

MicroRNAs: Control and Loss of Control in Human Physiology and Disease

Min Li · Christian Marin-Muller · Uddalak Bharadwaj ·
Kwong-Hon Chow · Qizhi Yao · Changyi Chen

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Abstract Analysis of the human genome indicates that a large fraction of the genome sequences are RNAs that do not encode any proteins, also known as non-coding RNAs. MicroRNAs (miRNAs) are a group of small non-coding RNA molecules 20–22 nucleotides (nt) in length that are predicted to control the activity of approximately 30% of all protein-coding genes in mammals. miRNAs play important roles in many diseases, including cancer, cardiovascular disease, and immune disorders. The expression of miRNAs can be regulated by epigenetic modification, DNA copy number change, and genetic mutations. miRNAs can serve as a valuable therapeutic target for a large number of diseases. For miRNAs with oncogenic capabilities, potential therapies include miRNA silencing, antisense blocking, and miRNA modifications. For miRNAs with tumor suppression functions, overexpression of those miRNAs might be a useful strategy to inhibit tumor growth. In this review, we discuss the current progress of miRNA research, regulation of miRNA expression, prediction of miRNA targets, and

regulatory role of miRNAs in human physiology and diseases, with a specific focus on miRNAs in pancreatic cancer, liver cancer, colorectal cancer, cardiovascular disease, the immune system, and infectious disease. This review provides valuable information for clinicians and researchers who want to recognize the newest advances in this new field and identify possible lines of investigation in miRNAs as important mediators in human physiology and diseases.

Introduction

After completion of the Human Genome Project (HGP) in 2003, about 20,000–25,000 genes were identified and the sequences of 3 billion base pairs (bp) of human DNA were determined. This revealed many opportunities for biological and biomedical research. The emerging field of genomics is the study of an individual's gene structure, including how the genes interact with one another and with the environment. It might be possible to predict the medical outcome of individuals diagnosed or threatened with disease by looking at their genomes. It is believed that genomics has the potential to change the current standard practice of medicine. The new concept, "personalized genomic medicine," includes the use of genomic information to improve the diagnosis, prognosis, prevention, and treatment of diseases. The goal of personalized medicine is to obtain the best medical outcomes by selecting treatments that best fit a person's genomic profile. Recently, the first department-wide report on the goal of Personalized Health Care was released by the U.S. Department of Health and Human Services, with the goal of delivering "the right treatment, at the right time" for

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M. Li · C. Marin-Muller · U. Bharadwaj · K.-H. Chow ·
Q. Yao · C. Chen (✉)

Molecular Surgeon Research Center, Division of Vascular
Surgery and Endovascular Therapy, Michael E. DeBakey
Department of Surgery-MARB 413, Baylor College
of Medicine, One Baylor Plaza, Mail Stop: BCM390,
Houston, TX 77030-3411, USA
e-mail: jchen@bcm.tmc.edu

Q. Yao · C. Chen
Michael E. DeBakey VA Medical Center,
Baylor College of Medicine, Houston, TX, USA

each individual patient. Personalized genomic medicine is best annotated by the completion of the James Watson project in 2007. This is the first full genome sequenced using the revolutionary rapid-sequencing 454 technology, which marked another milestone in human genome sequencing. This project took only two months to complete using massively parallel sequencing, at approximately one hundredth the cost of traditional capillary electrophoresis methods, at the Baylor College of Medicine Human Genome Sequencing Center and the 454 Life Sciences [1, 2].

Analysis of the human genome indicates that a large fraction of the genome sequences are RNAs that do not encode any proteins. These non-coding RNAs include microRNAs (miRNAs), small nucleolar RNAs, other small regulatory RNAs (many of them yet to be identified), and a large number of longer non-coding RNAs. miRNAs are a group of small non-coding RNA molecules, related to small interfering RNAs (siRNAs), which have been studied extensively [3–8]. Mature miRNAs are 20–22 nucleotides (nt) long; they originate from a longer molecule and are processed in the nucleus into hairpin RNAs of 70–100 nt by the RNA-specific ribonuclease Droscha. The hairpin RNAs are then transported to the cytoplasm via an exportin 5-dependent pathway, where they are further cleaved by a second ribonuclease called Dicer to generate the final mature miRNA [9–12]. Recently, miRNAs have rapidly emerged as important translational regulators of gene expression by partially base-pairing with their target mRNAs at 3'-untranslational region (3' UTR). In mammals, miRNAs are predicted to control the activity of approximately 30% of all protein-coding genes, and they have been shown to participate in the regulation of almost every cellular process examined to date [13]. So far, according to the miRBase database, more than 5,000 miRNAs have been annotated in vertebrates, invertebrates, and plants. In this review, we discuss the current progress of miRNA research, regulation of miRNA expression, prediction of miRNA targets, and the regulatory roles of miRNAs in human physiology and diseases, with a specific focus on miRNAs in cancer, cardiovascular disease, the immune system, and infectious disease. This provides valuable information for clinicians and researchers who want to recognize the newest advances in this new field and identify possible lines of investigation with miRNAs as important mediators in human physiology and diseases.

Discovery, nomenclature, and mechanisms of miRNAs

The gene-silencing mechanism by miRNA or siRNA was first elucidated by Dr. Andrew Fire, Dr. Craig Mello and

colleagues in 1998, who won the Nobel Prize in 2006 [14]. They found that injection of double-stranded (ds) RNA into adult *Caenorhabditis elegans* caused potent and specific interference of RNAs that are homologous to the dsRNA [14]. This has significant impact on specific gene regulation using miRNA or siRNA. The first identified miRNAs were lin-4 and let-7, which are believed to be heterochronic genes controlling the development of *Caenorhabditis elegans* [15, 16]. It has been indicated that lin-4 is upregulated in the first larval (L1) stage inhibiting the expression of its target genes lin-14 and lin-28, whereas the let-7 level is induced at the L3/L4 stage suppressing lin-41 gene expression [15, 16]. Since then, miRNAs have been identified in many species, and it seems that all eukaryotes utilize these miRNAs to regulate gene expression. As mentioned, more than 5,000 miRNAs have been identified in vertebrates, invertebrates, and plants, and this number is continuing to grow.

Given the large numbers of miRNAs and the fast growing discovery of new miRNA molecules, it is necessary to standardize their nomenclature. In the past two years, the number of miRNA hairpin loci documented in the miRBase database, the most commonly used database for miRNA, has grown rapidly, from 2,909 in 36 species (June 2005, release 7) to 5,071 in 58 species (August 2007, release 10), expressing 5,922 distinct mature miRNA sequences [17]. miRNAs are usually given numerical identifiers based on their sequence similarity. For example, if the last assigned miRNA is miR-500, the next miRNA with no similarity to previously identified 22-nt core sequences will be named miR-501. Homologous miRNAs in different species will receive the same name. Sequences with one or two base changes are usually assigned suffixes, such as miR-181a and miR-181b. If miRNAs originate from separate genomic loci in a given organism, they will be given numerical suffixes, such as mir-6-1 and mir-6-2 [18, 19].

The biogenesis of miRNAs has been extensively studied. Initially, miRNAs are transcribed by RNA polymerase II as long primary RNAs (pri-miRNAs), usually several kilobases in length, which are then processed by the RNase III enzyme Droscha into short hairpin structures (~80 bases) called precursor miRNAs (pre-miRNAs). Subsequently, pre-miRNAs are trimmed by the RNase III enzyme Dicer into mature miRNAs with ~22 bases. miRNAs loaded into the RNA-induced silencing complex (RISC) can guide either the translational repression or complete degradation of the target mRNA depending on the pairing complementarity. miRNAs usually base-pair to target mRNA's 3' UTR with imperfect complementarity, resulting in translational repression. With perfect pairing to the target mRNAs, miRNAs can also induce mRNA degradation [20, 21]. The mRNA targets of vertebrate miRNAs

were estimated by comparing conserved complementarity to the seed sequence of the miRNAs, suggesting that about 30% of human genes are regulated by miRNAs, with an average of ~200 mRNA targets per miRNA molecule.

