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REVIEW AND PERSPECTIVE

MicroRNA—implications for cancer

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Abstract MicroRNAs (miRNAs) are small RNA molecules that regulate gene expression post-transcriptionally. After the discovery of the first miRNA in the roundworm *Caenorhabditis elegans*, these short regulatory RNAs have been found to be an abundant class of RNAs in plants, animals, and DNA viruses. About 3% of human genes encode for miRNAs, and up to 30% of human protein coding genes may be regulated by miRNAs. MicroRNAs play a key role in diverse biological processes, including development, cell proliferation, differentiation, and apoptosis. Accordingly, altered miRNA expression is likely to contribute to human disease, including cancer. This review will summarize the emerging knowledge of the connections between human miRNA biology and different aspects of carcinogenesis. Various techniques available to investigate miRNAs will also be discussed.

Keywords MicroRNA · miRNA · Cancer · Pathogenesis · Development

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The “small RNA revolution”

Small ribonucleic acid (RNA) can act as a specific regulator of gene expression. This discovery has been an exciting breakthrough in Biological Sciences of the past decade, culminating in last year’s Nobel Prize in Physiology or Medicine awarded to Andrew Fire and Craig Mello. Building on previous work mainly in plants [50], Fire et al. [23] discovered that exogenous double-stranded RNA can be used to specifically interfere with gene function. This phenomenon was called RNA interference (RNAi). They also speculated that organisms might use double-stranded RNA naturally as a way of silencing genes. It was then shown that RNA interference was mediated by 22 nucleotide single-stranded RNAs termed small interfering RNAs (siRNAs) derived from the longer double-stranded RNA precursors [87]. The small interfering RNAs were found to repress genes by eliminating the corresponding messenger RNA transcripts, and thus, preventing protein synthesis.

Over the following years, many new small functional RNAs have been found. RNA is usually thought of as messenger RNA that serves as the template for translation of genes into proteins. In contrast, functional or non-coding RNA molecules are transcribed from a DNA sequence, but not translated into protein. The encoding DNA sequence is often referred to as an RNA gene. Functional RNA genes in the human genome include transfer RNA (tRNA), ribosomal RNA (rRNA), and various other small non-coding RNAs. Several hundred genes in our genome encode small functional RNA molecules collectively called microRNAs (miRNAs). Precursors of these miRNA molecules form structures of double-stranded RNA that can activate the RNA interference machinery. MicroRNAs downregulate

gene expression either by degradation of messenger RNA through the RNA interference pathway or by inhibiting protein translation.

The first miRNA was discovered in 1993 by Victor Ambros and colleagues Rosalind Lee and Rhonda Feinbaum [42]. A genetic screen in the roundworm *Caenorhabditis elegans*, a millimeter-long animal used as a model organism in biological research, identified genes involved in developmental timing [42]. Surprisingly, one of the genes, termed *lin-4*, did not encode a protein but instead a novel 22-nucleotide small RNA. Seven years later, Reinhart et al. [70] discovered a second 22-nucleotide small RNA of this type, *let-7*, a gene also involved in *C. elegans* developmental timing. The *lin-4* and *let-7* small regulatory RNAs soon became very exciting for two reasons. Firstly, homologs of the *let-7* gene were identified in other animals including humans [65]. The conservation of *let-7* across species suggested an important and fundamental biological role for this small RNA. Secondly, the mechanism of RNA interference (RNAi) was discovered at that time, and it became clear that miRNA and RNAi pathways were intricately linked and shared common components. Within the following year, more than 100 additional small regulatory RNAs similar to *lin-4* and *let-7* were identified in worms, the fruit fly *Drosophila*, and in humans [38, 40, 41]. These small non-coding RNAs were named microRNAs (miRNAs) [38, 40, 41].

