenotype [11]. Notably, this work went on to identify translational repression of E74A as the key event responsible for miR-34 effects on aging. E74A is a component of steroid hormone signaling pathways, a molecular network that had been previously associated with aging modulation [12]. In an elegant example of antagonistic pleiotropy [11], the authors have proposed that although E74A is required during juvenile development, silencing of E74A by miR-34 in adulthood is critical to avoid the harmful effects of this protein, which exhibits sharply opposing functions on animal fitness at different life stages. In addition, a different study identified a novel miRNA, named miR-282, and provided evidence of its involvement on viability, longevity, and egg production. A preliminary expression analysis of computationally predicted targets of miR-282 suggested that its effects on aging may be mediated by the modulation of a nervous system-specific adenylate cyclase (*rutabaga*) during metamorphosis [13].

In contrast to invertebrates, current knowledge about the role of miRNAs in mammalian aging is still very limited due to the increased complexity and longer life-span of these organisms. To date, no study has been able to demonstrate the ability of a single miRNA to modulate the rate of aging in rodents by loss- or gain-of-function modifications. However, cumulative observations during the last decade of miRNA research strongly indicate that these molecules contribute to mammalian aging. Profiling studies evaluated the miRNA expression levels of young, adult, and old rat brains, and reported 547 known and 171 candidate novel miRNAs that were differentially expressed among these groups [14]. Aged brains exhibit a predominant upregulation of approximately 70 miRNAs and a downregulation of their respective target genes [15]. Apart from the brain, muscle also degenerates with aging resulting in loss of muscle mass (sarcopenia) over time. miRNA expression profiling from mouse muscle at two different ages shows 34 miRNAs differentially expressed with age, including miR-206 and miR-434 [16]. Perhaps the strongest evidence supporting the putative role of miRNAs in mammalian aging is the activity of specific miRNAs in both short- and long-life animal models. Thus, a study in Ames dwarf mice,

which live 70% longer than wild-type mice due to deficiencies in pituitary hormones, has suggested a critical role for miR-27a in their characteristic life-span extension [17]. This miRNA is significantly increased in the liver of Ames mice, whereas its target genes, ornithine decarboxylase and spermidine synthase, are downregulated. Interestingly, both are important enzymes of the aging-associated polyamine biosynthetic pathway. Conversely, the upregulation of miR-1 and the miR-29 family have also been linked to the progeroid phenotype of a mouse model of Hutchinson-Gilford progeria syndrome by controlling multiple overlapping aging pathways [18, 19].

Several transcriptional studies in humans and other primates have also supported this notion of miRNA modulation of the aging process. Thus, the analysis of mRNA, miRNA, and protein expression in human and macaque brain evidenced regulatory relationships between miRNAs and mRNAs during aging of both species [20]. Further studies of miRNA expression in human serum from young and old individuals have found that the expression of miR-151a-5p, miR-181a-5p, and miR-1248 is significantly decreased in older individuals. Consistently, these miRNAs also show decayed levels in the serum of elderly rhesus monkeys [21]. Recently, one work has reported the first comparison of miRNAs expression profiles of cells from centenarians, octogenarians, and young individuals. Surprisingly, centenarians showed a narrow upregulation of miRNA levels compared to young individuals, but wide upregulated miRNA levels compared to octogenarians [22]. In addition, 15 platelet miRNAs were differentially regulated by age in a study performed in 154 healthy subjects, while their respective target mRNAs were inversely expressed [23].

Micro-managing the Hallmarks of Aging

Over the last years, advances in genetics and molecular biology have led to the identification of a subset of genes whose deregulation affects the aging process. In fact, the study of these genes has been instrumental in defining the hallmarks of aging [3]. In this section, we will discuss the functional relevance of miRNA-mediated regulation of these evolutionary conserved molecular and cellular processes.

Genomic Instability

Even under the most controlled environmental conditions a mortal organism ages, illustrating how exogenous and endogenous sources of damage strongly influence the aging process through the generation of DNA lesions. Cells display a broad repertoire of macromolecule turnover and repair systems that are matched with the variety and frequency of DNA damage. These mechanisms, responsible for genome integrity, include molecular circuits that detect damage and activate pathways aimed at repairing the damage and/or preventing abnormal cellular behaviors, in a process termed DNA damage response (DDR) [24]. Recent studies have unveiled different miRNAs that contribute to the modulation of DDR to try to maintain genome integrity (Fig. 11.2).

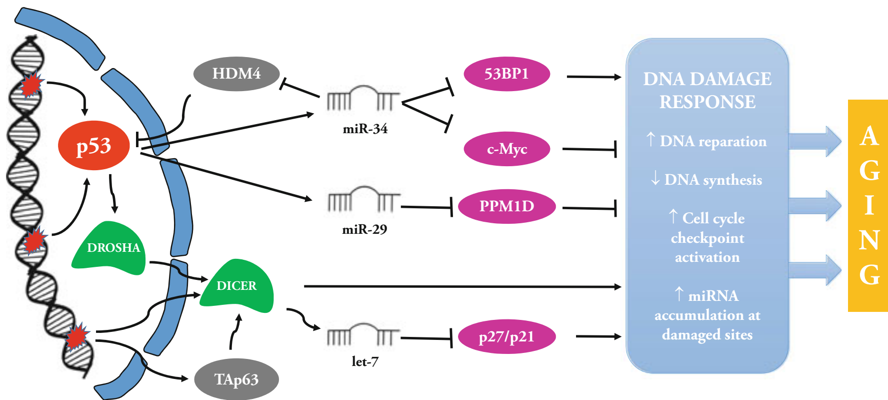

Fig. 11.2 Functional relevance of miRNAs in genomic instability. miRNAs regulate the DNA damage response that is triggered by genomic instability in aging

The miR-34 family members were the first group of miRNAs described to be transcriptionally regulated by p53 in response to DNA damage [25]. Thus, miR-34c expression is induced by p53 and inhibits c-Myc following DNA damage, preventing aberrant DNA synthesis and activating the S-phase checkpoint [26]. Additionally, miR-34 inhibits HDM4, a negative regulator of p53, and establishes a positive feedback loop [27]. In contrast to this protective role, miR-34a can act as a promoter of DNA damage and mitotic catastrophe, because it inhibits 53BP1 and counteracts its recruitment to DNA double-strand breaks, impairing DNA reparation [28]. In *Zmpste24*-deficient mice, a model of Hutchinson-Gilford progeria syndrome [29], altered chromatin architecture mediates the transcription of components of the miR-29 family in a p53-dependent manner [19]. Similar to the p53-dependent miR-34 feedback loop, miR-29 targets and represses PPM1D, a phosphatase that fine-tunes the DDR through inhibition of the activity and stability of p53. Similarly, Werner syndrome (WS), a premature aging disorder caused by mutations in a RecQ-like DNA helicase, can be phenocopied in both mice and worms by loss of function of the corresponding orthologous genes: *WRN* and *wrn-1*, respectively. This study also revealed that worms with mutations in *wrn-1* show a reduced expression of miR-124, and that, surprisingly, the loss of miR-124 phenocopies the *wrn-1* mutation [30].

Accumulating evidence also suggests control of miRNA biogenesis as an important regulatory element of the DDR pathway. For example, knockdown of *DICER*, a nuclease complex that contributes to miRNA maturation, reduced the ATM-dependent DDR through downregulation of miRNAs at damaged sites [31]. By contrast, *DICER* knockdown increases the resistance to camptothecin-induced DNA damage by reduction of let-7, which increased p21/p27 levels, again impairing DNA reparation [32]. Also in response to DNA damage, p53 interacts with the Drosha processing complex and coordinates the maturation of several miRNAs with growth-suppressive function, including miR-16-1, miR-143, and miR-145 [33].

Finally, DICER levels are also fine-tuned in response to DNA damage. TAp63, a member of the p53 family, directly binds to the *Dicer* promoter and activates its transcription in mice [34].

Telomere Attrition

Telomeres are repetitive DNA sequences located at the end of chromosomes that protect them from degradation. Telomeres recruit a multiprotein complex named shelterin [35] to form a chromatin structure that prevents its recognition as double strand breaks and the access of DNA repair proteins [36]. Only telomerase, a special DNA polymerase, can replicate telomeres. Adult cells do not express telomerase, which results in a progressive shortening of these protective fragments throughout aging until cells lose their replicative capacity.

miRNAs regulate diverse components of the shelterin or telomerase protein complexes. For example, miR-155 targets a conserved sequence motif in the 3′ UTR of the shelterin component TRF1, thereby mediating its translational repression and triggering telomere fragility and chromosome alterations [37]. As mentioned above, other miRNAs are able to regulate telomerase, for example, miR-498 regulates the catalytic subunit of telomerase by targeting the 3′ UTR of the encoding mRNA [38]. Conversely, shortening of telomeres may change the miRNA expression profile of different cells. Thus, miRNA expression profiles in cells with intact or with shortened telomeres revealed that 47 miRNAs were differentially expressed between these cell types. Some of these miRNAs are implicated in growth arrest or act as oncogene repressors [39]. In addition, chromosome instability driven by telomere shortening dramatically alters the pattern of miRNA expression in cells [40]. In summary, genomic instability and telomere attrition represent two molecular hallmarks that influence cancer and aging biology. This genetic damage is counteracted by the DDR systems, which in turn are heavily regulated by miRNAs. Therefore, modulating the activity of these miRNAs could either accelerate or decelerate tissue aging and age-related carcinogenesis [41].

Epigenetic Alterations

Sirtuins are important NAD-dependent protein deacetylases and ADP-ribosyltransferases implicated in histone modification, an essential epigenetic mechanism. The sirtuin pathway, whose upregulation extends longevity in yeast, worms, and flies [2], is also influenced by miRNAs. Among the different sirtuins, SIRT1 is widely recognized as a crucial regulator of metabolism, stress responses, replicative senescence, and inflammation [42]. In fact, SIRT1 is an important mediator of the beneficial metabolic effects of caloric restriction (CR) and the target effector of the antiaging molecule resveratrol. Among the miRNAs that might

regulate SIRT1 during aging, the aforementioned age-related miR-34 is one of the best examples, as assessed by its ability to directly target *SIRT1* mRNA in several in vitro and in vivo experiments [43]. Likewise, miR-217 upregulation in human endothelial cells during aging reduces SIRT1 activity and promotes senescence [44]. There is also evidence that several miRNAs, including miR-181a/b, miR-9, miR-204, miR-135a, and miR-199b, downregulate *SIRT1* during differentiation of mouse embryonic stem cells into different tissues [45]. Alternatively, other miRNAs modulate SIRT1 activity indirectly. For instance, miR-519 contributes to human fibroblasts senescence by decreasing the protein levels of SIRT1 through targeting the RNA binding protein HuR [46].

Apart from histone deacetylation, miRNAs are able to control other epigenetic mechanisms, like methylation of DNA. Thus, it has been shown that *Dicer1* deficiency in mice leads to decreased DNA methylation, along with increased telomere recombination and telomere elongation [47]. This methylation defect is a consequence of the reduced expression of the Dnmt1, Dnmt3a, and Dnmt3b DNA methyltransferases. Further analyses have identified miR-290 as an agent in this decrease of methyltransferase levels, through repression of the retinoblastoma-like 2 protein (Rbl2). Moreover, miRNAs can also be methylated, as illustrated by the finding in *Drosophila* of an increase in the 2′-O-methylation at the 3′ end of miRNAs with age. These epigenetic modifications guide the preferential loading of miRNAs into Ago2, and not in Ago1. Loss of methylation leads to accelerated neurodegeneration and shorter lifespan, suggesting the role of methylation of miRNAs in age-associated events [48].

Loss of Proteostasis

Protein folding and degradation of misfolded proteins are key processes that are closely related to aging. In addition, their experimental manipulation can precipitate or ameliorate this unavoidable process. Therefore, protein homeostasis or proteostasis is a *bona fide* aging hallmark [3, 49]. The main mechanisms implicated in proteostasis (autophagy, proteasomal degradation, and chaperone-mediating protein folding) are subjected to miRNA regulation.

In mammals, the regulation of the proteasome is especially important in the nervous system, as illustrated by the severe neurodegenerative diseases related to the accumulation of protein aggregates during aging [50]. In patients suffering from the spinocerebellar ataxia type 1 neurodegenerative disorder (SCA1), a group of upregulated miRNAs defines a pathological expression pattern. The targets of these miRNAs are enriched in members of the ubiquitin-proteasome system, suggesting that this system is deregulated in SCA1 [51]. More recently, another work has identified DNA damage as a mechanism that modifies the repertoire of proteasome-associated miRNAs, suggesting that in stress conditions these regulatory components modify proteasome function [52].

Autophagy is an evolutionarily conserved mechanism that allows the cell to digest its own components [53]. The activity of different factors belonging to this pathway can be fine-tuned by miRNAs after transcription. Thus, the tumor suppressor

miR-101 inhibits autophagy by targeting three different genes: *STMN1*, *RAB5A*, and *ATG4D* [54]. miR-376b modulates human autophagy by regulating intracellular levels of ATG4C and BECN1 [55], while miR-34a targets ATG4B [56]. Alvarez-Erviti et al. have demonstrated that at least eight different miRNAs can regulate chaperone-mediated autophagy, which has a key role in the pathogenesis of a wide range of diseases, especially in the nervous system [57]. These miRNAs decrease chaperone-mediated autophagy through downregulation of hsc70 and LAMP-2A protein levels in the brain [57]. Finally, miR-216a impairs the autophagic function in aging endothelial cells through inhibition of two autophagy-related genes: *Beclin1* and *ATG5* [58]. Likewise, chaperones, a group of proteins implicated in the correct protein folding, are also under miRNA regulation. Thus, miR-17-5p targets multiple endoplasmic reticulum stress-related chaperones during chronic oxidative stress [59].

