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A Macro View of MicroRNAs: The Discovery of MicroRNAs and Their Role in Hematopoiesis and Hematologic Disease

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Abstract

MicroRNAs (miRNAs) are a class of endogenously encoded ~22 nucleotide, noncoding, single-stranded RNAs that contribute to development, body planning, stem cell differentiation, and tissue identity through posttranscriptional regulation and degradation of transcripts. Given their importance, it is predictable that dysregulation of miRNAs, which target a wide variety of transcripts, can result in malignant transformation. In this review, we explore the discovery of miRNAs, their mechanism of action, and the tools that aid in their discovery and study. Strikingly, many of the studies that have expanded our understanding of the contributions of miRNAs to normal physiology and in the development of diseases have come from studies in the hematopoietic system and hematologic malignancies, with some of the earliest identified functions for mammalian miRNAs coming from observations made in leukemias. So, with a special focus on the hematologic system, we will discuss how miRNAs contribute to differentiation of stem cells and how dysregulation of miRNAs contributes to the development of malignancy, by providing examples of specific miRNAs that function as oncogenes or tumor suppressors, as well as of defects in miRNA processing. Finally, we will discuss the promise of miRNA-based therapeutics and challenges for the future study of disease-causing miRNAs.

1. INTRODUCTION

MicroRNAs (miRNAs) are just one in an expanding class of noncoding RNAs (ncRNAs), which contribute to diverse biological processes, and the continuous discovery of different classes of ncRNAs has disrupted the conception of commonly established roles for proteins and RNAs in the regulation of cellular activity (Cech and Steitz, 2014). ncRNAs include a diverse set of RNA transcripts that are not translated into proteins. The earliest ncRNAs identified were ribosomal RNAs, which are major constituents of the ribosome and contribute to translation as a ribozyme, and transfer RNAs (tRNAs), which are the adaptor molecules that translate the triplet codon of mRNAs into an amino acid. More recently discovered ncRNAs include small nuclear RNAs, which include splicing-associated RNAs and small nucleolar RNAs; small interfering RNAs (siRNAs); miRNAs; PIWI-associated RNAs (Aravin et al., 2006); and long noncoding RNAs (lncRNAs), which include

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competing endogenous RNAs (ceRNAs) (Tay et al., 2011), circular RNAs (circRNAs) (Zaphiropoulos, 1997), and transcribed pseudogenes (Poliseno et al., 2010).

miRNAs, which will be the focus of this review, are small, ~22 nucleotide (nt), single-stranded, endogenously encoded, ncRNAs that serve a critical role in posttranscriptional regulation of protein expression, and thus contribute to a wide range of biological processes and in the development of disease. This posttranscriptional regulation is so important to normal physiology that more than 60% of human protein-coding genes are under selective evolutionary pressure to maintain miRNA binding sites, also called miRNA response elements (MREs), in their 3′ untranslated regions (3′-UTRs) (Friedman et al., 2009). miRNAs are encoded and transcribed in the nucleus and are exported to the cytoplasm, where they become incorporated into ribonucleoprotein-silencing machinery. Serving as the target recognition component of the ribonucleoprotein-silencing machinery, miRNAs identify specific transcripts, in a sequence-specific manner, for translational repression and transcript destabilization. Thousands of putative miRNAs have been identified in the human genome, with hundreds having been experimentally validated to effect known targets.

Soon after the discovery of miRNAs in humans and other vertebrates, researchers began to recognize the contribution of miRNA dysregulation in the development of cancers, initially describing the role of miR-15 and miR-16 loss in del(13q) chronic lymphocytic leukemia (CLL) (Calin et al., 2002). With the appreciation of the important role of miRNAs in physiology and disease, the number of studies investigating miRNAs has exploded, with large-scale projects, such as the Cancer Genome Atlas (TCGA), collecting next-generation sequencing data for the examination of miRNA dysregulation in disease, and researchers developing complicated conditional models of miRNA overexpression and knockdown to more faithfully recapitulate miRNA dysregulation in experimental animals.

In the past decades, researchers have made tremendous strides toward understanding miRNA biogenesis and function, the structure of miRNA-encoding genes, and the sequence specificity of miRNA targeting. More recently, the predominant mechanisms that miRNAs use to silence their targeted transcripts have been elucidated, shedding light on that controversial topic (Eichhorn et al., 2014; Ricci et al., 2013). With many tools at their disposal, including target-site prediction algorithms, next-generation sequencing, and animal models, among many others, researchers are now well equipped for sophisticated studies of miRNAs, their function in normal physiology, and the mechanisms through which they can become dysregulated and contribute to disease.

With increasing attention on the role of miRNAs as essential regulators of cellular processes, and in the development and progression of disease, more attention has been focused on the posttranscriptional regulation of miRNAs themselves. Recent publications have suggested mechanisms through which miRNA activity may be modulated by changing the number of available MREs. mRNAs transcribed from pseudogenes (pseudo-mRNAs), circRNAs, and other ceRNAs have been shown to antagonize miRNA activity by competing with mRNAs sharing MREs for available miRNAs (Arvey et al., 2010; Cazalla et al., 2010; Hansen et al., 2013; Jeyapalan et al., 2011; Poliseno et al., 2010; Tay et al., 2011; Wang et al., 2008). While work is still being done to understand how miRNA expression is transcriptionally

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regulated, the emerging role of these endogenously encoded miRNA sponges makes understanding miRNA-regulatory networks even more complicated. Because ceRNAs can divert miRNAs from binding to their targets, total miRNA may not be a sufficient measure of miRNA efficacy; rather, miRNA availability may need to be calculated or experimentally observed using reporters. Still, in many contexts, miRNA number may drastically outweigh the availability of MREs on ceRNAs, so careful quantitation of miRNAs and ceRNAs is necessary for a complete understanding of miRNA regulation. The intricacy of miRNA regulation highlights the importance of these small RNAs to cellular processes.

In this review, we will examine the history of the discovery of miRNAs, including their first recognition, the experiments that elucidated their mechanisms of action in repressing translation, and the various bio-informatic tools that have facilitated the discovery of miRNAs and their targets. We will also discuss the emerging research that has demonstrated the essential roles of miRNAs in development and normal physiological processes, as well as the increasing studies that demonstrate the role of miRNAs in the pathogenesis of disease. As many of the earliest miRNA-associated diseases were leukemias and much research has been conducted in this area, we will apply specific focus to miRNAs that contribute to the regulation of the hematopoietic system and the dysregulations that contribute to hematologic malignancies. Finally, we will discuss the burgeoning research into miRNA-based therapeutic strategies, their potential and pitfalls, and some promising clinical data that suggest that miRNA-based therapeutics may become increasingly prevalent in the decades to come.

2. THE MicroRNA REVOLUTION: A HISTORY

In the past few decades, the scientific community has witnessed a revolution in our understanding of gene expression and posttranscriptional regulation. Previously, genome structure and subtypes of proteins, such as transcription factors and epigenetic mediators, were thought to be the only modulators of gene expression. However, in recent years, the examination of miRNAs and other ncRNAs, which participate in posttranscriptional regulation, has revealed additional layers of regulatory machinery that are critical to a variety of cellular functions. The discovery and study of miRNAs and their associated cellular machinery has disrupted the way in which we think about the control of gene expression and has provided new molecular tools for scientific inquiry. Further, this revolutionary period is paradigmatic of the capacity of the scientific community to adjust its beliefs and models when confronted with new information. In this section, we will review the early history of the discovery of miRNAs and the realization that miRNAs contribute to posttranscriptional regulation in a variety of organisms. We will also discuss the biogenesis of miRNAs, their mechanism of action, and tools that have been developed to aid in their discovery and in the identification of their targets.

2.1 The Discovery of MicroRNAs

Many researchers in the biological sciences will remember the discovery of a class of small ncRNAs now known as microRNAs or miRNAs, almost 25 years ago (Fig. 1). In a landmark paper published in the early 1990s, Lee et al. described their findings that *lin-4*, a gene

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important in the regulation of nematode larval development, encodes a small 22 nt RNA and a larger RNA thought to be its precursor (Lee et al., 1993). Cloning efforts led to the isolation of the genomic encoding region of *lin-4*, where the absence of start and stop codons revealed that it was unlikely that *lin-4* was protein coding, and probes developed based on the *lin-4* sequence were used to identify the *lin-4S* and *lin-4L* small RNAs, which we now know to be the miRNA and its precursor. Further, the Ambros group recognized that a short sequence within the lin-4 small RNA was complementary to the 3′-UTR of the heterochronic gene, *lin-14*, which had been previously identified as an important negative regulatory region for lin-14 translation (Arasu et al., 1991; Lee et al., 1993; Wightman et al., 1991). This observation of complementarity between the two sequences suggested a mechanism through which the small RNA, lin-4, might exert its function—by antisense binding in the 3′-UTR. This proposed mechanism was elucidated in work published alongside the Ambros group's discovery; Wightman et al. further examined the similar phenotypes between *lin-14* gain of function and *lin-4*-null *Caenorhabditis elegans* animals, identifying seven sites in the *lin-14* 3′-UTR containing 7 nt seed sequences with complementarity to the lin-4 small RNA (Wightman et al., 1993). The Ruvkun group found that the 3′-UTR of *lin-14* was necessary and sufficient for lin-4 to exert its temporal posttranscriptional control of lin-14 expression, firmly establishing the antisense regulation of *lin-14* by lin-4, but by an unknown mechanism (Wightman et al., 1993).

While lin-4 was the earliest discovered miRNA in animals, in the following years, with the aid of biological and sequencing tools, and with computational tools paired with the availability of sequenced genomes (*C. elegans* in 1991 (The C. elegans Sequencing Consortium, 1998), *Homo sapiens* in 2001 (McPherson et al., 2001; Venter et al., 2001), and *Mus musculus* in 2002 (Mouse Genome Sequencing Consortium et al., 2002)), an increasing number of potential miRNAs and putatively targeted transcripts were identified, the mechanisms of translational repression by miRNAs became further elucidated, and the importance of miRNAs to developmental and disease processes became much clearer. No homolog for lin-4 was identified outside of *C. elegans*; so until 2000, posttranscriptional regulation by small RNAs was thought to be restricted to nematodes. In fact, it was the discovery of another heterochronic miRNA, let-7, in *C. elegans* by the Ruvkun group (Pasquinelli et al., 2000; Reinhart et al., 2000), which does have homologs in vertebrates, including in humans and other animals, that took miRNAs from a niche developmental regulatory mechanism specific to nematodes, to a broader phenomenon that would affect our understanding of gene regulation and reshape the central dogma of molecular biology.

With the appreciation that small regulatory RNAs represent a more widely conserved phenomenon among metazoans, additional microRNAs were soon identified by intensive cloning efforts, first in *Drosophila*, *C. elegans*, and humans (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001), and shortly thereafter in a variety of other organisms (Ambros et al., 2003; Aravin et al., 2003; Lagos-Quintana et al., 2003; Lim et al., 2003a,b). Variable miRNA expression was also identified in specific tissues (Dostie et al., 2003; Houbaviy et al., 2003; Kim et al., 2004; Lagos-Quintana et al., 2002) and disease states (Michael et al., 2003). The rapid expansion of this class of regulatory molecules was facilitated by the establishment of a naming and cataloging system for microRNAs and their precursors (Griffiths-Jones, 2004), which prevented an overlap in naming of newly identified

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miRNAs and allowed for names to be consistent for homologs across organisms. The microRNA Registry, still in use today under the name miRBase (Griffiths-Jones et al., 2006), also allowed depositors to identify genomic locations of a given miRNA, to identify similar miRNAs, and to identify miRNAs that were derived from the different arms of the same hairpins. Further, this repository has facilitated the expansion of computational programs for miRNA prediction.