Subsequently, many more short regulatory RNAs were identified in almost all multicellular organisms, including flowering plants, worms, flies, fish, frogs, mammals [38, 40, 41, 48, 71], and in single cellular algae and DNA viruses [66, 75]. To date, more than 500 human miRNAs have been experimentally identified. Computational predictions of miRNA targets suggest that up to 30% of human protein coding genes may be regulated by miRNAs [46, 68]. This makes miRNAs one of the most abundant classes of regulatory genes in humans. MicroRNAs are now perceived as a key layer of post-transcriptional control within the networks of gene regulation.

MicroRNAs are sequentially processed from longer precursor molecules that are encoded by the miRNA genes [1] (Fig. 1). MiRNA genes are referred to by the same name (termed *mir*) written in italics to distinguish them from the corresponding mature miRNA (termed miR) followed by a number, e.g., *mir-1* or miR-1. The encoding DNA sequence is much longer than the mature miRNA. Two ribonuclease enzymes, Drosha and Dicer, subsequently process the primary transcripts (or pri-miRNA) to generate mature miRNAs. The primary transcripts contain one or more stem-loop structures of about 70 bases. Stem-loops are double-stranded RNA structures consisting of a nucleotide sequence that can fold back on itself to form a double helix with a region of imperfect base pairing that forms an open loop at the end (Fig. 1a). The ribonuclease Drosha excises the stem-loop structure to form the precursor miRNA (or pre-miRNA) [43]. After export into the cytoplasm, the pre-miRNA is cleaved by the ribonuclease Dicer to generate a short RNA duplex [6, 28]. After untwisting, one RNA strand becomes the mature single-stranded miRNA, while the complementary strand, termed miRNA*, is usually rapidly degraded (Fig. 1b).

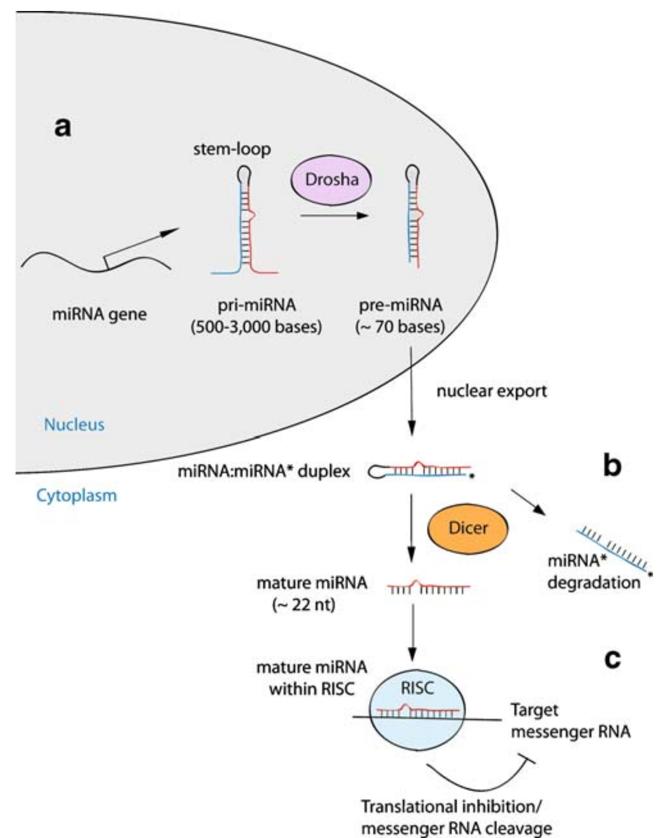

Fig. 1 The biogenesis and function of miRNAs. **a** Primary miRNAs (*pri-miRNA*) are transcribed from longer encoding DNA sequences (miRNA genes). The pri-miRNA contains one or more stem-loop structures of about 70 bases. In the nucleus, the ribonuclease enzyme Drosha excises the stem-loop structure to form the precursor miRNA (*pre-miRNA*). **b** After export into the cytoplasm, the pre-miRNA is cleaved by the ribonuclease Dicer to generate a short RNA duplex (miRNA:miRNA*). **c** The mature single-stranded miRNA is incorporated into the RNA-induced silencing complex (*RISC*), while the complementary strand (miRNA*) is usually rapidly degraded. The miRNA incorporated into the silencing complex can bind to the target messenger RNA by base pairing, causing inhibition of protein translation and/or degradation of the target messenger RNA