In summary, these first four hallmarks of aging, genomic instability, telomere attrition, epigenetic alterations, and loss of proteostasis, are included in the category of primary hallmarks because they represent the main causes of cellular damage that underlie the aging process. As described above, miRNAs regulate all these four processes and the molecular mechanisms that repair or counteract this damage, including those mediated by telomerase, chaperones, and epigenetic enzymes [3].

Deregulated Nutrient-Sensing

There is a close relationship between nutrient-sensing and aging. Among nutrient-sensing pathways, IGF-1 signaling stands out as a highly conserved regulatory module that influences longevity and also coordinates growth, development, and metabolism [2]. Like other aging hallmarks, nutrient-sensing pathways are also susceptible to miRNA regulation [60]. For example, the anomalous upregulation of miR-1, which targets the Igf-1 mRNA, is associated with the systemic deregulation of the somatotroph axis in premature aging mice [18]. Conversely, miR-470, miR-669b, and miR-681 are significantly upregulated in brain of long-lived Ames dwarf mice and their expression inversely correlates with the expression of several genes of the IGF-1/insulin pathway. Functional studies have demonstrated that these miRNAs target the IGF-1 receptor and contribute to reduce the levels of phosphorylated AKT and FOXO3a, two downstream targets of this signaling pathway, in the brain of mutant mice [61]. In addition, human miR-145 also represses the expression of IGF-1 receptor and its ligand, IRS-1 [62], while miR-206 and miR-320 target this somatotroph axis in rats [63, 64]. Similarly, miR-182 and miR-223 downregulation enhances IGF-1 signaling and mediates the estrogen impact on skeletal muscle. It has been proposed that IGF-1R and FOXO3a are the main targets whose overexpression triggers this positive effect [65]. In addition to regulating IGF-1, miR-17, miR-19b, miR-20a, and miR-106a target PTEN and inhibit the AKT-mTOR pathway [66], emphasizing the diversity of miRNAs which can contribute to modulate nutrient-sensing mechanisms implicated in the control of aging and longevity.

Mitochondrial Dysfunction

Mitochondrial dysfunction is another hallmark of aging [3]. The decline of autophagic clearance during aging affects the equilibrium between mitochondrial fusion and fission, leading to a build-up of dysfunctional mitochondria, oxidative stress, inflammation, and apoptosis. The miRNAs that exert their roles in the mitochondria are called mitomiRs and could act as vectors that sense and respond dynamically to the changing microenvironment in this organelle. These regulatory elements modulate nuclear and mitochondrial encoded targets [67]. In a recent study, let-7b, miR146a, miR-133b, miR-106a, miR-19b, miR-20a, miR-34a, miR-181a, and miR-221 have been identified as mitomiRs. Further analysis has revealed that targets of these miRNAs include genes that play important roles in processes like energy metabolism, mitochondrial transport, and apoptosis, whose deregulation is linked to aging [68]. Mitochondrial dynamics also plays a key role in some age-related pathologies, such as Parkinson's disease [69]. In addition, miR-34b and miR-34c, whose expression is reduced in brain from Parkinson's disease patients, alter the mitochondrial function in neuronal cells through the inhibition of DJ1 and parkin, two proteins associated with familial forms of the pathology [69]. An overview of miRs and mitochondrial function is available in Chap. 3.

Cellular Senescence

A growing number of miRNAs are arising as important modulators of senescence. This irreversible state of cellular growth arrest is an important effector of the cellular response against DNA damage that prevents the malignant proliferation of cells harboring oncogenic DNA mutations. However, as in the case of DDR, some aspects of cellular senescence have led to the consideration that it has a dual role through lifespan, protecting from cancer development but promoting inflammation and tissue exhaustion, which prompts age-related alterations [70]. Numerous works have revealed functional roles for miRNAs in senescence through a variety of mechanisms. For example, miR-21 was reported as the first oncomiR that is able to produce a hyper oncogenic signal sufficient to limit cell proliferation. Accordingly, miR-21 is upregulated in senescent cells and its overexpression leads to cell-cycle arrest [71]. Another well-known oncogene, *HRAS* (V-12), induces senescence in primary fibroblasts through the production of reactive oxygen species (ROS). Yang et al. first identified protein tyrosine phosphatase 1B (*PTP1B*) as a major target of RAS-induced inhibition by ROS [72]. In turn, phospho-Tyr 393 of argonaute 2 is a direct substrate of PTP1B and its phosphorylation impairs the loading of miRNAs and the formation of the RNA-induced silencing complex (RISC) [72]. Furthermore, a recent work has identified 22 senescence-associated miRNAs in human mammary epithelial cells. In this subset, miR-26b, miR-181a, miR-210, and miR-424 repress Polycomb group proteins CBX7, embryonic ectoderm development (EED), enhancer

of zeste homologue 2 (EZH2) and suppressor of zeste 12 homologue (Suz12), and activate p16, a key regulator of cellular senescence [73]. Moreover, macrophage polarization plays a key role in developing age-associated diseases such as macular degeneration. miR-33 impairs the ability of macrophages to efflux cholesterol, and this intracellular lipid polarizes older macrophages to an abnormal, alternatively activated phenotype that promotes pathologic vascular proliferation. miR-33 exerts this pathological role by downregulating the ATP binding cassette transporter ABCA1 and, consequently, mice deficient for *Abca1* exhibit accelerated aging [74]. Another geromiR, let-7, contributes to prevent senescence through the inhibition of the retinoblastoma/E2F repressor complex, allowing the expression of proliferation-promoting genes [75]. The biogenesis pathway of miRNAs plays a crucial role in the regulation of cellular senescence. Thus, in a recent work, the synthesis of canonical miRNAs was disrupted by knockdown of the microprocessor complex subunit DGCR8 [76]. In this experiment, DGCR8 inactivation results in a dramatic antiproliferative response, with the acquisition of a senescent phenotype [76].

In summary, antagonistic hallmarks (deregulated nutrient-sensing, mitochondrial dysfunction, and cellular senescence) establish the biological response to primary hallmarks and, at low doses, mediate positive effects. However, when they become very strong they can be deleterious. miRNAs contribute to fine-tune the intensity of these responses and keep them under physiological limits [3].

Stem Cell Exhaustion

In addition to senescence, the decline of adult stem cell self-renewal and pluripotency is also considered a key determinant in the age-associated deterioration of tissue homeostasis and maintenance. Notably, several reports have described senescence or age-related changes in miRNAs of human or rhesus macaque mesenchymal stem cells [77, 78]. Furthermore, numerous studies have reported essential roles for miRNAs in processes such as renewal, pluripotency, quiescent state maintenance, proliferation, and differentiation of adult stem cells in several tissues and organisms. For example, the loss of self-renewal potential in old neural stem cells has been linked to age-dependent upregulation of let-7b, which in turn inhibits HMGA2 expression, a repressor of the INK4a/ARF locus [79]. Another illustrative example involving let-7 is the testis stem-cell niche. In *Drosophila* testis, aging results in a marked decrease in the self-renewal factor Unpaired (Upd), leading to a loss of germline stem cells. The RNA binding protein Imp protects Upd from degradation by let-7. In the absence of Imp, Upd mRNA becomes unprotected and susceptible to degradation [80]. Hematopoietic stem cell self-renewal in mice is also regulated by miR-33 repression of *TP53* [81], and the maintenance of quiescent state in human muscle adult stem cells has been shown to be highly dependent on miRNA activity, being miR-489 one of the most prominent effectors [82]. Stem cell proliferation and neuronal differentiation in mice are also finely regulated by miRNAs of the miR-106b-25 cluster, which are in turn controlled by the aging-associated

FoxO transcription factors [83]. Additionally, the expression of miR-486-5p and miR-598 in human adipose tissue-derived mesenchymal stem cells (hADSCs) progressively increases with aging and regulates the expression of SIRT1, inducing a premature senescence-like phenotype. In the case of miR-486-5p, this mechanism blocks adipogenic and osteogenic differentiation [84, 85]. Studies of human embryonic stem cells (hESCs) have described a nonfunctional p53-p21 axis at the G1/S checkpoint, which has recently been reported to be regulated by the miR-302 family [86]. A progressive increase of miR-335 in ex vivo cultures of hMSCs, as well as forced expression of miR-335, resulted in early senescence-like alterations including senescence-associated secretory phenotype (SASP). Also in hMSCs, miR-141-3p direct binding to the 3′ UTR of *ZMPSTE24*, which is involved in maturation of lamin A, leads to accumulation of prelamin A in the nuclear envelope [87].

Altered Intercellular Communication

Aging also involves changes at the level of intercellular communication, be it endocrine, neuroendocrine, or neuronal. miRNAs influence intercellular communication by being included in exosomes or directly circulating through body fluids [88, 89]. In *C. elegans*, life-span is controlled by signaling between the germline and the soma. Different approaches have confirmed that miRNAs influence this process by targeting well-known aging signaling pathways. For example, germ cell removal extends life-span by triggering the activation of the *DAF-16/FOXO* transcription factor in the intestine, and it was reported that miR-71 and let-7 function to mediate this increase in longevity [90, 91]. In recent years, several lines of research have converged on the concept of inflammaging: an age-related systemic chronic inflammation that represents a prominent alteration in intercellular communication. miRNAs can fine-tune this activation of immune cells by regulating the SASP and the Toll-like receptors, two putative mechanisms underlying inflammaging [92, 93]. For example, miR-21 and miR-29a are able to bind to TLR8, activating TLR-mediated NF-kB signaling that leads to increased secretion of the proinflammatory cytokines interleukin-6 and TNF-a [94].

There are several possibilities for restoring defective intercellular communication underlying aging processes. These strategies include nutritional interventions such as caloric restriction, a condition that can promote longevity and protect against age-associated disease across species. An elegant example in this regard has been reported in *C. elegans* through the identification of miRNA-80 as a major regulator during CR. Thus, miR-80 deletion confers system-wide healthy aging in a mechanism that involves the factors cbp-1, daf-16/FOXO, and hsf-1 [95].

In summary, stem cells exhaustion and altered intercellular communication fall into the category of integrative hallmarks, as they are the main mediators of the aging phenotype and together influence the aging rate [3]. miRNAs regulate self-renewal, inflammation, and other important conditions concerning this group of integrative hallmarks of aging.

Conclusions and Perspectives

The discovery of miRNAs has opened a new chapter in aging research that could help to achieve a deeper knowledge of the molecular network underlying this complex process. Although we are far from understanding the precise involvement of these molecules in age-related alterations, solid evidence from the literature, discussed in this chapter, supports an important role for the growing group of geromiRs in aging modulation. miRNA-mediated regulation impacts all nine molecular and cellular hallmarks of aging, modifying the aging rate and age-related diseases both in invertebrates and mammals [96]. Developing mammalian in vivo models for ablation and gain of specific miRNAs will undoubtedly help us to answer many important questions pertaining to how individual geromiRs regulate tissue aging and organismal life-span. Nevertheless, diverse hallmarks are interconnected, which will necessitate broader, integrative strategies. For example, cells that enter into senescence acquire a SASP that leads to production of proinflammatory cytokines and the triggering of inflammaging, a status with altered intercellular communication associated with aging [92]. In addition, a single miRNA may regulate several processes affecting more than one hallmark. On top of this, the regulation of miRNA expression, epigenetically or by transcription factors during aging, will also be a topic of interest for future investigations. The recent advances in strategies to effectively block or delivery specific miRNA in vivo may also facilitate new therapeutic opportunities to delay or ameliorate age-related alterations as well as premature aging syndromes [97]. Alternatively, a promising new area for miRNAs is in diagnostics, where miRNAs have great potential as molecular biomarkers of longevity with ability to predict individual longevity better than chronological age [98].

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References

1. Vijg J, Campisi J. Puzzles, promises and a cure for ageing. Nature. 2008;454:1065–71.
2. Kenyon CJ. The genetics of ageing. Nature. 2010;464:504–12.
3. Lopez-Otin C, Blasco MA, Partridge L, Serrano M, Kroemer G. The hallmarks of aging. Cell. 2013;153:1194–217.
4. 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.
5. Ameres SL, Zamore PD. Diversifying microRNA sequence and function. Nat Rev Mol Cell Biol. 2013;14:475–88.
6. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol. 2009;10:126–39.

