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MicroRNA Cancer Regulation

Advanced Concepts, Bioinformatics and Systems Biology Tools

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Preface

Background

Over the last decade, the interest in microRNAs (miRNAs) and their function as post-transcriptional regulators has grown significantly. It is now known that tumour growth in diverse cancer types and the development of metastasis can be mediated, if not caused, by dysfunctional regulation by miRNAs. There is subsequently an ever growing number of publications that link miRNAs to cancer (Fig. 1), focussing on the involvement in tumorigenesis and metastagenesis, including discussions about their role as biomarkers and potential role as therapeutic targets.

MiRNAs play diverse roles in the cell and are involved in many cell functions, like the cell cycle or proliferation, but they also regulate processes, like development and angiogenesis. Therefore, dysregulation of miRNA expression or alterations in the miRNA biogenesis can lead to pathogenesis, including multiple types of cancer. Important to note is that there exist more than 1,000 different miRNAs in human, each of them capable of regulating the expression of multiple genes. Thus, this abundant class of non-coding RNAs has a remarkable impact on gene regulation on a post-transcriptional level. With respect to cancer, some miRNAs are classified as tumour suppressors because they negatively regulate oncogenes. Others are *oncomirs* because they negatively regulate tumour suppressors. Recently, some miRNAs have also been associated with the development of metastasis and are typically referred to as *metastamir*.

The Systems Perspective on microRNAs in Cancer

Since the first miRNAs have been discovered, bioinformatics and systems biology approaches have paved the path for advancements in the field. Computational tools have been developed and are used for the identification of new miRNA genes, the prediction of potential mRNA targets, the functional categorization of target sets and for building mathematical models aiming for the interpretation of miRNA regulation in complex cellular networks, e.g. those associated with cancer.

It becomes therefore more and more evident that the study of single miRNA target interactions (one-on-one miRNA target regulation) is not appropriate. Instead, the joint repression of targets by multiple miRNAs and the multiplicity of targets affected by single miRNAs need to be investigated. One comes to
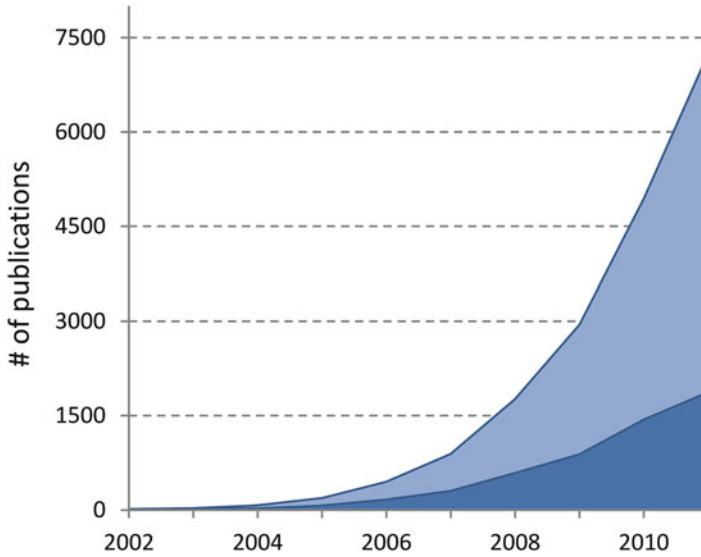
Fig. 1 Number of PubMed entries for 'microRNA + cancer' in total (*light blue*) and per year (*dark blue*)

the conclusion that understanding the role of miRNAs in cellular function and cancer is only possible when considering them as parts of complex regulatory networks.

Systems biology provides an appropriate approach to investigate the regulation of those complex miRNA-regulated networks composed of dozens to hundreds of proteins, genes and miRNAs. Moreover, a dynamical systems perspective becomes indispensable when dealing with networks enriched by non-linear motifs, including feedback and feedforward loops, which induce non-intuitive regulatory patterns like ultrasensitivity, bistability, or oscillations.

Scope of This Book and Target Audience

This edited volume reflects the current state of knowledge about the role of miRNAs in the formation and progression of solid tumours. The main focus lies on computational methods and applications, together with cutting edge experimental techniques that are used to approach all aspects of miRNA regulation in cancer. We are sure that the emergence of high-throughput quantitative techniques will make this integrative approach absolutely necessary in the near future.

This book will be a resource for researchers starting out with cancer miRNA research, but is also intended for the experienced researcher who wants to incorporate concepts and tools from systems biology and bioinformatics into his work. Bioinformaticians and modellers are provided with a general perspective on miRNA biology in cancer, and the state-of-the-art in computational miRNA biology.

Structure

Chapters 1–4. The first four chapters of this book provide an overview about miRNA genomics, biogenesis and mechanisms of target regulation and discuss state-of-the-art methods for the study of miRNAs in cancer, from transcriptomic profiling to applications in clinical practice. Computational tools, developed specifically for miRNA research, are introduced, including algorithms for target prediction, bioinformatics and model-based systems biology approaches to investigate miRNA-regulated networks.

Chapters 5–8. This part of the book presents case studies that discuss the role of miRNAs in various cancer types and their interaction with tumour suppressors and oncogenes. Cancer specific expression signatures and miRNA regulated systems, relevant for tumorigenesis, metastisation and resistance to treatment, are discussed. These chapters nicely illustrate the interdisciplinary approach that combines computational approaches with wet lab experiments, which nowadays forms an integral part of miRNA research.

Chapters 9–11. These three chapters deal with advanced computational approaches to investigate regulatory networks, composed of miRNAs, transcription factors and target genes with a focus on regulatory motifs, like feedback and feed-forwards loops. Moreover, approaches for model generation and simulation studying dynamical aspects of miRNA-target regulation are introduced.

Chapters 12–13. Chapter 12 provides an overview and classification of microRNA web resources dealing with miRNAs, their expression profiles, target genes, function and diseases associations. Chapter 13 introduces a web-based platform that integrates CLIP-Seq and Degradome-Seq data, for the detection of miRNA-target pairs and the identification of regulatory modules.

Chapters 15–16. These two chapters deal with methods for the analysis and integration of miRNA and mRNA expression profiles for the data driven prediction of miRNA-target relationships and the inference of phenotypic associations. Furthermore, methods for the analysis of combinatorial regulation of targets and pathways by miRNAs are discussed.

How to Read This Book

Biologists and Clinicians

Readers with prior knowledge of miRNA cancer biology can skip *Chap. 1* and can directly proceed to the second, third and fourth chapter (*Chaps. 2, 3 and 4*) in order to get a comprehensive overview about computational tools.

Depending on the reader's interest, one can proceed with one or more of the specialized chapters that discuss the interplay between miRNAs and p53

respectively E2F1 (*Chaps. 5* and *8*) and the role of miRNAs in melanoma and in the lung (*Chaps. 6 and 7*).

What follows are chapters that require the reader to have a basic understanding of principles in computational biology. Nevertheless, by reading these chapters one can get an idea of the value that bioinformatics and systems biology approaches have in unraveling underlying mechanisms of miRNA regulation in cancer.

Finally, web resources and tools that can be used to enrich the knowledge about all aspects of miRNA regulation are introduced in *Chap. 12*. *Chapter 13* deals with a particularly interesting resource for the identification of miRNA-target interactions.