MicroRNAs recognize their targets based on sequence complementarity [10]. The mature miRNA is partially complementary to one or more messenger RNAs. In humans, the complementary sites are usually within the 3′-untranslated region of the target messenger RNA. To become effective, the mature miRNA forms a complex with proteins, termed the RNA-induced silencing complex. The miRNA incorporated into the silencing complex can bind to

the target messenger RNA by base pairing. This base pairing subsequently causes inhibition of protein translation and/or degradation of the messenger RNA (Fig. 1c). The potential mechanisms underlying this process were recently reviewed [30, 67]. Protein levels of the target gene are consequently reduced, whereas messenger RNA levels may or may not be decreased. In humans, miRNAs mainly inhibit protein translation of their target genes and only infrequently cause degradation or cleavage of the messenger RNA [1].

The biological role and in vivo functions of most mammalian miRNAs are still poorly understood. In invertebrates, miRNAs regulate developmental timing (e.g., *lin-4*), neuronal differentiation, cell proliferation, growth control, and programmed cell death [9, 33, 42]. In mammals, miRNAs have been found to play a role in embryogenesis and stem cell maintenance [7], hematopoietic cell differentiation [17], and brain development [59, 60]. To date, knowledge of human miRNAs has been primarily descriptive. MicroRNA expression has been found to be deregulated in a wide range of human diseases including cancer. However, it remains uncertain whether altered miRNA expression is a cause or consequence of pathological processes. The underlying mechanisms of why and how miRNAs become deregulated are largely unknown. Although bioinformatics approaches can predict thousands of genes that are potentially targeted and regulated by miRNAs based on sequence complementarity, only very few miRNA target genes have been functionally validated. Our group is currently investigating the role of miRNAs in mammary gland development and breast cancer pathogenesis. A comparison of miRNA and gene expression identified miRNAs that classify molecular breast cancer subtypes [8]. As cancer is ultimately a consequence of disordered gene expression, miRNAs have been suggested to contribute to the development of cancer [11]. This review will focus on the connection between human miRNA biology and different aspects of carcinogenesis. Various techniques available to investigate miRNAs will also be discussed.

MicroRNAs and cancer

Three important observations early in the history of miRNAs suggested a potential role in human cancer. Firstly, the earliest miRNAs discovered in the roundworm *C. elegans* and the fruit fly *Drosophila* were shown to control cell proliferation and apoptosis [9, 42]. Their deregulation may therefore contribute to proliferative diseases such as cancer. Secondly, when human miRNAs were discovered, it was noticed that many miRNA genes were located at fragile sites in the genome or regions that are commonly amplified or deleted in human cancer [14]. Thirdly, malignant tumors and tumor cell lines were found to have widespread deregulated miRNA expression compared to normal tissues [12, 24, 52]. The question remained whether the altered miRNA expression observed in cancer is a cause or consequence of malignant transformation.

MicroRNAs as causal cancer genes at genomic breakpoints

Five years ago, the first direct evidence for an involvement of miRNAs in cancer was reported [13]. Calin et al. studied a well-known deletion on chromosome 13, which is the most frequent chromosomal abnormality in chronic lymphocytic leukemia (CLL). This deletion had long been suspected to contribute to leukemogenesis. However, extensive studies had failed to identify a causal gene. Calin et al. [13] found that two miRNA genes, *mir-15* and *mir-16*, were located within this 30-kb deletion. They subsequently analyzed the expression of miR-15 and miR-16 in blood samples from patients with CLL. Both miRNAs were absent or downregulated in the majority (68%) of cases when compared to normal tissue or lymphocytes. This finding suggested that these two miRNAs were causally involved in the pathogenesis of chronic lymphocytic leukemia.