Prediction of miRNA target genes

The speed at which miRNAs have been discovered during the last decade or so is really far greater than the speed at which researchers are able to elucidate their functions. At present, there are 5,071 miRNA loci from 58 species, expressing 5,922 distinct mature miRNA in various animal species (miRBase Database Release 10) [17]. Although reports have been describing roles of individual miRNAs in regulating cell physiology and functions, there is still a long way to go before we can comprehend their roles in cell physiology and disease in the context of the already complex network of proteins. The first step toward elucidating the regulatory role of miRNAs is using computer programs that can efficiently predict the binding site of individual miRNAs in the 3' UTRs of protein-coding mRNAs.

Broadly, there are two issues facing the researcher. The first is to find putative binding sites for a given miRNA, searching 3' UTRs for all genes (target search) and the second is to find putative binding sites for all possible miRNAs in the 3' UTR of a particular protein-coding mRNA (binding site prediction). A list of programs that can be used for the prediction of miRNA targets, as well as miRNA binding sites (most of the resources have both functions), is provided in Table 1 [22–28]. The basis of such search is like any other sequence-based searches, to try to look for Watson-Creek pairing, but has more

complexities associated to it than finding a simple complement [29]. Presently, most of the search algorithms rely heavily on an initial perfect match at the 5' seed region [22]. The hypothesis is supported by the fact that 5' end of miRNAs is better conserved [30] and is perfectly complementary to 3' UTR elements or its known miRNA targets in nonvertebrates [22, 30, 31]. However, the presence of a mere seed match does not ensure efficient binding as several miRNAs with mismatches at the seed are already validated in the literature as efficient blockers of the cognate genes [29]. That is why search algorithms now take into consideration compensatory binding within residues ~30 nucleotides upstream and ~30 nucleotides downstream of the seed site, with weighting tailing off with the inverse of the distance from the seed site so as not to result in false negatives [29]. The major problem in predicting a miRNA-3' UTR interaction correctly is false-positives (which occur at a rate of around 20%) [32]. The search algorithms now incorporate several other parameters to maximize the so-called signal-to-noise ratio. For example, sequence conservation among two (miRBase Target) or more (Targetscan) species is considered. The hypothesis is that highly conserved miRNAs are more likely to be involved in regulation. Unlike miRBase, Targetscan outputs all kinds of sequence conservations and states the degree of conservation for both miRNA families and predicted targets [22]. Additional weight (implemented by Targetscan) is given to miRNAs that present in multiple copies at the 3' UTR of the same gene, as these might have a greater chance in regulating a gene even with a less than perfect complement at the 3' UTR [33].

The Targetscan algorithm searches UTRs in one organism for the presence of conserved 8mer and 7mer sites that match the seed region of each miRNA. It initially

Table 1 List of software widely used to predict miRNA targets

| Software | URL | Input format | | Reference |
|-----------------|---|--------------------|--|-----------|
| miRBase targets | http://microrna.sanger.ac.uk/targets/v5/ | Sequence/gene name | miRNA target, miRNA binding sites | [17] |
| Targetscan | http://www.targetscan.org/ | Sequence/gene name | miRNA target, miRNA binding sites | [22] |
| PicTar | http://pictar.bio.nyu.edu/ | Sequence/gene name | miRNA target, miRNA binding sites | [23] |
| microrna.org | http://www.microrna.org/microrna/home.do | Gene name only | miRNA target, miRNA binding sites, expression profiles | [24] |
| TargetRank | http://hollywood.mit.edu/targetrank/ | Sequence only | miRNA target only | [25] |
| DIANA-MicroT | http://diana.pcbi.upenn.edu/cgi-bin/micro_t.cgi/ | Sequence only | miRNA target, miRNA binding sites | [26] |
| rna22 | http://cbcsrv.watson.ibm.com/rna22_targets.html | Sequence only | miRNA target, miRNA binding sites | [27] |
| TarBase | http://www.diana.pcbi.upenn.edu/tarbase.html | Sequence only | miRNA target, miRNA binding sites, expression profiles | [28] |

Note: In some cases the reference cited might not deal directly with human/vertebrate miRNA prediction but was quoted as the original article

looks for a perfect Watson-Crick complementarity at the seed region, disallowing any wobble pairs (G-U). At the next stage, it allows G-U (wobble) pairs in both directions to extend seed matches, and it stops at mismatches. It then uses the RNAfold program [34] to calculate the thermodynamic free energy of the interaction. After optimizing base-pairing in this way, it searches the remaining 3' region of miRNA, precisely 35 bases of UTR 5' to each seed match. At the next stage, it calculates the folding free energy (G) assigned to each putative miRNA:target interaction and assigns rank to each UTR. It then assigns context scores, calculated as the context of the site, which is important for site efficacy, and these scores can be used to predict site performance.

This process is repeated for each of the organisms with UTR data sets. By using increasingly stringent criteria, these various programs can reduce the number of predicted potential miRNA-3' UTR interactions. However, no program can guarantee with 100% assurance that the predicted mRNA is actually a target of the miRNA, and therefore there is a need for resources that can test these initial predictions against recorded experimentally validated miRNA targets. For example, microRNA.org (<http://www.microrna.org>) is a resource for miRNA target prediction and miRNA expression that is widely used by the research community [35]. Apart from predicting targets using the miRanda algorithm, it also investigates predicted miRNA targets and their binding sites, as well as miRNA expression in various tissues, using the miRNA expression profiles taken from a recent profiling study across 250 small RNA libraries collected from human, mouse, and rat tissues and cell lines [36]. This definitely enhances the ideas of researchers about miRNAs of interest with respect to function and/or preferred expression sites.

The key issue for a busy researcher is how quickly and efficiently it is possible to get an idea about the targets for a particular miRNA of interest or to find out which miRNAs could probably regulate the protein of interest. For example, Notch1 expression is already known to be regulated by miR-23b, miR-24, miR-27 [37], and miR-34 [38]. When we search the 3' UTR for Notch1 using three resources—miRBase Target, Targetscan, and microRNA.org—we get varied results (Table 2). As validated [38] for binding to Notch1 3' UTR, miR-34 is one of the predicted binding miRNAs when both Targetscan and microRNA.org are used (Table 2, shown in bold), but the miRBase Target fails to predict it. Similarly, we present the predicted targets for three other genes—endothelial nitric oxide synthase (eNOS), zinc transporter SLC39A4 and mesothelin—in Table 2. We found that the results from the different programs for the same gene are not completely similar although with variable overlaps. For searching targets of a miRNA, miRBase could be used for initial

screening, as the user interface is easy (inputting the name of miRNA) and the output screen has an option to look at the analysis using two other servers Targetscan and PicTar. As discussed earlier, the latter programs have an option to use higher stringency. Some other programs such as TarBase have ambitious and elaborate interfaces, recording and assimilating the sequence annotation such as genomic organization, precursor sequences and literature citations, expression, and transcriptional regulation of miRNAs [28].