Computational Scientists and Modellers

For computer scientists and modellers it is in any case wise to read the first two chapters to get a comprehensive introduction into the field of miRNAs in human cancers and an overview about established applications of computational algorithms in miRNA biology (*Chaps. 1 and 2*). Those, who are already familiar with miRNA-target prediction and validation methods might want to skip the third chapter. In any case we recommend reading the fourth chapter, introducing systems biology approaches that have only recently entered the scene of computational miRNA biology (*Chap. 4*). Further, state-of-the-art developments are covered after the chapters that describe some case scenarios of miRNA biology and cancer. Thus, we would recommend readers with more interest in computational approaches to proceed with *Chaps. 9, 10, 11, 14, 15 and 16*.

Acknowledgements

First of all we would like to thank all authors for making the effort and contributing to this book with their highly engaging chapters.

We would like to thank Xin Lai for offering his help whenever needed and to all the other colleagues in the Department of Systems Biology and Bioinformatics at the University of Rostock who create a unique working environment.

Rostock, Germany
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</div>

Contents

MicroRNAs in Human Cancer

1

Thalia A. Farazi, Jessica I. Hoell, Pavel Morozov,
and Thomas Tuschl

Abstract

Mature microRNAs (miRNAs) are single-stranded RNA molecules of 20–23-nucleotide (nt) length that control gene expression in many cellular processes. These molecules typically reduce the translation and stability of mRNAs, including those of genes that mediate processes in tumorigenesis, such as inflammation, cell cycle regulation, stress response, differentiation, apoptosis, and invasion. miRNA targeting is initiated through specific base-pairing interactions between the 5′ end ("seed" region) of the miRNA and sites within coding and untranslated regions (UTRs) of mRNAs; target sites in the 3′ UTR lead to more effective mRNA destabilization. Since miRNAs frequently target hundreds of mRNAs, miRNA regulatory pathways are complex. To provide a critical overview of miRNA dysregulation in cancer, we first discuss the methods currently available for studying the role of miRNAs in cancer and then review miRNA genomic organization, biogenesis, and mechanism of target recognition, examining how these processes are altered in tumorigenesis. Given the critical role miRNAs play in tumorigenesis processes and their disease specific expression, they hold potential as therapeutic targets and novel biomarkers.

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1.1 miRNA Overview

miRNAs were originally shown to be important in timing of larval development in *C. elegans*, leading to the identification of the miRNAs *lin-4* and *let-7* [1, 2]. Our initial understanding of miRNA-mRNA target recognition came from

observations of sequence complementarity of the *lin-4* RNA to multiple conserved sites within the *lin-14* 3′ UTR [1, 3]; molecular genetic analysis had shown that this complementarity was required for the repression of *lin-14* by *lin-4* [4]. Homologues of *let-7* or *lin-4/mir-125* were thereafter shown to have temporal expression patterns in other organisms, including mammals, and to regulate mammalian development [5–8]. Given their integral role in development, it was no surprise that miRNAs were soon found to be important in tumorigenesis, and since their discovery close to 5,000 publications associate miRNAs to cancer, including over 1,000 reviews (recent examples include [9–11]). miRNAs were initially linked to tumorigenesis due to their apparent proximity to chromosomal breakpoints [12] and their dysregulated expression levels in many malignancies [13, 14].

Given the wealth of rapidly accumulating information implicating miRNAs in cancer, to allow the reader to critically assess the reports exploring the function of miRNAs in malignancies, we first review the methods used to study the expression and role of miRNAs in tumors, and then review the evidence that relates miRNA genomic organization, biogenesis, target recognition and function to tumorigenesis. An overview of miRNA cistronic expression and sequence similarity allows a better understanding of the regulation of miRNA expression and the factors contributing to technical limitations in accuracy of miRNA detection. Understanding the regulatory potential of miRNAs based on sequence similarity families and miRNA abundance allows evaluation of which miRNAs are important regulators of tumorigenesis pathways.

1.2 Methods for Studying miRNA Genetics and Expression

1.2.1 miRNA Profiling

The main methods currently used for miRNA profiling are sequencing, microarray and real-time RT-PCR based approaches (reviewed in [15–17]). The input material initially used for these studies comprised high quality preserved fresh frozen samples, but recently it has been possible to obtain reproducible and comparable profiles using formalin-fixed paraffin-embedded tissues (FFPE), making these archived tumor collections accessible for study [18–20]. Microarrays generally provide fold-changes in miRNA expression between samples, with members of miRNA sequence families prone to cross-hybridization [21–24]. More recently, calibration cocktails of synthetic miRNAs were used in array experiments to derive absolute abundance of miRNAs [25]. RT-PCR methods are lower throughput and require normalization (i.e. candidate reference genes including other small noncoding RNAs [26, 27]). Mean expression normalization has been suggested as an alternative RT-PCR normalization method for reduction of technical variation to allow appreciation of biological changes [28]. If external miRNA standards are used for quantification (i.e. [29, 30]), the most abundant miRNA, which may vary in length due to 3′ end heterogeneity, should be used as a calibration standard. Sequencing methods, besides their obvious potential to identify new miRNAs, editing and mutation events, estimate miRNA abundance based on frequency of sequence reads (e.g. [5, 7, 8, 31–34]). Given the dramatic increase in sequencing power, bar-coding samples can allow multiple specimens to be processed at the same time, reducing the cost and effort of profiling, and paving the way for large specimen studies [34–36]. Ligation biases between miRNAs and 5′ and 3′ adapters for RT-PCR amplification exist in sequencing methods, and miRNA read frequencies may not always reflect the absolute expression levels, but these variations are irrelevant when monitoring fold-changes between samples. A study with a synthetic pool of 770 miRNA sequences showed that overall, these biases do not prevent identification of miRNAs, and allowed estimation of these biases [36]. For example, certain miRNAs could be over-represented due to higher ligation efficiency (such as miR-21, which was ~2-fold over-represented), while other miRNAs could be under-represented (such as miR-31, which was >5-fold under-represented). However, given the increasing depth of sequencing, most under-represented miRNAs are identified with sufficient sequence reads to

allow for a statistically significant comparison across parallel processed samples.

Recent studies have compared the results obtained using multiple platforms [37]. A study of miRNA expression in liposarcoma revealed excellent agreement between bar-coded next generation sequencing and microarray profiles [38], while another study of miRNA expression in breast cancer showed good agreement between bar-coded sequencing and another hybridization-based method, Northern blotting [39].

Finally, choosing the appropriate statistical analysis to evaluate the data depends on the methodology used to obtain the profiles, ranging from established SAM analysis for microarray data [40], to newly developed techniques for sequencing data [34, 41, 42]. Recent in situ hybridization (ISH) advances allowed sensitive detection of miRNAs in heterogeneous tissues, defining miRNA cellular localization [43–45]. The potential of miRNA localization to suggest function for a subpopulation of cells was demonstrated early on, as in the case of *lsy-6* expressed in less than ten neurons in *C. elegans* controlling left/right asymmetry [46].