In 2005, three reports provided the first mechanistic insight into how miRNAs might contribute to carcinogenesis. Two independent studies described the relationship between a miRNA cluster, *mir-17-92*, and the Myc oncogenic pathway [27, 63]. A third report demonstrated an interaction between *let-7* miRNA and the RAS proto-oncogene [32].

The mir-17-92 cluster—small RNAs with oncogenic potential

A cluster of six miRNAs, the *mir-17-92* cluster, was found to be located within a region on chromosome 13 that is commonly amplified in human B-cell lymphomas [64]. He et al. [27] demonstrated that the miRNAs from the *mir-17-92* cluster were overexpressed in lymphoma cell lines carrying this amplification, and expression levels correlated with gene copy number of the *mir-17-92* locus [27]. Further, the miR-17-92 primary transcript was found to be overexpressed in tumor samples from lymphoma patients. To test their hypothesis that *mir-17-92* actively contributes to lymphomagenesis, the authors took advantage of a mouse model of human B-cell lymphoma. These mice develop lymphomas due to an overexpression of the Myc oncogene. The Myc oncogene encodes the transcription factor c-Myc that regulates cell proliferation, growth, and apoptosis, and overexpression of c-Myc is common in cancer. He et al. [27] demonstrated that additional expres-

sion of the *mir-17-92* cluster accelerated c-Myc-induced tumorigenesis in mice. The authors therefore suggested that *mir-17-92* was the first potential non-coding oncogene, referred to as oncomir-1.

The cellular function of miR-17-92 was not identified in these experiments. Nevertheless, the pathology of the tumors indicated lower rates of apoptosis as compared to tumors with Myc overexpression alone. Three recent studies contributed towards our understanding of the oncogenic potential of miR-17-92. Two reports demonstrated an anti-apoptotic effect of miR-17-92 through various pathways that promote cell proliferation and growth [55, 76]. A third study identified mir-17-92 as a mediator of angiogenesis in tumors induced by the oncogene c-Myc [19].

O'Donnell et al. [63] independently identified the same cluster of miRNAs, *mir-17-92*, to be regulated by the transcription factor c-Myc. The transcription factor Myc induces expression of E2F1 growth factor. The *mir-17-92* cluster which is also induced by c-Myc does, in contrast, inhibit E2F1 expression. The authors therefore suggested a novel regulatory mechanism by which c-Myc fine-tunes gene expression by activating the transcription of target genes and by simultaneously inducing inhibitory miRNAs that reduce their translation.

The example of the *mir-17-92* cluster highlights that a distinction between oncogenic and tumor suppressor miRNAs is likely to be an oversimplification. The same miRNAs may have oncogenic or tumor suppressor activity depending on the context and the cell type they are expressed in. A single miRNA may regulate various unrelated target genes and thereby control opposing activities such as cellular proliferation and apoptosis. The ultimate function of a miRNA may depend on the tissue type they are expressed in and what target genes are present.

MicroRNAs with tumor suppressor potential

The *let-7* family of miRNAs was the first group of miRNAs shown to regulate expression of a proto-oncogene, the RAS protein. RAS proteins are membrane-associated signaling proteins that regulate cell growth and differentiation. A miRNA that controls expression of these potentially oncogenic proteins would be predicted to possess tumor suppressor activity.

Mutations in the RAS oncogene are present in approximately 15–30% of all human cancers, and overexpression of the RAS oncogene is common in lung cancer. Johnson et al. [32] showed that overexpression of RAS protein in lung cancer tissue correlated with reduced expression of *let-7* miRNA. They experimentally confirmed that *let-7* can inhibit RAS expression in human cancer cell lines. Loss or reduction of *let-7* in lung cancer leads to RAS overexpression, thus, promoting cellular growth and contributing to tumorigenesis. The authors therefore suggested that *let-7* acts as tumor suppressor [32]. Another group independently reported reduced expression of *let-7* in lung cancers and found that this correlated with a poor prognosis [77].