2.2 miRNA Biogenesis in Animals

miRNAs are encoded in the metazoan genome in a variety of ways. miRNA processing from primary miRNAs (pri-miRNAs) allows for some miRNAs to be encoded in clusters, such as the *miR-17~92* polycistron, in which several miRNAs are cotranscribed; thus they are coregulated. Other miRNAs are encoded in introns, also called miRtrons, or in nonprotein-coding exons (e.g., 3′-UTRs) of host protein-coding genes where they may be cotranscribed and coregulated with the host gene (Berezikov et al., 2007; Cai et al., 2004; Rodriguez et al., 2004; Weber, 2005). The remaining miRNAs are encoded in their own genes, where they can be individually transcriptionally regulated. Many miRNAs are intergenic and are frequently found in the opposite orientation to neighboring genes, suggesting that miRNA transcription is typically independently regulated (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001). Early studies led to the hypothesis that miRNAs are processed from transcripts generated by RNA Pol II because fusion constructs generated for the study of miRNA regulation in which protein-coding reporter genes were placed downstream of the presumed 5′-regulatory elements of miRNA genes led to very robust protein expression (Johnson et al., 2003; Johnston and Hobert, 2003; Lagos-Quintana et al., 2002). As evidenced by the 5′-7-methylguanosine (m^7G) caps and poly(A) tails that have been demonstrated on pri-miRNA transcripts, as well as the loss of miRNA production upon treatment with the potent RNA polymerase II (Pol II) inhibitor, α-aminitin, most miRNA precursors are, indeed, transcribed by Pol II (Lee et al., 2004), suggesting that the structure of miRNA host genes and promoters resembles those of protein-coding genes. The similarities between miRNA-encoding genes and protein-coding genes have been substantiated by further studies (Zhou et al., 2007). miRNA transcription by RNA polymerase III (Pol III) is much rarer than Pol II transcription and occurs when miRNA genes are near *Alu* sites, which contain a Pol III promoter (Borchert et al., 2006). As pri-miRNAs generated by Pol III undergo similar processing to Pol II generated pri-miRNAs (Faller and Guo, 2008), they do not require separate consideration.

miRNAs that are processed from retained portions of their host transcript, such as independent miRNA genes, miRNA polycistrons, and miRNAs encoded in nonprotein-coding regions of a transcript, but not miRtrons, are processed as pri-miRNAs (Lee et al., 2002), whether they are transcribed by Pol II, as is most common, or Pol III (Faller and Guo, 2008) (Fig. 2). pri-miRNAs are processed to precursor miRNAs (pre-miRNAs), which are ~70 nt hairpin loop structures, by the Microprocessor complex, minimally composed of Drosha Ribonuclease III (DROSHA) and DiGeorge syndrome critical region 8 (DGCR8) in vertebrates, which are localized in the nucleus (Gregory et al., 2004). The hairpin loop structures that will become pre-miRNAs are recognized by double-stranded RNA (dsRNA) binding regions in the DGCR8 protein, which is the vertebrate homolog of the Pasha protein

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(Han et al., 2004, 2006). DROSHA, a class 2 Ribonuclease III, is responsible for cleaving the DGCR8-recognized pri-miRNA to the ~70 nt pre-miRNA at sufficient distance from the base of the hairpin, defining one end of the mature miRNA (Lee et al., 2003). Cleavage by the Microprocessor complex can be a site of posttranscriptional miRNA regulation, with some miRNAs requiring specific cofactors for efficient processing and regulation, such as SMAD proteins in the processing of pri-*miR-21* and Lin-28, and RNA binding protein, in the processing of *pri-let-7* (Davis et al., 2008; Viswanathan et al., 2008). In contrast, the miRtron-processing pathway, which is better studied in invertebrates, bypasses the Microprocessor complex to generate pre-miRNAs that can be exported from the nucleus for further processing (Berezikov et al., 2007; Okamura et al., 2007; Ruby et al., 2007). Hairpin-containing lariats, the result of splicing, must be debranched, but then they behave as pre-miRNAs and are ready for nuclear export (Okamura et al., 2007; Ruby et al., 2007).

Pre-miRNAs, processed from pri-miRNAs or miRtrons, are exported from the nucleus by the EXPORTIN-5–Ran-GTP shuttle (Bohnsack et al., 2004; Lund et al., 2004; Yi et al., 2003). Knockdown of EXPORTIN-5 impairs miRNAs from being exported from the nucleus, but does not result in their accumulation in the nucleus, suggesting that improperly processed and improperly exported miRNAs are quickly degraded (Bohnsack et al., 2004; Yi et al., 2003; Zeng and Cullen, 2004). Export is also an important checkpoint of pre-miRNA processing—only pre-miRNAs with stem loops of sufficient length and with 3′-overhangs are recognized by EXPORTIN-5 and exported from the nucleus for cytoplasmic processing (Lund and Dahlberg, 2006; Zeng and Cullen, 2004).

Pre-miRNAs are further processed in the cytoplasm, where they are cleaved to their final ~22 nt form by DICER1, a class 3 Ribonuclease III, which defines the other end of the mature miRNA. DICER1 liberates the mature miRNA and its opposing arm from the pre-miRNA hairpin in the form of an imperfectly complementary miRNA:miRNA* duplex (Grishok et al., 2001; Hutvagner et al., 2001; Ketting et al., 2001; Lee et al., 2003). DICER1 cleavage is another checkpoint site in mature miRNA processing, with some pre-miRNAs requiring posttranscriptional base modification by adenosine deaminases for efficient cleavage by DICER1 (Kawahara et al., 2007). In summary, genomically encoded miRNAs are transcribed by RNA polymerases to pri-miRNAs, which are processed to pre-miRNAs in the nucleus by the DCGR8–DROSHA-containing Microprocessor complex. Pre-miRNAs are exported from the nucleus by the EXPORTIN-5–Ran-GTP shuttle and are processed to mature miRNAs, and can then exert their biological function, which we will discuss in the next section.

2.3 Mechanism of Action of miRNAs in Animals

The biological function of miRNAs is exerted in a way that is highly similar to the RNA interference (RNAi) mechanism described by Fire et al., work that would win Andrew Fire and Craig Mello the Nobel Prize in Physiology or Medicine in 2006 (Fire et al., 1998). Our understanding of RNAi was preceded by knowledge that injection of antisense RNA caused inhibition of protein expression in plants and *C. elegans* (Ecker and Davis, 1986; Fire et al., 1991; Guo and Kemphues, 1995; Izant and Weintraub, 1984; Nellen and Lichtenstein, 1993). But, in 1998 Fire et al. achieved much more potent RNA silencing by injecting

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dsRNA into *C. elegans*, whereas injection of single-stranded RNA (ssRNA) yielded only a moderate inhibitory effect (Fire et al., 1998). The observation that dsRNA yielded such a potent effect, even when diluted to only a few molecules per cell, led to the hypothesis that RNAi works by some catalytic mechanism, rather than by simple anti-sense binding (Fire et al., 1998). This ultimately led to the proposal of the existence of an RNA-induced silencing complex (RISC) with sequence-specific nuclease activity by Gregory Hannon's group in 2000, which demonstrated that RNAi could be carried out by *Drosophila* cell lysates (Hammond et al., 2000). Through fractionation studies of their *Drosophila* cell lysates, the Hannon group was the first to isolate the RISC, to confirm that the RISC was responsible for specific endonuclease activity, and to demonstrate that the RISC was a ribonucleoprotein complex containing the small RNA that was responsible for sequence-specific cleavage specificity (Hammond et al., 2000).

The RISC is a miRNA ribonucleoprotein (miRNP) complex that is loaded with a miRNA and is responsible for mediating its sequence-specific repressive effects (Schwarz and Zamore, 2002). The mammalian RISC contains components that are similar to those contained in the RISC responsible for RNAi in lower organisms. As DICER1 processes the pre-miRNA to the miRNA:miRNA* duplex, it unwinds the duplex and selects a strand, the strand with weaker 5′-end base pairing, for insertion into the RISC (Khvorova et al., 2003; Krol et al., 2004; Schwarz et al., 2003), with the opposite strand, the passenger strand, typically getting degraded by the C3PO (component 3 promoter of RISC) endonuclease (Liu et al., 2009; Ye et al., 2011). The primary functional constituents of the RISC are the Argonaute (AGO) family of proteins, which are responsible for mediating the function of a miRNA against its target through sequence-specific binding.

Humans have four AGO family members, AGO1–4 (Ipsaro and Joshua-Tor, 2015). Though all four AGO family members have been demonstrated to participate in miRNA-mediated repression (Ipsaro and Joshua-Tor, 2015), only AGO2 demonstrates the endonucleolytic activity necessary to perform siRNA-mediated slicing (Liu et al., 2004; Meister et al., 2004b), an important function that has been co-opted to a great effect by molecular biologists, but not thought to be an important mechanism of miRNA-mediated action in mammals. AGO proteins contain four domains: N-terminal, PAZ, MID, and PIWI (Song et al., 2004). Structural analysis of AGO proteins has revealed that the 5′-end of miRNAs, including the seed sequence, are held by the N-terminal and PIWI domains by nonspecific interactions with the 2′OH groups of the RNA sugar-phosphate backbone, making them RNA-specific binders, while the PAZ domain is responsible for holding the 3′-end of the miRNA (Ipsaro and Joshua-Tor, 2015). Further studies have demonstrated that the tight packing of AGO and the forming RNA duplex are responsible for the perfect base-complementarity necessary in the seed sequence—bubbles formed by mismatches cannot be tolerated in such close quarters (Schirle et al., 2014). AGO undertakes a series of progressive conformational changes as the seed sequence proceeds to recognize its target through base pairing (Schirle et al., 2014). miRNA availability affects AGO stability and degradation and, ultimately, the miRISC miRNP that is formed is highly stable and persistent in the cell (Elkayam et al., 2012; Schirle et al., 2014). The miRISC has been reported to have an exceedingly long half-life in cells, suggesting that miRNA regulation may serve as a form of

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cellular memory, able to respond quickly to stimuli, bypassing responses that would require transcription or translation (Ipsaro and Joshua-Tor, 2015).

In contrast to AGO2-catalyzed mRNA cleavage, which occurs when there is perfect complementarity between a small RNA and an mRNA transcript, most human miRNAs do not have perfect complementarity to their transcripts and translational repression must be carried out by other means. A number of mechanisms have been proposed through which miRNAs could mediate translational repression, but recently consensus has coalesced around two methods of repression in most cellular contexts in mammals: inhibition of cap-dependent translation initiation, and mRNA destabilization and degradation (Jonas and Izaurralde, 2015), which is the dominant means of repression by miRNAs (Eichhorn et al., 2014) (Fig. 3). Some other mechanisms of repression have been identified in other organisms and in the context of early developmental processes (Bazzini et al., 2012; Subtelny et al., 2014), but will not be discussed here.

Though the miRNA component is responsible for mRNA target recognition and recruitment of the miRISC to the targeted transcript, it is the protein–protein interactions with the AGO component of the miRISC that are responsible for mediating translational repression. Interaction of AGO with glycine-tryptophan 182 (GW182) family members is essential for miRNA-mediated repression, as demonstrated by studies in which GW182 is depleted or the AGO–GW182 interaction is blocked (Eulalio et al., 2008; Fabian et al., 2009; Takimoto et al., 2009; Yao et al., 2011). GW182 proteins serve as the scaffolds for the recruitment of the effector proteins of mRNA destabilization (Fabian and Sonenberg, 2012), namely the CCR4–NOT deadenylase complex (Braun et al., 2011; Chekulaeva et al., 2010; Fabian et al., 2011). Following deadenylation, further target destabilization and degradation are achieved by decapping and normal 5′-to-3′ mRNA degradation (Behm-Ansmant et al., 2006; Eulalio et al., 2007; Rehwinkel et al., 2005).

Though miRNA-mediated mRNA destabilization is the predominant mechanism of miRNA repression (Eichhorn et al., 2014), experiments using artificial mRNAs that are resistant to deadenylation have shown that miRNA-mediated translational repression is potent and robust (Humphreys et al., 2005; Mathonnet et al., 2007; Pillai et al., 2005). Ribosomal profiling allows for the measurement of ribosome occupancy on a given transcript—when this is compared to the total levels of that transcript, one can arrive at the transcript's "translational efficiency." The advent of effective ribosomal profiling in mammalian cells (Guo et al., 2010a) has largely settled the issue of the type of translational repression mediated by miRNAs—dramatically decreased translational efficiencies in the presence of miRNAs suggest that miRNA-mediated translational repression occurs through inhibition of translation initiation, rather than some mechanism of postinitiation inhibition (Guo et al., 2010a). In short, in the presence of miRISCs, ribosomes cannot assemble on a targeted transcript; thus translation cannot initiate. In fact, more recent studies utilizing cell-free extracts and various toxins and proteases to regulate different steps of translation have demonstrated that miRNA-mediated translational repression occurs very early, at the level of ribosome scanning, and requires cooperation from poly(A) binding proteins (PABPs) and eukaryotic translation initiation factor 4G (Ricci et al., 2013) likely through the disruption of mRNA circularization, though the precise mechanism is unclear.