Roles and mechanisms of miRNAs in cancer

miRNAs play important roles in many biological processes including cell growth, apoptosis, hematopoietic lineage differentiation, and gene regulation, and miRNAs are also involved in a wide variety of human diseases such as cancer, vascular disease, immune disease, and infections. Recently, aberrant expression patterns of miRNA have been reported in many human cancers. More than 50% of human miRNA genes are thought to be located in cancer-associated regions or at fragile sites of chromosomes, which are hot spots for gene deletion, amplification, and mutations. Many genomic profiling studies have indicated a general downregulation of miRNAs in various tumors, including pancreatic cancer, breast cancer, prostate cancer, liver cancer, colon cancer, and ovarian cancer, suggesting a more negative regulation of cancer growth by miRNAs [39–47]. miR-17-92 cluster, which is amplified in human B-cell lymphomas, and miR-155, which is upregulated in Burkitt's lymphoma, were reported as the first human miRNA oncogenes [48–50]. Interestingly, miRNA-based classification of poorly differentiated tumors seems to have been more accurate than mRNA profiles in the same group of patients [51]. These studies suggest that miRNA profiling could be a useful tool in cancer diagnosis and prognosis, and miRNAs may also serve as effective therapeutic targets.

Recent studies on the tumor-suppressing or tumor-promoting activities of miRNAs using both cell culture and animal models support the hypothesis that miRNAs can serve as either oncogenes or tumor suppression genes. miR-15 and miR-16 are the first defined miRNAs with tumor suppressor functions. Previous studies have shown that B-cell chronic lymphocytic leukemia (B-CLL) is associated with loss of chromosomal region 13q14. Calin et al. found that mi-R15 and mi-R16 are located within a 30-kb region at chromosome 13q14, a region deleted in more than half of B-CLL (68%) [52]. miR-15- and miR-16-induced tumor suppression appears to be mediated through downregulating the anti-apoptotic protein Bcl2. Bcl2 is frequently over-expressed in CLL and the 3' UTR of the Bcl2 mRNA contains potential binding sites for

Table 2 Comparison of predictive powers of various programs using validated miR-Target gene interactions and genes without any reported miRNA validation

| Gene | miRBase | Targets | Targetscan | miRNA.org | |
|-------------------------|----------------------|----------------|----------------------|-----------------------------|----------------|
| eNOS (unreported) | <i>Predicted: 44</i> | hsa-miR-507 | <i>Predicted: 24</i> | <i>Predicted: Not found</i> | |
| | hsa-miR-125a-3p | hsa-miR-150* | hsa-miR-492 | | |
| | hsa-miR-362-5p | hsa-miR-885-3p | hsa-miR-765 | | |
| | hsa-miR-650 | hsa-miR-516b | hsa-miR-335 | | |
| | hsa-miR-500 | hsa-miR-627 | hsa-miR-593 | | |
| | hsa-miR-185* | hsa-miR-555 | hsa-miR-506 | | |
| | hsa-miR-502-5p | hsa-miR-543 | hsa-miR-124 | | |
| | hsa-miR-214 | hsa-miR-609 | hsa-miR-502 | | |
| | hsa-miR-506 | hsa-miR-492 | hsa-miR-362 | | |
| | hsa-miR-526b | hsa-miR-623 | hsa-miR-155 | | |
| | hsa-miR-525-3p | hsa-miR-184 | hsa-miR-765 | | |
| | hsa-miR-155 | hsa-miR-220b | hsa-miR-583 | | |
| | hsa-miR-31 | hsa-miR-889 | hsa-miR-524 | | |
| | hsa-miR-299-3p | hsa-miR-576-5p | hsa-miR-124a | | |
| | hsa-miR-518c* | hsa-miR-639 | hsa-miR-488 | | |
| | hsa-miR-524-3p | hsa-miR-325 | hsa-miR-525 | | |
| | hsa-miR-105* | hsa-miR-564 | hsa-miR-34a | | |
| | hsa-miR-593* | hsa-miR-886-5p | hsa-miR-449b | | |
| | hsa-miR-369-3p | hsa-miR-631 | hsa-miR-34c | | |
| | hsa-miR-571 | hsa-miR-523 | hsa-miR-449 | | |
| hsa-miR-886-3p | | hsa-miR-543 | | | |
| hsa-miR-330-5p | | hsa-miR-31 | | | |
| hsa-miR-576-3p | | hsa-miR-552 | | | |
| hsa-miR-510 | | hsa-miR-584 | | | |
| hsa-miR-744 | | hsa-miR-558 | | | |
| SLC39A4 (unreported) | <i>Predicted: 13</i> | hsa-miR-92a-1* | <i>Predicted: 8</i> | <i>Predicted: 16</i> | hsa-miR-487b |
| | hsa-miR-593 | hsa-miR-571 | hsa-miR-625 | hsa-miR-214 | hsa-miR-181a |
| | hsa-miR-92a-2* | hsa-miR-299-3p | hsa-miR-224 | hsa-miR-15b | hsa-miR-361-5p |
| | hsa-miR-29b-2* | hsa-let-7e* | hsa-miR-769-3p | hsa-miR-15a | hsa-miR-424 |
| | hsa-miR-511 | hsa-miR-758 | hsa-miR-638 | hsa-miR-497 | hsa-miR-16 |
| | hsa-miR-29b-1* | | hsa-miR-758 | hsa-miR-503 | hsa-miR-449a |
| | hsa-miR-769-3p | | hsa-miR-612 | hsa-miR-383 | hsa-miR-486-5p |
| | hsa-miR-187 | | hsa-miR-571 | hsa-miR-195 | hsa-miR-708 |
| hsa-miR-450b-3p | | hsa-miR-511 | hsa-miR-486-3p | | |
| Mesothelin (unreported) | <i>Predicted: 27</i> | hsa-miR-542-5p | <i>Predicted: 9</i> | <i>Predicted: Not found</i> | |
| | hsa-miR-608 | hsa-miR-515-3p | hsa-miR-214 | | |
| | hsa-miR-744 | hsa-miR-769-3p | hsa-miR-542-5p | | |
| | hsa-miR-663 | hsa-miR-638 | hsa-miR-663 | | |
| | hsa-miR-615-5p | hsa-miR-363* | hsa-miR-608 | | |
| | hsa-miR-92a-2* | hsa-miR-187 | hsa-miR-635 | | |
| | hsa-miR-542-5p | hsa-miR-220b | hsa-miR-17-3p | | |
| | hsa-miR-632 | hsa-miR-92a-1* | hsa-miR-632 | | |
| | hsa-miR-194* | hsa-miR-621 | hsa-miR-346 | | |
| | hsa-miR-887 | hsa-miR-325 | hsa-miR-187 | | |
| | hsa-miR-187 | hsa-miR-145* | | | |
| | hsa-miR-491-5p | hsa-miR-611 | | | |
| | hsa-miR-632 | hsa-miR-99b | | | |
| | hsa-miR-636 | | | | |
| hsa-miR-214 | | | | | |