Global loss of miRNA expression in cancer

A global decrease in miRNA levels has been observed in human cancers, indicating that small RNAs may have an intrinsic function in tumor suppression. Lu et al. [52] were the first to show that the expression levels of many miRNAs were significantly reduced in cancers compared to the corresponding normal tissues. They analyzed a total of 217 human and mouse miRNAs across 334 human cancers, cancer cell lines, and normal tissues. Cancers had significantly reduced global miRNA expression. Poorly differentiated tumors had lower miRNA levels compared with more-differentiated tumors. The authors hypothesized that miRNAs can function to drive terminal differentiation and prevent cell division. Global changes in miRNA expression may reflect the degree of cell differentiation [52]. A recent study examined the expression of 241 human miRNAs in a comprehensive panel of human cancer cell lines, the NCI-60 panel, and in normal tissues [24]. The authors confirmed the finding that most miRNAs were expressed at lower levels in human tumor-derived cell lines compared with the corresponding normal tissue [24].

Until recently, considerable uncertainty remained as to whether the altered miRNA expression observed in cancer was a cause or consequence of malignant transformation. Earlier this year, a study by Kumar et al. [37] proved for the first time that widespread reduction in miRNA expression does, indeed, promote tumorigenesis. The authors globally reduced the production of mature miRNAs through a knockdown of the miRNA-processing enzymes Drosha and Dicer in cell lines. The mouse and human cancer cells consequently showed decreased steady-state miRNA levels. These cells with global miRNA loss showed enhanced cellular growth in vitro [37]. When injected into nude mice, these cells generated faster growing and more invasive tumors compared to controls. To assess the effect of global miRNA loss in vivo, the authors deleted the miRNA-processing enzyme Dicer in a mouse model of lung cancer. The Dicer mutant mice who had impaired miRNA processing developed an increased tumor burden, with an expansion in tumor number and tumor size, as well as tumors which were less well differentiated compared to controls [37]. Overall, these data clearly suggest that global miRNA loss enhances tumorigenesis. Kumar et al. demonstrated that loss of miRNAs leads to upregulation of proto-oncogenes such as RAS and c-Myc. However, it remains to

be elucidated whether loss of all miRNAs is necessary or whether reduction of a subgroup of key tumor suppressor miRNAs, such as *let-7*, is the event that promotes malignant transformation.

MicroRNAs in the p53 tumor suppressor network

Transcriptional networks are often deregulated in cancer cells and may lead to altered transcription of miRNA genes. Two recent studies identified a miRNA, miR-34, to be regulated by the p53 transcription factor [16, 26]. The p53 protein, also called “the guardian of the genome”, regulates the cellular response to stress and cancer-initiating events such as DNA damage. He et al. [26] found that a miRNA, miR-34, is directly activated by the transcription factor p53 after DNA damage. Expression of miR-34 induces cell cycle arrest and thereby acts together with other effectors of the p53 tumor suppressor network to inhibit inappropriate cell proliferation. Another group independently demonstrated that miR-34 is upregulated by p53 upon DNA damage and promotes apoptosis [16].

Together, these data indicate that altered expression of miRNAs is not simply a secondary event that reflects the less differentiated state of cancer cells. In contrast, at least in some cases, miRNA expression is specifically driven by tumor suppressors and oncogenes.

MicroRNAs with a role in tumor invasion and metastasis

Transcriptional networks may drive miRNA expression in cancers. Recent work from Ma et al. [54] suggested a model by which a pleiotropic transcription factor, Twist, induces expression of a specific miRNA, which suppresses its direct target and in turn activates a pro-metastatic gene, leading to tumor cell invasion and metastasis. The expression of miR-10b induced by the transcription factor Twist promoted cell migration and invasion in mouse and human breast cancer cells. Furthermore, the expression level of miR-10b in primary human breast carcinomas correlated with clinical progression [54]. These findings, if confirmed, suggest that specific miRNAs may have a role beyond the tumor-initiating event and directly participate in tumor progression and metastasis.