1.2.2 miRNA Databases and Validation

It is critical to know which miRNAs are validated and have the potential to regulate cellular functions, especially given the frequent revisions of the miRNA database, miRBase (www.mirbase.org) [47], and the dramatic increase in the number of novel and re-annotated miRNAs through the use of deep-sequencing technologies. It is extremely challenging to establish the validity of novel miRNAs, particularly when their definition is based on a handful of sequence reads. The latest release of miRBase (version 17) includes 1,424 human miRNA precursors. Compared to version 16, version 17 includes 385 novel human miRNA precursors, 45 name changes, 1 sequence revision, and the removal of 2 precursors. Given the recent explosion in acquisition of next generation sequencing profiles, miRBase has now added features to

allow evaluation of microRNA annotation [48]. The database mapped reads from short RNA deep-sequencing experiments to miRNAs and developed web interfaces to view these mappings. This is an important step in characterizing the newly identified miRNAs as prototypical miRNAs (consisting of a hairpin structure and processing sites consistent with RNase III cleavage steps).

The challenge of constantly revising and curating existing databases based on newly acquired sequencing data is illustrated in two recent studies re-evaluating mouse and human miRNAs. A recent study of 60 million small RNA sequence reads generated from a variety of adult and embryonic mouse tissues confirmed 398 annotated miRNA genes and identified 108 novel miRNA genes but was unable to find sequencing evidence for 150 previously annotated mouse miRNAs. Ectopic expression of the confirmed and newly identified miRNA hairpin sequences yielded small RNAs with the classical miRNA features but failed to support other previously annotated sequences (of the 17 tested miRNAs with no read evidence, only one yielded a single sequence read, while of 28 tested miRNAs with insufficient number of reads, only 4 were verified) [49]. A more recent study has re-annotated human miRNAs based on read evidence from over 1,000 human samples [39]. miRNAs were curated both on the basis of read counts, as well as patterns compatible with traditional miRNA processing, re-defining prototypical miRNAs (557 precursors, corresponding to 1,112 mature and star sequences (miRNA*, described in the following section), miR-451 and miR-618 being the only miRNAs without a star sequence). 269 not yet reported star sequences were added (compared to miRBase 16), putative miRNAs from miRBase, for which read evidence was not obtained, were ignored, and specific miRNAs were renamed according to the read ratio between mature and star sequences. The importance of curated miRNA databases is especially evident in assessing the statistical significance of differentially expressed miRNAs to identify potential biomarkers based on microarray studies. Including miRNAs without

strong read evidence in such comparisons could skew the results.

1.3 Mechanisms of Alteration of miRNA Levels in Malignancy

We review miRNA biogenesis (Fig. 1.1) and illustrate which steps of the biogenesis pathway are linked to malignancy, starting from miRNA genomic localization, transcriptional regulation, processing steps and post-transcriptional modification. There is evidence supporting the association of the first three processes and/or the factors that control them with tumorigenesis, whereas evidence relating post-transcriptional miRNA modifications to cancer is not clear-cut.

1.3.1 General Principles of miRNA Genomic Organization

miRNAs are frequently expressed as polycistronic transcripts. To date, 1,424 human miRNA precursor sequences have been deposited in miRBase [47]. Approximately one-third (497) of these miRNAs are located in 156 clusters, each measuring ≤51 kb in the human genome (51 kb being the longest distance between miRNAs belonging to the same cluster, Fig. 1.2). These miRNA clusters are co-expressed based on evidence from miRNA profiling data from a variety of tissues and cell lines [22, 33, 34, 49]. The genomic organization of representative oncogenic (miR-17 and miR-21) and tumor suppressor (let-7 and miR-141) sequence families (described in following section) is illustrated in Fig. 1.2. Presentation of miRNA profiles in the form of expression clusters provides a readily interpretable summary of expression data and stresses the importance of cistronic expression regulation; dysregulation of one member of the cluster should be accompanied by similar dysregulation of other cluster members [39]. Since miRNA genes are frequently multi-copy, determining the relative contribution of each genomic location to mature miRNA expression is challenging.

1.3.2 Alterations in Genomic miRNA Copy Numbers and Location

Changes in miRNA expression between normal and tumor specimens are often attributed to the location of miRNAs in regions of chromosomal instability (amplification, translocation or deletion), or nearby chromosomal breakpoints, initially locating 52.5% of miRNA genes in cancer-associated regions or fragile sites [12]. The miRNA cluster *mir-15a/16-1* is located in a frequently deleted genomic locus containing a putative tumor suppressor containing region in chronic B-cell lymphocytic leukemia (B-CLL) [50]. Other examples include deletion of *let-7g/mir-135-1* in a variety of human malignancies [12], amplification of *mir-17-92* cluster in lymphoma [51], translocation of *mir-17-92* in T-cell acute lymphoblastic leukemia (T-ALL) [52] and amplification of *mir-26a* in glioblastoma [53].

1.3.3 Alterations in miRNA Transcriptional Regulation

Some autonomously expressed miRNA genes have promoter regions that allow miRNAs to be highly expressed in a cell-type-specific manner, and can even drive high levels of oncogenes in cases of chromosomal translocation. The *mir-142* gene, strongly expressed in hematopoietic cells, is located on chromosome 17 and was found at the breakpoint junction of a t(8;17) translocation to *MYC*, which causes an aggressive B-cell leukemia [54]. The translocated *MYC* gene, which was also truncated at the first exon, was located only four nucleotides from the 3′ end of the *mir-142* precursor, placing it under the control of the upstream *mir-142* promoter. In an animal model for Hepatocellular Carcinoma (HCC), a similar event placed *C-MYC* downstream of the *mir-122a* promoter, which is active only in hepatocytes [55].