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The question of whether the dominant effect of miRNAs on translation is mediated by translational repression or mRNA destabilization is a significant one to researchers studying miRNAs—if miRNAs act primarily by mRNA destabilization, then steady-state measures of mRNA levels, which are easily carried out by quantitative PCR, are a good approximation of the levels of miRNA-mediated repression. In contrast, if translational repression predominates, researchers would need to assess protein levels to determine the efficacy of miRNA repression, which proves more difficult than measuring transcript levels, especially when working with rarified cell populations. The data on translational repression and mRNA destabilization suggest a temporal order—first miRNAs act by translational repression, while mRNA destabilization is initiated. Once mRNA destabilization begins, it quickly becomes the predominant method of miRNA action (Eichhorn et al., 2014). Fortunately, because mRNA destabilization and degradation are the primary mechanism through which miRNAs regulate their targeted transcripts, steady-state measurement of transcript levels yields a reasonable approximation of miRNA effect.

We have described the transcription and synthesis of miRNAs, their one-way transport out of the nucleus to the cytoplasm, and their role in mediating transcriptional repression by blocking translation initiation and by causing mRNA destabilization. However, a number of studies have begun to identify other regulatory functions for miRNAs, outside of their canonical role in posttranscriptional repression. In late 2007, the Steitz group reported that miR-369-3 was instrumental in the recruitment of AGO2 and fragile X mental retardation (FXR1) related to AU-rich elements and MREs in the 3′-UTR of tumor necrosis factor α (TNFα), a necessary signal for translation activation in the context of serum starvation and growth arrest (Vasudevan et al., 2007). This group also reported that let-7 and an artificial miRNA mimic that they introduced contributed to translation activation upon cell cycle arrest, suggesting that miRNA-directed translational activation might be a more broadly conserved phenomenon. Similarly, miR-206 was found to promote translation of Kruppel-like factor 4 (Klf4) in the immortalized rat kidney epithelial cells upon reaching confluence and cell cycle arrest (Lin et al., 2011). miRNA import into the nucleus has also been demonstrated (Hwang et al., 2007) and miRNA-associated proteins, AGO and DICER1, have been detected in nuclear lysates (Gagnon et al., 2014; Robb et al., 2005), suggesting that miRNAs may play an additional role as transcriptional regulators. For example, miR-373 and miR-205 have been associated with transcriptional activation through the recruitment of transcriptional machinery to complementary regions in gene promoters and associated activating chromatin changes (Huang et al., 2012; Majid et al., 2010; Place et al., 2008) through a mechanism similar to RNA activation (Huang et al., 2010). A number of additional functions for nuclear miRNA have been proposed, but are poorly understood and have yet to be substantively implicated in normal physiology or the pathogenesis of disease. For the remainder of this review, we will focus on canonical miRNA-based posttranscriptional repression, and proposed noncanonical functions of endogenous miRNAs will not be discussed further. Noncanonical regulation by exogenously introduced small RNAs and endogenous miRNAs is well reviewed elsewhere (Cipolla, 2014; Huang and Li, 2012; Jiao and Slack, 2014; Roberts, 2014; Vasudevan, 2012).

Armed with an understanding of miRNA biogenesis and the mechanisms through which miRNAs exert posttranscriptional repression, it is important to briefly discuss how the

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targets of miRNAs are predicted and identified, and the tools available to researchers for assessing these putative targets.

2.4 miRNA Target Recognition and Tools for Identifying miRNAs and Their Targets

We have already discussed how the identification of lin-4 complementary sites in the *lin-14* 3′-UTR gave rise to the hypothesis that sequence complementarity is somehow necessary for repression of specific targets (see Section 2.1) (Lee et al., 1993; Wightman et al., 1993). In contrast to the perfect complementarity of siRNA-based silencing, the imperfect complementary of miRNAs to their targets makes it more difficult to identify targeted transcripts with confidence, yielding a challenging computational problem for genome-wide miRNA target prediction (Bartel, 2009), as well as the greater necessity to validate in situ miRNA–mRNA interactions before asserting miRNA-based effects. Poor overlap between miRNA predictions by different groups in the same organisms, such as in humans, led to the conclusion that early prediction algorithms failed to successfully identify miRNAs and their targeted transcripts. However, the prediction algorithms were quickly improved by adding the capability to assess sites within 3′-UTRs for evolutionary conservation, which suggests biological functionality (Lewis et al., 2005). As algorithms for miRNA target prediction across species and utilizing different methodologies improved, three critical features emerged as essential for accurate target prediction: (1) the 5′-region of the miRNA, specifically nucleotides 2–7, called the “seed sequence,” must maintain perfect Watson–Crick complementarity to improve target prediction. The importance of the seed sequence is supported experimentally by identification of validated miRNAs and their targets in other organisms, reporting that the 5′-end of the miRNAs are the most highly conserved across species, and that disrupting base paring in the seed region with nucleotide substitution disrupts miRNA function (Brennecke et al., 2005; Doench and Sharp, 2004; Lim et al., 2003b). We have already discussed the steric hindrance within the miRISC that necessitates perfect base-pair complementarity in seed region and has no tolerance for mismatches (also see Section 2.3; Schirle et al., 2014). (2) Assessment of the 7–8 nt seed sequence is sufficient for miRNA target prediction. (3) miRNAs with high evolutionary conservation have a high number of conserved targets, and a significant percentage of human genes are under selective pressure to maintain their 3′-UTR miRNA target sites (Brennecke et al., 2005; Friedman et al., 2009; Krek et al., 2005; Lewis et al., 2005). Target prediction algorithms such as TargetScan (Agarwal et al., 2015), PicTar (Krek et al., 2005), and Miranda (Betel et al., 2010) have been reviewed extensively (Bartel, 2009), but suffice it to say that these tools, used alone or in combination, represent invaluable resources to miRNA researchers.

The challenges associated with reliable target prediction highlight the importance of confirming miRNA:target interactions experimentally. A number of experimental techniques have been used to experimentally validate these interactions, the most common technique being the luciferase reporter assay, which is applied to miRNA biology by cloning the 3′-UTR in question, containing MRE to the end of a recombinant firefly *lucif-erase* gene. If the miRNA:target interaction is genuine, luciferase activity is decreased upon overexpression of the miRNA or is increased upon knockdown of the miRNA. Similar reporter assays can be conducted with fluorescent proteins. High-throughput assays based on this reporter strategy,

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such as Sensor-seq, have revealed that many miRNAs that can be detected in a cell by sequencing or by real-time PCR are not active in repressing transcripts (Mullokandov et al., 2012). Other large-scale methods for detecting miRNA:target interactions have relied on cross-linking miRISCs with their targets, immunoprecipitation, and next-generation sequencing. For example, the Tuschl group reported on photoactivatable ribonucleoside-enhanced cross-linking and immunoprecipitation (PAR-CLIP), to experimentally validate miRNA targets (Hafner et al., 2010). When modified photoactivatable nucleoside analogs are introduced to cultured cells in vitro, they can be cross-linked into their miRISCs along with the targeted transcript. Expression of FLAG-tagged AGO facilitates the pull-down of cross-linked miRISCs and their targets, which can then be processed into sequencing libraries so that miRNA targets can be identified. PAR-CLIP is similar to high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation (HITS-CLIP), which does not utilize photoactivatable nucleosides (Chi et al., 2009). Both techniques identify protein–RNA interactions and rely on the analysis of miRNA seed sequences to predict miRNA: mRNA interactions from two independent datasets: AGO–mRNA binding sites and AGO–miRNA binding sites. For this reason, they fail to directly identify any noncanonical miRNA interactions, such as with bulged seed sequences or with ncRNAs. To solve this dilemma, the Tollervey group modified their cross-linking, ligation, a sequencing of hybrids (CLASH) method to allow for the direct sequencing of miRNA–target chimeras (Helwak et al., 2013; Kudla et al., 2011). In CLASH, cross-linked miRNAs and targets can be ligated to each other before sequencing library preparation—the resulting library contains chimeric reads of a miRNA and its target, allowing for direct high-resolution mapping of these interactions.

Though seed binding rules have proven very useful in predicting and identifying many miRNA:mRNA interactions, the detailed, genome-wide mapping studies utilizing HITS-CLIP and CLASH have revealed that a significant percentage of miRNA:mRNA interactions cannot be identified by canonical seed matching rules (Chi et al., 2012; Helwak et al., 2013). For example, careful examination of AGO binding sites in HITS-CLIP data that cannot be paired with a miRNA or a mRNA target by seed matches, called “orphan clusters,” revealed that upward of 15% of miRNA:mRNA interactions are characterized by a bulged-G in the target sequence, corresponding to miRNA positions 5–6 (Chi et al., 2012). This work demonstrated that bulged seed matching, which had only been observed on an individual basis, is a broader phenomenon that is conserved across nematodes and mammals (Didiano and Hobert, 2006; Ha et al., 1996; Tay et al., 2008; Vella et al., 2004). This brings attention to a number of noncanonical miRNA:mRNA pairing interactions that have been observed. miR-24 is, for instance, upregulated in terminal hematopoietic differentiation and has been demonstrated to inhibit cell proliferation (Lal et al., 2009). While many direct targets of miR-24 contain canonical miR-24 seed sequences, a number of important cell cycle genes, including *E2F2* and *MYC*, are direct targets of miR-24, but do not contain canonical miR-24 seed sequences. These targets were predicted to bind miR-24 at downstream sites that tolerate mismatches, wobbles, and bulged sites. “Centered sites,” which bind their targets by complementarity to miRNA nucleotides 4–15, but without seed sequence or 3′-binding, have also been identified as functional, noncanonical miRNA binding sites (Shin et al., 2010). As more researchers apply high-throughput methods for identifying miRNA:mRNA

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interactions in a variety of cell types and organisms, it is possible that additional mechanisms of noncanonical miRNA target recognition will be identified.

Large-scale methods for identifying miRNA targets highlight another interesting aspect of miRNA biology—a given miRNA can target multiple genes and a given gene can be targeted by multiple miRNAs, adding a level of complexity to the study of miRNAs that can be tedious to unravel. The initial assessment of miRNAs and their targets can be made by examining the target prediction algorithms we discussed at the beginning of this section, and we have explored various techniques, such as luciferase assays and CLASH, which can be used to validate these interactions in cell lines. For functional assessment of miRNAs, researchers need other tools (Akbari Moqadam et al., 2013; Bernardo et al., 2012; van Rooij, 2011). Transfection of miRNA mimics, viral overexpression, and transgenic models can all be used to study the effect of miRNA overexpression on their targets in the relevant cellular contexts. Transfection of miRNA antagonists, virally introduced sponge constructs, and genetic knockout models can be used to study the effect of miRNA downregulation and loss. Conditional genetic models can be used to study the role of miRNAs in specific tissues or during specific developmental processes. Mutation of MREs in the 3′-UTR of targets can be used to specifically study the effects of a miRNA on a specific target. All of these models can be used in the context of other functional assays for processes such as differentiation, proliferation, apoptosis, cell migration, disease-free survival, and many others.

2.5 The Competing Endogenous MicroRNA Hypothesis and the Emerging Role of Endogenous MicroRNA Inhibition

In our introduction, we briefly presented the concept of ceRNAs. The ceRNA hypothesis is that RNA species with MREs can inhibit the activity of expressed miRNAs by sequestering them, preventing them from exerting their activity on their protein-coding mRNA target (Salmena et al., 2011; Thomson and Dinger, 2016). Researchers have long been aware that competition of miRNAs could inhibit their function, and the use of antisense miRNA antagonists (e.g., antagomiRs) and miRNA sponges has been used in experimentation and, increasingly, in the development of therapeutics, suggesting that inhibition of miRNAs by endogenously encoded ceRNAs is similarly possible. Exogenous miRNA antagonism and the development of miRNA-based therapeutics will be discussed more extensively in Section 5. More recently, endogenously expressed lncRNAs, including transcribed pseudogenes and circRNAs, have been demonstrated to contribute to miRNA inhibition as ceRNAs. Even endogenously expressed, protein-coding mRNAs, which are often highly expressed as compared to lncRNAs, have been shown to contribute to the ceRNA phenomenon. In this section, we will briefly discuss the groundbreaking works that have identified different ceRNA species and the potential for bioinformatic identification of ceRNA interactions, and address questions of the broad applicability of the ceRNA hypothesis across the highly uncharacterized lncRNA landscape.