7. Ugalde AP, Espanol Y, Lopez-Otin C. Micromanaging aging with miRNAs: new messages from the nuclear envelope. Nucleus. 2011;2:549–55.
8. Boehm M, Slack F. A developmental timing microRNA and its target regulate life span in *C. elegans*. Science. 2005;310:1954–7.
9. Kato M, Chen X, Inukai S, Zhao H, Slack FJ. Age-associated changes in expression of small, noncoding RNAs, including microRNAs, in *C. elegans*. RNA. 2011;17:1804–20.
10. Lucanic M, Graham J, Scott G, Bhaumik D, Benz CC, Hubbard A, Lithgow GJ, Melov S. Age-related micro-RNA abundance in individual *C. elegans*. Aging. 2013;5:394–411.
11. Liu N, Landreh M, Cao K, Abe M, Hendriks GJ, Kennerdell JR, Zhu Y, Wang LS, Bonini NM. The microRNA miR-34 modulates ageing and neurodegeneration in *Drosophila*. Nature. 2012;482:519–23.
12. Simon AF, Shih C, Mack A, Benzer S. Steroid control of longevity in *Drosophila melanogaster*. Science. 2003;299:1407–10.
13. Vilmos P, Bujna A, Szuperak M, Havelda Z, Varallyay E, Szabad J, Kucerova L, Somogyi K, Kristo I, Lukacsovich T, Jankovics F, Henn L, Erdelyi M. Viability, longevity, and egg production of *Drosophila melanogaster* are regulated by the miR-282 microRNA. Genetics. 2013;195:469–80.
14. Yin L, Sun Y, Wu J, Yan S, Deng Z, Wang J, Liao S, Yin D, Li G. Discovering novel microRNAs and age-related nonlinear changes in rat brains using deep sequencing. Neurobiol Aging. 2015;36:1037–44.
15. Li N, Bates DJ, An J, Terry DA, Wang E. Up-regulation of key microRNAs, and inverse down-regulation of their predicted oxidative phosphorylation target genes, during aging in mouse brain. Neurobiol Aging. 2011;32:944–55.
16. Kim JY, Park YK, Lee KP, Lee SM, Kang TW, Kim HJ, Dho SH, Kim SY, Kwon KS. Genome-wide profiling of the microRNA-mRNA regulatory network in skeletal muscle with aging. Aging. 2014;6:524–44.
17. Bates DJ, Li N, Liang R, Sarojini H, An J, Masternak MM, Bartke A, Wang E. MicroRNA regulation in Ames dwarf mouse liver may contribute to delayed aging. Aging Cell. 2010;9:1–18.
18. Marino G, Ugalde AP, Fernandez AF, Osorio FG, Fueyo A, Freije JM, Lopez-Otin C. Insulin-like growth factor 1 treatment extends longevity in a mouse model of human premature aging by restoring somatotroph axis function. Proc Natl Acad Sci U S A. 2010; 107:16268–73.
19. Ugalde AP, Ramsay AJ, de la Rosa J, Varela I, Marino G, Cadinanos J, Lu J, Freije JM, Lopez-Otin C. Aging and chronic DNA damage response activate a regulatory pathway involving miR-29 and p53. EMBO J. 2011;30:2219–32.
20. Somel M, Guo S, Fu N, Yan Z, Hu HY, Xu Y, Yuan Y, Ning Z, Hu Y, Menzel C, Hu H, Lachmann M, Zeng R, Chen W, Khaitovich P. MicroRNA, mRNA, and protein expression link development and aging in human and macaque brain. Genome Res. 2010;20:1207–18.
21. 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. 2013;5:725–40.
22. Serna E, Gambini J, Borras C, Abdelaziz KM, Belenguer A, Sanchis P, Avellana JA, Rodriguez-Manas L, Vina J. Centenarians, but not octogenarians, up-regulate the expression of microRNAs. Sci Rep. 2012;2:961.
23. Simon LM, Edelstein LC, Nagalla S, Woodley AB, Chen ES, Kong X, Ma L, Fortina P, Kunapuli S, Holinstat M, McKenzie SE, Dong JF, Shaw CA, Bray PF. Human platelet microRNA-mRNA networks associated with age and gender revealed by integrated plateletomics. Blood. 2014;123:e37–45.
24. Lord CJ, Ashworth A. The DNA damage response and cancer therapy. Nature. 2012;481:287–94.
25. Rokavec M, Li H, Jiang L, Hermeking H. The p53/miR-34 axis in development and disease. J Mol Cell Biol. 2014;6:214–30.
26. Cannell IG, Kong YW, Johnston SJ, Chen ML, Collins HM, Dobbyn HC, Elia A, Kress TR, Dickens M, Clemens MJ, Heery DM, Gaestel M, Eilers M, Willis AE, Bushell M. p38 MAPK/

MK2-mediated induction of miR-34c following DNA damage prevents Myc-dependent DNA replication. Proc Natl Acad Sci U S A. 2010;107:5375–80.
27. Okada N, Lin CP, Ribeiro MC, Biton A, Lai G, He X, Bu P, Vogel H, Jablons DM, Keller AC, Wilkinson JE, He B, Speed TP, He L. A positive feedback between p53 and miR-34 miRNAs mediates tumor suppression. Genes Dev. 2014;28:438–50.
28. Kofman AV, Kim J, Park SY, Dupart E, Letson C, Bao Y, Ding K, Chen Q, Schiff D, Larner J, Abounader R. microRNA-34a promotes DNA damage and mitotic catastrophe. Cell Cycle. 2013;12:3500–11.
29. Varela I, Cadinanos J, Pendas AM, Gutierrez-Fernandez A, Folgueras AR, Sanchez LM, Zhou Z, Rodriguez FJ, Stewart CL, Vega JA, Tryggvason K, Freije JM, Lopez-Otin C. Accelerated ageing in mice deficient in *Zmpste24* protease is linked to p53 signalling activation. Nature. 2005;437:564–8.
30. Dallaire A, Garand C, Paquel ER, Mitchell SJ, de Cabo R, Simard MJ, Lebel M. Down regulation of miR-124 in both Werner syndrome DNA helicase mutant mice and mutant *Caenorhabditis elegans wrn-1* reveals the importance of this microRNA in accelerated aging. Aging. 2012;4:636–47.
31. Francia S, Michelini F, Saxena A, Tang D, de Hoon M, Anelli V, Mione M, Carninci P, d'Adda di Fagagna F. Site-specific DICER and DROSHA RNA products control the DNA-damage response. Nature. 2012;488:231–5.
32. Liu B, Liu M, Wang J, Zhang X, Wang X, Wang P, Wang H, Li W, Wang Y. DICER-dependent biogenesis of let-7 miRNAs affects human cell response to DNA damage via targeting p21/p27. Nucleic Acids Res. 2015;43:1626–36.
33. Suzuki HI, Yamagata K, Sugimoto K, Iwamoto T, Kato S, Miyazono K. Modulation of microRNA processing by p53. Nature. 2009;460:529–33.
34. Su X, Chakravarti D, Cho MS, Liu L, Gi YJ, Lin YL, Leung ML, El-Naggar A, Creighton CJ, Suraokar MB, Wistuba I, Flores ER. TAp63 suppresses metastasis through coordinate regulation of Dicer and miRNAs. Nature. 2010;467:986–90.
35. Palm W, de Lange T. How shelterin protects mammalian telomeres. Annu Rev Genet. 2008;42:301–34.
36. Fumagalli M, Rossiello F, Clerici M, Barozzi S, Cittaro D, Kaplunov JM, Bucci G, Dobreva M, Matti V, Beausejour CM, Herbig U, Longhese MP, d'Adda di Fagagna F. Telomeric DNA damage is irreparable and causes persistent DNA-damage-response activation. Nat Cell Biol. 2012;14:355–65.
37. Dinami R, Ercolani C, Petti E, Piazza S, Ciani Y, Sestito R, Sacconi A, Biagioni F, le Sage C, Agami R, Benetti R, Mottolese M, Schneider C, Blandino G, Schoeftner S. miR-155 drives telomere fragility in human breast cancer by targeting TRF1. Cancer Res. 2014;74:4145–56.
38. Kasiappan R, Shen Z, Tse AK, Jinwal U, Tang J, Lungchukiet P, Sun Y, Kruk P, Nicosia SV, Zhang X, Bai W. 1,25-Dihydroxyvitamin D3 suppresses telomerase expression and human cancer growth through microRNA-498. J Biol Chem. 2012;287:41297–309.
39. Uziel O, Yosef N, Sharan R, Ruppin E, Kupiec M, Kushnir M, Beery E, Cohen-Diker T, Nordenberg J, Lahav M. The effects of telomere shortening on cancer cells: a network model of proteomic and microRNA analysis. Genomics. 2015;105:5–16.
40. Castro-Vega LJ, Jouravleva K, Liu WY, Martinez C, Gestraud P, Hupe P, Servant N, Albaud B, Gentien D, Gad S, Richard S, Bacchetti S, Londono-Vallejo A. Telomere crisis in kidney epithelial cells promotes the acquisition of a microRNA signature retrieved in aggressive renal cell carcinomas. Carcinogenesis. 2013;34:1173–80.
41. Sperka T, Wang J, Rudolph KL. DNA damage checkpoints in stem cells, ageing and cancer. Nat Rev Mol Cell Biol. 2012;13:579–90.
42. Haigis MC, Sinclair DA. Mammalian sirtuins: biological insights and disease relevance. Annu Rev Pathol. 2010;5:253–95.
43. Lee J, Kemper JK. Controlling SIRT1 expression by microRNAs in health and metabolic disease. Aging. 2010;2:527–34.
44. Menghini R, Casagrande V, Cardellini M, Martelli E, Terrinoni A, Amati F, Vasa-Nicotera M, Ippoliti A, Novelli G, Melino G, Lauro R, Federici M. MicroRNA 217 modulates endothelial cell senescence via silent information regulator 1. Circulation. 2009;120:1524–32.

45. Saunders LR, Sharma AD, Tawney J, Nakagawa M, Okita K, Yamanaka S, Willenbring H, Verdin E. miRNAs regulate SIRT1 expression during mouse embryonic stem cell differentiation and in adult mouse tissues. Aging. 2010;2:415–31.
46. Marasa BS, Srikantan S, Martindale JL, Kim MM, Lee EK, Gorospe M, Abdelmohsen K. MicroRNA profiling in human diploid fibroblasts uncovers miR-519 role in replicative senescence. Aging. 2010;2:333–43.
47. Benetti R, Gonzalo S, Jaco I, Munoz P, Gonzalez S, Schoeftner S, Murchison E, Andl T, Chen T, Klatt P, Li E, Serrano M, Millar S, Hannon G, Blasco MA. A mammalian microRNA cluster controls DNA methylation and telomere recombination via Rbl2-dependent regulation of DNA methyltransferases. Nat Struct Mol Biol. 2008;15:268–79.
48. Abe M, Naqvi A, Hendriks GJ, Feltzin V, Zhu Y, Grigoriev A, Bonini NM. Impact of age-associated increase in 2′-O-methylation of miRNAs on aging and neurodegeneration in *Drosophila*. Genes Dev. 2014;28:44–57.
49. Morimoto RI, Cuervo AM. Proteostasis and the aging proteome in health and disease. J Gerontol A Biol Sci Med Sci. 2014;69 Suppl 1:S33–8.
50. Aguzzi A, O'Connor T. Protein aggregation diseases: pathogenicity and therapeutic perspectives. Nat Rev Drug Discov. 2010;9:237–48.
51. Persengiev S, Kondova I, Bontrop RE. Functional annotation of small noncoding RNAs target genes provides evidence for a deregulated ubiquitin-proteasome pathway in spinocerebellar ataxia type 1. J Nucleic Acids. 2012;2012:672536.
52. Tsimokha AS, Kulichkova VA, Karpova EV, Zaykova JJ, Aksenov ND, Vasilishina AA, Kropotov AV, Antonov A, Barlev NA. DNA damage modulates interactions between microRNAs and the 26S proteasome. Oncotarget. 2014;5:3555–67.
53. Fernandez AF, Lopez-Otin C. The functional and pathologic relevance of autophagy proteases. J Clin Invest. 2015;125:33–41.
54. Frankel LB, Wen J, Lees M, Hoyer-Hansen M, Farkas T, Krogh A, Jaattela M, Lund AH. microRNA-101 is a potent inhibitor of autophagy. EMBO J. 2011;30:4628–41.
55. Korkmaz G, le Sage C, Tekirdag KA, Agami R, Gozuacik D. miR-376b controls starvation and mTOR inhibition-related autophagy by targeting ATG4C and BECN1. Autophagy. 2012;8:165–76.
56. Rothe K, Lin H, Lin KB, Leung A, Wang HM, Malekesmaeili M, Brinkman RR, Forrest DL, Gorski SM, Jiang X. The core autophagy protein ATG4B is a potential biomarker and therapeutic target in CML stem/progenitor cells. Blood. 2014;123:3622–34.
57. Alvarez-Erviti L, Seow Y, Schapira AH, Rodriguez-Oroz MC, Obeso JA, Cooper JM. Influence of microRNA deregulation on chaperone-mediated autophagy and alpha-synuclein pathology in Parkinson's disease. Cell Death Dis. 2013;4, e545.
58. Menghini R, Casagrande V, Marino A, Marchetti V, Cardellini M, Stoehr R, Rizza S, Martelli E, Greco S, Mauriello A, Ippoliti A, Martelli F, Lauro R, Federici M. MiR-216a: a link between endothelial dysfunction and autophagy. Cell Death Dis. 2014;5, e1029.
59. Wang Q, Hu W, Lei M, Wang Y, Yan B, Liu J, Zhang R, Jin Y. MiR-17-5p impairs trafficking of H-ERG K+ channel protein by targeting multiple ER stress-related chaperones during chronic oxidative stress. PLoS One. 2013;8, e84984.
60. Jung HJ, Suh Y. Regulation of IGF-1 signaling by microRNAs. Front Genet. 2014;5:472.
61. Liang R, Khanna A, Muthusamy S, Li N, Sarojini H, Kopchick JJ, Masternak MM, Bartke A, Wang E. Post-transcriptional regulation of IGF1R by key microRNAs in long-lived mutant mice. Aging Cell. 2011;10:1080–8.
62. La Rocca G, Badin M, Shi B, Xu SQ, Deangelis T, Sepp-Lorenzinoi L, Baserga R. Mechanism of growth inhibition by MicroRNA 145: the role of the IGF-I receptor signaling pathway. J Cell Physiol. 2009;220:485–91.
63. Shan ZX, Lin QX, Fu YH, Deng CY, Zhou ZL, Zhu JN, Liu XY, Zhang YY, Li Y, Lin SG, Yu XY. Upregulated expression of miR-1/miR-206 in a rat model of myocardial infarction. Biochem Biophys Res Commun. 2009;381:597–601.
64. Wang XH, Qian RZ, Zhang W, Chen SF, Jin HM, Hu RM. MicroRNA-320 expression in myocardial microvascular endothelial cells and its relationship with insulin-like growth factor-1 in type 2 diabetic rats. Clin Exp Pharmacol Physiol. 2009;36:181–8.