Many transcription factors regulate miRNA expression in a tissue-specific and disease state-specific fashion, and some miRNAs are regulated by well-established tumor suppressor or oncogene pathways such as TP53, MYC, and RAS (reviewed in [56]). The miRNA and its transcriptional
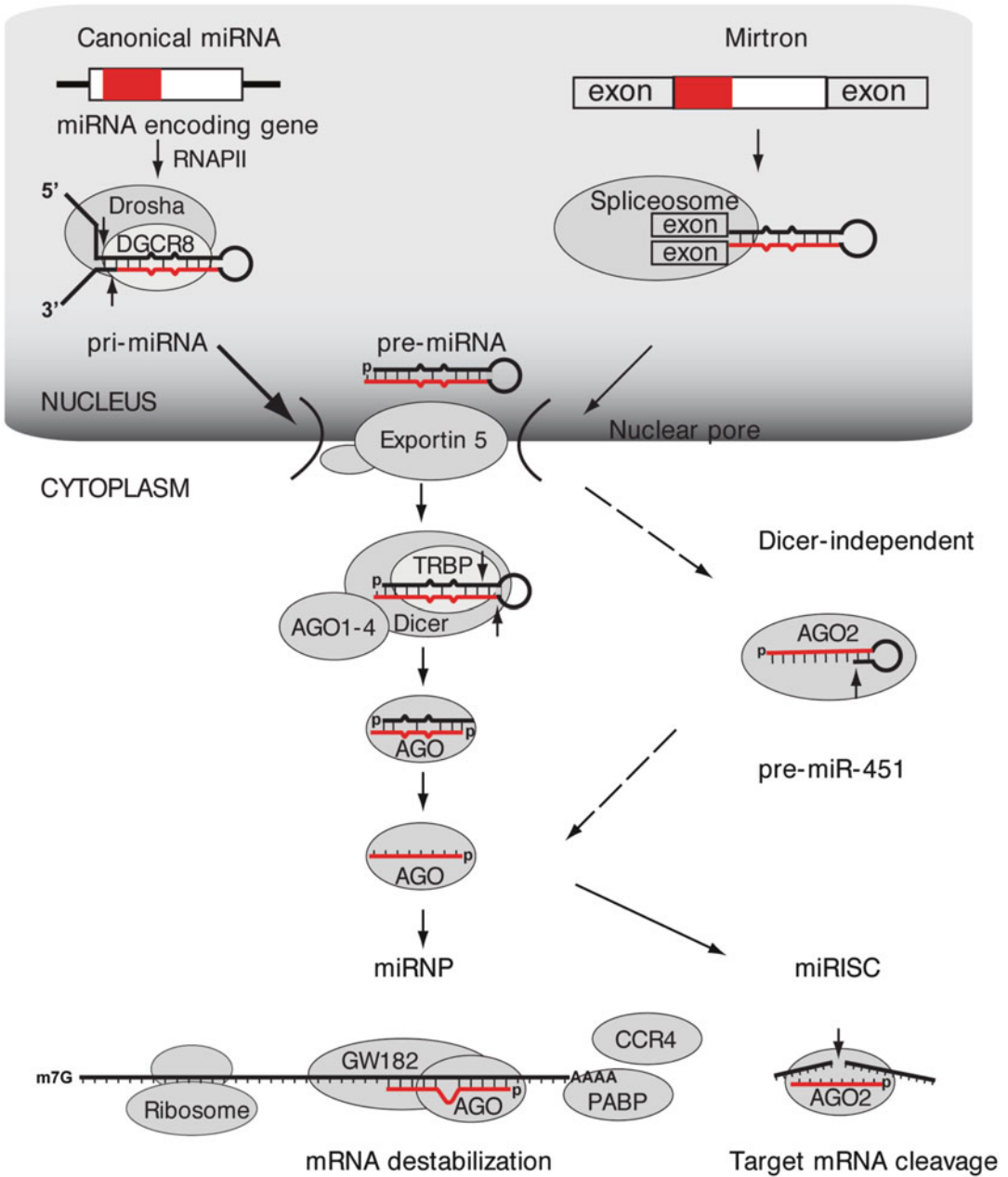
Fig. 1.1 miRNA biogenesis pathway. miRNAs are transcribed by RNAPII to produce pri-miRNAs. Canonical miRNAs are processed by the endoribonuclease Drosha in partnership with its RBP partner DGCR8; mirtrons are instead processed by the spliceosome. The processed pre-miRNA is transported to the cytoplasm through an export complex consisting of exportin 5. The pre-miRNA is subsequently processed in the cytoplasm by another endoribonuclease Dicer in partnership with its RBP partner TRBP to form the final 21–23 nucleotide miRNA product. miR-451 is not processed by Dicer, but is rather cleaved by AGO2. Mature miRNAs (indicated in *red*) are then incorporated into AGO 1 through 4, forming miRNPs, also known as miRISC. miRNPs also incorporate other proteins, such as GW182. miRNPs are thought to direct miRNA mediated destabilization (i.e. through interaction with CCR4) or miRNA mediated translational repression (i.e. through interaction with ribosomes) of miRNAs without perfectly complementary mRNA targets. miRISC is thought to direct AGO2 catalyzed target mRNA cleavage of miRNA fully or nearly fully complementary mRNA targets

a

chr21

mir-99a   let-7c   50.3kb   mir-125b-2   *C21ORF34*

chr19

mir-99b let-7e   mir-125a

chr11

mir-100   5.6 kb   let-7a-2   46.7 kb   mir-125b-1   *LOC39959*

chr9

let-7a-1   let-7f-1   2.4 kb   let-7d

chr22

let-7a-3   let-7b   *LOC400931*

chrX

let-7f-2   mir-98   *HUWE1*

chr3   *WDR82*

let-7g

chr12

let-7i

■ let-7 family

```
let-7a    TGAGGTAGTAGGTTGTATAGTT
let-7f    TGAGGTAGTAGAATTGTATAGTT
let-7b    TGAGGTAGTAGGTTGTGTGGTT
let-7i    TGAGGTAGTAGTTTGTGCTGTT
let-7g    TGAGGTAGTAGTTTGTACAGTT
let-7c    TGAGGTAGTAGGTTGTATGGTT
let-7d    AGAGGTAGTAGGTTGCATAGTT
let-7e    TGAGGTAGGAGGTTGTATAGTT
miR-98    TGAGGTAGTAAGTTGTATTGTT
          ******* * *** ***
```

■ miR-99 family

```
miR-99a   AACCCGTAGATCCGATCTTGTG
miR-100   AACCCGTAGATCCGAACTTGTG
miR-99b   CACCCGTAGAACCGACCTTGCG
          ********* **** **** *
```

■ miR-125 family

```
miR-125b  TCCCTGAGACCCT--AACTTGTGA
miR-125a  TCCCTGAGACCCTTTAACCTGTGA
          ************* *** *****
```

b

chr1

mir-200b   mir-200a   mir-429

chr12

mir-200c   mir-141

■ miR-141 family

```
miR-141   TAACACTGTCTGGTAAAGATGG-
miR-200c  TAATACTGCCGGGTAATGATGGA
miR-200b  TAATACTGCCTGGTAATGATGA-
miR-200a  TAACACTGTCTGGTAACGATGT-
miR-429   TAATACTGTCTGGTAAAACCGT-
          *** **** * ***** *
```

c

chr17

TMEM49   0.7 kb   mir-21

■ miR-21

```
miR-21    TAGCTTATCAGACTGATGTTGA
```

■ miR-17 family

```
miR-93    CAAAGTGCTGTTCGTGCAGGTAG
miR-106b  TAAAGTGCTGACAGTGCAGAT--
miR-17    CAAAGTGCTTTACAGTGCAGGTAG
miR-20a   TAAAGTGCTTATAGTGCAGGTAG
miR-18a   TAAGGTGCATCTAGTGCAGATAG
miR-106a  AAAAGTGCTTACAGTGCAGGTAG
miR-20b   CAAAGTGCTCATAGTGCAGGTAG
miR-18b   TAAGGTGCATCTAGTGCAGTT--
          ** **** ****** *
```

d

chr13

mir-18a   mir-20a   mir-92a-1

mir-17   mir-19a   mir-19b-1   *C13orf25*

chrX

mir-18b   mir-19b-2   mir-363

mir-106a   mir-20b   mir-92a-2

chr7

mir-106b mir-93 mir-25   *MCM7*

1 kb

■ miR-19 family

```
miR-19b   TGTGCAAATCCATGCAAAACTGA
miR-19a   TGTGCAAATCTATGCAAAACTGA
          ********** ************
```

■ miR-25 family

```
miR-92a   TATTGCACTTGTCCCGGCCTGT-
miR-25    CATTGCACTTGTCTCGGTCTGA-
miR-363   AATTGCACG-GTATCCATCTGTA
          ******* ** * ***
```

regulators can participate in complex feedback regulation loops. Examples include the TP53 regulated *mir-34a* [57, 58], the RAS regulated *mir-21* [33, 59, 60] and the MYC regulated *mir-17-92* gene cluster [61, 62].

miRNA dysregulation has also been linked to changes in epigenetic regulation, such as the methylation status of miRNA genes, which results in alterations in their expression levels [63, 64]. Examples of methylated miRNA genes include *mir-127* in bladder cancer cells [65] and *mir-9-1* in breast cancer [66].