Numerous pseudogenes have been identified as ceRNAs, including *OCT4P4* (Wang et al., 2013), *BRAFP1* (Karreth et al., 2015), *CYP4Z2P* (Zheng et al., 2015), and *Pbcas4* (Marques et al., 2012), with *PTENP1* (Johnsson et al., 2013; Poliseno et al., 2010; Yu et al., 2014) being the best characterized. The Dr. Pandolfi's group was the first to identify a noncoding

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role for a pseudogene in the regulation of miRNA activity (Poliseno et al., 2010). Previous studies of *PTEN* dosage in malignancy have led researchers to examine the importance of *PTEN*-targeting miRNAs and contributed to the hypothesis that a miRNA-sponging pseudogene could have a significant impact on *PTEN* translation (Huse et al., 2009; Kato et al., 2009; Yang et al., 2008). The *PTENP1* pseudogene is not translated due to a mutation to its initiating methionine and retains a number of shared MREs with the *PTEN* transcript. Dysregulation of those miRNAs, either by overexpression or by inhibition, affects *PTEN* and *PTENP1* expression similarly. Likewise, over-expression of the *PTENP1* 3′-UTR leads to the upregulation of *PTEN* and repression of the PI3K/AKT phosphorylation cascade and reduces cell proliferation and colony formation in vitro, demonstrating the role for *PTENP1* as a tumor suppressor. Consistently, focal *PTENP1* loss was found in a number of prostate cancer samples, suggesting that loss of the *PTENP1* tumor suppressor can contribute to the development of malignancy. Subsequent studies have identified a role for *PTENP1* loss in the pathogenesis of renal cell carcinoma, and for a *PTENP1* antisense transcript in stabilizing the *PTENP1* transcript for export to the cytoplasm, where it can exert its ceRNA function to sponge *PTEN*-targeting miRNAs (Johnsson et al., 2013; Yu et al., 2014).

Other pseudogenes have also been characterized for their function as ceRNAs. Repression of the mouse *Bcas4* pseudogene, *Pbcas4*, led to changes in gene expression consistent with *Bcas4* repression (Marques et al., 2012). An earlier study identified *CYP4Z2P*, a pseudogene of *CYP4Z2*, as promoting breast cancer angiogenesis, which is similar to the role of *CYP4Z2* in breast cancer (Zheng et al., 2014). A subsequent study utilized luciferase reporter assays to demonstrate that *CYP4Z2* and *CYP4Z2P* are regulated by the same miRNAs and that overexpression of the *CYP4Z2P* results in the downregulation of the *CYP4Z2* reporter, consistent with the ceRNA hypothesis (Zheng et al., 2015). Further, overexpression of the *CYP4Z2P* 3′-UTR promotes angiogenesis in ex vivo models and the activation of downstream pathways of *CYP4Z2. OCT4P4*, a pseudogene of the oncogene *OCT4*, has also been found to be frequently dysregulated in hepatocellular carcinoma (HCC) (Wang et al., 2013). The *OCT4P4* transcript serves as a sponge for miR-145, which contributes to the derepression of *OCT4*. Consistently, *OCT4P4* and *OCT4* levels were found to be positively correlated in HCC samples. Overexpression of *OCT4P4* increases cell proliferation and colony formation in vitro, and tumorigenicity in transplanted tumor models in mice. Most strikingly, *OCT4P4* expression can significantly stratify disease-free survival and overall survival in HCC patients, with high *OCT4P4* expression being associated with poorer outcomes. Interestingly, in trying to expand their studies and examine the broader applicability of the ceRNA hypothesis, the Pandolfi group had previously recognized the conservation of the miR-145 binding site between *OCT4* and its pseudogenes (Poliseno et al., 2010). This group further succeeded in expanding their hypothesis in their subsequent examination of the *BRAF* pseudogene, *BRAFP1*, and its contribution to the development of lymphoma in vivo (Karreth et al., 2015). In vitro, *BRAFP1* is demonstrated to regulate BRAF expression in a DICER1-dependent manner, suggesting that miRNAs are involved in this regulation, consistent with the ceRNA hypothesis. Transgenic mice that can be made to inducibly and transiently overexpress the murine *Braf-rs1* pseudogene develop a malignancy consistent with diffuse large B-cell lymphoma (DLBCL), which regresses when *Braf-rs1*

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overexpression is withdrawn. Further, *BRAFP1* has been found to be upregulated in DLBCL and multiple human cancers, suggesting that it may play a role in oncogenesis.

Other RNA species have also been shown to contribute to the ceRNA phenomenon. A number of noncanonical splicing mechanisms have been proposed for the generation of circRNAs (Jeck and Sharpless, 2014). Regardless, an essential feature of circRNAs is that they lack 5′- and 3′-ends—therefore, they are resistant to exonuclease degradation and might be effective endogenous miRNA sponges if they contain MREs. Though not many circRNAs contain miRNA binding sites (Guo et al., 2014), several examples of circRNAs serving as ceRNAs have been identified. A circRNA derived from the *Sex-determining region Y* (*SRY*) gene, which was the first discovered circRNA (Capel et al., 1993), was found to contain 16 binding MREs for miR-138 (Hansen et al., 2013). The efficacy in the *SRY*-generated circRNA in sequestering miR-138 was demonstrated by luciferase assay and coimmunoprecipitation of miR-138 and the circRNAs with tagged AGO. Another circRNA, ciRS-7 (circular RNA sponge for miR-7, a.k.a. *CDR1-AS*), has been shown to sequester miR-7 (Hansen et al., 2013; Memczak et al., 2013). Approximately 70 potential miR-7 binding sites were identified in ciRS-7 (Hansen et al., 2013). Coexpression of miR-7 and ciRS-7 results in increased immunoprecipitation of ciRS-7 with AGO than when ciRS-7 is expressed alone, demonstrating that the two RNA species interact, and expression of ciRS-7 protected miR-7 targets from repression upon miR-7 transfection. The Dr. Kjems' group also stressed the utility of circRNA, as compared to linear RNA, in serving as a miRNA sponge —ciRS-7 bound by miR-7 was resistant to degradation as compared to linear sponge constructs of the same sequence. Further characterization of ciRS-7 demonstrated that ciRS-7 and miR-7 are coexpressed in the mammalian brain and that ciRS-7 is localized to the cytoplasm, where it can exert its effect, and introduction of ciRS-7 in zebrafish disrupts brain development comparably to miR-7 inhibition (Memczak et al., 2013).

One ongoing criticism of the ceRNA hypothesis is that pseudogenes and circRNAs are not frequently transcribed at high enough levels to exert a significant sequestration effect on miRNAs. In contrast, protein-coding mRNAs can be highly expressed. Recent excitement about the ceRNA hypothesis has yielded increased interest in the capacity of mRNAs to participate in miRNA sequestration. In trying to understand how protein-coding mRNAs can regulate each other through competition over miRNAs, we can return to *PTEN*, which has been studied by the Pandolfi group as a protein-coding mRNA that may also serve as a ceRNA. First, they sought to identify genes that are coexpressed with *PTEN* and share MREs, and thus may serve as ceRNAs, through a computational approach that they called mutually targeted MRE enrichment (MuTaME) (Tay et al., 2011). Knockdown of potential ceRNAs against *PTEN* resulted in down-regulation of *PTEN*, downregulation of PTEN protein, and activation of the PI3K/AKT pathway, in a DICER1-dependent manner, suggesting that this regulation is dependent on their competition over miRNAs. The MuTaME approach was also used to identify ceRNAs against *PTEN* in the context of a mouse model of melanoma, driven by *B-Raf* mutation, in the context of an in vivo forward genetic screen to identify cooperating mutations (Karreth et al., 2011). This method identified *ZEB2* as a ceRNA targeting *PTEN*; when *ZEB2* expression is lost, *PTEN* expression is decreased. Low *PTEN* expression in human tumor samples is associated with low expression of *ZEB2*, consistent with the ceRNA hypothesis. A number of other mRNAs

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have been identified as ceRNAs, including *VCAN* in the development of HCC (Fang et al., 2013; Lee et al., 2010), and *FOXO1* in the development of breast cancer (Yang et al., 2014), among others.

When one considers that many protein-coding mRNA transcripts can compete for miRNAs and serve as ceRNAs, it quickly becomes clear that, in most cases, focusing on the role of a single mRNA as a ceRNA might not be sufficiently informative. This has been made even clearer through a transcriptome-wide analysis of RNA–RNA interactions conducted by the Califano group, in which they revealed a broad network of miRNA-mediated posttranscriptional regulation, much of which is mediated by sponge activity (Sumazin et al., 2011). Another example comes through the Sensor-seq approach, in which miRNA activity, rather than abundance, was measured for a large number of miRNAs by introducing a library of miRNA-activity reporter constructs (Mullokandov et al., 2012). miRNAs with a high number of expressed competing MREs were found to have low activity, as measured by the reporter, as compared to their abundance, indicating that their function was being diluted because of the large pool of targets available to them. Mathematical modeling of the ceRNA concept has suggested that derepression of a target requires near-equimolar expression of the miRNA and ceRNA, and that ceRNA competition for abundant miRNAs would require drastic changes in MRE availability (Ala et al., 2013; Bosia et al., 2013; Figliuzzi et al., 2013; Jens and Rajewsky, 2015). Other studies have experimentally demonstrated that changes to individual ceRNA transcripts are unlikely to affect the activity of most miRNAs (Bosson et al., 2014; Denzler et al., 2014); thus the changing levels of ceRNAs would most dramatically be felt on lowly expressed miRNAs.

It is clear that there is a role for RNA competition in the regulation of miRNA activity, but studies identifying the effect of individual ceRNAs must be critically evaluated for the appropriate validation studies and assessed for the physiological relevance of performed experiments.

Equipped with an understanding of how miRNAs are transcribed and processed, how they exert their function and how putative miRNA targets can be identified and validated, how miRNA function can be assessed, and the possible mechanisms through which miRNAs may be posttranscriptionally regulated, we will spend the next several sections discussing specific miRNAs and their roles in normal hematopoietic development, and as oncogenes and tumor suppressors whose dysregulation can contribute to malignant transformation.

3. MicroRNAs IN HEMATOPOIETIC DEVELOPMENT

Hematopoiesis is a highly regulated process of differentiation that is responsible for providing mature blood cells for the lifetime of an individual. The rigidly regulated stepwise differentiation process that characterizes hematopoiesis, as well as a sophisticated toolbox of cell type identifying surface markers and a broad variety of in vitro and in vivo assays, makes the hematopoietic system paradigmatic for assessing the mechanisms that regulate differentiation processes. At a time when there were no discovered functions for mammalian miRNAs, the role for miRNAs in hematopoietic differentiation became an early target of study because several identified miRNA genes were found at sites of genomic translocation

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in human leukemia: *miR-15* and *miR-16* are located at chromosome 13q14 and are found to be downregulated or lost in a majority of B-cell CLLs (Calin et al., 2002); and *miR-142* is located on chromosome 17 (Lagos-Quintana et al., 2002), at the junction of the t(8;17) translocation that results in an aggressive B-cell leukemia associated with upregulated MYC expression (Gauwerky et al., 1989). Preliminary studies, in which the Bartel group sought to identify differential expression of known miRNAs in different hematopoietic cell types (fetal liver, bone marrow, spleen, and thymus), yielded miR-181, miR-142, and miR-223 as differentially expressed in the various tissues (Chen et al., 2004). Differential expression of these miRNAs suggested that they may play a role in hematopoietic differentiation, which was supported by ectopic expression studies. Overexpression of miR-142 and miR-223 contributed to the expansion of T cells in vitro in culture conditions supportive of lymphoid differentiation, while overexpression of miR-181 contributed to the expansion of B cells in vitro and upon in vivo transplantation (Chen et al., 2004). This study, of course, suggested a role for miRNAs in hematopoietic differentiation and also supported a hypothesis that gene silencing may play an important role in the decision of stem and progenitor cells to self-renew or to differentiate into a specific cell type. In this section, we will discuss several miRNAs that have been shown to be important contributors to hematopoietic differentiation and to hematopoietic stem cell (HSC) maintenance. We will also discuss large-scale microRNA screening, which has been facilitated by our ability to fractionate the hematopoietic system into specific cell types based on their surface markers.