Regulation of miRNAs in cancer—who regulates the regulators?

In few cases, the underlying cause of miRNA deregulation in cancer is clear. As discussed above, the overexpression of miR-17-92 correlates with amplification of its gene locus [27]. Similarly, decreased expression of miR-15 and miR-16 is associated with a corresponding chromosomal deletion [13].

Transcriptional or epigenetic regulation of miRNAs has been recently reported [53, 73]. The transcription of a miRNA gene, *mir-124a*, was shown to be inactivated by hypermethylation of its promoter in various human tumors. This process of epigenetic silencing is a well-known mechanism to inactivate protein-coding genes in cancer cells and may similarly apply to miRNAs. The miRNA gene *mir-127* is usually expressed in normal cells but not in cancer cells. Saito et al. [73] demonstrated that miR-127 was highly induced in cultured human cancer cells after treatment with demethylating drugs, suggesting that it is subject to epigenetic silencing through promoter hypermethylation.

A novel mechanism of miRNA regulation was suggested by Mayr et al. [56] and Lee and Dutta [44]. They demonstrated that miRNA function could be regulated through loss of miRNA binding sites in the target gene. Both groups independently demonstrated that chromosomal translocations in a known oncogene, high mobility group A2 (Hmga2), led to loss of the *let-7* miRNA binding sites in its messenger RNA. Disrupted repression of Hmga2 by *let-7* promoted oncogenic transformation and growth in mammalian cells. These two studies provide the first evidence that disrupting the interaction of a single miRNA and its target can produce an abnormal phenotype in mammalian cells [44, 56].

In addition, there is evidence that miRNAs are regulated indirectly through control of their processing enzymes. Thomson et al. [81] showed that a downregulation of miRNAs in human cancer was not associated with reduced levels of the primary miRNA transcripts. The authors therefore suggested regulation of miRNAs during subsequent processing steps, e.g., through altered function of the enzyme Drosha [81].

MicroRNA profiling—implications for cancer diagnosis

Lu et al. [52] asked the question whether global miRNA expression profiles could classify human cancer. MicroRNA expression profiles clearly differentiated human cancers according to their developmental origin.

Cancers of epithelial and hematopoietic origin had distinct miRNA profiles. A subgroup of gastrointestinal tumors, which arise from endoderm, was distinguished by miRNA expression patterns. Furthermore, tumors within a single cell lineage such as acute lymphoblastic leukemia were further differentiated according to their underlying genetic abnormality into BCR/ABL-positive tumors, T-cell tumors, and those with MLL gene rearrangement [52]. Finally, the authors applied the miRNA expression profiles they had established to an independent series of 17 poorly differentiated tumors of unknown origin. Based on the differential expression of 217 miRNAs, a correct diagnosis could be established in 12 out of 17 of the tumors. In

contrast, gene expression profiling based on ~16,000 messenger RNAs did not accurately classify the tumors [52]. This has potential important clinical implications. If miRNAs prove useful for clinical diagnosis, their key advantage might be their high stability. In contrast to most messenger RNAs, they are long-lived in vivo [49] and very stable in vitro [78], which might allow analysis of paraffin-embedded samples for routine diagnostic applications.

MicroRNAs—novel therapeutic targets?

Regulatory RNAs may also have therapeutic applications by which disease-causing miRNAs could be antagonized or functional miRNAs restored. The most intuitive choice of molecules to correct altered miRNA–messenger RNA interactions are RNA oligonucleotides. These oligonucleotides need to be chemically modified to allow for stability in serum and cellular uptake. Modified antisense oligonucleotides are already being developed to utilize the intrinsic RNAi pathway for delivery of gene therapy. If the delivery problem can be overcome, then miRNA therapies may also be possible.