Table 2 continued

| Gene | miRBase | Targetscan | miRNA.org |
|-------------------|----------------------|---------------------|-----------------------|
| Notch1 (reported) | <i>Predicted: 10</i> | <i>Predicted: 5</i> | <i>Predicted: 39</i> |
| | hsa-miR-608 | hsa-miR-34a | hsa-miR-200b |
| | hsa-miR-516b | hsa-miR-34b | hsa-miR-582-5p |
| | hsa-miR-490-3p | hsa-miR-34c | hsa-miR-433 |
| | hsa-miR-577 | hsa-miR-449 | hsa-miR-509-3p |
| | hsa-miR-582-5p | hsa-miR-449b | hsa-miR-101 |
| | hsa-miR-139-5p | | hsa-miR-449b |
| | hsa-miR-200b | | hsa-miR-425 |
| | hsa-miR-433 | | hsa-miR-429 |
| | hsa-miR-509-3p | | hsa-miR-139-5p |
| | hsa-miR-100 | | hsa-miR-200c |
| | | | hsa-miR-603 |
| | | | hsa-miR-449a |
| | | | hsa-miR-374a |
| | | | hsa-miR-34c-5p |
| | | | hsa-miR-34a |
| | | | hsa-miR-374b |
| | | | hsa-miR-421 |
| | | | hsa-miR-548c-3p |
| | | hsa-miR-150 | |
| | | hsa-miR-155 | |
| | | hsa-miR-371-5p | |
| | | hsa-miR-873 | |
| | | hsa-miR-340 | |
| | | hsa-miR-181d | |
| | | hsa-miR-19a | |
| | | hsa-miR-19b | |
| | | hsa-miR-154 | |
| | | hsa-miR-183 | |
| | | hsa-miR-369-3p | |
| | | hsa-miR-144 | |
| | | hsa-miR-221 | |
| | | hsa-miR-128a | |
| | | hsa-miR-128b | |
| | | hsa-miR-196b | |
| | | hsa-miR-423-5p | |
| | | hsa-miR-598 | |
| | | hsa-miR-106a | |
| | | hsa-miR-146b-5p | |
| | | hsa-miR-802 | |

* A miRNA which is derived from the same precursor miRNA as the regular miRNA (without asterisk), but is less abundant and having a different mature sequence as compared with the regular miRNA

Note: Boldface indicates a predicted miRNA which has already been experimentally validated

miR-15 and miR-16. Expression of these miRNAs causes downregulation of Bcl2 and induces apoptosis in a leukemia cell line [53, 54]. Other examples include miR-29, which suppresses DNA methyltransferase (DNMT)-3A and -3B in lung cancer [55]; let-7, which regulates the expression of *RAS* and other genes involved in cell cycle and cell division functions in lung cancer [56]; and miR-34, which suppresses cell growth in ovarian cancer and colon cancer [57, 58].

In addition to the tumor suppressor function, miRNAs can also serve as oncogenes to promote cancer growth. B-cell integration cluster (BIC)/miR-155 is the first miRNA shown to have the tumor-promoting activity. Overexpression of BIC/miR-155 was observed in a variety of cancers, and a transgenic mice line with BIC/miR-155 developed a polyclonal B-cell malignancy, suggesting that BIC/miR-155 is an oncogene [59]. Although a molecular target, angiotensin II type I receptor, has been indicated for miR-155, the mechanisms by which BIC/miR-155 promotes tumorigenesis remain unknown. miR-10b is another oncogene that is highly associated with cancer metastasis; it is over-expressed in metastatic breast cancer cells and promotes cell migration and invasion. The transcription of miR-10b is regulated by the transcription factor Twist, and the downstream targets of miR-10b include homeobox D10. The inhibition of homeobox D10 by miR-10b increases the

expression of RHOC, a well-characterized pro-metastatic gene, leading to tumor cell invasion and metastasis [60]. Other miRNAs as oncogenes include miR-17 clusters in B-cell lymphoma [61], miR-21 in glioblastoma [62], and miR-373 and miR-520c as metastasis-promoting miRNAs [63].

miRNA profiling studies in tumor and normal tissues have revealed the essential role of these molecules, and dysregulation of miRNAs in various cancers suggests an additional regulation mechanism by miRNAs in human cancers, including pancreatic cancer, liver cancer, and colorectal cancer among many other cancer types.

miRNAs and pancreatic cancer

Pancreatic cancer is the fourth leading cause of cancer-related death in America, and it holds the number 1 fatality rate of all cancers. Previous mRNA profiling studies have suggested many promising markers for early diagnosis and therapy in pancreatic cancer. However, the accuracy in distinguishing the tumor from normal samples is yet to improve [64–67]. With the discovery of miRNAs, more and more investigators are studying the miRNA signatures in pancreatic cancer. A recent profiling study using 28 pancreatic tumors, 15 adjacent benign tissues, 4 chronic pancreatitis specimens, 6 normal pancreas tissues, and 9 pancreatic cancer cell lines identified several miRNAs

aberrantly expressed in pancreatic cancer or desmoplasia, including miRNAs previously reported as differentially expressed in other human cancers, such as miR-155, miR-21, miR-221, and miR-222, as well as some not previously reported in cancers, such as miR-376a and miR-301. Most of the top aberrantly expressed miRNAs are downregulated in tumor tissues [39].

Szafrańska et al. [68] found that miR-205, -18a, -31, -93, -221, and -224 are over-expressed in both primary neoplastic ductal cells and in established pancreatic cancer cell lines. Therefore, these miRNAs may represent potential biomarkers for pancreatic cancer. They also identified 26 miRNAs most prominently misregulated in pancreatic cancer, and a reverse transcriptase-polymerase chain reaction (RT-PCR) index using only miR-217 and -196a was found to discriminate between normal pancreas, chronic pancreatitis, and cancer tissues, suggesting a potential utility for miRNAs in diagnostic procedures for pancreatic cancer.

Another study investigating the global miRNA expression patterns in normal pancreas, pancreatic endocrine tumors, and acinar carcinomas indicated that the expression of miR-103 and miR-107, associated with lack of expression of miR-155, discriminates tumor from normal tissues [69]. A set of 10 miRNAs distinguishes endocrine from acinar tumors and is possibly associated with either normal endocrine differentiation or endocrine tumorigenesis. miR-204 is primarily expressed in insulinomas and correlates with immunohistochemical expression of insulin. They also found that the overexpression of miR-21 is strongly associated with cell proliferation and liver metastasis. The same study suggests that alteration in miRNA expression is related to endocrine and acinar neoplastic transformation and progression of malignancy.

Bloomston et al. [70] found that 21 miRNAs with increased expression and 4 with decreased expression could differentiate pancreatic cancer from benign pancreatic tissues in 90% of samples by cross validation. Fifteen over-expressed and 8 downregulated miRNAs could differentiate pancreatic cancer from chronic pancreatitis with 93% accuracy. A subgroup of 6 miRNAs was able to distinguish long-term survivors with node-positive disease from patients who would die within 24 months. They also found that high expression of miR-196a-2 could predict poor survival (14.3 months versus 26.5 months) of pancreatic cancer patients.

miRNAs and hepatocellular carcinoma

Hepatocellular carcinoma (HCC) ranks as the fifth most common cancer worldwide and is the third leading cause of cancer deaths. The high fatality rate is due to diverse etiology, lack of diagnostic markers for early diagnosis, and the highly variable clinical course of HCC [71]. Several

signaling pathways involved in HCC development and progression have been recognized, and specific molecular signatures have been associated with different etiologic factors, biological characteristics, and clinical evolution of HCC [72, 73]. Recent studies have indicated that aberrant expression of miRNAs also plays an important role in HCC pathogenesis.

miR-122 was first identified as the most frequent miRNA in the liver in 2002 [74], and was reported to be significantly and specifically downregulated in HCC in both humans and rodents. A genome-wide miRNA microarray study indicated that miR-122a was downregulated in 70% of HCCs and in all HCC-derived cell lines [41]. miR-122a can regulate cyclin G1 expression in HCC-derived cell lines, and an inverse correlation between miR-122a and cyclin G1 expression exists in primary liver carcinomas [41, 74]. Other putative target genes of miR-122 include N-Myc and DRLM in liver cancer [75, 76].

miR-1 was also suggested as a tumor suppressor in HCC. miR-1-1 was methylated in HCC cell lines and in primary human HCCs, but not in matching liver tissues. miR-1 expression was also markedly reduced in primary human hepatocellular carcinomas compared with matching normal liver tissues. Ectopic expression of miR-1 in HCC cells inhibited cell growth and reduced replication potential and clonogenic survival. FoxP1 and MET are predicted targets for miR-1 and contain three and two miR-1 binding sites in their 3' UTR, respectively. The inhibition of cell cycle progression and induction of apoptosis after restoration of miR-1 expression are possible mechanisms by which DNA hypomethylating agents suppress hepatocarcinoma cell growth [77].