3.1 miR-223

Following the revelation that miR-223 is differentially expressed in the hematopoietic system, miR-223 has been further studied for its role in differentiation, particularly granulocytic cell maturation (Fazi et al., 2005; Johnnidis et al., 2008). Soon after the Ambros group reported the expression of miR-223 in the bone marrow (Chen et al., 2004), the competing granulopoiesis-associated transcription factors, NFI-A and C/EBPα, were shown to regulate miR-223 expression in human granulocyte maturation, suggesting that miR-223 is associated with granulocyte maturation or function (Fazi et al., 2005). Using reporter constructs and retinoic acid (RA)-based granulocytic differentiation in human leukemia cell lines, Fazi et al. demonstrated that NFI-A and C/EBPα compete for binding at the *miR-223* promoter. Further, a luciferase reporter assay identified NFI-A as a direct target of miR-223, which forms a feedback loop that reinforces granulocyte differentiation (Fazi et al., 2005). NFI-A competes for binding with C/EBPα in the promoters of granulocytic differentiation genes, including *miR-223*. When C/EBPα binding predominates, *miR-223* and other pro-granulocytic genes can be expressed. miR-223 represses NFI-A expression, reducing its blockade on granulocytic differentiation and completing the positive feedback loop that promotes granulopoiesis. Consistently, ectopic overexpression of miR-223 in leukemic cell lines induces their differentiation, in the absence of RA (Fazi et al., 2005). miR-223's role is also highlighted by its upregulation in RA-induced differentiation of acute promyelocytic leukemia cell lines (Garzon et al., 2007). miR-223 is expressed in very low levels in HSCs and steadily increased through granulocytic differentiation to bone marrow and peripheral blood neutrophils, while its expression plummets through monocyte differentiation (Johnnidis et al., 2008). Surprisingly, experiments conducted with genetically engineered mice deficient in *miR-223* revealed an expansion of granulocytes (Johnnidis et

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al., 2008), suggesting that miR-223's contribution to granulopoiesis may be more complicated than originally thought. In the context of constitutive *miR-223* deficiency, the Camargo group revealed that the transcript of Myocyte-specific enhancer factor 2c (Mef2c), whose expression promotes myeloid progenitor expansion and enhanced granulocytic differentiation, is a specific target of miR-223 (Johnnidis et al., 2008). The inconsistencies between miR-223 as either a promoter or a repressor of granulopoiesis can be explained by the different experimental systems used in these publications. Whereas the Camargo group used a knockout of the *miR-223* locus, Fazi et al. overexpressed miR-223 in leukemic cell lines; overexpression vs knockout may each reveal different aspects of miR-223 function. Additionally, different experimental systems may reveal temporal differences of miR-223 function; the leukemic cell lines that were used by Fazi et al. represent an early granulocytic progenitor, while miR-223 was disrupted throughout the hematopoietic system by the Camargo group, suggesting that miR-223 may play different roles, and by different mechanisms, at different stages of differentiation (Fazi et al., 2005; Garzon et al., 2007).

Additional studies have identified another regulatory mechanism for miR-223 expression—consistent with its role in hematopoietic differentiation, miR-223 is regulated, much like other "myeloid genes," by the C/EBPs and another important myeloid transcription factor, PU.1 (Fukao et al., 2007). Fukao et al. identified a highly conserved region upstream of the *miR-223*-encoding region, which contains two PU.1 binding sites, as well as one C/EBP binding site and one GATA-1 binding site. Chromatin immunoprecipitation (ChIP) studies have confirmed binding of PU.1, C/EBP, and GATA1 at the core promoter, and expression studies indicated that while expression of PU.1 or C/EBP alone mildly induces miR-223 expression, they dramatically increase miR-223 transcription when expressed in combination (Fukao et al., 2007). As expected, GATA1 expression inhibits miR-223 expression, as is expected of a myeloid gene. The study was able to replicate the increased expression of miR-223 upon RA treatment that was previously identified (Fazi et al., 2005) and demonstrated that this regulation is also mediated by PU.1 and C/EBP (Fukao et al., 2007). Interestingly, it has been revealed that downregulation of miR-223 in acute myeloid leukemia (AML) can occur by an unrelated mechanism. The t(8;21) translocation is the most common karyotypic abnormality in AML and generates the *AML1–ETO* fusion oncogene, a potent epigenetic disruptor that represses transcription through interactions with histone deacetylase (HDAC) and DNA methyltransferase (DNMT) (Liu et al., 2005, 2006). The miR-223-coding region is downregulated and packaged into heterochromatin in leukemic blasts and in cell lines harboring the t(8;21) translocation by the recruitment of AML1–ETO to an AML1 binding site near the *miR-223* locus (Fazi et al., 2007). Ectopic overexpression of miR-223 or RNAi against the AML1–ETO fusion protein restores miR-223 expression and induces differentiation in leukemic cell lines (Fukao et al., 2007). Though the exact role of miR-223 in hematopoietic differentiation remains unclear, in large part due to the contradictory findings that miR-223 promotes granulopoiesis (Fazi et al., 2005), but that loss of *miR-223* results in an expansion of mouse granulocytes (Johnnidis et al., 2008), it is clear that miR-223 plays an important role in hematopoietic differentiation, and that its dysregulation can promote oncogenesis. Further studies of the role of miR-223 in specific cell types and contexts will surely elucidate the mechanisms through which miR-223 contributes to hematopoietic differentiation of multiple cell types.

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We briefly discussed miRNA strand selection and loading into the RISC in Section 2.3. Typically, the strand with weaker 5′-end base pairing in the miRNA:miRNA* duplex is loaded into the RISC and the miRNA*, also known as the passenger strand, is degraded by C3PO (Khvorova et al., 2003; Krol et al., 2004; Liu et al., 2009; Schwarz et al., 2003; Ye et al., 2011). The advent of deep sequencing for small RNAs has made it easier to detect miRNA* species and examine their relative abundance to miRNAs. Examination of sequencing libraries generated from a variety of mouse and human tissues identified a number of highly abundant miRNA* species, including miR-223* (Kuchenbauer et al., 2011). Typically, overexpression of a miRNA involves the overexpression of both arms of the miRNA:miRNA* duplex, making it difficult to untangle their separate functions. In order to study the specific function of miR-223*, the researchers developed a retroviral construct in which the miR-223 seed sequence was mutated so that miR-223* activity could be studied independently. Overexpression of miR-223* abrogates the increase in colony formation exhibited in *miR-223*^{KO} bone marrow. The *insulin-like growth factor receptor* 1 (*Igf1r*) gene is dysregulated in *miR-223*^{KO} bone marrow and is predicted to be a target of miR-223 and miR-223*. Overexpression of miR-223* in the context of *miR-223*^{KO} restored the repression of *Igf1r*, suggesting that miR-223* also contributes to its regulation. Overexpression of miR-223* was also shown to repress a number of predicted miR-223* targets, further supporting its role as an important regulatory miRNA*. Interestingly, miR-223* expression levels were able to stratify AML survival outcomes, with increased miR-223* expression being associated with a superior outcome, while miR-223 expression was not associated with any differences in survival. A number of other miRNA* species have been identified for their contribution to physiology and malignant transformation. For example, expression of miR-9* has also been demonstrated to be associated with a favorable outcome in AML (Nowek et al., 2016), and overexpression of miR-27/miR-27* has been associated with increased metastatic potential in osteosarcoma (Salah et al., 2015). Clearly, there is an emerging appreciation of the role of miRNA* species, which will surely be the subjects of further studies.

3.2 miR-155

miR-155 is another miRNA whose role in hematopoiesis was established early, due to the observation that miR-155 is overexpressed in some B-cell lymphomas (Eis et al., 2005; Kluiver et al., 2005). Proviral integration by Avian sarcoma leukosis virus activates a gene entitled “BIC,” B-cell integration cluster, which encodes *miR-155* in a gene that generates a fully spliced and polyadenylated transcript (Tam, 2001; Tam et al., 1997). Like miR-223, miR-155 is only modestly expressed in HSCs, and its expression increases upon differentiation to some mature cell types, particularly upon exposure to immunogenic stimuli. miR-155 has been shown to target activation-induced cytidine deaminase (AID), an enzyme which participates in somatic hypermutation and class-switch recombination, which is essential for B-cell maturation in germinal centers (de Yebenes et al., 2008; Teng et al., 2008). INPP5D (phosphatidylinositol-3,4,5-trisphosphate 5-phosphatase 1) is a 5′-inositol phosphatase that is a tumor suppressor in B-cell lymphoma—when *INPP5D* and *PTEN* are both deleted, it results in an aggressive B-cell lymphoma (Pedersen et al., 2009). *INPP5D* is a target of miR-155, which has been shown to participate in a TNFα/miR-155/INPP5D pathway in DLBCL (Pedersen et al., 2009), and miR-155 levels can be reduced upon TNFα

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antagonism. miR-155 has also been shown to contribute to the pathogenesis of DLBCL through the repression of SMAD5 (Rai et al., 2010). In the hematopoietic system, SMAD5 is responsive to transforming growth factor-beta (TGFβ) and bone morphogenetic protein (BMP) signaling and works to inhibit cell proliferation. Overexpression of miR-155 in DLBCL cells represses SMAD5 expression, making them unresponsive to TGFβ or BMP-signaled growth arrest, contributing to lymphomagenesis (Rai et al., 2010). Together, these data suggest that miR-155 is an important regulator of B-cell development, and a potent oncogene in lymphoid malignancies.

3.3 miR-181, Lin-28, and let-7

As we have previously discussed, let-7, a heterochronic miRNA involved in developmental timing, was the second discovered miRNA in *C. elegans* (see Section 2.1). Identification of let-7 homologs in humans and other organisms led to the realization that the miRNA phenomenon was conserved in many species. Interestingly, *pri-let-7* is detectable in undifferentiated embryonic stem cells (ESCs) and in mature cells, whereas mature let-7 is only detectable in mature cells, suggesting that it is posttranscriptionally regulated. *Lin-28* is an RNA binding protein that is conserved from *C. elegans* to humans, and is also associated with developmental timing (Ambros and Horvitz, 1984). Lin-28 has been identified as a posttranscriptional regulator of *let-7* processing (Newman et al., 2008; Viswanathan et al., 2008). Lin-28 binds to the loop of *pre-let-7* and recruits additional factors that contribute to its degradation and the blockade of further processing. Lin-28 is highly expressed in ESCs, and the antagonism between Lin-28 and *let-7* maturation is thought to play a role in maintaining the undifferentiated state—in fact, the Thomson group included LIN-28 among their cocktail of factors used to generate induced pluripotent stem cells from human somatic cells (Yu et al., 2007). let-7 is also capable of repressing Lin-28 expression, suggesting that let-7 and Lin-28 participate in an autoregulatory loop (Rybak et al., 2008).

It was not until 2012 that a role for the let-7/LIN-28 autoregulatory loop was identified in hematopoiesis (Li et al., 2012). Overexpression of LIN-28 prevented megakaryocytic differentiation in K562 erythroleukemia cells. To demonstrate that this effect was mediated through the repression of let-7, the researchers overexpressed a *let-7* construct that was mutated to resist binding by LIN-28, which rescued megakaryocytic differentiation. Further, the researchers identified miR-181 as the upstream regulator of LIN-28, and the switch that represses LIN-28 expression to allow for differentiation. Reporter assays demonstrated that *LIN-28* is a direct target of miR-181, and expression studies of differentiating K562 cells and human umbilical cord blood $CD34^+$ progenitor cells revealed that miR-181 upregulation precedes *LIN-28* downregulation and let-7 upregulation (Li et al., 2012). Additional studies have supported these findings. let-7 was found to be downregulated in a number of leukemic cell lines, including HL-60, NB4, and U937, and enforced expression of let-7 improved RA-induced granulocytic differentiation (Pelosi et al., 2013).