Two studies have successfully applied 2′-*O*-Methyl-modified antisense RNAs to inhibit miRNA function in cultured cells [29, 57]. Recent work by Krutzfeldt et al. [36] demonstrated that modified cholesterol-conjugated antisense RNAs designated “antagomirs” could effectively inhibit miRNA function in vivo in the adult mouse. The authors applied three daily intravenous injections of antagomirs and achieved effective inhibition of four miRNAs over a period of weeks in most tissues except brain [36]. A novel approach was recently reported by Ebert et al. [21]. They developed miRNA inhibitors that can be transiently expressed in cultured mammalian cells. These competitive inhibitors termed “miRNA sponges” derepressed miRNA targets at least as strongly as chemically modified antisense oligonucleotides [21]. A different approach was taken by Tsuda et al. [83]. The authors designed synthetic miRNAs to target overexpressed tumor proteins, such as HER-2 protein. A synthetic miRNA targeting HER-2 messenger RNA successfully inhibited HER-2 protein expression in ovarian cancer cells [83]. Together, these studies hold some promise of miRNAs as future therapeutic targets.

One limitation of antisense RNA therapies is the restricted number of cells that can be targeted. Any approach to knock down a particular miRNA with antisense oligonucleotides will only result in partial knockdown. This may represent a limitation for cancer therapies. It remains to be seen whether indirectly mediated bystander effects on cancer cells that have not been directly targeted may partly overcome this limitation. In contrast, a partial effect on function may be of therapeutic value in neurodegenerative diseases, such as Parkinson’s or Alzheimer’s disease. A partial restoration of dopamine production by antisense therapy might result in a significant clinical improvement in Parkinson patients. Similarly, a partial reduction of the disease-causing proteins in Alzheimer’s disease may lead to a clinical improvement and might be achievable by RNA based or miRNA gene therapy.

Techniques and approaches to study miRNAs

All known miRNAs are registered in a public web-based registry, the “miRBase” database that provides up-to-date information on all published miRNAs [25]. Novel miRNA genes can be discovered by bioinformatics approaches searching for evolutionary conserved stem-loop structures in the genome (reviewed in [3, 5]). Experimentally, miRNAs are discovered by cloning all small RNAs from a certain tissue type or developmental stage and subsequent sequencing to identify the subgroup of small RNAs that fulfill the criteria for miRNAs [38, 51]. Both computational and experimental approaches indicate that many more miRNAs are likely to be identified [4, 5], which is reflected by the rapidly increasing number of annotated miRNAs which increased from less than 300 to more than 4,000 over the past 4 years [58].

MicroRNA expression studies

Northern blot analysis is a well-established technique for studying messenger RNA expression and was soon adapted to detect miRNAs in cells or tissues [42, 84]. Subsequently, conventional DNA microarray technology was modified to form miRNA microarrays, allowing for the detection of multiple miRNAs simultaneously across various samples [15, 60, 62, 82].

Lu et al. [52] developed a novel microarray strategy to improve probe specificity, which is critical due to the short nature of mature miRNAs. They performed hybridization in solution using polystyrene capture beads that are coupled to oligonucleotide probes complementary to the miRNAs of interest. The solution hybrids are then analyzed using a multicolor flow cytometer measuring bead color, denoting miRNA identity, and labeling intensity, denoting miRNA abundance [52].

In parallel to microarray platforms, commercial assays for quantitative reverse transcriptase polymerase chain reaction (RT-PCR) have become available. These allow for the analysis of miRNAs in small tissue samples or even single cells [79], as well as validation of microarray data. In addition to mature miRNAs, these quantitative RT-PCR assays can be applied to analyze miRNA precursors and primary transcripts [31]. In situ hybridization for the

detection of mature miRNAs has recently become possible by using special high-affinity locked nucleic acid (LNA)-modified DNA oligonucleotide probes and holds promise for the application on human formalin-fixed and paraffin embedded tissue [35, 61].