65. Olivieri F, Ahtiainen M, Lazzarini R, Pollanen E, Capri M, Lorenzi M, Fulgenzi G, Albertini MC, Salvioli S, Alen MJ, Kujala UM, Borghetti G, Babini L, Kaprio J, Sipila S, Franceschi C, Kovanen V, Procopio AD. Hormone replacement therapy enhances IGF-1 signaling in skeletal muscle by diminishing miR-182 and miR-223 expressions: a study on postmenopausal monozygotic twin pairs. Aging Cell. 2014;13:850–61.
66. Patel M, Gomez NC, McFadden AW, Moats-Staats BM, Wu S, Rojas A, Sapp T, Simon JM, Smith SV, Kaiser-Rogers K, Davis IJ. PTEN deficiency mediates a reciprocal response to IGFI and mTOR inhibition. Mol Cancer Res. 2014;12:1610–20.
67. Bandiera S, Mategot 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.
68. Rippo MR, Olivieri F, Monsurro V, Prattichizzo F, Albertini MC, Procopio AD. MitomiRs in human inflamm-aging: a hypothesis involving miR-181a, miR-34a and miR-146a. Exp Gerontol. 2014;56:154–63.
69. Minones-Moyano E, Porta S, Escaramis G, Rabionet R, Iraola S, Kagerbauer B, Espinosa-Parrilla Y, Ferrer I, Estivill X, Marti E. MicroRNA profiling of Parkinson's disease brains identifies early downregulation of miR-34b/c which modulate mitochondrial function. Hum Mol Genet. 2011;20:3067–78.
70. Rodier F, Campisi J. Four faces of cellular senescence. J Cell Biol. 2011;192:547–56.
71. Dellago H, Preschitz-Kammerhofer B, Terlecki-Zaniewicz L, Schreiner C, Fortschegger K, Chang MW, Hackl M, Monteforte R, Kuhnel H, Schosserer M, Gruber F, Tschachler E, Scheideler M, Grillari-Voglauer R, Grillari J, Wieser M. High levels of oncomiR-21 contribute to the senescence-induced growth arrest in normal human cells and its knock-down increases the replicative lifespan. Aging Cell. 2013;12:446–58.
72. Yang M, Haase AD, Huang FK, Coulis G, Rivera KD, Dickinson BC, Chang CJ, Pappin DJ, Neubert TA, Hannon GJ, Boivin B, Tonks NK. Dephosphorylation of tyrosine 393 in argonaute 2 by protein tyrosine phosphatase 1B regulates gene silencing in oncogenic RAS-induced senescence. Mol Cell. 2014;55:782–90.
73. Overhoff MG, Garbe JC, Koh J, Stampfer MR, Beach DH, Bishop CL. Cellular senescence mediated by p16INK4A-coupled miRNA pathways. Nucleic Acids Res. 2014;42:1606–18.
74. Sene A, Khan AA, Cox D, Nakamura RE, Santeford A, Kim BM, Sidhu R, Onken MD, Harbour JW, Hagbi-Levi S, Chowers I, Edwards PA, Baldan A, Parks JS, Ory DS, Apte RS. Impaired cholesterol efflux in senescent macrophages promotes age-related macular degeneration. Cell Metab. 2013;17:549–61.
75. Benhamed M, Herbig U, Ye T, Dejean A, Bischof O. Senescence is an endogenous trigger for microRNA-directed transcriptional gene silencing in human cells. Nat Cell Biol. 2012;14:266–75.
76. Gomez-Cabello D, Adrados I, Gamarra D, Kobayashi H, Takatsu Y, Takatsu K, Gil J, Palmero I. DGCR8-mediated disruption of miRNA biogenesis induces cellular senescence in primary fibroblasts. Aging Cell. 2013;12:923–31.
77. Wagner W, Horn P, Castoldi M, Diehlmann A, Bork S, Saffrich R, Benes V, Blake J, Pfister S, Eckstein V, Ho AD. Replicative senescence of mesenchymal stem cells: a continuous and organized process. PLoS One. 2008;3, e2213.
78. Yu JM, Wu X, Gimble JM, Guan X, Freitas MA, Bunnell BA. Age-related changes in mesenchymal stem cells derived from rhesus macaque bone marrow. Aging Cell. 2011;10:66–79.
79. Nishino J, Kim I, Chada K, Morrison SJ. Hmga2 promotes neural stem cell self-renewal in young but not old mice by reducing p16Ink4a and p19Arf expression. Cell. 2008;135:227–39.
80. Toledano H, D'Alterio C, Czech B, Levine E, Jones DL. The let-7-Imp axis regulates ageing of the *Drosophila* testis stem-cell niche. Nature. 2012;485:605–10.
81. Herrera-Merchan A, Cerrato C, Luengo G, Dominguez O, Piris MA, Serrano M, Gonzalez S. miR-33-mediated downregulation of p53 controls hematopoietic stem cell self-renewal. Cell Cycle. 2010;9:3277–85.
82. Cheung TH, Quach NL, Charville GW, Liu L, Park L, Edalati A, Yoo B, Hoang P, Rando TA. Maintenance of muscle stem-cell quiescence by microRNA-489. Nature. 2012;482:524–8.

83. Brett JO, Renault VM, Rafalski VA, Webb AE, Brunet A. The microRNA cluster miR-106b~25 regulates adult neural stem/progenitor cell proliferation and neuronal differentiation. Aging. 2011;3:108–24.
84. Kim YJ, Hwang SH, Lee SY, Shin KK, Cho HH, Bae YC, Jung JS. miR-486-5p induces replicative senescence of human adipose tissue-derived mesenchymal stem cells and its expression is controlled by high glucose. Stem Cells Dev. 2012;21:1749–60.
85. Shin KK, Kim YJ, Hong CP, Yang JW, Bae YC, Jung JS. Retracted article: miR-598 induces replicative senescence in human adipose tissue-derived mesenchymal stem cells via silent information regulator 1. Mol Cell Biochem. 2013;372(1–2):285.
86. Dolezalova D, Mraz M, Barta T, Plevova K, Vinarsky V, Holubcova Z, Jaros J, Dvorak P, Pospisilova S, Hampl A. MicroRNAs regulate p21(Waf1/Cip1) protein expression and the DNA damage response in human embryonic stem cells. Stem Cells. 2012;30:1362–72.
87. Yu KR, Lee S, Jung JW, Hong IS, Kim HS, Seo Y, Shin TH, Kang KS. MicroRNA-141-3p plays a role in human mesenchymal stem cell aging by directly targeting ZMPSTE24. J Cell Sci. 2013;126:5422–31.
88. Baixauli F, Lopez-Otin C, Mittelbrunn M. Exosomes and autophagy: coordinated mechanisms for the maintenance of cellular fitness. Front Immunol. 2014;5:403.
89. Robbins PD, Morelli AE. Regulation of immune responses by extracellular vesicles. Nat Rev Immunol. 2014;14:195–208.
90. Boulias K, Horvitz HR. The C. elegans microRNA mir-71 acts in neurons to promote germline-mediated longevity through regulation of DAF-16/FOXO. Cell Metab. 2012;15:439–50.
91. Shen Y, Wollam J, Magner D, Karalay O, Antebi A. A steroid receptor-microRNA switch regulates life span in response to signals from the gonad. Science. 2012;338:1472–6.
92. Olivieri F, Rippo MR, Monsurro V, Salvioli S, Capri M, Procopio AD, Franceschi C. MicroRNAs linking inflamm-aging, cellular senescence and cancer. Ageing Res Rev. 2013;12:1056–68.
93. Olivieri F, Rippo MR, Prattichizzo F, Babini L, Graciotti L, Recchioni R, Procopio AD. Toll like receptor signaling in “inflammaging”: microRNA as new players. Immun Ageing. 2013;10:11.
94. 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.
95. Vora M, Shah M, Ostafi S, Onken B, Xue J, Ni JZ, Gu S, Driscoll M. Deletion of microRNA-80 activates dietary restriction to extend *C. elegans* healthspan and lifespan. PLoS Genet. 2013;9, e1003737.
96. Dimmeler S, Nicotera P. MicroRNAs in age-related diseases. EMBO Mol Med. 2013;5:180–90.
97. Gordon LB, Rothman FG, Lopez-Otin C, Misteli T. Progeria: a paradigm for translational medicine. Cell. 2014;156:400–7.
98. Pincus Z, Smith-Vikos T, Slack FJ. MicroRNA predictors of longevity in *Caenorhabditis elegans*. PLoS Genet. 2011;7:e1002306.

Chapter 12
Computational Prediction of microRNA Targets

Alessandro Laganà

Abstract Computational prediction of microRNA (miRNA) targets is a fundamental step towards the characterization of miRNA function and the understanding of their role in disease. A single miRNA can regulate hundreds of different gene transcripts through partial sequence complementarity and a single gene may be regulated by several miRNAs acting cooperatively. The remarkable advances made in recent years have allowed the identification of key features for functional miRNA binding sites. A plethora of prediction tools are now available, but their accuracies remain rather poor, as miRNA target recognition has revealed itself to be a very complex and dynamic mechanism, still only partially understood.

In this chapter, the principles of miRNA target prediction in animals are presented, together with the most up-to-date and effective computational approaches and tools available.

Keywords microRNA • Target prediction • Post-transcriptional gene regulation

Introduction

microRNAs (miRNAs) are short endogenous RNA molecules, typically 18–22 bp long, that function as post-transcriptional regulators of gene expression in higher eukaryotes [1, 2]. More than 35,000 miRNAs from more than 200 species have been identified so far according to miRBase, the official miRNA database [3, 4]. miRNAs are the mature products of longer hairpin-shaped precursors called pre-miRNAs. Pre-miRNAs come, in turn, from primary transcripts, called pri-miRNAs, which are transcribed from miRNA genes. A pre-miRNA can encode one or two mature products, one from each arm of the hairpin (5p or 3p), and a single mature

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miRNA can derive from multiple precursors, either identical or highly similar [5]. Mature miRNAs are incorporated into effector protein complexes called RISC (RNA-Induced Silencing Complex) and exert their function by binding specific target mRNAs through perfect or, more often, partial sequence complementarity.

Several models have been proposed to describe the mechanisms underlying miRNA-mediated gene regulation, mainly mRNA destabilization, mRNA cleavage, or translational inhibition [6–14]. Although all these models are plausible, none of them are fully supported by evidence, thus the subject remains highly controversial.

Several experimental techniques have been successfully employed for the identification of miRNA target, whether high-throughput (microarray and RNA-Seq, associated or not with immunoprecipitation) or low-throughput (qRT-PCR, reporter assays, western blot) [15]. Nevertheless, miRNA research relies heavily on computational methods from miRNA detection, annotation, and quantification to target prediction and downstream functional analysis. Computational tools are built based on experimental evidence and help generate new evidence which, in turn, provides feedback to refine computational tools, implementing a fruitful albeit inevitably biased loop.

In this chapter, principles of miRNA targeting and computational methods for target prediction in animals are reviewed, highlighting advantages and limitations of the current approaches as well as discussing challenges.

miRNA Targeting Features

The interaction of miRNAs with their targets is primarily based on sequence complementarity, either full or partial, thus many features and determinants of targeting involve the base composition of miRNA and target sequences. Numerous sequence features of miRNA binding sites and their flanking regions have been identified as critical determinants of targeting activity, the most important being the presence of a short *seed* area in the 5′ end of the miRNA sequence, which is considered essential for target recognition [2]. Many miRNAs are extensively conserved across evolution and can be classified into families, and evidence shows that the binding sites of highly conserved miRNAs are conserved as well [3, 4, 16–19]. Therefore, evolutionary conservation represents another important feature for target prediction. A third layer of targeting features is represented by thermodynamics, which concerns the structural accessibility of miRNA binding sites and the stability of miRNA–target duplexes. Finally, miRNA and gene expression data can be used to improve the performance of target prediction methods.

In the next sections of this chapter, each category of features introduced above will be covered in more detail, along with the tools which implement them. Tools and features are summarized in Tables 12.1 and 12.2.