In another study, in which researchers were investigating factors that were lost in the postnatal transition of HSCs, Lin-28 was identified as increasing the self-renewal capacity of mouse HSCs (Copley et al., 2013). A microarray revealed that *Lin-28* was the most highly differentially expressed between fetal liver and adult bone marrow, being highly expressed in

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the fetal liver. Consistently, let-7 expression is low in fetal liver and high in adult bone marrow, and these findings held when examining hematopoietic stem/progenitor cells (HSPCs) and HSCs isolated from the fetal liver and adult bone marrow. let-7 binding sites were identified in a number of genes that were highly expressed in fetal liver, as compared to adult bone marrow, including in *Hmga2* (High-mobility group AT-hook 2), a known direct target of let-7 (Mayr et al., 2007), and *Hmga2* transcript levels were shown to decline through differentiation, as let-7 levels increased. Increased chimerism upon primary bone marrow transplantation, and a limiting-dilution assay of secondarily transplanted adult HSCs with enforced *Lin-28* expression, revealed that Lin-28 drastically increases the self-renewal capacity of HSCs. Similarly, enforced expression of *Hmga2* results in increased self-renewal capacity of HSCs, while fetal liver HSCs isolated from *Hmga2* knockout mice were shown to have impaired self-renewal. Taken together, these data suggest that a Lin-28/let-7/Hmga2 regulatory axis mediates HSC maintenance, with *Lin-28* downregulation upon postnatal transition resulting in a decrease in self-renewal capacity in adult HSCs (Copley et al., 2013). Other studies have also demonstrated that enforced expression of Lin-28 in adult HSCs confers broader differentiation capacity, allowing for the generation of a variety of B cells, T cells, and natural killer T cells, a capacity intrinsic to fetal liver HSCs and typical of fetal lymphopoiesis (Yuan et al., 2012). A more detailed examination of this phenomenon, using a barcoding strategy to trace the descendants of marked fetal liver HSCs upon transplantation, has confirmed that Lin-28 expression is essential for preserving the potential of fetal liver HSCs to differentiate into a variety of lymphoid cell types (Kristiansen et al., 2016). Fetal liver HSCs that still express Lin-28 can differentiate to B1-a type B cells, which can only be generated in the fetal liver, but this capacity is lost, and HSCs become B2-type restricted, as Lin-28 expression decreases. Enforced expression of Lin-28 in adult HSCs restores the capacity to differentiate B1-a type cells, the hallmark of a fetal liver HSC.

3.4 miRNA Profiling in the Hematopoietic System

With the role of miRNAs in hematopoietic differentiation firmly established in the earlier studies that we have discussed, a number of groups began to take advantage of the ability to fractionate human HSCs, progenitor cells, and mature cells isolated from bone marrow, mobilized peripheral blood, and umbilical cord blood for miRNA profiling, revealing interesting patterns of differential expression between subpopulations (Bissels et al., 2011; Jin et al., 2008; Liao et al., 2008; Merkerova et al., 2010; Raghavachari et al., 2014). For example, miRNA expression profiling was conducted on HSC-enriched $CD34^+CD38^-$ umbilical cord blood cells against total $CD34^+$ cells, which include committed progenitor cells (Liao et al., 2008). The study identified 9 miRNAs that were fourfold over-expressed in $CD34^+CD38^-$ cells compared to $CD34^+$ cells, including miR-520h, and 22 miRNAs that were fourfold underexpressed, including miR-129 (Liao et al., 2008). $CD34^+$ cells transfected with miR-520h yielded increased colonies in colony-forming cell assays, suggesting that miR-520h contributes to the maintenance of HSCs, though no target of miR-520h responsible for the phenotype was identified in the study (Liao et al., 2008).

miRNA profiling was also carried out on $CD133^+$ HSCs, $CD34^+CD133^-$ progenitors, and $CD34^-CD133^-$ differentiated cells from G-CSF (granulocyte-colony-stimulating factor) mobilized peripheral blood (Jin et al., 2008) and from isolated bone marrow (Bissels et al.,

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2011). Among other differentially regulated miRNAs, both studies identified miR-146a as being upregulated in CD133^{+} HSCs.

3.5 miR-146a

miR-146a expression was originally identified as a target of NF-κB regulation, and a participant in a negative-feedback loop with NF-κB expression (Taganov et al., 2006). Expression of miR-146a in various immune effector cells suggests that it plays an important role in innate immunity (Boldin et al., 2011). But following its identification as a highly expressed miRNA in HSCs, researchers questioned whether or not it contributed to HSC maintenance and differentiation of various hematopoietic lineages. Promyelocytic zinc finger (PLZF)-overexpressing leukemic cell lines, which have a propensity for unileage megakaryocytic differentiation, were assessed for dysregulated miRNA expression (Labbaye et al., 2008). miR-146a was identified as significantly downregulated in PLZF-overexpressing cell lines, and its expression was further reduced upon induction of megakaryocytic differentiation in human cord blood progenitor cells. ChIP experiments confirmed that PLZF binds the promoter of miR-146a, suggesting that it is an inhibitor of miR-146a expression. CXCR4, the SDF1 chemokine receptor that is essential for thrombopoiesis in vivo (Avecilla et al., 2004), was identified as a putative target of miR-146a and confirmed to be a direct target of miR-146a by the luciferase assay (Labbaye et al., 2008). Through a series of rescue experiments with either miR-146a overexpression or enforced expression of *CXCR4* with a mutated 3′-UTR, the researchers clearly demonstrated a PLZF/miR-146a/CXCR4 axis for regulation of megakaryocyte differentiation, in which miR-146a must be negatively regulated by PLZF to allow for CXCR4 expression and promotion of megakaryopoiesis (Labbaye et al., 2008).

In subsequent studies, David Baltimore's group generated constitutive *miR-146a* knockout mice to study the effect of miR-146a on NF-κB regulation and inflammation (Zhao et al., 2011). *miR-146a* deficiency leads to the development of transplantable myeloid malignancies, as well as lymphomas, over a long 6- to 18-month time course (Zhao et al., 2013). Over-expression of NF-κB was responsible for the oncogenesis, suggesting that miR-146a inhibition of NF-κB is tumor suppressive (Zhao et al., 2011). Further examination of the *miR-146a* knockout model demonstrated that loss of *miR-146a* resulted in chronic inflammatory stress in HSCs, and a loss in HSC number and quality over time. Reciprocal transplantation experiments, in which *miR-146a*-deficient bone marrow is transplanted to lethally irradiated wild-type mice or wild-type bone marrow is transplanted to irradiated *miR-146a*-deficient mice, revealed that the effect of *miR-146a* loss is a cell intrinsic, rather than the result of a defect in the hematopoietic niche, suggesting that miR-146a is a bona fide regulator of HSC maintenance (Zhao et al., 2013). The miR-146a/NF-κB feedback loop responsible for this phenotype has been further elucidated—inflammatory stimuli promote the expression of interleukin-1 receptor-associated kinase 1/2 (IRAK1/2) and TRAF6, which in turn stimulates NF-κB expression (Hou et al., 2009). NF-κB induces miR-146a expression, which is responsible for the negative feedback by inhibiting IRAK1/2 and TRAF6 expression by directly targeting their 3′-UTRs (Hou et al., 2009).

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The study of the contribution of miRNAs to hematopoietic differentiation continues, often led by investigation of a disease-causing miRNA and how its dysregulation disrupts normal processes. While we have discussed the contribution of some physiologically important miRNAs originally identified for their contribution to disease in previous sections, in the following sections we will examine a number of miRNAs that have been implicated in hematologic disease, whether as oncogenes, due to their overexpression, or as tumor suppressors, due to their downregulation.

4. MicroRNAs IN HEMATOLOGIC MALIGNANCIES

By now, dysregulation of miRNA expression has been detected in many types of cancers, with miRNA dysfunction being implicated increasingly in the pathogenesis of certain malignancies. With miRNAs being so important to hematopoiesis, it is no surprise that miRNAs play a role in the development and pathogenesis of hematologic malignancies (Table 1). In fact, we have already discussed the identification of the first miRNAs shown to play physiologically important roles in mammals, miR-15, miR-16, and miR-142, which are encoded at translocation sites in lymphocytic leukemias. Further studies of the genomic locations of miRNAs have hinted at their importance in the development hematologic disease—in a survey of six human AML and myelodysplastic syndrome (MDS) cell lines, >75% of miRNAs were mapped to sites of genomic alterations in these cell lines, resulting in expression changes to nearly 20% of these miRNAs (Starczynowski et al., 2011), consistent with previous studies that have mapped miRNAs to fragile sites in the genome (Calin et al., 2004b). Other studies examining blasts from AML patients have been able to detect various disrupted miRNA expression signatures that can be associated with AML prognosis, and known cytogenetic and karyotypic classifications (Dixon-McIver et al., 2008; Garzon et al., 2008b; Jongen-Lavrencic et al., 2008). Additionally, disruption of the miRNA-processing machinery, such as *DICER1* deletion, can also result in the development of hematologic disease. For example, conditional deletion of *Dicer1* in mouse mesenchymal osteoprogenitor cells results in a niche defect that contributes to the development of myelodysplasia and secondary leukemia (Raaijmakers et al., 2010).

While the aforementioned studies are important for identifying miRNAs for further investigation as mediators of disease or prognostic indicators, none of these genome-wide studies can identify specific disease-associated miRNAs or the mechanism through which they cause malignancy. In understanding studies of dysregulation of specific miRNAs that result in hematologic disease, it is helpful to consider a number of categories: (1) oncomiRs or miRNAs that act as oncogenes by causing disease when they are overexpressed; (2) tumor-suppressive miRNAs, which normally inhibit expression of known oncogenes and result in malignancy when their expression is lost; and (3) disruption to miRNA-processing machinery, which has more global effects on the expression of miRNAs, but can cause malignancy depending on cellular context (Fig. 4). These divisions are important in considering how to study and evaluate disease-associated miRNAs, but also how to approach miRNA-based therapeutic strategies.

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4.1 OncomiRs

OncomiRs represent the most expansive class of known disease-associated miRNAs. The term "oncomiR" was first coined by Scott Hammond's group in a study in which they reported on the potent oncogenic effect of the *miR-17~92* polycistron, which is amplified in B-cell lymphomas (He et al., 2005). As the portmanteau implies, oncogenic miRNAs contribute to malignant transformation when they are overexpressed, typically because they downregulate the expression of tumor-suppressive proteins. Some cancers that result from the overexpression of an oncomiR have been reported to be addicted to the continued expression of the oncomiR, as is the case with miR-21 (Medina et al., 2010), which presents the opportunity for miRNA-based therapeutics.

4.1.1 The miR-17~92 Polycistron—Amplification of 13q31–q32, and specifically *C13orf25*, has been identified as contributing to the transformation of malignant B-cell lymphoma (Rao et al., 1998). One of the transcripts that is found in *C13orf25* encodes seven miRNAs in what we now know as the *miR-17~92* polycistron (Ota et al., 2004), which is also encoded in two homologs, *miR-106a~92* and *miR-106b~25* (Tanzer and Stadler, 2004). The Hammond group screened lymphoma samples and discovered upregulation of the five miRNAs encoded in the *miR-17~92* polycistron in 65% of the isolates (He et al., 2005). When *miR-17~92* is ectopically expressed in a transgenic model of B-cell lymphoma, in which *c-Myc* is expressed from the immunoglobulin heavy chain enhancer (*Eμ-myc*), transplantable lymphomas develop with a dramatically decreased latency, and animal succumb to disease in 65 days (He et al., 2005). The *miR-17~92* cluster has therefore been implicated in a number of malignancies, including a variety of hematologic malignancies beyond B-cell lymphoma (Lawrie, 2013).

ChIP experiments have identified c-Myc as a positive regulator of the *miR-17~92* cluster, suggesting that it may contribute to oncogenesis (O'Donnell et al., 2005), and a number of targets of microRNAs encoded in the *miR-17~92* polycistron that may contribute to its oncogenic potential have been identified. For example, *miR-17~92* contributes to B-cell development and B-cell maintenance through its inhibition of *Bim*, a proapoptotic protein (Ventura et al., 2008). The *miR-17~92* has also been identified to negatively regulate a number of other important tumor suppressors, including E2F1 (Woods et al., 2007), cyclin D1 (CCND1) (Chen et al., 2008a; Deshpande et al., 2009), and PTEN protein phosphatase 2 (PP2) (Mavrakis et al., 2010).