Functional characterization of miRNAs

Various strategies have been used to investigate the function of specific miRNAs. In worms and flies, loss-of-function mutants for specific miRNAs or miRNA families allow us to draw conclusions regarding possible physiological functions of miRNAs from the resulting abnormal phenotype [34, 39, 86]. The knockdown of miRNAs or pre-miRNAs using modified antisense oligonucleotides has proven particularly useful in cell lines [29, 45, 57]. LNA-modified antisense oligonucleotides have been successfully utilized to knock down specific miRNAs in cultured cells [22]. This approach allowed identification of a crucial role for a miRNA, miR-223, in granulocytic differentiation [22]. In addition, the modified antisense RNAs (antagomirs) described by Krutzfeldt et al. [36], which inhibit miRNA function in the adult mouse, may provide a potential research tool to study miRNA function in vivo.

In mammals, induced defects in miRNA biogenesis are a useful tool for investigating the biological roles of miRNAs, as loss-of-function mutants are not available for most miRNA genes. Dicer knockout mouse models have revealed essential roles for miRNAs in murine organogenesis [88]. A recent study utilized a combined knockdown of the miRNA-processing enzymes Drosha, Dicer1, and DGCR8 to study the consequences of a global decrease in mature miRNAs in cancer cell lines and in a mouse model for lung cancer [37].

Earlier this year, four independent groups have, for the first time, deleted genes for single miRNAs in mice [72, 80, 85, 88]. Two of the groups deleted the same DNA sequence for *mir-155* and described severe immune defects [72, 80]. Mice lacking miR-155 showed impaired function of B and T lymphocytes and dendritic cells [72]. In particular, T helper cell differentiation and the germinal center reaction to produce a T-cell-dependent antibody response were defective [80]. Together, these two studies demonstrated a key role for miR-155 in normal immune function. The two other groups deleted different miRNAs, miR-1-2 and miR-208, and reported cardiac defects. Mice lacking miR-208 showed inadequate cardiac growth in response to stress [85], while mice lacking miR-1-2 had defects in cardiac morphogenesis and electrical conduction [88].

MicroRNA target sites

A validated biochemical strategy for identifying miRNA targets would be highly desirable. Two groups have recently reported promising approaches to experimentally identify miRNA targets. Both approaches apply biochemical methods to purify the effector complexes of miRNAs associated with proteins and bound messenger RNA targets [2, 20].

An increasing number of sophisticated bioinformatics approaches are being developed to predict putative miRNA target genes [3, 10, 69, 74]. This is based on the fact that miRNA target recognition is at least partly based on simple sequence complementarity. Interestingly, exact base pairing between miRNAs and their targets commonly appears to be required only in the first six to eight bases from the 5′ end of the miRNA. The short nature of this designated “seed region” allows a single miRNA to act on up to a hundred different target sites, and all human miRNAs together may regulate up to one third of protein coding genes [10, 47]. A different approach to discovering miRNA target genes is to knock out or overexpress a particular miRNA and use conventional microarrays to identify genes that show changes in expression. This approach is based on the observation that some miRNAs can also downregulate messenger RNA levels in addition to downregulating protein levels of their target genes [49]. Experimental validation of miRNA target sites has been limited to date. A common approach has been to express a miRNA in vivo while simultaneously expressing and monitoring the target messenger RNA linked to a reporter gene, i.e., Luciferase [10, 18, 55, 56, 76]. The fact that a single miRNA can regulate multiple targets and a particular target may be regulated by various miRNAs suggests a highly complex network of miRNA-target interactions, which is only beginning to be unraveled.

Conclusions

Over recent years, miRNAs have emerged as major players in the complex networks of gene regulation and have been implicated in various aspects of human disease. Only 5 years after the first study reported a direct involvement of miRNAs in cancer, these small RNAs have already significantly improved our understanding of carcinogenesis. In addition to protein-coding oncogenes and tumor suppressor genes, we will have to take into account miRNAs and their regulatory networks if we aim to understand the complex processes underlying malignant transformation.

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