Table 12.1 Summary of miRNA target prediction tools with the features that they incorporate

Tool	Seed	Mismatch	Context	Cons	Access	Expr	Energy	3′UTR	CDS	IP	Other	Ref
ComiR	•	•	•	•	•	~	•	•			Refine other pred	[77, 78]
CoSMic	•	•	•	•	•	•	•	•			Refine other pred	[58]
DIANA microT	•	•	•	•	•		•	•				[79]
DIANA microT-CDS	•	•	•	•	•		•	•	•	•		[42]
doRiNA	•	•	•	•			•	•		•	Uses PicTar and TargetScan	[63]
EIMMo	•	~		•				•				[80]
EMBL	•	•		•			•	•				[81]
ExprTargetDB	•	•	•	•		•		•			Refine other pred	[82]
Findtar3	•	•		•			•	•				[83]
GenMiR++						•						[55]
HOCTAR	•	•	•	•	•	•	•	•			Refine other pred	[56]
MBSTAR	•	~	•		•		•	•				[41]
MicroCosm	•	•		•			•	•			Uses miRanda	[35]
Microinspector		•					•	•				[84]
microTar	•				~		•	•				[85]
minoTar	•			•					•			[44]
miRanda	•	•		•			•	•				[34, 35]
miRanda-mirSVR	•	•		•			•	•				[36]
miRcode	•			•				•	•		Includes lncRNA	[86]
miRiam		•			•		•					[47]
miRmap	•	•	•	•	•		•	•				[51]
mirMark	•	•	•	•	•		•	•				[38]
miRTarget2	•		•	•	•		•	•				[87]

(continued)

Table 12.1 (continued)

Tool	Seed	Mismatch	Context	Cons	Access	Expr	Energy	3′UTR	CDS	IP	Other	Ref
mirWip	•				•		•	•		•		[59]
MIRZA	•	•			•		•	•		•		[37]
MultiMiTar	•	~	•					•				[88]
PACCMIT	•			•	•		•	•				[89, 90]
PACCMIT-CDS	•			•	•		•	•	•			[43]
PicTar	•	•		•			•	•				[23, 40]
PITA	•	•			•		•	•				[50]
ProbmiR											Based on pattern discovery	[91]
RepTar	•	•						•			Include viral miRNAs	[92]
RNA22							•	•			Based on pattern matching	[93]
RNAHybrid	~	•			~		•	•				[46]
Starbase	•	•	•	•	•		•	•		•	Uses other tools	[64, 65]
StarmiR	•	•			•		•	•	•	•		[49, 60]
SVMicrO	~	•	•	•	•		•	•				[94]
TaLasso	•	•		•		•	•	•			Refine other pred	[57]
TargetMiner	•	~	•	•	•		•	•				[95]
TargetScan	•	•	•	•				•				[16, 17, 21, 24]
TargetSpy	•	~	•		•		•	•				[96]

Seed: perfect seed match required. Mismatch: mismatches in the seed area allowed. Context: sequence context features, e.g., AU, site position. Cons: evolutionary conservation. Access: structural accessibility. Expr: expression data. Energy: free energy. 3′ UTR: predictions available for 3′ UTR sequences. CDS: predictions available for CDS. IP: immunoprecipitation data. The symbol • indicates that the tool makes use of the corresponding feature. The symbol ~ indicates that the corresponding feature is partially used or used in a less stringent way

Table 12.2 Availability and user features for the tools summarized in Table 12.1

Tool	Species	Web	Custom	Download pred	Download tool	Last upd	URL
ComiR	h,m,f,w	•		•	•	2014	www.benoslab.pitt.edu/comir/
CoSMic	h		•		•	2012	www.weizmann.ac.il/complex/compphys/software/cosmic
DIANA microT	h,m,f,w	•				2012	DIANA.imis.athena-innovation.gr/DIANATools/
DIANA microT-CDS	h,m,f,w	•				2012	DIANA.imis.athena-innovation.gr/DIANATools/
doRiNA	h,m,f,w	•	•			2014	dorina.mdc-berlin.de
EIMMo	h,m,f,w,fi	•	~	•		2007	www.mirz.unibas.ch/ElMMo2/
EMBL	f			•		2006	www.russelllab.org/miRNAs/
ExprTargetDB	h	•		•		2010	www.scandb.org/apps/microrna/index.html
Findtar3	h,m,r	•	•			2014	bio.sz.tsinghua.edu.cn
GenMiR++	h		•		•	2008	www.psi.toronto.edu/genmir/
HOCTAR	h	•				2011	hoctar.tigem.it
MBSTAR	h		•	•	•	2014	www.isical.ac.in/~bioinfo_miu/MBStar/MBStar_download20.htm
MicroCosm	multiple	•		•		2008	www.ebi.ac.uk/enright-srv/microcosm/htdocs/targets/v5
Microinspector	multiple	•	•			2012	bioinfo.uni-plovdiv.bg/microinspector/
microTar	m,f,w		•		•	2008	tiger.dbs.nus.edu.sg/microtar/
minoTar	h,f	•		•		2010	www.flyrnai.org/cgi-bin/DRSC_MinoTar.pl
miRanda	h,m,r,f,w	•		•	•	2010	www.microrna.org
miRanda-mirSVR	h,m,r,f,w	•		•		2010	www.microrna.org
miRcode	h	•		•		2012	mircode.org/index.php
miRiam	any		•		•	2010	ferrolab.dmi.unict.it/miriam.html

(continued)

Table 12.2 (continued)

Tool	Species	Web	Custom	Download pred	Download tool	Last upd	URL
miRmap	h,m		•		•	2013	mirmap.ezlab.org/docs/
mirMark	h		•		•	2014	sgithub.com/lanagarmire/MirMark
miRTarget2	h,m,r,d,c	•	•	•		2014	mirdb.org/miRDB/
mirWip	w	•				2008	146.189.76.171/query.php
MIRZA	h	•	•			2013	www.clipz.unibas.ch/index.php?r=tools/mirza/Submission/index
MultiMiTar	h	•	•	•	•	2011	www.isical.ac.in/~bioinfo_miu/multimitar.htm
PACCMIT	h		•	•	•	2012	lcpt.epfl.ch/MicroRNA_target_predictions
PACCMIT-CDS	h		•	•	•	2012	lcpt.epfl.ch/MicroRNA_target_predictions
PicTar	v,fl,w	•		•		2007	pictar.mdc-berlin.de
PITA	h,m,f,w	•	•	•	•	2007	genie.weizmann.ac.il/pubs/mir07/
ProbmiR	any		•		•	2011	www.baskent.edu.tr/~hogul/probmir/
RepTar	h,m	•		•		2010	reptar.ekmd.huji.ac.il
RNA22	h,m	•	•	•		2012	scm.jefferson.edu/rna22v2/
RNAHybrid	any		•		•	2006	bibiserv.techfak.uni-bielefeld.de/rnahybrid
Starbase	h,m	•		~		2014	starbase.sysu.edu.cn/index.php
StarmiR	h,m,w		•			2013	sfold.wadsworth.org/cgi-bin/starmir.pl
SVMicrO	h		•	•	•	2010	compgenomics.utsa.edu/svmicro.html
TaLasso	h	•				2012	talasso.cnb.csic.es
TargetMiner	h	•		•	•	2012	www.isical.ac.in/~bioinfo_miu/targetminer20.htm
TargetScan	h,ma,m,w,f,fi	•	•	•	•	2012	www.targetscan.org
TargetSpy	h,m,r,c,f	•	•	•	•	2009	www.targetspy.org

Species: species considered (h=human, m=mouse, f=fly, w=worm, fi=fish, ma=mammals, r=rat, v=vertebrates, d=dog, c=chicken, multiple=any species, or more than five/six species). Web: program available through web interface. Custom: custom miRNA and/or target sequences accepted. Download pred: collection of predictions is available for download. Download tool: tool is available for download and local use. The symbol • indicates that the tool implements the corresponding feature. The symbol ~ indicates that the corresponding feature is partially implemented

Seed Match

The seed sequence of an miRNA is defined as the 6/7 nucleotides (nt) region at the 5′ end of the mature miRNA. miRNA targets usually exhibit perfect Watson–Crick (WC) complementarity (i.e., A-U and G-C) to the seed region, as evidence shows it is the area used by the RISC as a nucleation signal for target recognition. Thus, seed match constitutes a fundamental feature of miRNA/mRNA binding that most prediction algorithms rely on. There are four types of canonical seed matches (see Fig. 12.1a):

- 6mer: the basic seed type, a perfect WC match between nucleotides 2 and 7 of the miRNA and the target site.
- 7mer-A1: a 6mer augmented by an A at target position 1.

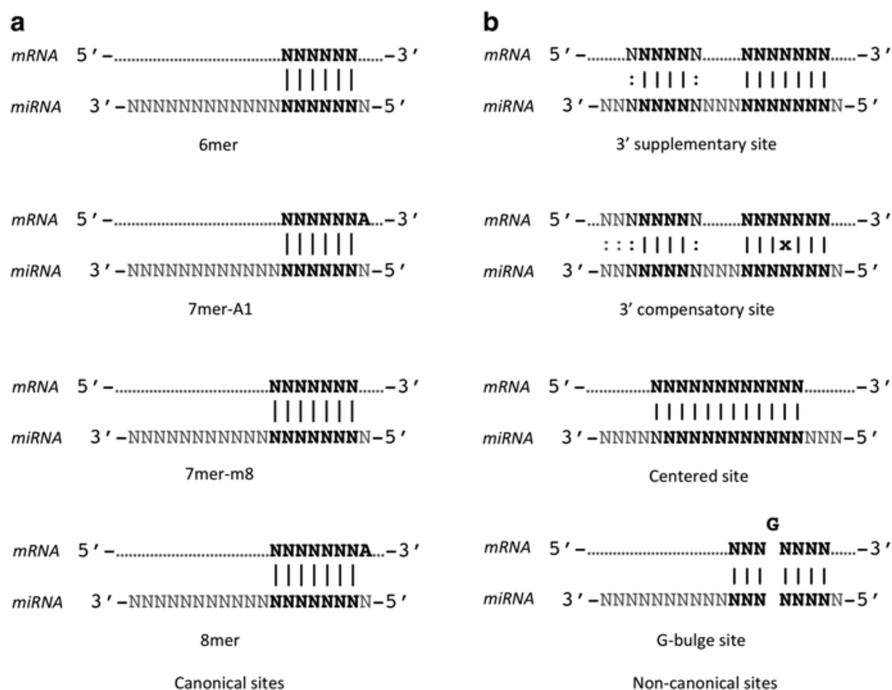

Fig. 12.1 Different types of seed match. (**a**) Canonical sites. They involve perfect complementarity of the seed region of the miRNA, a consecutive stretch of 6/7 nucleotides starting at position 2. The characters in *bold* indicate the miRNA seed sequence and its binding site. The symbol "|" indicates WC binding. (**b**) Noncanonical sites. The characters in *bold* indicate sufficient minimal stretches of paired nucleotides. The symbol "|" indicates WC binding. The symbol "x" indicates a mismatch. *Non-bold* and *grey* characters associated with the symbol ":" indicate optional binding

- 7mer-m8: a perfect WC match between nucleotides 2 and 8 of the miRNA and the target site.
- 8mer: a 7mer-m8 augmented by an A at target position 1.

These types of seed match were first described and characterized by Bartel, and implemented in TargetScan, one of the most popular tools for miRNA target prediction [2, 16, 17, 20, 21]. Seed match is the most widely employed feature for target recognition, often used on its own for fast estimation of miRNA targets in preliminary studies [16, 22, 23]. Further research has revealed the presence of an extended set of seed matches, which includes the offset 6mer site, that is a shifted 6mer at positions 3–8 of the miRNA, and 3′ supplementary and compensatory sites (see Fig. 12.1b) [2].

In particular, 3′-supplementary sites are an extension of the 6mer, 7mer-A1, 7mer-m8, and 8mer sites where the additional pairing of at least 3 nucleotides, usually starting at positions 13 of the miRNA, is observed. In 3′-compensatory sites, instead, pairing of at least 4 nucleotides starting at miRNA nucleotides 12 or 13 can compensate for a seed mismatch and thereby create a functional site.

Seed match type usually correlates with miRNA repression efficacy, according to the following hierarchy: 8mer > 7mer-m8 > 7mer-A1 > 6mer [24]. Several studies have shown that mRNAs, especially their 3′ UTRs (UnTranslated Regions), are particularly enriched with miRNA-seed matching sites highly conserved across evolution [16, 17].

The seed match feature has been accepted as a rule and is implemented by the majority of target prediction tools [25]. As a consequence, binding sites exhibiting a perfect match to an miRNA seed are more likely to be predicted and validated. This, in turn, leads to a positive reinforcement of the dominance of the seed site, as the datasets of experimentally validated targets contribute to refine target prediction methods [26]. Nevertheless, the presence of a seed match for an miRNA does not necessarily imply a true binding site. A recent work on artificial miRNA has shown that miRNAs which were specifically designed to have one or multiple perfect seed matches to their targets had no repression activity whatsoever, even in the presence of 3′ compensatory sites [27].

Thus, the seed match is not a sufficient indicator of a functional binding site and it can even lead to false negatives as there is evidence of functional sites that don't exhibit perfect complementarity to the miRNA seed. Indeed, recent studies have proven alternative models of miRNA–target interaction. Shin et al. identified a class of miRNA target sites that lack both perfect seed pairing and 3′-compensatory pairing and instead have at least 11 nt of contiguous WC base pairing to the center region of the miRNA at either position 4–14 or 5–15, without substantial pairing to either the 5′ or the 3′ ends of the miRNA (Fig. 12.1b) [28].

Other studies based on Cross-Linking Immunoprecipitation (CLIP) methods such as HITS-CLIP (High-throughput sequencing of RNA isolated by cross-linking immunoprecipitation) and PAR-CLIP (Photoactivatable-Ribonucleoside-Enhanced Crosslinking Immunoprecipitation) showed other types of noncanonical interactions involving seed mismatches and bulges in the seed region [29–32].

Evidence suggests a functional role in the occurrence of bulged seed sites,[1] which feature an extra G in the mRNA between positions five and six of the miRNA (the "pivot" pairing rule) (Fig. 12.1b) [29]. A work by Helwak et al., based on a variant of the CLIP method called CLASH (cross-linking, ligation, and sequencing of hybrids), revealed five classes of interactions with distinct base-pairing patterns [33]. Three of these classes featured canonical seed binding with or without the presence of supplementary base pairing involving the middle or the 3′ end of the miRNA. The other two classes showed binding limited to a region located in the middle and 3′ end of the miRNA, or distributed and even less stable base pairing. Moreover, despite the abundance of literature supporting preferential binding to the 3′ UTR, around 60 % of binding sites were detected in the coding sequence (CDS).

Most tools for miRNA target prediction implement the canonical seed match rule and only a few of them, such as miRanda/mirSVR, MIRZA, and mirMark, are specifically designed to include noncanonical matches [34–38]. However, many tools allow users to relax the constraints on the matches, thus enabling the identification of noncanonical binding sites (see Table 12.1).

In light of what has been discussed so far, sequence complementarity is not sufficient to correctly determine miRNA binding sites and therefore other features must be taken into account.