Highly dysregulated miR-223 could also distinguish HCC from adjacent normal liver tissues. Overexpression of miR-223 in hepatitis B virus (HBV), hepatitis C virus (HCV), and non-HBV non-HCV-related HCC cell lines caused inhibitory effect on cell viability. Stathmin 1 (STMN1) was suggested as a downstream target of miR-223, because a strong inverse relationship between STMN1 mRNA and miR-223 expressions was observed. These data indicate a specific role of miR-223 downregulation in the development of HCC [78].

miRNAs and colorectal cancer

Colorectal cancer remains a leading cause of mortality worldwide. It is second only to lung cancer as the leading cause of death from cancer. Chemotherapy has significant therapeutic effects, but the only curative treatment is still surgical resection [47]. New diagnostic markers and therapeutic targets are needed for this malignancy. Because of the accurate discrimination between tumor and normal tissues, the specific expression patterns of miRNAs can

make them promising candidates for a new marker in colorectal cancer.

A recent study found that 37 miRNAs were differentially expressed in colon cancer samples from a cohort. Five miRNAs (miR-20a, miR-21, miR-106a, miR-181b, and miR-203) were selected for validation in tumors. Higher miR-21 expression was present in adenomas and in tumors with more advanced TNM staging, and was found to be associated with poor survival and poor therapeutic outcome [47]. This association may help predict the benefit of therapy in individual patients whose miR-21 expression status is known, and it may help identify patients who are candidates for more aggressive initial therapies. If high miR-21 expression is the reason for the poor therapeutic outcome, the antisense therapeutics targeting miR-21 may have benefits in patients with high expression of miR-21 [47]. Indeed, inhibition of miR-21 decreases cell growth both in vitro and in xenograft mouse models through downregulation of Bcl-2 [79]. Other targets of miR-21 include the tumor suppressor genes, the phosphatase and tensin homolog (PTEN), and tropomyosin 1 (TPM1) [80, 81].

Two independent studies have examined the miRNA profile of colorectal cancer compared with normal colonic epithelium [82, 83]. Both studies described a general downregulation of miRNAs in tumor cells, and two miRNAs, miR-143 and miR-145, were found to be significantly lower in colorectal tumor cells than in normal colon cells [21]. The cell growth of human colon cancer cell lines was significantly inhibited in a dose-dependent manner after transfection with either miR-143 or miR-145 precursor miRNA [84]. One of the target genes of miR-143 is ERK5 in the human colon cancer cell line DLD-1, indicating that miR-143-induced tumor suppression is mediated, at least in part, by inhibition of the mitogen-activated protein kinase (MAPK) pathway. Other miRNAs involved in colorectal cancer pathogenesis include miR-34b and miR-34c, two components of the p53 network, as well as miR-342, all of which are epigenetically silenced in colorectal cancer [85, 86].

Roles and mechanisms of miRNAs in cardiovascular disease

A number of studies have shown that miRNAs are responsible for regulating such biological processes as metabolism, proliferation, differentiation, apoptosis, development, hematopoiesis, and oncogenesis. In addition, many miRNAs are differentially expressed in different tissues, suggesting that they may be involved in regulating tissue-specific functions. A few studies have looked at the role of miRNAs in vascular tissue. An earlier study showed that mouse mutants with the first and second exons of the

dicer gene deleted had severe impairments in blood vessel formation of the developing embryo and altered expression of the angiogenic regulators vegf, flt1, kdr, and tie-1 [87]. One group of investigators found that lowering miRNA levels by knocking down Dicer expression in human microvascular endothelial cells results in decreased production of reactive oxygen species and decreased angiogenesis [88]. Another group found that downregulation of Dicer results in decreased levels of miR-27b and let-7f, which leads to decreased capillary sprouting of endothelial cells [89]. Another study showed that expression of miR-221 and miR-222 in human umbilical vein endothelial cells decreases c-Kit and angiogenesis induced by its ligand stem cell factor [90]. All of these studies indicate that miRNA plays a role in regulating normal vascular development and function.

Normal functioning of the vascular system involves dynamic interactions between endothelial cells, vascular smooth muscle cells (VSMCs), and monocyte-derived macrophages. Consequently, vascular disease occurs when those cells are not functioning properly. The term “vascular disease” encompasses many conditions, such as atherosclerosis, coronary artery disease, peripheral artery disease, venous disease, stroke, and post-angioplasty restenosis. The formation of vascular lesions in these diseases can occur via a number of pathophysiological processes. One investigative group showed that a specific polymorphism in the 3' UTR of the human angiotensin II type 1 receptor expressed in endothelial and vascular smooth muscle cells may contribute to vascular thickening and atherosclerosis because binding of miR-155 and subsequent translational repression is disrupted [91].

It is widely accepted that atherosclerosis is an inflammatory process [92]. Monocytes, monocyte-derived macrophages, and T lymphocytes are critical in the development of atherosclerosis and are frequently found even in the earliest atherosclerotic lesions. An important component of this inflammatory process is leukocyte adhesion to endothelial cells mediated by the vascular cell adhesion molecule 1 (VCAM-1). One study has shown that miR-126 in endothelial cells inhibits VCAM-1 expression and blocks tumor necrosis factor- α (TNF- α)-induced leukocyte binding [93]. miR-155 has been shown to be substantially upregulated in monocytes and macrophages after stimulation with various inflammatory molecules [94]. These findings suggest that miRNAs may play an important role in mediating inflammation-driven vascular lesion formation. In addition, antisense therapy targeting miR-122 significantly decreases hepatic fatty-acid and cholesterol synthesis rates in a diet-induced obesity mouse model, leading to decreased plasma cholesterol levels and a significant improvement in liver steatosis [95]. This study implicates miR-122 as a key regulator of cholesterol and

fatty-acid metabolism in the adult liver, indirectly affecting atherosclerosis formation.

One study looked at the role of miRNA in the vascular system using a rat carotid artery balloon injury model [96]. Specifically, the miRNA expression pattern in rat carotid arteries is altered after angioplasty. In addition, downregulation of miR-21 was found to be highly upregulated post-injury, via anti-sense depletion that decreased neointimal lesion formation. Consistent with this finding, inhibition of miR-21 also decreased proliferation and increased apoptosis in VSMCs. Formation of neointimal lesions represents a common pathophysiological response in the spectrum of vascular diseases. Understanding the molecular mechanism underlying this process may help to elucidate more therapeutic targets for the treatment of vascular disease.

Roles and mechanisms of miRNAs in the immune system

miRNAs are involved in the regulation of at least 30% of the human genome [20, 97]. It is therefore not surprising that over the past few years a powerful role for miRNA regulation of the immune system has come to light. miRNAs have been implicated in varied aspects of the immune response in animals, including hematopoietic lineage differentiation and maintenance of cell identity [98, 99], modulation of T cell sensitivity, and cell effector functions [99]. Because miRNAs are responsible for regulating multiple genes, a dysregulation in their expression levels can have far-reaching consequences with respect to gene regulation; this holds true for miRNAs involved in immune function.

miRNAs have been implicated in the proliferation of quiescent naïve T cells and their differentiation into actively dividing effector T cells capable of producing the various cytokines involved in an effective immune response [100]. This occurs as a result of the progressive differentiation of the immune cells, a process affected by a marked change in gene expression profiles of these cells during and after infection [101, 102]. These changes in gene expression are paralleled by altered miRNA expression profiles, evidencing their role in this regulation, where they are expected to influence these fundamental cellular processes [103].