1.3.4 miRNA Biogenesis Pathway in Tumorigenesis

miRNA biogenesis has been reviewed extensively [56, 67–73] (Fig. 1.1). miRNA pathway components could either be mis-expressed in tumors or mutated (reviewed in [74, 75]). Post-transcriptional regulation of miRNAs themselves through RNA editing or terminal modifications was shown to alter miRNA targeting, processing and stability, but connection of these modifications to tumorigenesis has not yet been definitive (reviewed in [56, 75, 76]).

1.3.4.1 miRNA Biogenesis
Briefly, the mature 20–23-nt miRNA molecules are excised in a multi-step process from primary transcripts (pri-miRNAs) that contain one or more 70-nt hairpin miRNA precursors (pre-miRNA) and have their own promoters or share promoters with coding genes. These hairpin structures are recognized in the nucleus by DGCR8, a double-stranded RNA-binding protein (dsRBP), and RNASEN, also known as RNase III Drosha, and excised to yield pre-miRNAs. These molecules are subsequently transported by XPO5 (exportin 5) to the cytoplasm where they are further processed by DICER1 (Dicer) in complex with the dsRBPs TARBP2 (TRBP) and/or PRKRA to yield an RNA duplex processing intermediate composed of mature miRNA and miRNA* sequences. Some miRNAs bypass the general miRNA processing and their maturation can be independent of DGCR8 and RNASEN, such as miR-320 or miR-484 [77], or are DICER1 independent, such as erythropoiesis-related miR-451 [78, 79]. DGCR8 and RNASEN independent miRNAs include mirtrons and tailed mirtrons, which release their pre-miRNA by splicing and exonuclase trimming [80, 81]. A recent review describes alternative processing pathways and enumerates settings in which alternative miRNA pathways contribute to distinct phenotypes among miRNA biogenesis mutants [82].

While the mature miRNA is loaded into the Argonaute/EIF2C (AGO) proteins that are at the core of the miRNA-containing ribonucleoprotein complex (miRNP), sometimes also referred to as RNA-induced silencing complex (miRISC), the miRNA* is released and degraded. miR-451 is generated from an unusual hairpin structure that is processed by AGO2 instead of DICER1 [78, 79]. The miRNPs contain a member of the AGO family (1–4), which binds the miRNA and mediates target mRNA recognition. Several other RBPs have been implicated in miRNA biogenesis, including DHX9, DDX6, MOV10, DDX5, DDX17, LIN28A, HNRNPA1 and KSRP [56, 83]. Following transcription, miRNAs can be modified by several enzymes, including deaminases, resulting in miRNA editing, and terminal uridylyl transferases (TUTases), leading to pre-miRNA uridylylation, potentially affecting the

◄

Fig. 1.2 miRNA genomic and functional organization. The genomic and functional organization of four miRNA clusters is clarified: (**a**) *let-7/mir-98* cluster, (**b**) *mir-141/mir-200a* cluster, (**c**) *mir-21* cluster and (**d**) *mir-17-92* cluster. The genomic locations for each of the miRNA members are defined. *Grey* lines denote intronic regions. miRNA mature sequences are color coded according to the sequence family they belong to (i.e. in the *let-7/mir-98* cluster *red* signifies the let-7 sequence family). The star sequence is defined with a *grey* bar. The sequence families are depicted as sequence alignments compared to the most highly expressed miRNA family member shown on *top*, based on profiles of over 1,000 human specimens [39]. *Shaded residues* denote differences from the most highly expressed miRNA family member

amount and ratio of miRNA and miRNA* (e.g. [84]), or their sequences (e.g. [85]).

1.3.4.2 Alterations in RNASEN/DGCR8 and DICER1/TARBP2

Inhibition of the miRNA biogenesis pathway leads to severe developmental defects and is lethal in many organisms (reviewed earlier in [86], recent examples include [77, 78]), and perturbations of this pathway predispose to tumorigenesis [87]. Initial miRNA expression profiling experiments suggested that miRNAs are less abundant in tumors compared to their normal tissue counterparts [14], leading to the proposal that miRNAs are predominantly tumor suppressors rather than oncogenes. Quantification of absolute miRNA levels, not only relative abundance, in miRNA profiling methods is necessary to clarify these observations. 27% of various tumors are found to have a hemizygous deletion of the gene that encodes DICER1 [88]. Global knockdown of mature miRNAs by targeting DICER1, RNASEN and its cofactor DGCR8 increases the oncogenic potential of already transformed cancer cell lines and accelerates tumor formation [87]. Reductions in the amount of DICER1 resulting in impaired miRNA processing have also been shown to increase the rate of tumor formation in two different cancer mouse models, a K-RAS-driven lung cancer [88] and an Rb-driven retinoblastoma [89]. DICER1 is therefore considered a haploinsufficient tumor suppressor, requiring partial deletion for its associated tumorigenesis phenotype [89]. The phosphorylation of the DICER1 cofactor TARBP2 by the mitogen-activated protein kinase Erk enhances pre-miRNA processing of oncogenic miRNAs, such as miR-21, and decreases production of tumor suppressor let-7a [90]. Moreover, TARBP2 is mutated in some colon and gastric cancers with microsatellite instability, and TARBP2 frameshift mutations correlate with DICER1 destabilization; in cell lines and xenografts with TARBP2 mutations, reintroduction of wild type TARBP2/DICER1 slowed tumor growth [91, 92]. Finally, DICER1 was also recently implicated as a metastasis suppressor (reviewed in [93]).

1.3.4.3 Alterations in Other Pathway-Related RBPs

Firstly, expression of LIN28A blocks processing of tumor suppressor pri- and pre-let-7 [94–98], thus maintaining expression of genes that drive self-renewal and proliferation (reviewed in [99]); tumors that express LIN28A were indeed shown to be poorly differentiated and more aggressive than LIN28A-negative tumors. Secondly, the helicases DDX5 and DDX17 are thought to stimulate processing of one third of all murine miRNAs by acting as a scaffold and recruiting factors to the RNASEN complex and thereby promoting pri-miRNA processing [100]. Association of DDX17 and DDX5 RNA helicases through interactions mediated by the tumor suppressor TP53 with the RNASEN/DGCR8 complex facilitates the conversion of pri- to pre-miRNAs [101]. Specifically, the DDX5-mediated interaction of the RNASEN complex with the tumor suppressor TP53 was shown to have a stimulatory effect on the tumor suppressor pri-miR-16-1, pri-miR-143 and pri-miR-145 processing in response to DNA damage in cancer cells [101]. Thus, TP53 mutations, often observed in malignancies, led to a decrease in pre-miRNA production. Thirdly, oncogenic SMADs, downstream effectors of the TGF-β superfamily pathways, have been shown to control RNASEN-mediated miRNA maturation through interaction with DDX5, promoting expression of oncogenic miR-21 [102]. KSRP promotes the biogenesis of a subset of miRNAs, including let-7a, by serving as a component of both DICER1 and RNASEN complexes affecting proliferation, apoptosis and differentiation [103]. In a final example, inactivating mutations of XPO5 in tumors with microsatellite instability result in the nuclear retention of miRNAs [104]. Restoration of XPO5 function reverses the impaired export of pre-miRNAs and has tumor suppressor features.