Conservation of the Binding Site

Many miRNAs are evolutionary conserved, that is, their sequence is maintained across species, and can therefore be classified into families. In particular, mature sequences are often fully conserved, while pre-miRNAs may exhibit a variable degree of conservation. Not surprisingly, the most conserved region of a mature miRNA is the seed sequence [16, 17]. This is a significant feature in the prediction of miRNA targets, as conserved miRNA sequences often imply conserved targets. Many prediction tools employ multiple sequence alignments of 3′UTRs from several species to identify conserved sites for miRNA seeds. This feature can be used in combination with other features to better identify a functional binding site, as in mirSVR or miRMark, or it can be employed as a filtering criterion, as in TargetScan [16, 17, 20]. The latter searches for the presence of 8mer and 7mer sites with various degrees of conservation (broadly conserved across most vertebrates, conserved across mammals or poorly conserved) and then ranks them based on other sequence features which are described in the next section, such as the AU content of the binding area and its position in the 3′UTR. Sites with mismatches in the seed region that are compensated by conserved 3′ pairing are also predicted.

Although conservation can significantly reduce the number of false positives, it is of no use in predicting nonconserved sites or sites for species-specific miRNAs. Moreover, conservation is an indicator of the functional relevance of miRNA

[1] Bulges are unpaired stretches of nucleotides located within one strand of a nucleic acid duplex.

binding sites and, as such, it represents an indirect way to detect them. However, it does not reflect what happens inside a cell, as the molecular machinery has no knowledge of the evolutionary conservation of a given site. Evidence shows that a site can be functional no matter whether it is conserved or not [39]. Therefore, the use of conservation should be limited to specific applications and, preferably, in combination with other features.

Binding Site Location and Sequence Context

As already discussed previously, a large amount of evidence suggests that miRNA binding sites are preferentially located in the 3′UTR of target genes, thus many tools are specifically designed to detect this kind of sites. It has been demonstrated that the presence of multiple binding sites for the same miRNA or for co-expressed miRNAs in 3′UTRs can strongly enhance the degree of repression. This is one of the key features of the PicTar algorithm, which implements a probabilistic model in which miRNAs compete with each other for binding, taking into account synergistic effects of multiple binding sites of one miRNA or several miRNAs acting together [23, 40]. Later works characterized the optimal distance between binding sites for cooperativity and showed that functional sites are preferentially located within a locally AU-rich context, near both ends of the 3′UTR, but not too close to the stop codon [24]. These features were first implemented in TargetScan but were later refined and extended by several other tools based on machine learning approaches such as mirSVR, mirMark, and MBSTAR [36, 38, 41]. In particular, the authors of mirMark were able to identify several discriminant site-level and UTR-level features including matches, mismatches, and bulges at specific positions of the miRNA sequence, the total number of AU matches in the binding sites, and the distance of the binding site from the beginning of the UTR. MBSTAR, instead, considers the number and size of bulges as well as specific nucleotide motifs flanking the binding site as relevant features of functional interactions.

As previously mentioned, recent works based on immunoprecipitation have revealed a significant presence of miRNA binding sites outside of the 3′UTR, in particular within the CDS. Some tools allow users to provide their own target and/or miRNA sequences, thus enabling prediction of binding sites in arbitrary regions, such as the 5′ UTR and the CDS (see Table 12.2). However, DIANA microT-CDS, PACCMIT-CDS, and MinoTar were specifically designed to predict binding sites in the CDS [42–44]. Specifically, PACCMIT-CDS and MinoTar find candidate binding sites within the CDS by searching for conserved motifs complementary to the miRNA sequence. This represents a challenge because of the strong selective pressure aimed at preserving the amino acid sequence and codon usage. Both methods rely on background models which account for and remove bias introduced by conservation at the protein level in order to identify motifs overrepresented specifically due to miRNA function. DIANA microT-CDS, instead, is based on a machine learning approach applied to CLIP data to identify the most relevant features associated with miRNA binding to the CDS, 3′UTR, or both.

Thermodynamic Stability and Structural Accessibility

Unlike conservation, thermodynamic properties of miRNA–target duplexes can be used to explain and model the mechanisms underlying target recognition and stable binding. Most target prediction tools use minimum free energy (MFE) as a measure of the stability of the candidate miRNA–target duplex. Lower energy values indicate stronger binding, and therefore more plausible predictions. Tools such as miRanda, PicTar, and TargetScan make use of RNA folding prediction software, such as Vienna RNA Fold, to estimate free energy of predicted miRNA–target duplexes and filter out candidates above a certain threshold [45]. RNAHybrid, instead, is based on a more sophisticated approach which identifies the energetically most favorable hybridizations of a small RNA to a large RNA [46]. The hybridization MFE of the miRNA and its candidate target is computed by dynamic programming. Canonical interactions can be predicted by forcing a perfect match of the seed. Intramolecular hybridizations, that is, base pairings between target nucleotides or between miRNA nucleotides, are not considered.

In reality, mRNA molecules can fold into highly elaborated secondary and tertiary structures, and a perfect sequence match for an miRNA might not be structurally accessible for binding. The amount of AU nucleotides flanking the binding sites can be used as a proxy to estimate the structural accessibility of the site, as a higher content of AU may be associated with weaker mRNA secondary structure and thus increased accessibility. Several tools attempt to provide more robust and accurate models to evaluate the structural accessibility of miRNA binding sites. miRiam uses a model based on local base pairing probabilities, as computed by the software RNAplfold to identify mRNA regions with a higher probability of being accessible for binding [47, 48]. STarMir primarily relies on the secondary structure of the target as predicted by the tool Sfold [49]. The miRNA–target interaction is modelled as a two-step hybridization reaction: the nucleation at a small accessible site (4 nt long) and the hybrid elongation to disrupt local target secondary structure and form the complete duplex. PITA is based on a slightly different model, which computes the difference between the free energy gained from the formation of the miRNA–mRNA duplex and the energetic cost of unpairing the target to make it accessible to the miRNA [50].

The tool miRmap combines structural accessibility with sequence context features from TargetScan and other probabilistic and conservation features, such as the probability of motif overrepresentation in the 3′UTR and the probability that part of a sequence is under negative selection [51]. The algorithm is based on a multiple linear regression approach, which ranks target site accessibility as the most predictive feature.

A recent work showed that tertiary structure-based modeling of miRNA interactions can reveal structural mechanisms not accessible with current secondary structure-based methods and suggested that the integration of secondary and tertiary structure-based methods can help achieve greater accuracy in miRNA–target prediction [52].

Such models reveal the importance of including structural constraints in the prediction of miRNA–target interactions and, in some cases, successfully explain variability in target repression due to differences in accessibility. However, despite the recent progress, the prediction of secondary and tertiary structure of long mRNA molecules still represents a significant challenge. Furthermore, miRNA–target interactions occur in a dynamic, complex environment in which multiple RNA molecules interact with one another and with proteins; thus, current models are likely to only partially describe the targeting mechanisms and the reactions that take place inside a cell.

The Use of Expression Data

It is commonly accepted that miRNAs can exert their function by either inhibiting the translation of target transcripts or by promoting their degradation. Despite the plausibility of both mechanisms, neither can fully explain the experimental evidence accumulated so far [26]. Two recent articles, based on fish and fly models, described miRNA targeting as a two-step mechanism involving translational repression followed by mRNA decay [53, 54]. According to this model, mRNA translation and degradation are so intimately connected that modulating one can dramatically affect the other.

Although the mechanisms of miRNA action may not appear to be fully relevant for target prediction, changes in the expression of mRNA transcripts and proteins can help discriminate between true and false targets. mirSVR was trained on a dataset of microarray experiments measuring changes in the transcriptome following over-expression of specific miRNAs. Downregulated genes exhibiting a single potential binding site for the transfected miRNA were considered true targets. In general, negative correlation of miRNA and mRNA expression across multiple samples can be an indicator of miRNA targeting and can be used to filter predictions made by any computational methods. The main drawback of this approach is that it is difficult to discriminate between direct and indirect targets. Anticorrelation of the expression of an miRNA–gene pair doesn't necessarily imply that the gene is a direct target of the miRNA, even in the presence of potential binding sites. In fact, the observed anticorrelation might be the effect of a chain of events which does not involve any direct interaction between the miRNA and the candidate target. Nevertheless, expression data can be a particularly helpful resource for refining target prediction in specific cellular context.

For example, GenMiR++ employed a Bayesian approach to generate high-confidence target predictions supported by RNA expression across 88 tissues and cell types, sequence complementarity and comparative genomics [55].

HOCTAR, instead, used expression data to predict targets of intronic miRNAs[2] considering the expression of the host gene as a proxy for the expression of the miRNA

[2] miRNAs encoded within the introns of coding genes.

itself [56]. Targets for intronic miRNAs were first predicted with existing tools, such as miRanda, TargetScan, and PicTar. Then, predicted targets whose expression was anticorrelated with the expression of miRNA host genes were selected through an expression correlation analysis performed by using the tool g:Sorter.

A more recent work called TaLasso proposed the use of LASSO regression with nonpositive constraints to integrate sequence-based prediction with miRNA and gene expression measurement [57]. Results obtained on two public datasets that have paired expression levels of human miRNAs and mRNAs showed that the top ranking interactions recovered by TaLasso were especially enriched in experimentally validated targets and functionally meaningful.

Similarly, the CoSMic algorithm combines sequence-based prediction, from tools such as PITA, TargetScan or miRanda, with miRNA and mRNA expression data. Spearman correlation is used to identify anticorrelated miRNA-target pairs and combined with the prediction scores in order to generate the optimal set of miRNA targets [58].

Inferring Targets from CLIP Data

Immunoprecipitation techniques, such as HITS-CLIP, PAR-CLIP, and CLASH, allow the analysis of protein–RNA interactions. These techniques can be used to isolate RNA bound by the Argonaute protein 2 (Ago2), which is an essential catalytic component of the RISC, and determine AGO–miRNA and AGO–mRNA interactions. These datasets are then combined and analyzed by computational methods in order to predict interaction sites between miRNA and target mRNA. As already mentioned in section “Seed Match,” CLIP methods have allowed the identification of canonical and noncanonical binding sites in 5′ UTR, CDS, and 3′ UTR of target mRNAs.

Several tools have been developed to analyze CLIP data and predict targets for miRNA. mirWIP was designed to infer miRNA binding sites in *Caenorhabditis elegans* based on the immunoprecipitation of the RISC components AIN-1 and AIN-2 [59]. An initial set of miRNA binding sites was generated by RNAhybrid and then filtered on the basis of minimal free energy, phylogenetic conservation, and seed pairing configuration. This dataset was analyzed for features enriched in the 3′ UTR sequences of AIN-IP transcripts. These features were then used to score individual predicted binding sites, and the scores were combined into total miRNA family scores as well as a total target score for each transcript.

Another tool, called MIRZA, was based on a biophysical model of miRNA–target interaction with energy parameters inferred from AGO2 CLIP data [37]. These parameters were position specific and were calculated by maximizing the binding probabilities of miRNAs with the mRNA fragments observed in an AGO2-CLIP sample. MIRZA is able to predict both canonical and noncanonical binding sites.

The most recent versions of DIANA microT and STarmiR are both based on machine learning methods trained on CLIP datasets [42, 60]. In particular, DIANA

microT-CDS makes use of generalized linear models, while STarmiR is based on logistic prediction models.

miRTarCLIP is a system for mining miRNA–target sites from user provided CLIP-Seq and PAR-CLIP sequencing data [61]. It includes a module for pre-processing of RNA-Seq raw data, which automatically removes adaptor sequences and filters low quality reads. The reads are then aligned to 3′ UTR and annotated with validated miRNA binding sites from miRTarBase [62] and predicted sites from TargetScan.

Several databases combining miRNA target predictions and CLIP data are also available online. doRiNA integrates miRNA and RNA Binding Proteins (RBP) from different species (human, mouse, and worm) into one framework [63]. Users are required to select a species, a genome assembly, a target region (e.g., any, CDS, 5′UTR, 3′UTR, intron, intergenic), a data source (e.g., HITS-CLIP, PAR-CLIP, TargetScan/PicTar predictions etc.), and a set of specific regulators from the selected data source (e.g., hsa-let-7a|CLASH, AGO2 PAR-CLIP). A combinatorial search allows users to perform two different queries and filter the results based on their *union* (sites present in either sets), *intersection* (sites present in both sets), *difference* (sites present in one set but not in the other), and *exclusive disjunction* (sites present in either sets but not in both).

StarBase is a web resource which provides users with RNA–RNA and protein–RNA interaction networks inferred from 108 different CLIP datasets [64, 65]. Predictions of miRNA–mRNA interactions supported by CLIP data are available, as well as CLIP supported interactions of miRNAs with other types of ncRNA molecules, such as lncRNA (long noncoding RNA), circRNA (circular RNA), pseudogenes, and sncRNA (small noncoding RNA, including snoRNA, rRNA, etc.). More general CLIP-based data is also available, including binding sites for several RBPs on mRNA, lncRNA, pseudogene, and sncRNA. Other features implemented in StarBase include cancer-related analysis, functional analysis of miRNA, and the prediction of competing endogenous RNA (ceRNA) for a given mRNA[3] [66–68]. StarBase contains ceRNA pairs and regulatory ceRNA networks predicted by overlapping potential microRNA targets, calculated by miRanda/mirSVR, with CLIP data.

Databases of Validated Targets and Consensus Tools

Several resources are available online to assist users in the selection of miRNA targets, including manually curated databases of experimentally validated targets (see Table 12.3). miRTarBase contains over 50,000 miRNA–target interactions from 18 species, collected by manually surveying miRNA literature retrieved through text mining [62]. For each miRNA–target pair, information on the validation methods is

[3] ceRNA are coding or noncoding transcripts that regulate other transcripts by competing for shared miRNAs.