The importance of miRNAs in cell-mediated immunity can be ascertained when Dicer is conditionally knocked out in mice, resulting in impaired T-cell development and aberrant T-helper-cell differentiation [104]. In addition to cell-mediated immunity, miRNAs have also been associated with the innate immune response, where they activate Toll-like receptors and function in the inflammatory response [105, 106], and with the humoral immune response, where they affect antibody switching and

diversity [107]. Recent studies have demonstrated the importance of individual miRNAs in the immune system as they relate to lineage differentiation of immune cells, elucidating their role in hematopoietic cell development [108]. A role for miRNAs has also been established with regard to mRNA decay as it relates to immune cell turnover [109, 110].

The posttranslational control of gene expression exerted by miRNAs is more than just a general fine-tuning of regulation. It is defined by specific patterns of expression analogous to the differential expression of protein-coding genes. miRNAs are expressed, via the miRNA-generating Dicer enzyme, in a manner dependent on cell type and developmental stage [111]. Deletion of Dicer in the T-cell lineage has revealed a role for this enzyme in thymic development and the differentiation of T-helper and T-regulatory lymphocytes [112]. The exact role of miRNAs in this process is not known [102]. However, natural T-regulatory cells and CD4+ T cells have distinct miRNA profiles [111], and Dicer-deficient CD4+ T lymphocytes fail to differentiate and proliferate into effective helper T cells, expanding only 4- to 6-fold during differentiation *in vitro* compared with a 30-fold expansion in control cells. Dicer is also required for optimal maturation and homeostasis of peripheral T lymphocytes, particularly those of CD8+ cytotoxic T-cell lineage [104]. Dicer ablation also affects antibody diversity and B lymphocyte survival [107].

Several miRNAs specifically appear as key players in the most recent work in the immune field. miR-155 is perhaps the most widely studied; yet it is not the only miRNA involved in immune response. For instance, miR-125b is also involved in the response to endotoxin shock, where it is downregulated in contrast to miR-155 [113]. miR-146a has also been implicated in the innate responses associated with inflammation [114]. miR-181a expression was shown to increase interleukin (IL)-2 release following T-cell activation through phosphatase downregulation [115]. miR-223 works in concert with transcription factors NFI-A and C/EBP to regulate granulopoiesis [116, 117], and it is also over-expressed in the lung in response to lipopolysaccharide (LPS)-induced inflammation [118]. The miR-17-92 cluster is involved in the promotion of lymphomagenesis and autoimmunity through the dysregulation of Bim, which is essential for the deletion of self-reactive thymocytes, mature T cells, and immature and mature B cells [119]. Analysis of miRNA expression in the hematopoietic system indicates that, along with miR-155, at least four other miRNAs (miR-142, miR-144, miR-150, and miR-223) are highly specific for hematopoietic cells [110]. Some of the key players are discussed in more detail below. For a detailed description of current methodologies for studying miRNA-mediated gene regulation and function in the immune system see Mao et al. [120].

miR-155

The importance of miRNAs in immune function has been clearly elucidated by several separate groups that created immunocompromised knockout mice through the deletion of miR-155, produced from the non-protein coding transcript of the *bic* gene, which has been implicated as a collaborator with *c-myc* in B lymphomagenesis [121, 122]. miR-155 has emerged as a key player among miRNAs that have a significant impact on the biology of lymphocytes [123]. The expression of one strand of BIC RNA leads to the generation of one strand of precursor miR-155 [124]. Thai et al. [125] reported the role of this miRNA in regulating T helper cell differentiation and the germinal center reaction to produce an optimal T-cell-dependent antibody response. They found that *bic*/miR-155 expression was absent in nonlymphoid organs as well as in resting CD4+ T cells; yet it became strongly upregulated upon activation of these cells. Furthermore, in results that were simultaneously confirmed by Rodriguez et al. [126], the decreased immune response of these mice was attributed to a reduced number of germinal center B cells, which normally intensely proliferate in the germinal centers prior to undergoing somatic hypermutation of rearranged variable immunoglobulin (Ig) segments (for a review of the role of germinal centers in B-cell physiology, see Klein and Dalla-Favera [127]). Rodriguez et al. [126] demonstrated that *bic*-deficient mice produced significantly reduced amounts of IgM and switched-antigen specific antibodies, indicative of impaired B-cell responses. They, along with other groups of investigators, have also identified a wide spectrum of target genes for miR-155 with diverse molecular roles, such as T-cell costimulation, chemotaxis, and cell signaling, in particular including the *c-Maf* mRNA transcript, which is responsible for IL-4, IL-5, and IL-10 production by Th2 cells [126, 128, 129].

Microarray analysis of B cells activated under conditions to promote class switching to IgG1 has demonstrated that miR-155 regulates a vast number of genes [130]. For example, the gene *Sfp11*, which encodes Pu.1 transcription factor, is a direct target of miR-155. Overexpression of this gene in miR-155 deficient cells results in reduced numbers of IgG1-switched cells [130] and an impaired memory response. In addition to its role in the humoral immune response, the *bic*/miR-155 gene locus has been mapped to a region on chromosome 21q21, which is established as a susceptibility region for atopic dermatitis, seasonal pollen allergy, and asthma [126, 131, 132]. Furthermore, miR-155 is present in 10–30-fold higher copy number in diffuse, large B-cell lymphomas than in normal B cells [59, 122], indicating the importance of proper regulation of its levels [123].

Adding to the complex character of this particular miRNA, emerging data have begun to reveal a role for miR-155 in innate immune responses, with the expression of high

levels in monocytes and macrophages [106], particularly after exposure to inflammatory stimuli, including polyriboinosinic:polyribocytidylic acid or the cytokine interferon (IFN)- β [94]. Inhibition of the kinase JNK pathway and MyDD-88 blocked induction of miR-155 in both cases indicates that MAPK signaling is a factor in miR-155 signaling.

In lymphocytes, miR-155 expression is tightly regulated in myeloid cells, suggesting a specialized function during times of inflammatory stress [123]. This role was observed by Tili et al. [113] whose study established an additional role for miR-155 in the regulation of the response to endotoxin shock following LPS stimulation. Septic shock occurs when an overproduction of cytokines in response to an overwhelming bacterial infection leads to fever and disseminated intravascular coagulation, followed by organ failure and death [133]. miR-155 overexpression results in enhanced translation and increased production of TNF- α , which is the major cytokine produced by macrophages in response to LPS [134]. Tili et al. [113] hypothesize this may occur through the disruption of the self-regulatory effects of TNF- α . When mice over-expressing miR-155 are exposed to LPS, the result is septic shock and death. Thus, miR-155 can be stimulated by both bacterial (LPS) and viral (IFN- β) stimuli [94], demonstrating the broad range of miRNA interaction within the immune system.

Recently, miR-155 has been observed to drive granulocyte/monocyte expansion during inflammation in vivo [123]. In response to LPS presentation, the inflammatory process increases the production of granulocytes and monocytes, reciprocally replenishing those lost during the battle against infection [135]. The findings of O'Connell et al. [123] indicate that miR-155 is induced by LPS in the bone marrow and is sufficient to increase the numbers of granulocytes and monocytes throughout hematopoietic development, at the expense of B lymphocytes and erythroid precursors in the bone marrow.

miR-146a/b

miR-146 is represented in two human genes (miR-146a on chromosome 5 and miR-146b on chromosome 10), which differ by only two nucleotides in the 3' region [105, 136]. Taganov et al. [105] performed an analysis of miR-146a and miR-146b gene expression that unveiled a pattern of induction in response to a variety of microbial components and proinflammatory cytokines. miR-146a has also been shown to regulate the innate immune response by interfering with the nuclear factor (NF)- κ B pathway, acting as a negative regulator of expression of NF- κ B components such as IRAK1 and TRAF6 [94, 105], both of which encode key adapter molecules downstream of Toll-like receptors [137, 138].