1.4 Dysregulation of miRNA-mRNA Target Recognition

1.4.1 miRNA Function/Mechanism

As described above, miRNAs function through the AGO proteins, containing both RNA-binding

domains and RNase H domains (reviewed in [105]). The four human *Ago* genes are co-expressed and bind to miRNAs irrespective of their sequence. AGO2, in contrast to the other members, retains an active RNase H domain and thus is able to directly cleave target RNAs with extensive complementarity to the bound miR-NAs. The assembly of the miRNP complex involves multiple AGO conformational transitions captured in a series of crystal structures (reviewed in [106]). The mRNA target is recognized by pairing of the miRNA seed region (position 2–8) to complementary sequences located mainly in the target 3′ UTR, but also in the coding regions. Target mRNA recognition and regulation involves members of the GW182/TNRC6 family. TNRC6 proteins act at the effector step of silencing, downstream of AGO proteins, and play a crucial role in miRNA silencing in animals (reviewed in [107]). Proteomic approaches identified additional AGO-interacting proteins, some of which likely represent mRNA-interacting partners that co-purified with miRNA-targeted mRNPs; their function in RNA silencing processes and potentially tumorigenesis remains to be established.

In mammalian cells under steady state conditions, miRNAs have been shown to destabilize targeted transcripts [108–111] through a variety of mechanisms, including de-capping and de-adenylation; target mRNA and protein abundance changes track closely [108, 109, 112, 113]. These studies also showed that miRNAs destabilize mRNAs preferably through binding sites located in their 3′ UTRs [114–118]. Ribosome profiling studies demonstrated that the ribosome density of miRNA targets was unaltered, while changes in miRNA levels were inversely correlated to mRNA and protein abundance, emphasizing the role of miRNAs in regulation of mRNA stability but not translation [119]. Translational regulation by miRNA targeting is considered to predominantly act at the level of translation initiation. Identification of miRNA/mRNA ribonucleoprotein components in processing bodies (P-bodies) also implies their role in mRNA storage and RNA turnover. An excellent recent review describes the differ-

ent mechanisms implicated in miRNA function, highlighting the different experiments supporting translational repression versus mRNA decay and the evolution in our current thinking [107].

1.4.2   Organization of miRNAs into Sequence Families

Certain miRNAs share sequence similarity in regions that are critical for mRNA target recognition, specifically the seed region, and are best viewed as a family when considering mRNA target regulation and functional consequences of altered miRNA expression. miRNAs can be grouped in sequence families, based not only on their seed sequence similarity but also overall sequence similarity given that the miRNA 3′ end also contributes to miRNA targeting, although to a lesser extent (reviewed in [68]) (Fig. 1.2). Changes in the overall abundance of miRNA sequence families relate directly to target regulation. In a MYC-driven B-cell lymphoma mouse model, a conditional knockout of the oncogenic *miR-17-92* gene cluster induces apoptosis, which can be reduced by reintroduction of only one of the four sequence families produced from the cluster [120].

1.4.3   miRNA-mRNA Stoichiometry

The majority of miRNA profiling studies do not provide an estimate of miRNA abundance, which is critical in our understanding of the role of miRNA-mRNA mediated regulation in tumorigenesis. Only the most abundantly expressed miRNAs occupy a substantial fraction of their available mRNA target sites and affect target mRNA stability [118]. Abundant miRNAs that behave as "switches", turned on or off during the tumorigenesis process, as shown in developmental processes, have the most significant regulatory potential, given that miRNAs usually only lead to modest 1.5- to 4-fold regulation of their target expression [112, 113, 115]. However, given that specific mRNAs are subject to regulation by multiple miRNAs of unrelated families, cumulative effects of lower expressed miRNAs may be

relevant [67, 121, 122]. Furthermore, in the rare circumstance that miRNAs share near perfect complementarity to mRNAs, they may act in a siRNA-like catalytic mode, cleaving mRNA targets even at low miRNA abundance. To conclude, the interplay between miRNAs expressed in particular tissues, the levels of their respective expressed targets, as well as other post-transcriptional gene regulatory mechanisms (such as regulation by RBPs or other competing interactions – see below) is likely responsible for balancing miRNA conferred regulation.

1.4.4 Changes in the miRNA Targets

The binding sites of miRNAs in mRNAs can be altered through a variety of mechanisms, such as point mutations, translocations, shortening of the 3′ UTR, competition with other RBPs or decoy molecules for mRNA binding. Point mutations in miRNA targets can both create or destroy a miRNA binding site [123–125]. Chromosomal translocations can remove miRNA binding sites from their regulated oncogenes, such as in the case of let-7 targeting of the 3′ UTR of the *Hmga2* gene [126]. Shortening of the 3′ UTR through alternative polyadenylation can relax miRNA mediated regulation of known oncogenes, such as *IGF2BP1/IMP1*, and lead to oncogenic transformation [127], as does use of decoy pseudogenes, as in the case of *PTEN*, by saturating miRNA binding sites [128]. Finally, cooperativity or competition of miRNAs for mRNA target site binding with other RBPs, such as ELAVL1 (HuR), DND1 and PUM1, can also de-repress target expression [129–132]. This topic is discussed in a recent review [83].

1.5 Cancer Tissues Have Distinct miRNA Profiles

We will first discuss the state of current miRNA profile databases, and then explore the issue of tissue heterogeneity in the tissue profiles before summarizing the role of miRNA dysregulation in malignancies.

1.5.1 miRNA Cancer Database

The development of miRNA microarrays, RT-PCR platforms and deep sequencing methodologies has resulted in an exponential acquisition of miRNA profiles. Some of the published miRNA profiles are available in the NCBI Gene Expression Omnibus, similarly to mRNA profiles (other resources include www.microrna.org, http://www.mirz.unibas.ch). Larger cancer and blood-borne disease collections have recently been published using various platforms [133–135]. However, there is no database or viewer that allows for cross-platform comparison of existing data.

1.5.2 Tissue Heterogeneity

Tissues are generally composed of multiple cell types, each with their distinct gene expression program. Disease not only alters the expression programs of the affected cell type, but often also its cell type composition. To best separate these effects in the profiling of heterogeneous tumor samples, it may be useful to profile tumor cell lines and individual cell types that may be present in a tumor sample, or define miRNA cellular localization by performing RNA ISH. Figure 1.3 compares miRNA abundance profiles of normal breast, an estrogen receptor positive invasive ductal breast carcinoma, the estrogen receptor positive ductal cell line MCF7, human fat and blood [38, 39]. Strikingly, we can model the profile of a human cancer by simply combining tumor cell line and human fat profiles at equal ratio. This demonstrates that the MCF7 tumor cell line may be a good disease model for deciphering miRNA regulatory networks, as it expresses many of the miRNAs present in the predominant tumor derived cell type and highlights the need for individual cell type miRNA profiles.