Table 12.3 Availability and user features for target databases and consensus prediction tools

Tool	Species	Type	Download	Last upd	URL	Ref
DIANA TarBase	multiple	Validated	Regist only	2014	DIANA.imis.athena-innovation.gr/DIANATools/index.php?r=tarbase/index	[69]
miRDip	h	Consensus pred	Regist only		dorina.mdc-berlin.de	[71]
miRecords	multiple	Validated +consensus	•	2013	mirecords.biolead.org	[70]
miRTarBase	multiple	Validated	•	2013	mirtarbase.mbc.nctu.edu.tw	[62]

Species: species considered (h=human, multiple=any species or more than two species). Type: validated targets and/or consensus predictions. Download: predictions are downloadable—Regist only indicates that only registered users can download the data. The symbol • indicates that the tool implements the corresponding feature

provided and classified as *strong* and *less strong* evidence. Strong evidence consists of Reporter Assay, Western Blot, and qPCR, while less strong evidence includes microarray, NGS, pSILAC, and other high-throughput techniques. The tool provides detailed information on the binding sites, which is either retrieved from the literature, if available, or predicted by miRanda. Other relevant data provided by the tool includes miRNA–target expression profiles retrieved from experiments deposited in GEO and miRNA target interaction networks.

DIANA-TarBase is another database of experimentally validated targets [69]. The latest release contains more than half a million miRNA–gene interactions curated from published experiments in 356 different cell types from 24 species. For each miRNA–target pair basic information on the validation method, validation type (direct, indirect, or unknown), tissue, and experimental conditions are given.

Another resource which provides users with experimentally validated targets is miRecords [70]. The latest release contains over 2500 interactions from 9 species. Like miRTarBase and TarBase, miRecords contains information on the validation methods and binding data from the original articles.

All three databases described above can be consulted online and downloaded as a flat file. In addition to validated targets, miRecords integrates predictions from 11 different tools. This is a powerful feature that allows users to obtain consensus predictions from multiple sources. In a similar way, the tool mirDIP integrates 12 miRNA prediction datasets from six miRNA prediction tools, allowing users to customize their searches based on targeting features such as conservation and structural accessibility [71].

Finally, tools such as miRo′, miRGator, and miRWalk make use of consensus prediction to infer potential miRNA association with disease, processes, functions, and pathways [72–74] (see Chap. 21 of volume “microRNA and Medical Evidence” for more details on miRNA expression profiling).

The Choice of the Appropriate Tool(s)

Target prediction is a fundamental step in miRNA research. A single miRNA can regulate multiple coding and noncoding transcripts which in turn can be regulated by multiple miRNAs, implementing dynamic and extremely intricate regulatory networks that affect a multitude of biological processes. The disruption of such networks can dramatically alter the phenotype and contribute to disease pathogenesis. Increasingly powerful and sophisticated computational tools are available today for functional analysis of miRNA regulation at different levels and in different contexts. They allow to associate miRNAs with specific phenotypes based on a variety of data such as genomic context, sequence variation, and differential expression of miRNAs and their targets. The correct identification of miRNA targets is therefore crucial for the generation of biologically meaningful hypotheses.

Despite the progress made in the past decade, the molecular mechanisms underlying miRNA-mediated regulation are not yet fully understood, and consequently finding true functional miRNA targets still represent a challenge. A plethora of prediction algorithms are publicly available online, and choosing one can be a really difficult task. All tools implement different (or sometimes just slightly different) computational models based on different combinations of the same targeting features, so the sets of predicted targets may present significant overlaps. Consensus of multiple tools can increase confidence in a prediction. However, if the tools considered are all based on evolutionary conservation, the consensus might be due to a redundant prediction based purely on a single feature. Conversely, an interaction supported by tools based on different features may have higher chances of being true.

Several factors should be taken into account when investigating miRNAs and their targets. First of all, as obvious as it may sound, it is essential to consider the biological system of interest and the data available. Many miRNAs and genes are tissue specific, thus a potentially strong interaction may occur in specific cellular contexts or conditions only (e.g., disease vs. normal tissue) [75]. Expression profiles of miRNA and mRNA or CLIP data can help narrow down the search space and focus on the relevant molecules (e.g., differentially expressed miRNAs and genes), and can be used to select interesting miRNAs and/or genes to study or to filter predictions. Tools which make use of expression and CLIP data can be useful in such cases. However, whether expression data is available or not, it is always advisable to consult multiple tools, and consensus databases make life easier in that regard. In particular, one should consider tools that take into account different targeting features and focus on the interactions with the highest support. This is a common practice, especially in the exploratory phase of a project.

In other cases, one might focus on specific interactions and check if there is a model that could explain them. For example, over-expression or knock out of a specific miRNA might reveal a strong anticorrelated gene, which could then be considered for further analysis. One or more predictors might confirm the targeting or not find any signal of possible interaction. In addition, it may be useful to check

for noncanonical interactions or for binding sites occurring in the 5′UTR or the CDS of the potential target.

Other practical aspects, which are summarized in Table 12.2, may contribute to the choice of prediction tools. Some tools are available through user-friendly web interfaces, where users can browse predicted targets and binding sites and apply appropriate filters based on different parameters. Prediction results are often downloadable as a flat file or Excel sheets. Other tools also allow users to input their own miRNA and/or target sequences and perform the prediction in real time. This option can be really useful when dealing with specific variants of miRNAs and genes (e.g., mutated or edited). Most tools which offer this functionality can be downloaded and used locally, and in some cases it is the only available option. The use of such tools requires issuing commands from a terminal, which may discourage some users.

Finally, performances of the tools should also be taken into consideration. Each algorithm was described in at least one published article and, in many cases, some form of experimental validation of predicted interactions was reported. In other cases, performances were assessed by probability tests and/or by the ability of the tool to correctly identify a set of experimentally validated interactions. Comparisons with other tools were often reported, but usually limited to the most popular or the most similar ones in terms of features incorporated. The importance of the findings and/or the number of original experimentally validated cases reported in the corresponding publications are in most cases considered reasonable and reliable indicators of the quality of a prediction tool and generally contribute to its popularity. Unfortunately, a systematic and unbiased comparison of all available tools is rather difficult and has not been done to date. However, a very recent review presented an empirical evaluation of seven representative miRNA target predictors: PicTar, TargetScan, DIANA microT-CDS, miRanda/mirSVR, EIMMo, mirTarget2, and miRmap [76]. The tools were selected based on several criteria, such as the availability as a web server or a database or pre-computed predictions, the inclusion of human and mouse miRNA, and the generation of a probability score. As the most recent of the selected tools was published in 2012, the tests were performed using a dataset of experimentally validated miRNA targets that were published after 2012. This reduced the bias caused by the potential overlap between the test set and the data used to develop the tools. Performances were assessed at the gene and the duplex level[4] based on different measures, such as AUC (Area Under the ROC curve), MCC (Matthews Correlation Coefficient), sensitivity, specificity, precision, signal-to-noise ratio, and predicted-to-native functional target ratio. Although TargetScan and miRmap showed high overall predictive quality, no tool was consistently better than the others according to all the considered measures. This shows the evident limitations of current approaches and the intrinsic difficulties in the evaluation of their performances.

[4]The gene level test consisted in the prediction of interaction between mRNAs and a given miRNA. The duplex level test consisted in the prediction of interaction between a given fragment of mRNA and a given miRNA.

Conclusion

Despite the advances made in recent years, miRNA target prediction still remains a challenge. Understanding advantages and limitations of currently available methods can help identify areas of further improvement. A few practical aspects should always be taken into consideration in the development of miRNA target predictors, regardless of the methods implemented. Tools should be accessible through user-friendly interfaces and kept up to date with the latest miRNAs reported in miRBase. They should work on both reference and novel miRNA/gene sequences and provide users with detailed result reports. They should also have optimized default settings and allow expert users to customize the search parameters.

More importantly, predictive quality needs to be dramatically improved. This could be achieved not only by adequately combining current knowledge on target recognition, but also by inferring new features from novel data generated by increasingly accurate experimental techniques.

Lastly, unbiased benchmark datasets and standardized evaluation procedures are much needed, in order to fill the gap that currently prevents proper assessment of tools' performances.

References

1. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell. 2004;116:281–97.
2. Bartel DP. microRNAs: target recognition and regulatory functions. Cell. 2009;136:215–33. doi:10.1016/j.cell.2009.01.002.
3. Kozomara A, Griffiths-Jones S. miRBase: integrating microRNA annotation and deep-sequencing data. Nucleic Acids Res. 2011;39:D152–7.
4. Kozomara A, Griffiths-Jones S. miRBase: annotating high confidence microRNAs using deep sequencing data. Nucleic Acids Res. 2013;42:D68–73. doi:10.1093/nar/gkt1181.
5. Du T. microPrimer: the biogenesis and function of microRNA. Development. 2005;132: 4645–52. doi:10.1242/dev.02070.
6. Pillai RS, Bhattacharyya SN, Artus CG, et al. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science. 2005;309:1573–6. doi:10.1126/science.1115079.
7. Maroney PA, Yu Y, Fisher J, Nilsen TW. Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat Struct Mol Biol. 2006;13:1102–7. doi:10.1038/nsmb1174.
8. Eulalio A, Huntzinger E, Nishihara T, et al. Deadenylation is a widespread effect of miRNA regulation. RNA. 2009;15:21–32. doi:10.1261/rna.1399509.
9. Wu L, Fan J, Belasco JG. MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci. 2006;103:4034–9. doi:10.1073/pnas.0510928103.
10. Meister G, Landthaler M, Patkaniowska A, et al. Human Argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs. Mol Cell. 2004;15:185–97. doi:10.1016/j.molcel.2004.07.007.
11. Tuschl T, Zamore PD, Lehmann R, et al. Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev. 1999;13:3191–7.
12. Selbach M, Schwanhäusser B, Thierfelder N, et al. Widespread changes in protein synthesis induced by microRNAs. Nature. 2008;455:58–63. doi:10.1038/nature07228.

13. Baek D, Villén J, Shin C, et al. The impact of microRNAs on protein output. Nature. 2008;455:64–71. doi:10.1038/nature07242.
14. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. Nature. 2010;466:835–40. doi:10.1038/nature09267.
15. Tarang S, Weston MD. Macros in microRNA target identification: a comparative analysis of in silico, in vitro, and in vivo approaches to microRNA target identification. RNA Biol. 2014;11(4):324–33. doi:10.4161/rna.28649.
16. 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. doi:10.1016/j.cell.2004.12.035.
17. Friedman RC, Farh KK-H, Burge CB, Bartel DP. Most mammalian mRNAs are conserved targets of microRNAs. Genome Res. 2008;19:92–105. doi:10.1101/gr.082701.108.
18. Ding J, Zhou S, Guan J. miRFam: an effective automatic miRNA classification method based on n-grams and a multiclass SVM. BMC Bioinformatics. 2011;12:216. doi:10.1186/1471-2105-12-216.
19. Zou Q, Mao Y, Hu L, et al. miRClassify: an advanced web server for miRNA family classification and annotation. Comput Biol Med. 2014;45:157–60. doi:10.1016/j.compbiomed.2013.12.007.
20. Grimson A. A targeted approach to miRNA target identification. Nat Methods. 2010;7:795–7. doi:10.1038/nmeth1010-795.
21. Garcia DM, Baek D, Shin C, et al. Weak seed-pairing stability and high target-site abundance decrease the proficiency of lsy-6 and other microRNAs. Nat Struct Mol Biol. 2011;18:1139–46. doi:10.1038/nsmb.2115.
22. Brennecke J, Stark A, Russell RB, Cohen SM. Principles of microRNA-target recognition. PLoS Biol. 2005;3, e85. doi:10.1371/journal.pbio.0030085.
23. Krek A, Grün D, Poy MN, et al. Combinatorial microRNA target predictions. Nat Genet. 2005;37:495–500. doi:10.1038/ng1536.
24. Grimson A, Farh KK-H, Johnston WK, et al. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell. 2007;27:91–105. doi:10.1016/j.molcel.2007.06.017.
25. Peterson SM, Thompson JA, Ufkin ML, et al. Common features of microRNA target prediction tools. Front Genet. 2014;5:23. doi:10.3389/fgene.2014.00023.
26. Cloonan N. Re-thinking miRNA-mRNA interactions: intertwining issues confound target discovery. Bioessays. 2015;37:379–88. doi:10.1002/bies.201400191.
27. Lagana A, Acunzo M, Romano G, et al. miR-Synth: a computational resource for the design of multi-site multi-target synthetic miRNAs. Nucleic Acids Res. 2014;42:5416–25. doi:10.1093/nar/gku202.
28. Shin C, Nam J-W, Farh KK-H, et al. Expanding the microRNA targeting code: functional sites with centered pairing. Mol Cell. 2010;38:789–802. doi:10.1016/j.molcel.2010.06.005.
29. Chi SW, Hannon GJ, Darnell RB. An alternative mode of microRNA target recognition. Nat Struct Mol Biol. 2012;19:321–7. doi:10.1038/nsmb.2230.
30. Hafner M, Lianoglou S, Tuschl T, Betel D. Genome-wide identification of miRNA targets by PAR-CLIP. Methods. 2012;58:94–105. doi:10.1016/j.ymeth.2012.08.006.
31. Loeb GB, Khan AA, Canner D, et al. Transcriptome-wide miR-155 binding map reveals widespread noncanonical microRNA targeting. Mol Cell. 2012;48:760–70. doi:10.1016/j.molcel.2012.10.002.
32. Martin HC, Wani S, Steptoe AL, et al. Imperfect centered miRNA binding sites are common and can mediate repression of target mRNAs. Genome Biol. 2014;15:R51. doi:10.1186/gb-2014-15-3-r51.
33. Helwak A, Kudla G, Dudnakova T, Tollervey D. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. Cell. 2013;153:654–65. doi:10.1016/j.cell.2013.03.043.
34. John B, Enright AJ, Aravin A, et al. Human microRNA targets. PLoS Biol. 2004;2, e363. doi:10.1371/journal.pbio.0020363.