Examination of miRNA function by overexpression and inhibition showed that increased mi-146a expression also negatively regulated the release of the proinflammatory chemokines IL-8 and RANTES at the translational level [114], providing a novel mechanism for regulation of severe inflammation during the innate immune response.

In addition, a recent investigation of the distribution of miRNAs has implicated miR-146 and miR-203 as regulators in psoriasis, a disease resulting from impairment in the crosstalk between the immune system and structural skin cells [139], via modulation of TNF- α and regulation of keratinocyte function, respectively [140]. miR-146a was also found to be highly expressed in synovial tissue in patients with rheumatoid arthritis, where it could be induced by stimulation with TNF- α and IL-1 β [141], indicating a potential link. However, its function in this system has yet to be elucidated.

miR-150

The involvement of miRNAs in lymphocyte development can also be seen through studies of miR-150. miR-150 is highly upregulated during the development of mature T and B cells, being expressed in mature lymphocytes, but not their progenitors [142], and it is crucial for terminal stages of differentiation [142–144]. miR-150 exerts its regulatory control by modulating c-Myb, a transcription factor that controls multiple steps of lymphocyte development [142], the overexpression of which has been associated with colorectal cancer [145]. The wide-reaching implications of the association between c-Myb regulation and miR-150 have recently indicated that miRNAs may play a greater role than that of just fine-tuning lineage fate, both inside and outside of immune cells [146].

miR-150 shows a sharp rise in expression levels at the immature B-cell stage. However, if expressed prematurely, it blocks early B-cell development by interrupting the transition from the pro-B cells, the first B-lineage-restricted cells, which initiate rearrangement at the Ig heavy chain locus, to the pre-B stage, when the heavy chain assembles with a surrogate light chain [144, 147]. As a result, ectopic expression of miR-150 led to reduced numbers of B cells in the circulation, spleen, and lymph nodes, but had little or no change in the levels of T cells and myeloid cells [144]. This was in direct contrast to the effects of ectopic expression of miR-181a, which led to an increase in B-cell production and a decrease in CD8 T-cell production [98].

miR-181a

miR-181 is differentially expressed in B cells [148], as well as in the thymus, brain, lung, bone marrow, and spleen. It

appears to be induced during lineage differentiation. Ectopic expression of miR-181 led to a doubling of cells of B-lymphoid lineage [98].

A successful immune system must be able to protect against foreign pathogens while at the same time avoiding collateral damage to its own self tissues [149]. T-cell responsiveness is regulated by T-cell receptor (TCR) signaling, through which T-cell sensitivity to antigens is intrinsically modulated. Li et al. [115] demonstrated that the sensitivity of mature T cells was modified by miR-181a. Increased miR-181a expression augmented TCR signaling strength and resulted in an enhanced sensitivity to a T-cell agonist, probably through increased CD28 expression and decreased CTLA-4 expression.

miR-223

miR-223 is nearly exclusively expressed in bone marrow, the primary hematopoietic organ, which consists of hematopoietic stem cells and myeloid, erythroid, and lymphoid cells at various differentiation stages [150], and it is implicated in myeloid production and granulocyte formation [151]. Johnnidis et al. [151] observed that mice deficient in miR-223 have an expanded granulocytic compartment, likely due to the lack of regulation of Mef2c transcription factor, which promotes progenitor expansion. Their results indicate a role for miR-223 that is similar to that of miR-181a with regard to T cells, in that miR-223 acts as a fine tuner of neutrophil sensitivity.

In contrast, Fukao et al. [117] determined that miR-223 works in concert with transcription factors NFI-A and C/EBP to regulate granulopoiesis, indicative of multiple regulatory mechanisms for a single miRNA. The two factors NFI-A and C/EBP compete for binding to the miR-223 promoter. NFI-A maintains miR-223 at low levels while C/EBP plays the opposite role [116].

miR-17-92

The human miR-17-92 cluster transcript encoded on chromosome 13 is the precursor of seven miRNA molecules (miR-17-5p, miR-17-3p, miR-18a, miR-19a, miR-20a, miR-19b, and miR-92). It is involved in lymphomagenesis and autoimmunity through the dysregulation of Bim, which is essential for the deletion of self-reactive thymocytes, mature T cells, and immature and mature B cells [119]. Absence of miR-17-92 leads to increased levels of Bim, inhibiting B-cell development at the pro-B to pre-B transition [152]. Overexpression of this cluster leads to premature death associated with autoimmunity [119].

miR-16

miR-16 is part of a family of related miRNAs known to be involved in regulating cell cycle progression [153]. It is typically found in high levels throughout many types of cells, including immune system cells such as B and T cells [36, 54, 100], and it has been implicated in human leukemia [150]. Recent data presented by Asirvatham et al. [154] indicate that miRNAs such as miR-16 target the genes involved in regulating message stability via AU-rich elements (AREs), which lie at the 3' UTR of mRNAs and dictate their degradation. miR-16 binds to ARE and degrades the mRNA by an Argonaute two-dependent mechanism [110]. This mechanism of action implicates a broad regulation of immune genes by this ubiquitous miRNA, which may result in the failure of certain immune genes to respond to external stimuli [154].

miRNAs as a direct defense against viruses

The antiviral effects of miRNA are well documented in plants, where the viral double-stranded RNA is processed into miRNAs by Dicer. These small RNAs are then introduced into the RISC complex to target the pathogen's genome for destruction [108, 155, 156]. Such direct antiviral effects have also been observed in human cells. A cellular miRNA, miR-32, was found to effectively restrict the accumulation of the retrovirus primate foamy virus type 1 in human cells. However, these data have been controversial, with certain investigative groups directly contradicting the findings [157]; thus the direct interaction of viruses with the host miRNA machinery remains elusive.

However, Pedersen et al. [158] showed compelling evidence that IFN- β modulates the expression of numerous cellular miRNAs, eight of which have sequence-predicted targets within the hepatitis C virus genome. Through introduction of synthetic miRNA-mimics, they were able to reproduce the antiviral effects of IFN- β induction. At the same time, they demonstrated that neutralization of these miRNAs reduced the antiviral effects. In addition to these findings, they also reported that IFN- β treatment significantly reduces the liver-specific miR-122, which was previously shown to be essential for HCV replication in the liver [159], thereby increasingly supporting the theory that cellular miRNAs have direct antiviral effects.

Regulation of miRNA expression

In addition to the regulatory activities of miRNA to mRNA transcription and protein translation, the expression of

miRNA itself can also be controlled, which adds another layer of regulation to gene expression. Epigenetic modification, DNA copy alteration, and mutations are common mechanisms of miRNA regulation. miRNA epigenetic changes such as DNA methylation and histone modification are important in chromatin remodeling and gene regulation, including miRNA regulation in human cancer. In human breast cancer cells, alteration of miRNA levels was observed in response to histone deacetylase (HDAC) inhibitor LAQ824 [160]. In a human bladder cancer cell line T24, expression of 17 miRNAs such as miR-127 was significantly upregulated upon simultaneous treatment with chromatin-modifying drugs, 5-aza-2'-deoxycytidine and 4-phenylbutyric acid [161]. These studies suggest that epigenetic alteration may play a critical role in regulating miRNA expression in human cancers, and epigenetic treatment might provide a novel strategy for cancer therapy [161–163].