4.1.2 miR-125—miR-125 has been studied for not only its role in regulation of normal HSC self-renewal but also its role in leukemogenesis, particularly in AML. We now know that the miR-125 family is actually homologous to lin-4, in *C. elegans*, the first identified miRNA (Lagos-Quintana et al., 2002). miR-125 was first identified as an oncomiR in prostate cancer, where it was found to promote androgen-independent tumor growth by inhibiting the proapoptotic regulator BAK1 (Shi et al., 2007). Later, an oncogenic role for miR-125 in the hematopoietic system was identified when overexpression studies revealed that miR-125 inhibits terminal differentiation of human leukemia cell lines, such as HL-60 and NB4 (Bousquet et al., 2008). The t(2;11)(p21;q23) translocation is a rare abnormality that results in AML or MDS, often as a primary or single event. A number of protein-coding

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and miRNA-encoding genes are located near the breakpoints, including miR-100, let-7a-2, and miR-125b, and miR-125b expression was found to be dramatically upregulated in samples from affected patients (Bousquet et al., 2008). Using models of HL-60 and NB4 differentiation induced by treatment with DMSO and RA, respectively, Bousquet et al. demonstrated that transfection of miR-125b can prevent differentiation of these cell lines to mature monocytic and granulocytic cells. Later, in vivo, studies conducted by the Lodish group demonstrated that when fetal liver cells overexpressing miR-125b are transplanted into mice, they develop a macrocytic anemia that converts to various types of leukemia. Additionally, overexpression of miR-125b can cooperate with the *BCR–ABL* fusion oncogene to accelerate leukemia development (Bousquet et al., 2010). miR-125b transfection prevents differentiation of primary, patient-isolated $CD34^+$ blasts, suggesting that miR-125b can contribute to a differentiation blockade under leukemic conditions. Overexpression of miR-125b, which is also encoded in a second gene on chromosome 21, has also been associated with trisomy 21/Down syndrome-associated acute megakaryoblastic leukemia (Klusmann et al., 2010). miR-125b cooperates with *GATA1* mutation to cause increased proliferation and self-renewal of megakaryocyte progenitors. Additionally, this study identified a number of targets of miR-125b that may contribute to its oncogenic function, including tumor suppressor, ST18, and DICER1, which will be discussed in a following section (Klusmann et al., 2010).

Building off data observed in miR-125b overexpression in leukemia, a number of studies have identified a role for miR-125 in normal hematopoiesis. In 2010, David Scadden's and Christopher Park's groups demonstrated that miR-125 is highly expressed in HSCs and plays a role in regulating HSC number (Guo et al., 2010b; Ooi et al., 2010). The Scadden group observed that conditional *Dicer* deletion in the hematopoietic compartment resulted in HSPC failure due to apoptosis (Guo et al., 2010b). With the increasing evidence for the contribution of miRNAs to hematopoiesis, this result was surely not surprising, but it was one of the earlier results to demonstrate that miRNAs are important to HSC function, and not just in reinforcing a lineage identity during differentiation. Importantly, the Scadden group performed miRNA profiling on the $CD34^-Flk2^-c\text{-}Kit^+Sca1^+Lineage^-$ population, which is highly enriched for long-term HSCs (LT-HSCs), and identified miR-99b, let-7e, and miR-125a, all expressed from a single miRNA cluster, as highly enriched in LT-HSCs. Ectopic overexpression of miR-125a resulted in increased multilineage reconstitution upon bone marrow transplantation, due to a specific increase in HSC self-renewal mediated by a reduction in HSC apoptosis (Guo et al., 2010b). Reduction of apoptosis in miR-125a-overexpressing HSCs is mediated by inhibition of the proapoptotic protein Bcl-2 (B-cell lymphoma 2) homologous antagonist/killer (Bak1), which was previously identified as a target of miR-125 in prostate cancer. Interestingly, the expansion of HSCs upon miR-125a overexpression must be mediated by additional unidentified targets, as well, because *Bak1* knockout was not sufficient to recapitulate the observed phenotypes (Guo et al., 2010b), highlighting a difficulty in the study of miRNAs—in any given cellular context, miRNAs may target multiple transcripts, to achieve unified or disparate ends.

The finding that miR-125 contributes to HSC maintenance and expansion has been replicated and extended. Through targeting of additional anti-apoptotic machinery, enforced miR-125b expression biases HSCs toward lymphoid differentiation (Ooi et al., 2010).

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miR-125b-overexpressing LT-HSCs demonstrated increased bone marrow reconstitution on primary and secondary transplantation, with a particular increase in lymphoid cells and progenitors upon secondary transplantation. Consistently, enforced miR-125b expression led to an increase in the number of lymphoid-biased Slamf1$^{low/neg}$ HSCs upon transplantation. Cell cycle analysis of cells overexpressing miR-125b demonstrated that the increase in reconstitution is not the result of increased self-renewal, but a decrease in apoptosis (Ooi et al., 2010), as was previously observed (Guo et al., 2010b). Two anti-apoptotic proteins that are potential targets of miR-125 and could contribute to the observed phenotype were also identified—*Bcl-2-modifying factor* (*Bmf*) and *Kruppel-like factor* (*Klf13*) expression was decreased upon miR-125 overexpression and increased upon miR-125 antagonism, though direct binding of miR-125 to the 3′-UTRs was not observed (Ooi et al., 2010). Many additional targets of miR-125 have been identified in other studies, in the hematopoietic system, and in other tissues (Klusmann et al., 2010; Le et al., 2009; Zhong et al., 2010). Though there is more to study, miR-125 is clearly an important regulator of HSC maintenance and, as such, a critical oncomiR upon its dysregulation.

4.1.3 miR-21—miR-21 is well studied for its contribution to malignant transformation and has been found to be upregulated in nearly all types of cancers that have been analyzed (Ling et al., 2013). miR-21's oncogenic capacity was first demonstrated in glioblastoma multiforme (GBM), where it was found to be required for survival of GBM-derived cell lines—when miR-21 was inhibited using antisense locked nucleic acids (LNAs), the cell lines underwent apoptotic cell death, demonstrating that these cell lines were addicted to persistent miR-21 expression (Chan et al., 2005). However, no responsible miR-21 target gene was identified in this study. In 2010, Slack's group extended the study of miR-21 in vivo by generating a mouse for doxycycline-controlled, tissue-specific overexpression of miR-21, making them only the second group to demonstrate the contribution of a miRNA to cancer development in a transgenic mouse model (Costinean et al., 2009). After birth, transgenic animals were driven to overexpress miR-21 in the hematopoietic system, and within 3 months the animals developed a transplantable malignancy closely mimicking pre-B-cell lymphoblastic lymphoma/leukemia. Strikingly, when transgene expression was terminated in transgenic animals, full tumor regression was observed after only 7 days, suggesting that miR-21 was sufficient for tumor initiation, but also necessary for cancer maintenance, again demonstrating oncomiR addiction for miR-21 (Medina et al., 2010). While Medina et al. observed that tumor regression was the result of apoptosis, consistent with miR-21's demonstrated role as an antiapoptotic factor, they did not report any specific targets of miR-21 that may have been responsible for the dramatic phenotype they observed. Other studies have identified targets of miR-21, including phosphate and tensin homolog (PTEN), a known tumor suppressor, and programmed cell death 4 (PDCD4), a nuclear protein thought to contribute to apoptosis (Yamanaka et al., 2009). Treatment of K562 cells with antisense oligonucleotides against miR-21 sensitizes the cells to apoptosis, confirming an antiapoptotic and oncogenic role for miR-21 (Hu et al., 2010; Li et al., 2010). miR-21 has been studied as an indicator of poor prognosis in CLL (Rossi et al., 2010), and when qPCR for miR-21 is added to the other diagnostic criteria for CLL, it improves prognostic risk stratification.

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miR-21 inhibition has also been investigated as a mechanism for restoring the SMAD7–TGFβ axis in MDS (Bhagat et al., 2013). TGFβ is a myelosuppressive cytokine that has been found to be upregulated in MDS, in which it is responsible for hematopoietic suppression. SMAD7, which is a negative regulator of the TGFβ receptor kinase, is downregulated in $CD34^+$ cells isolated from some MDS patients, and *SMAD7* loss results in increased TGFβ signaling, even in the absence of increased TGFβ expression (Bhagat et al., 2013). miR-21 has also been shown to be dysregulated in samples isolated from MDS patients (Sokol et al., 2011). The Verma group demonstrated that miR-21 binds the putative miR-21 binding site in the 3′-UTR of *SMAD* (Bhagat et al., 2013). Overexpression of miR-21 in $CD34^+$ cells leads to a reduction in their ability to form erythroid colonies, while miR-21 antagonism increased the ability of $CD34^+$ cells to form erythroid colonies in response to TGFβ. In a genetic mouse model of anemia, miR-21 antagonism improves TGFβ-induced bone marrow failure, consistent with its role in myelosuppression in MDS.

4.1.4 miR-22—By now, many other miRNAs have been identified as oncogenes in hematologic malignancies, some of which are reviewed elsewhere (Lawrie, 2013; Ling et al., 2013; Spizzo et al., 2009). One such oncomiR, *miR-22*, has become a focus of Dr. Pandolfi's group (Song et al., 2013a,b). miR-22 was identified as a potent oncogene simultaneously in metastatic breast cancer and in MDS. In both cases, miR-22 works by causing epigenetic dysregulation in affected cells, by repressing the ten–eleven translocation (TET) family of enzymes. DNMTs are responsible for transferring methyl groups to cytosines in the genome, creating a repressive mark that blocks transcription. TET enzymes oxidize 5-methylcytosine (5-mC) to 5-hydroxymethylcytosine (5-hmC), which may serve as an epigenetic mark in its own right. More importantly, this oxidation is the first step in removing the repressive 5-mC, making the TET enzymes antagonists of the DNMTs. In a model of metastatic breast cancer, miR-22 overexpression results in the repression of all three Tet enzymes, Tet1/2/3 (Song et al., 2013b). Loss of this important epigenetic regulator results in the silencing of the antimetastatic *miR-200* gene, and the promotion of breast cancer stemness, resulting in a more aggressive malignant metastatic phenotype.

miR-22 has also been found to be overexpressed in bone marrow isolated from MDS patients. The importance of TET2 in the hematopoietic system is well established, with *TET2* mutations and inhibition of the TET2 enzyme being very frequent defects in AML and MDS (Abdel-Wahab et al., 2009; Tefferi et al., 2009). Similar to what was observed in breast cancer, conditional miR-22 overexpression in the hematopoietic system results in downregulation of Tet2 (Song et al., 2013a). As a result, hematopoietic cells had global loss of 5-hmC and the reduced expression of known Tet2 target genes. HSCs overexpressing miR-22 exhibit increased self-renewal in vitro and increased reconstitution in vivo upon bone marrow transplantation. Over time, mice overexpressing miR-22 in the hematopoietic compartment develop a hematologic malignancy resembling MDS, which sometimes progresses to an AML-like disease. Further, antagonizing miR-22 overexpression in vitro increases Tet2 expression, decreases growth in cell lines, and decreases colony-forming capacity in explanted HSPCs, suggesting that there may be potential for miR-22 decoying in human disease (Song et al., 2013a).

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Though these data clearly identify an oncogenic role for miR-22 in MDS and in breast cancer, other studies have suggested tumor-suppressive properties for miR-22, highlighting the importance of cellular context on miRNA function. For example, miR-22, which is downregulated in some lung cancer specimens, has been shown to repress the receptor tyrosine kinase, ERBB3 (Ling et al., 2012), and reduced expression of miR-22 is associated with poor prognosis in colorectal cancer (Zhang et al., 2012). More recently, the miR-22's role in the hematopoietic system has also grown more complex (Wurm et al., 2017). Two studies have explored a role for miR-22 in promoting myeloid differentiation and serving as a tumor suppressor in de novo AML through the repression of known oncogenic pathways (Jiang et al., 2016; Shen et al., 2016). Further study is necessary to clarify the role of miR-22 in the various cellular contexts of hematopoiesis and leukemogenesis, and to determine if modulation of miR-22, either through miR-22-replacement or antagonism, would be beneficial in the management of hematologic malignancies. In the next section, we will discuss miRNAs with known tumor-suppressive function in hematologic disease.

4.2 Tumor-Suppressive MicroRNAs

While the class of known tumor-suppressive miRNAs is smaller than the known oncogenic miRNAs, and tumor-suppressive miRNAs do not have a clever nickname, like the "oncomiRs," they are no less important in the study of human malignancies nor in the history of the discovery of disease-associated miRNAs. miR-15 and miR-16, for example, were identified as lost in 13q14 deleted CLL (Calin et al., 2002), making them the first miRNAs with a demonstrated role in human physiology or disease. It should come as no surprise that tumor-suppressive miRNAs are responsible for repressing oncogenic genes. Tumor-suppressive miRNAs exhibit their oncogenic function when their expression is lost or downregulated, resulting in the uninhibited expression of the normally suppressed oncogenes. For example, the *let-7* miRNA, whose homology between *C. elegans* and humans prompted the discovery of miRNAs in mammals (Pasquinelli et al., 2000; Reinhart et al., 2000) and we have discussed more detail in Section 3.3, is a tumor-suppressive miRNA that represses the known oncogenes RAS and HMGA2 (Johnson et al., 2005; Mayr et al., 2007). In this section, we will discuss tumor-suppressive miRNAs, such as miR-15, miR-16, miR-203, and miR-29b.