35. Enright AJ, John B, Gaul U, et al. MicroRNA targets in Drosophila. Genome Biol. 2003;5:R1–14. doi:10.1186/gb-2003-5-1-r1.
36. Betel D, Koppal A, Agius P, et al. Comprehensive modeling of microRNA targets predicts functional non-conserved and non-canonical sites. Genome Biol. 2010;11:R90. doi:10.1186/gb-2010-11-8-r90.
37. Khorshid M, Hausser J, Zavolan M, van Nimwegen E. A biophysical miRNA-mRNA interaction model infers canonical and noncanonical targets. Nat Methods. 2013;10:253–5. doi:10.1038/nmeth.2341.
38. Menor M, Ching T, Zhu X, et al. mirMark: a site-level and UTR-level classifier for miRNA target prediction. Genome Biol. 2014;15(10):500. doi:10.1186/s13059-014-0500-5.
39. Farh KK-H, Grimson A, Jan C, et al. The widespread impact of mammalian MicroRNAs on mRNA repression and evolution. Science. 2005;310:1817–21. doi:10.1126/science.1121158.
40. Lall S, Grün D, Krek A, et al. A genome-wide map of conserved microRNA targets in C. elegans. Curr Biol. 2006;16:460–71. doi:10.1016/j.cub.2006.01.050.
41. Bandyopadhyay S, Ghosh D, Mitra R, Zhao Z. MBSTAR: multiple instance learning for predicting specific functional binding sites in microRNA targets. Sci Rep. 2015;5:8004–12. doi:10.1038/srep08004.
42. Reczko M, Maragkakis M, Alexiou P, et al. Functional microRNA targets in protein coding sequences. Bioinformatics. 2012;28:771–6. doi:10.1093/bioinformatics/bts043.
43. Marin RM, Sulc M, Vanicek J. Searching the coding region for microRNA targets. RNA. 2013;19:467–74. doi:10.1261/rna.035634.112.
44. Schnall-Levin M, Zhao Y, Perrimon N, Berger B. Conserved microRNA targeting in Drosophila is as widespread in coding regions as in 3′UTRs. Proc Natl Acad Sci U S A. 2010;107:15751–6. doi:10.1073/pnas.1006172107.
45. Lorenz R, Bernhart SH, Höner Zu Siederdissen C, et al. ViennaRNA Package 2.0. Algorithms Mol Biol. 2011;6:26. doi:10.1186/1748-7188-6-26.
46. Rehmsmeier M. Fast and effective prediction of microRNA/target duplexes. RNA. 2004;10:1507–17. doi:10.1261/rna.5248604.
47. Lagana A, Forte S, Russo F, et al. Prediction of human targets for viral-encoded microRNAs by thermodynamics and empirical constraints. J RNAi Gene Silenc. 2010;6:379–85.
48. Bernhart SH, Hofacker IL, Stadler PF. Local RNA base pairing probabilities in large sequences. Bioinformatics. 2006;22:614–5. doi:10.1093/bioinformatics/btk014.
49. Long D, Lee R, Williams P, et al. Potent effect of target structure on microRNA function. Nat Struct Mol Biol. 2007;14:287–94. doi:10.1038/nsmb1226.
50. Kertesz M, Iovino N, Unnerstall U, et al. The role of site accessibility in microRNA target recognition. Nat Genet. 2007;39:1278–84. doi:10.1038/ng2135.
51. Vejnar CE, Zdobnov EM. miRmap: comprehensive prediction of microRNA target repression strength. Nucleic Acids Res. 2012;40:11673–83. doi:10.1093/nar/gks901.
52. Gan HH, Gunsalus KC. Tertiary structure-based analysis of microRNA-target interactions. RNA. 2013;19:539–51. doi:10.1261/rna.035691.112.
53. Bazzini AA, Lee MT, Giraldez AJ. Ribosome profiling shows that miR-430 reduces translation before causing mRNA decay in zebrafish. Science. 2012;336:233–7. doi:10.1126/science.1215704.
54. Djuranovic S, Nahvi A, Green R. miRNA-mediated gene silencing by translational repression followed by mRNA deadenylation and decay. Science. 2012;336:237–40. doi:10.1126/science.1215691.
55. Huang JC, Babak T, Corson TW, et al. Using expression profiling data to identify human microRNA targets. Nat Methods. 2007;4:1045–9. doi:10.1038/nmeth1130.
56. Gennarino VA, Sardiello M, Mutarelli M, et al. HOCTAR database: a unique resource for microRNA target prediction. Gene. 2011;480:51–8. doi:10.1016/j.gene.2011.03.005.
57. Muniategui A, Nogales-Cadenas R, Vázquez M, et al. Quantification of miRNA-mRNA interactions. PLoS One. 2012;7:e30766. doi:10.1371/journal.pone.0030766.
58. Bossel Ben-Moshe N, Avraham R, Kedmi M, et al. Context-specific microRNA analysis: identification of functional microRNAs and their mRNA targets. Nucleic Acids Res. 2012;40:10614–27. doi:10.1093/nar/gks841.

59. Hammell M, Long D, Zhang L, et al. mirWIP: microRNA target prediction based on microRNA-containing ribonucleoprotein–enriched transcripts. Nat Methods. 2008;5:813–9. doi:10.1038/nmeth.1247.
60. Liu C, Mallick B, Long D, et al. CLIP-based prediction of mammalian microRNA binding sites. Nucleic Acids Res. 2013;41:e138. doi:10.1093/nar/gkt435.
61. Chou C-H, Lin F-M, Chou M-T, et al. A computational approach for identifying microRNA-target interactions using high-throughput CLIP and PAR-CLIP sequencing. BMC Genomics. 2013;14:S2. doi:10.1186/1471-2164-14-S1-S2.
62. Hsu S-D, Tseng Y-T, Shrestha S, et al. miRTarBase update 2014: an information resource for experimentally validated miRNA-target interactions. Nucleic Acids Res. 2014;42:D78–85. doi:10.1093/nar/gkt1266.
63. Anders G, Mackowiak SD, Jens M, et al. doRiNA: a database of RNA interactions in post-transcriptional regulation. Nucleic Acids Res. 2011;40:D180–6. doi:10.1093/nar/gkr1007.
64. Yang J-H, Li J-H, Shao P, et al. starBase: a database for exploring microRNA-mRNA interaction maps from Argonaute CLIP-Seq and Degradome-Seq data. Nucleic Acids Res. 2010;39:D202–9. doi:10.1093/nar/gkq1056.
65. Li J-H, Liu S, Zhou H, et al. starBase v2.0: decoding miRNA-ceRNA, miRNA-ncRNA and protein-RNA interaction networks from large-scale CLIP-Seq data. Nucleic Acids Res. 2013;42:D92–7. doi:10.1093/nar/gkt1248.
66. Salmena L, Poliseno L, Tay Y, et al. A ceRNA hypothesis: the Rosetta Stone of a hidden RNA language? Cell. 2011;146:353–8. doi:10.1016/j.cell.2011.07.014.
67. Poliseno L, Salmena L, Zhang J, et al. A coding-independent function of gene and pseudogene mRNAs regulates tumour biology. Nature. 2010;465:1033–8. doi:10.1038/nature09144.
68. Sen R, Ghosal S, Das S, et al. Competing endogenous RNA: the key to posttranscriptional regulation. Sci World J. 2014;2014:1–6. doi:10.1155/2014/896206.
69. Vlachos IS, Paraskevopoulou MD, Karagkouni D, et al. DIANA-TarBase v7.0: indexing more than half a million experimentally supported miRNA:mRNA interactions. Nucleic Acids Res. 2015;43:D153–9. doi:10.1093/nar/gku1215.
70. Xiao F, Zuo Z, Cai G, et al. miRecords: an integrated resource for microRNA-target interactions. Nucleic Acids Res. 2009;37:D105–10. doi:10.1093/nar/gkn851.
71. Shirdel EA, Xie W, Mak TW, Jurisica I. NAViGaTing the micronome—using multiple microRNA prediction databases to identify signalling pathway-associated microRNAs. PLoS One. 2011;6:e17429. doi:10.1371/journal.pone.0017429.
72. Lagana A, Forte S, Giudice A, et al. MiRo: a miRNA knowledge base. Database. 2009;2009:bap008. doi:10.1093/database/bap008.
73. Cho S, Jang I, Jun Y, et al. miRGator v3.0: a microRNA portal for deep sequencing, expression profiling and mRNA targeting. Nucleic Acids Res. 2012;41:D252–7. doi:10.1093/nar/gks1168.
74. Dweep H, Sticht C, Pandey P, Gretz N. MiRWalk—database: prediction of possible miRNA binding sites by "walking" the genes of three genomes. J Biomed Inform. 2011;44:839–47. doi:10.1016/j.jbi.2011.05.002.
75. Guo Z, Maki M, Ding R, et al. Genome-wide survey of tissue-specific microRNA and transcription factor regulatory networks in 12 tissues. Sci Rep. 2014;4:1–9. doi:10.1038/srep05150.
76. Fan X, Kurgan L. Comprehensive overview and assessment of computational prediction of microRNA targets in animals. Brief Bioinformatics. 2014:bbu044. doi: 10.1093/bib/bbu044
77. Coronnello C, Benos PV. ComiR: combinatorial microRNA target prediction tool. Nucleic Acids Res. 2013;41:W159–64. doi:10.1093/nar/gkt379.
78. Coronnello C, Hartmaier R, Arora A, et al. Novel modeling of combinatorial miRNA targeting identifies SNP with potential role in bone density. PLoS Comput Biol. 2012;8:e1002830. doi:10.1371/journal.pcbi.1002830.
79. Paraskevopoulou MD, Georgakilas G, Kostoulas N, et al. DIANA-microT web server v5.0: service integration into miRNA functional analysis workflows. Nucleic Acids Res. 2013;41:W169–73. doi:10.1093/nar/gkt393.
80. Gaidatzis D, van Nimwegen E, Hausser J, Zavolan M. Inference of miRNA targets using evolutionary conservation and pathway analysis. BMC Bioinformatics. 2007;8:248. doi:10.1186/1471-2105-8-69.

81. Stark A, Brennecke J, Russell RB, Cohen SM. Identification of Drosophila microRNA targets. PLoS Biol. 2003;1:e60. doi:10.1371/journal.pbio.0000060.
82. Gamazon ER, Im H-K, Duan S, et al. ExprTarget: an integrative approach to predicting human microRNA targets. PLoS One. 2010;5:e13534–8. doi:10.1371/journal.pone.0013534.
83. Ye W, Lv Q, Wong C-KA, et al. The effect of central loops in miRNA: MRE duplexes on the efficiency of miRNA-mediated gene regulation. PLoS One. 2008;3:e1719. doi:10.1371/journal.pone.0001719.
84. Rusinov V, Baev V, Minkov IN, Tabler M. MicroInspector: a web tool for detection of miRNA binding sites in an RNA sequence. Nucleic Acids Res. 2005;33:W696–700. doi:10.1093/nar/gki364.
85. Thadani R, Tammi MT. MicroTar: predicting microRNA targets from RNA duplexes. BMC Bioinformatics. 2006;7:S20–9. doi:10.1186/1471-2105-7-S5-S20.
86. Jeggari A, Marks DS, Larsson E. miRcode: a map of putative microRNA target sites in the long non-coding transcriptome. Bioinformatics. 2012;28:2062–3. doi:10.1093/bioinformatics/bts344.
87. Wang X, El Naqa IM. Prediction of both conserved and nonconserved microRNA targets in animals. Bioinformatics. 2008;24:325–32. doi:10.1093/bioinformatics/btm595.
88. Mitra R, Bandyopadhyay S. MultiMiTar: a novel multi objective optimization based miRNA-target prediction method. PLoS One. 2011;6, e24583. doi:10.1371/journal.pone.0024583.s012.
89. Marin RM, Vanicek J. Efficient use of accessibility in microRNA target prediction. Nucleic Acids Res. 2011;39:19–29. doi:10.1093/nar/gkq768.
90. Marín RM, Vaníček J. Optimal use of conservation and accessibility filters in microRNA target prediction. PLoS One. 2012;7, e32208. doi:10.1371/journal.pone.0032208.
91. Oğul H, Umu SU, Tuncel YY, Akkaya MS. A probabilistic approach to microRNA-target binding. Biochem Biophys Res Commun. 2011;413:111–5. doi:10.1016/j.bbrc.2011.08.065.
92. Elefant N, Berger A, Shein H, et al. RepTar: a database of predicted cellular targets of host and viral miRNAs. Nucleic Acids Res. 2011;39:D188–94. doi:10.1093/nar/gkq1233.
93. Miranda KC, Huynh T, Tay Y, et al. A pattern-based method for the identification of microRNA binding sites and their corresponding heteroduplexes. Cell. 2006;126:1203–17. doi:10.1016/j.cell.2006.07.031.
94. Liu H, Yue D, Chen Y, et al. Improving performance of mammalian microRNA target prediction. BMC Bioinformatics. 2010;11:476. doi:10.1186/1471-2105-11-476.
95. Bandyopadhyay S, Mitra R. TargetMiner: microRNA target prediction with systematic identification of tissue-specific negative examples. Bioinformatics. 2009;25:2625–31. doi:10.1093/bioinformatics/btp503.
96. Sturm M, Hackenberg M, Langenberger D, Frishman D. TargetSpy: a supervised machine learning approach for microRNA target prediction. BMC Bioinformatics. 2010;11:292. doi:10.1186/1471-2105-11-292.

ERRATUM

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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 chapters, and have been corrected in the book.

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Chapter 4: microRNAs Distinctively Regulate Vascular Smooth Muscle and Endothelial Cells: Functional Implications in Angiogenesis, Atherosclerosis, and In-Stent Restenosis

Chapter 5: Mechanistic Role of MicroRNAs in Coupling Lipid Metabolism and Atherosclerosis

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