1.5.3 miRNAs as Tumor Suppressors and Oncogenes

miRNA dysregulation could be used as a diagnostic tool even if the particular miRNAs do not serve any regulatory function. Alternatively,

Fig. 1.3 miRNA breast tumor and cell line profiles. Comparison of abundance profiles of the *top* expressed miRNA sequence families of normal breast, an estrogen receptor positive invasive ductal carcinoma breast tumor (ER+), the MCF7 ductal derived cell line, human subcutaneous adipose tissue and red blood cells
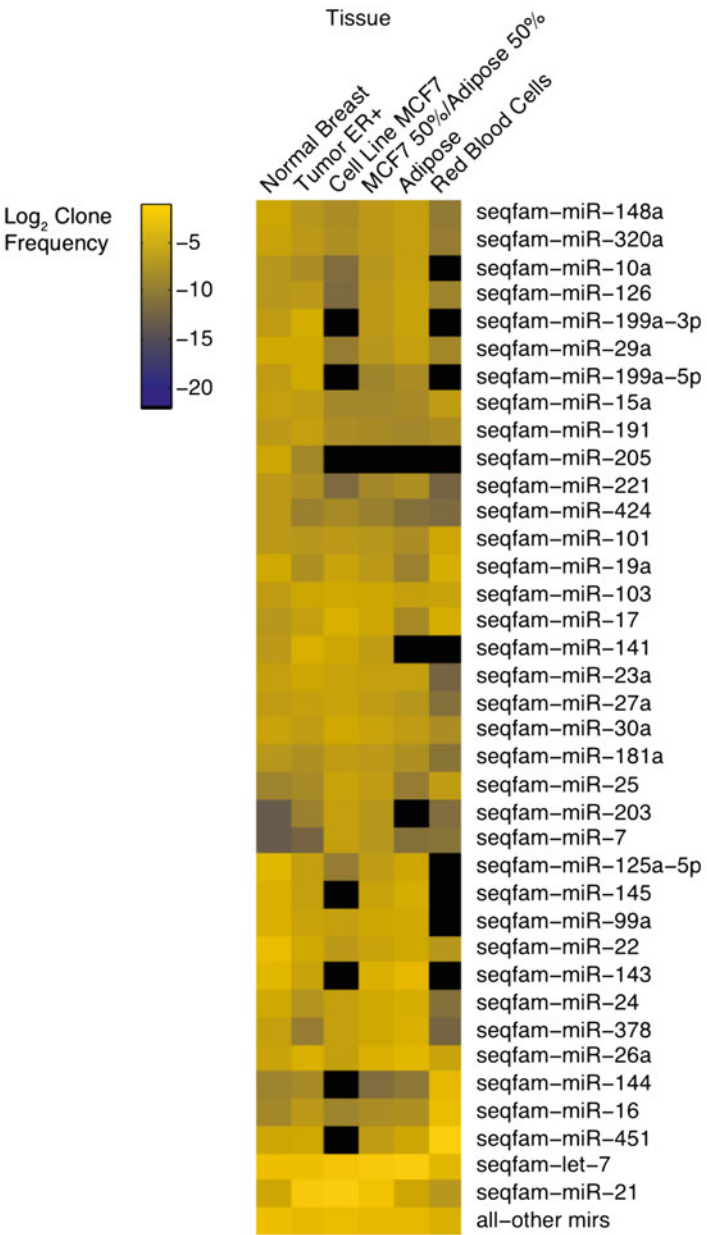
miRNA dysregulation could drive tumorigenesis through the roles miRNAs can adopt as tumor suppressors or oncogenes. miRNAs that are up- or down-regulated in malignancies are respectively referred to as oncogenic or tumor-suppressor miRNAs, sometimes even if there is no evidence for their causative role in tumorigenesis. Some of the most commonly dysregulated miRNAs are summarized in Table 1.1 (reviewed in [11]).

Functional studies performed in cancer cell lines or mouse models of various malignancies through over-expression or knockdown of miRNAs have supported a role for some of these miRNAs in tumorigenesis. Over-expression of tumor suppressor miRNAs, such as let-7g, reduced tumor burden in a K-RAS murine lung cancer model [172]. Over-expression of the oncogenic *mir-17-92* gene cluster led to a lymphoproliferative

Table 1.1 Some of the most common cancer-associated miRNAs

miRNA	Tissue type specificity	Chromosomal location	Property	Malignancy
let-7/98 cluster	Ubiquitous	Multiple members (chromosomes 3, 9, 11, 19, 21, 22, X)	TS	CLL [136], lymphoma [137], gastric [138], lung [139], prostate [9], breast [140], ovarian [138], colon [138], leiomyoma [138], melanoma [138]
mir-15a/16-1 cluster	Ubiquitous	13q14.2	TS	CLL [141], lymphoma [9], multiple myeloma [9], pituitary adenoma [142], prostate [142], pancreatic [142]
mir-17-92 cluster	Ubiquitous	Multiple members (chromosomes 7, 13, X)	OG	Lymphoma [143], multiple myeloma [9], lung [139], colon [143], medulloblastoma [144], breast [140], prostate [145]
miR-21	Ubiquitous	17q23.1	OG	Lymphoma, breast, lung, prostate, gastric, cervical, head and neck, colorectal, glioblastoma (for all: [146])
miR-26a	Ubiquitous	3p22.2 (−1) 12q14.1 (−2)	TS	Lymphoma [147], hepatocellular carcinoma [148], thyroid carcinoma [149]
			OG	Glioblastoma [53, 150]
miR-34a/b/c	Ubiquitous	1p36.22 (a) 11q23.1 (b) 11q23.1 (c)	TS	CLL [136], lymphoma [9], pancreatic [9], colon [9], neuroblastoma [151], glioblastoma [152]
miR-155	Hematopoietic system	21q21.3	OG	Lymphoma (i.e. Burkitt's, Hodgkin's, non-Hodgkin's) [9], CLL ([9], [18]), breast [140], lung [9], colon [9], pancreatic [9]
mir-141/200a cluster	Epithelial specific	Multiple members (chromosomes 1, 12)	TS	Breast [140, 153], renal clear cell carcinoma [154], gastric [155], bladder [156]
			OG/TS	Ovarian [157–159]
miR-205	Epithelial specific	1q32.2	TS	Prostate [160, 161], bladder [162], breast [153, 163, 164], esophageal [165]
			OG	Ovarian [166]
miR-206	Skeletal muscle specific	6p12.2	TS	Rhabdomyosarcoma [30], breast [167]
miR-9	Nervous system specific	1q22 (−1) 5q14.3 (−2) 15q26.1 (−3)	TS	Medulloblastoma [168], ovarian [169]
			OG/TS	Breast [66, 170, 171]

miRNAs that are up- or down-regulated in malignancies are respectively referred to as oncogenic (*OG*) or tumor-suppressor (*TS*), but their role in malignancy is not always experimentally validated. Given the number of manuscripts providing evidence for the role of each miRNA based on patient, cell culture or animal model studies, reviews are often cited instead of original reports to limit the number of references, and only a few selected reports are presented if no review is presented

disorder, and higher level expression of the cluster in MYC-driven B-cell lymphomas dramatically increased tumorigenicity [62, 173]. Over-expression of another oncogene, miR-21, frequently highly expressed in solid and hematologic malignancies, resulted in a pre-B malignant lymphoid like phenotype whereas subsequent miR-21 inactivation in the same model led to apoptosis and tumor regression [174]. Transgenic mice models with loss and gain of function of miR-21 combined with a model of lung cancer confirmed the role of miR-21 as an enhancer of tumorigenesis when over-expressed, or a partial protector when genetically deleted [59]. Ectopic expression

of miR-155 in bone marrow induced polyclonal pre-B cell proliferation progressing to B-cell leukemia or myeloproliferation in mice [175, 176].