Alteration in DNA copy number is another mechanism to modify the expression and function of miRNAs, which is a frequent contributor to cancer. The first miRNA gene with DNA copy number change in cancer was reported in CLL patients in 2002. miR-16-1 and miR-15a genes at chromosome 13q14 are deleted in more than 50% of chronic lymphocytic leukemia (CLL) patients [52]. Further studies indicated that these two miRNAs may serve as tumor suppressors in many cancers [54]. Amplification of C13orf25 at 13q31-32 was first reported in lymphoma patients in 2004 [60]. This amplified region contains seven miRNAs as a polycistronic cluster, and the expression of the miRNAs derived from this locus is significantly increased in this disease. This miRNA cluster is a well recognized oncogene in human cancer. Recent genomic and informatic studies indicate that a large proportion of miRNA gene-containing genomic loci exhibit DNA copy number alterations in ovarian cancer (37.1%), breast cancer (72.8%), and melanoma (85.9%) [164]. These findings support the hypothesis that DNA copy number changes of miRNAs are predominant in cancer and may account, at least in part, for the frequent miRNA gene dysregulation.

miRNA genes are usually clustered on the genome and are prone to mutations. A recent miRNA profiling study using 94 samples of CLL patients has identified a germ-line mutation in the miR-16-1-miR-15a primary precursor, leading to low levels of miRNA expression in vitro and in vivo, and associated with deletion of the normal allele [165]. Germ-line or somatic mutations were found in 5 of 42 sequenced miRNAs in 11 of 75 CLL patients, but no such mutations were found in 160 subjects without cancer. These studies indicate that mutations in miRNA genes are common events and may have functional importance [165].

miRNAs as therapeutic targets

Many studies have indicated that miRNAs can serve as valuable therapeutic targets for a large number of diseases, including cancer. For miRNAs with oncogenic capabilities, potential therapies include miRNA silencing, antisense blocking, and miRNA modifications. For miRNAs with tumor-suppression functions, overexpression of those miRNAs (such as the let-7 family) might be a useful strategy to inhibit tumor growth [166, 167].

miRNA silencing

miRNA silencing is a method to prevent mature miRNA production by inhibiting its processing. This could be done by interfering with the activities of Droscha, Dicer, or other components of the miRNA biogenesis pathway. Because these enzymes control the synthesis of all miRNAs, targeting them could be problematic, and it could generate many side effects. As an alternative method, oligonucleotide complementary to individual pri-miRNAs could disrupt the hairpin structure and specifically inhibit the miRNA function. Inhibition of a pre-miRNA with a siRNA is also possible, but less efficient [168, 169].

Antisense blocking

Antisense blocking is the most commonly used method to inhibit the function of miRNAs [170, 171]. Krutzfeldt et al. [171] were the first group to discover a new class of chemically modified oligonucleotides called “antagomirs” as specific and effective silencers of miRNA expression. Antagomirs are cholesterol-conjugated single-stranded RNA molecules 21–23 nt in length that are complementary to the mature target miRNA. Intravenous administration of antagomirs against miR-16, miR-122, miR-192, and miR-194 caused a marked reduction in corresponding miRNA levels in liver, lung, kidney, heart, intestine, and fat, among other tissues. The silencing of the endogenous miRNAs by antagomirs is specific, efficient, and long-lasting. Silencing miRNA-122, an abundant liver-specific miRNA, also resulted in upregulation of hundreds of genes predicted to be targets of miR-122 because these genes had a miR-122 recognition site in the 3' UTR region [95, 171]. These studies suggest that antagomirs are powerful tools with which to silence specific miRNAs, and they may represent a novel therapeutic strategy for silencing miRNAs in cancer.

Locked nucleic acid (LNA)

Locked nucleic acid (LNA) contains one or more nucleotide building blocks in which an extra methylene bridge

fixes the ribose moiety either in the C3'-endo (β -D-LNA) or C2'-endo (α -L-LNA) conformation. The LNA modification results in significant increases in melting temperature and stabilizes the duplexes. Locked nucleic acid oligonucleotides are synthesized in different formats, such as all-LNA, LNA/DNA mixmers, or LNA/DNA gapmers, all of which have the features of high affinity, good or even improved mismatch discrimination, low toxicity, and increased metabolic stability. Locked nucleic acid is particularly attractive for in vivo applications that are inaccessible to RNA interference technology, such as inhibition of oncogenic miRNAs. Furthermore, the extreme antisense-target duplex stability conferred by β -D-LNA also contributes to the capacity to invade stable secondary structures of RNA targets. Because LNA has features that result in very high hybridization affinity toward complementary single-stranded RNA without compromising the specificity of the interaction, and because it shows improved antisense efficacy and higher melting temperature toward complementary RNA than do 2'-O-methyl oligonucleotides of the same sequence. These studies suggest that LNA can serve as a promising antisense therapy with which to treat cancer [172, 173].

miRNA sponge

The effect of chemically modified antisense oligonucleotides for inhibiting miRNAs is usually transient, and the inhibition in animals is only achieved with a high dose. Developing a novel strategy to obtain long-term silencing of miRNAs at a reasonable concentration of the inhibitor is necessary. Ebert et al. [174] developed miRNA inhibitors called “microRNA sponges” that can be expressed in cells, as RNAs produced from transgenes under strong promoters. The transgene contains multiple, tandem binding sites to a miRNA of interest at the 3' UTR region after a green fluorescent protein (GFP) reporter gene. When the plasmids encoding these sponges are transiently transfected into HEK293T cells, sponges significantly inhibit miRNA expression at least as strongly as chemically modified antisense oligonucleotides. Because the sponges specifically inhibit miRNAs with a complementary heptameric seed, a single sponge can be used to block an entire miRNA seed family. The stably expressed sponges might have great potential in animal models to achieve long-lasting inhibition of miRNAs.

Epigenetic therapy

Considering the highly epigenetic modifications of many miRNAs, such as methylations, the epigenetic therapy with drugs that inhibit DNA methylation and with histone deacetylase (HDAC) may have important clinical potential

for anticancer therapy. 5-aza-2'-deoxycytidine and 5-azacytidine are commonly used DNA methylation inhibitors that have been approved by the U.S. Food and Drug Administration for clinical use. Many HDAC inhibitors are also undergoing clinical trials [175]. Activation of tumor-suppressor miRNAs, such as miR-127, by chromatin-modifying drugs may inhibit tumor growth through downregulation of their target oncogenes. Because the number of miRNA genes is increasing, and because most miRNAs are downregulated in cancer, there might be a large number of potential targets for anticancer therapy [172].

Summary and future directions

Given the enormous discoveries and rapid progress over the past few years on miRNAs, it is likely that miRNAs have great potential in the diagnosis and treatment of many diseases, including cancer, vascular disease, and immune disease. miRNA profiling associated with particular outcomes may provide insights into the underlying mechanisms of disease and suggest novel therapeutic strategies. Additionally, mutant or dysregulated miRNAs and their control pathways might also be useful therapeutic targets. Direct targeting to miRNAs that are amplified or upregulated in cancer patients holds great promise in anticancer therapy [9, 176]. Furthermore, miRNAs, unlike mRNAs, remain largely intact in routinely collected formalin-fixed paraffin-embedded clinical samples, and their overall levels may be less likely to be affected by collection and storage procedures [177], making it highly practical to use miRNA profiling as a marker in tumor classification, early detection, disease prognosis, and therapeutic decision making. However, there are still technical hurdles for miRNA research. For example, the cost of miRNA profiling is still high, achieving long-term and stable silencing of miRNA expression is still technically difficult, the total number of human miRNAs has yet to be determined, and the targets of miRNAs and their roles in cellular pathways are largely unclear. These problems are likely to be resolved with new developing technologies to make miRNA profiling, screening, and therapy faster, more reliable, and cost effective.

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