Metastasis-related miRNAs have been identified in various malignancies mainly from cell line and xenograft experiments (reviewed in [177]). Examples include breast cancer-related miR-10b, miR-9, miR-31 and miR-335 among others. The interesting regulatory roles of these miRNAs cannot easily be validated in large clinical studies. Two clinical studies with long-term follow-up data instead identified miR-210 to be associated with tumor aggressiveness [178, 179], pointing to difficulties reconciling cell line, xenograft model and patient materials, due to tissue heterogeneity discussed earlier, the heterogeneous nature of the malignancy and timing of clinical specimen acquisition. Tumor miRNA profiles cannot dissect contributions from subpopulations of cells that may be important for tumor characteristics such as metastasis, while cell line miRNA profiles cannot capture the cellular interactions from supporting cell types in the tumor microenvironment. Patient samples are often collected at time of diagnosis, by which time a tumor is already well established and cannot unravel early changes that may be critical in tumor initiation or later changes important in metastasis.

1.5.4   miRNA-Regulated Pathways

The observed effects of miRNA mis-expression on tumor initiation, maintenance or metastasis can be explained by the mRNA targets and pathways they regulate, which include known tumor suppressors and oncogenes (reviewed in [11]). miRNAs regulate a large number of genes, some estimates reporting miRNA regulation of up to 60% of the human genome, making it challenging to attribute a phenotype after mis-expression of a particular miRNA through its action on only a subset of targets [111, 180]. If a few of these targets control rate-limiting steps in the studied tumorigenesis processes within the specified tissues and cell types, such as metastasis, then miRNA regulation of a handful of targets could

potentially explain the phenotype resulting from miRNA mis-expression [181].

Examples of miRNA regulated cancer pathways include differentiation, apoptosis, proliferation and stem cell maintenance, a process important for disease relapse and/or metastasis. The skeletal muscle-specific miR-206 blocks human rhabdomyosarcoma growth in mouse xenograft models by inducing myogenic differentiation [30], while the mir-141/200a cluster is critical in the epithelial to mesenchymal transition (EMT) in various malignancies (reviewed in [182]). Sustained expression of endogenous *mir-17-92* cluster is required to suppress apoptosis in Myc-driven B-cell lymphomas in a conditional knockout allele of *mir-17-92* cluster [120]. TP53-regulated, ectopically expressed miR-34 induced cell cycle arrest in both primary and tumor derived cell lines, downregulating genes promoting cell cycle progression (reviewed in [58]). In a final example of miRNA regulated cancer pathways, isolation of a subset of highly tumorigenic breast cancer cells that were thought to have stemness properties showed that these cells do not express let-7 family members and that expression of let-7 or its known target RAS leads to loss of self renewal [183].

1.6   Alterations of miRNA Sequence

miRNA dysregulation could be a result of mutations in miRNA genes in well-conserved regions in their mature sequence affecting mRNA targeting, or the remainder of the miRNA precursor potentially affecting processing and stability of the mature miRNA (reviewed in [75]). For example, a mutation in the seed region of *mir-96* was shown to lead to hearing loss in a mouse model [184] and was identified in families with non-syndromic progressive sensorineural hearing loss [185], while a point mutation in the viral *mir-K5* precursor stem loop was shown to interfere with its processing and reduce mature miR-K5 accumulation [186]. Germline deletion of the *mir-17-92* gene cluster was another recent example causing skeletal growth defects in humans [187]. If miRNAs are drivers of oncogenic and tumor

suppressor pathways we would expect to find miRNA mutations that can also be causative of the disease. So far the only mutation identified in a miRNA that could lead to malignancy is miR-16, where a germline mutation potentially affects miR-16 biogenesis and abundance in a kindred with familial CLL [188] and New Zealand black mice that naturally develop CLL-like disease [189]. Single nucleotide polymorphisms (SNPs), located both in precursor and mature miRNA sequences, have been examined in the context of disease risk for various malignancies but have not been validated as causative (reviewed in [75]).

1.7 miRNA Target Identification

The currently available target prediction databases (reviewed in [68]) do not easily allow prioritizing the involvement of reported targets in certain phenotypes, thus necessitating the selection of a few targets from a list of hundreds for further study and validation, based on a priori knowledge of potentially involved biological pathways. Since the prediction algorithms do not always produce identical target lists, use of multiple algorithms and comparison or intersection of their results narrows the list to higher confidence targets. Targets are only relevant to a specific phenotype if they are expressed in the studied tissue, an issue not addressed by most computational prediction algorithms. Recently, new algorithms are trying to prioritize computationally predicted targets using integrated miRNA and mRNA profiles [134]. Biochemical identification methods in cell lines and tissues are being established and further refine our understanding of miRNA-mRNA target binding recognition. These methods involve two approaches: over-expression or down-regulation of studied miRNAs followed by assessment of transcriptome-wide mRNA levels by mRNA microarray analysis (e.g. [118]) or deep sequencing technology after immunoprecipitation of miRNAs and mRNAs complexed with AGO, the main component of the miRNA effector complex, to not only identify mRNA targets, but also localize their precise binding sites [190, 191].

1.8 miRNAs as Diagnostics

miRNAs demonstrated their potential as diagnostic tumor markers early on when their profiles were shown to correlate with the tumor embryonic origin, thus defining tumors of unknown origin indistinguishable by histology and assigned based on clinical information [14]. miRNA expression patterns have been linked to clinical outcomes given that miRNAs modulate tumor behavior such as tumor progression and metastasis. Expression of let-7 is downregulated in non-small cell lung cancer patients [192] and is associated with poor prognosis [125, 193], whereas a miRNA signature was identified to be associated with prognosis in CLL [188]. Advances in miRNA detection, such as ISH or RT-PCR, may allow miRNAs to be used as diagnostic and prognostic markers in the clinic.

1.9 miRNAs as Therapeutics

Because miRNAs affect the expression of multiple genes and thereby tune multiple points in disease pathways, miRNAs and their regulated genes represent interesting drug targets. Antisense oligonucleotide targeting experiments in human cell lines, mice [117, 194–197] and non-human primates [198] have demonstrated the feasibility of manipulating miRNA levels. miR-143 was initially shown to promote adipocyte differentiation and could be a target for therapies in obesity and metabolic diseases [194]. Alternatively, "miRNA sponges" have been exploited to reduce miRNA expression in mammalian cells and mouse models by using RNA transcripts expressed from strong promoters containing miRNA-complementary binding sites (reviewed in [199]). Systemic administration of antisense oligonucleotide therapeutics to miR-122, a liver-enriched miRNA, in mice and primates was shown to alter lipid metabolism and hepatitis C viral load, resulting in reduced liver damage [117, 195–197, 200, 201]. At the same time, systemic delivery of a miRNA mimic for miR-26a in a murine model of HCC reduced tumor size [148]. The new and exciting advances in delivery of miRNA inhibitors

and mimics hold the promise of quickly translating our knowledge of miRNAs into treating disease.

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