4.2.1 miR-15 and miR-16—As previously mentioned, miR-15 and miR-16 became early targets of study in the hematopoietic system because of their dysregulation in B-cell chronic lymphocytic leukemia (B-CLL). These miRNAs are encoded in the miR-15a–miR-16-1 cluster, which is lost with the 13q14 deletion or can be epigenetically silenced (Calin et al., 2002), suggesting that miR-15 and miR-16 represent tumor-suppressive miRNAs, whose loss can result in malignancy. While Calin et al. were able to identify arginyl-tRNA synthetase (RARS) as a putative target of miR-15/16, with RARS levels decreased in a number of samples with increased miR-15/16 expression, they were not able to associate this defect with a pathogenic process (Calin et al., 2002). Later, however, a number of miR-15/16 oncogenic targets were identified, helping to explain how their downregulation can result in oncogenesis. For example, miR-15 and miR-16 were found to repress the antiapoptotic Bcl-2 protein, which is characteristically overexpressed in CLL (Cimmino et al., 2005). Though Bcl-2 is frequently overexpressed due to a translocation in other malignancies, that

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is not frequently the case in CLL and the mechanism of overexpression had been previously unknown. The Croce group was able to demonstrate that when miR-15/16 levels were high, such as in healthy $CD5^+$ lymphoid cells, Bcl-2 expression was minimal, and that Bcl2 expression was inversely correlated with miR-15/16 expression in cells isolated from CLL patients (Cimmino et al., 2005). Additionally, the *Bcl-2* 3′-UTR is responsive to miR-15/16 in a luciferase assay, and overexpression of miR-15/16 induces apoptosis in leukemia cell lines, suggesting that loss of miR-15/16 expression can result in leukemia and that miR-15/16 are tumor-suppressive miRNAs in the hematopoietic system (Cimmino et al., 2005). A number of other oncogenic targets for miR-15 and miR-16 have been identified. Earlier studies identified a role for *CCDN1* 3′-UTR in the pathogenesis of mantle cell lymphoma (MCL)—mutations that results in the loss of the 3′-UTR result in MCL with a more aggressive clinical course (Wiestner et al., 2007). Chen et al. and Deshpande et al. recognized that stabilization of *CCDN1* occurred when a miR-16 target site was lost along with the rest of the UTR (Chen et al., 2008a; Deshpande et al., 2009).

4.2.2 miR-203—miR-203 dysregulation in malignancy was first identified in studies examining a 7-Mb fragile site at chromosome 14q32, which is highly enriched in miRNAs, and is frequently lost in T-cell malignancies (Bueno et al., 2008). The homologous region is also lost in an irradiation-induced model of T-cell lymphomas in mice. Interestingly, when a single copy of miR-203 is lost due to deletion at the fragile site, miR-203 expression is further reduced by epigenetic silencing of the other allele. In murine T-cell lymphoma samples and in a number of human T-cell tumor cell lines, the miR-203 locus is dramatically hypermethylated (Bueno et al., 2008). In the context of *miR-203* loss a number of putative miR-203 targets are upregulated, including ABL1, a protein tyrosine kinase, and potent oncogene, which was demonstrated to be a bona fide target of miR-203 through reporter assays. The Philadelphia chromosome, t(9;22)(q34;q11), is present in 95% of chronic myelogenous leukemia (CML) cases and results in the *BCR–ABL1* fusion oncogene. The *BCR–ABL1* transcript retains the *ABL1* 3′-UTR and is thus a target of miR-203. Interestingly, in cell lines in which ABL1 is overexpressed or BCR–ABL1 is expressed, the miR-203 locus is hypermethylated, suggesting a pressure to maintain low levels of miR-203, so that ABL1 or BCR–ABL1 can exert their function. Conversely, when affected cell lines are treated with demethylating agents, such as 5′-azacytidine (Aza), expression of ABL1 and BCR–ABL1 is reduced (Bueno et al., 2008). Together, these data suggest that miR-203 replacement in certain malignancies may function much like tyrosine kinase inhibitors, such as imatinib, in the treatment of CML. Hypermethylation of the miR-203 tumor suppressor has now been demonstrated in a variety of hematologic malignancies and in HCC (Chim et al., 2011; Furuta et al., 2010).

4.2.3 miR-29b—miR-29 was first identified as tumor suppressive in B-CLL (Pekarsky et al., 2006). T-Cell leukemia/lymphoma-1 (TCL1), which is a potent activator of the PI3K/AKT prosurvival pathway, is an oncogene that is frequently overexpressed in B- and T-cell leukemias (Pekarsky et al., 2001) and is associated with aggressive B-CLL (Pekarsky et al., 2006). miR-29 and miR-181 were shown to be downregulated in CLL and are putative regulators of TCL1, and *miR-29* loss has been shown to be associated with poor prognosis in CLL (Calin et al., 2005), suggesting its role as a tumor-suppressive miRNA in CLL. miR-29

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was also demonstrated to act as a tumor suppressor in cholangiocarcinoma (Mott et al., 2007). Myeloid cell leukemia 1 protein (MCL1) is a potent antiapoptotic protein in the BCL-2 family, which has low expression in cholangiocytes. In contrast, miR-29 is highly expressed in cholangiocytes and was shown to repress MCL1 expression. miR-29 expression is reduced in a number of cholangiosarcoma cell lines, and enforced expression of miR-29 reduced MCL1 expression and sensitizes cell lines to apoptosis (Mott et al., 2007).

As we discussed in the context of miR-203 (Section 4.2.2), hypermethylation is a frequent mediator of oncogenesis. The miR-29 family was identified as downregulated in lung cancer (Fabbri et al., 2007). In contrast, DNMT3a/b are frequently upregulated in lung cancer—upregulation of DNMT and hypermethylation is associated with poor prognosis in lung cancer. Intriguingly, the *DNMT3a/b* transcripts contain miR-29 binding sites, and expression of DNMT3a/b and miR-29 is inversely correlated with patient samples. Further, overexpression of miR-29 reduces the level of hypermethylation in cell lines, as detected by mass spectrometry (MS), and upregulation of reporter genes that are frequently downregulated in lung cancer due to hypermethylation (Fabbri et al., 2007).

Aberrant DNA hypermethylation is also typical of myeloid malignancies. *DNMT3a/b* transcripts have also been demonstrated to be direct targets of miR-29 expression in leukemic cell lines, and enforced expression of miR-29 results in repression of DNMT expression (Garzon et al., 2009). There are no miR-29 binding sites in the *DNMT1* 3′-UTR, suggesting that it is not a direct target of miR-29. Interestingly, miR-29 directly targets the zinc finger transcription factor, SP1, a positive regulator of DNMT1 expression. Taken together, miR-29 represses the expression of all of the DNMTs. Consistently, ectopic miR-29 overexpression in leukemic cell lines results in a reduction in global DNA methylation and partial differentiation, consistent with its role as a tumor suppressor (Garzon et al., 2009).

Further studies have placed miR-29 downregulation within a complicated, multicomponent regulatory circuit in KIT-driven AML (Liu et al., 2010). KIT is a receptor tyrosine kinase that is expressed on hematopoietic stem and progenitor cells and is critically important to the maintenance of HSCs and normal hematopoiesis, primarily through its interaction with the stem cell factor (or the KIT ligand). SP1 and NFκB cooperate to increase KIT expression by binding at the *KIT* promoter (Liu et al., 2010). The *SP1* transcript is a direct target of miR-29 (Garzon et al., 2009), and forced expression of miR-29 leads to decreased KIT expression (Liu et al., 2010). Interestingly, specific chemical inhibition of SP1 or NFκB results in decreased KIT expression and increased miR-29 expression, suggesting the existence of a feedback circuit that regulates the expression of miR-29, SP1, and KIT. ChIP reveals that SP1/NFκB recruit HDACs to the miR-29 locus, completing the autoregulatory loop whereby SPI/NFκB cooperate with HDACs to repress miR-29 expression, further enhancing expression of SP1, which promotes the expression of KIT (Liu et al., 2010).

4.3 MicroRNA-Processing Mutations

We have discussed that DICER is crucial to the cytoplasmic processing of the pre-miRNA to the mature miRNA (Section 2.2), the selection of the miRNA strand from the passenger strand, and loading the miRNA into the RISC so that it can affect its target transcripts. With

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DICER's importance to miRNA processing, and the ability for miRNA dysregulation to result in malignant transformation, we should expect that DICER1 dysregulation can contribute to the development of various types of cancers (Foulkes et al., 2014). We have already seen how the effect of a given miRNA's dysregulation is cell context specific—a miRNA that can serve as an oncomiR in some cell types can serve as a tumor suppressor in other cell types. Similarly, reduced DICER1 expression has been associated with oncogenesis in some malignancies, such as in lung, breast, skin, and ovarian cancer (Dedes et al., 2011; Karube et al., 2005; Merritt et al., 2008; Pampalakis et al., 2010; Sand et al., 2010), and DICER1 overexpression is associated with prostate cancer metastases, and in cervical and esophageal cancers (Chiosea et al., 2006; Muralidhar et al., 2007; Sugito et al., 2006). Germline DICER1 mutations have been associated with many familial and childhood cancer syndromes, including differentiated familial pulmonary blastoma (Hill et al., 2009), Wilms' tumor (Foulkes et al., 2011), ovarian sex-chord stromal tumors (Schultz et al., 2011), and many others constituting what is commonly referred to as "DICER1 syndrome" (Foulkes et al., 2014).

In the hematopoietic system, deletion of *Dicer1* in mouse osteoprogenitor cells, driven specifically by the *Osterix-Cre Recombinase* transgene, results in the development of a myelodysplasia and AML, in an example of niche-induced leukemogenesis (Raaijmakers et al., 2010). Loss of *Dicer1* in these mice results in the failure of osteoblastic differentiation, an expansion of HSPCs, and leukopenia, anemia, thrombocytopenia, and dysplasia in cells from various lineages, consistent with a diagnosis of MDS, which converted to AML in a number of animals. Reciprocal transplantation experiments confirm that this leukemogenesis is not the result of an intrinsic defect in the HSCs, rather from defects to the HSC niche. The Scadden group identified reduced expression of the *Shwachman–Diamond–Bodian* (*Sbds*) gene, associated with stress response, and the clinical syndrome associated with MDS and AML, as mediating the observed phenotype in *Dicer1* knockout mice, though they were not able to construct a mechanism connecting Dicer1 loss and downregulation of Sbds.

Because loss of both alleles of DICER1 is necessary to result in malignancy, somatic DICER1 mutations rarely result in malignancy. However, mutations to other members of the miRNA-processing axis have been reported to contribute to malignant transformation. Tumors exhibiting microsatellite instability due to mutations in the *Musashi 1/2* (*MSI1/2*), which is frequently mutated in hematologic malignancies and contributes to poor prognosis myeloid leukemias (Kharas et al., 2010), frequently develop somatic truncating mutations in *TARBP2*, which encodes TAR RNA binding protein, a protein essential for loading of the RISC (Melo et al., 2009). Microsatellite-unstable tumors have also exhibited mutations in XPO5, the exportin protein responsible for exporting pre-miRNAs from the nucleus (Melo et al., 2010). Msi1 has also been shown to contribute to Lin-28-mediated miRNA processing, which we discussed in Section 3.3, in early neural differentiation (Kawahara et al., 2011). Activating mutations or overexpression of *Msi1* might contribute to oncogenesis through the repression of let-7, and the derepression of let-7's oncogenic targets, though further study of this mechanism is required.

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OncomiRs and tumor suppressors both provide opportunities for therapeutic intervention. In the next section, we will discuss miRNA-based therapeutic strategies including antagonism of oncogenic miRNAs and replacement of lost tumor-suppressive miRNAs.

5. THERAPEUTIC STRATEGIES

As we have discussed in Section 3, miRNAs are important regulators of numerous physiological functions, including HSC maintenance and hematopoietic differentiation. miRNA dysregulation, either overexpression of oncogenic miRNAs or downregulation of tumor-suppressive miRNAs, can result in hematologic malignancy, as we have discussed in Section 4. miRNA-based cancer therapeutics are, in the simplest terms, a strategy of combatting pathogenesis by restoring miRNA function of tumor-suppressive miRNAs that are lost or downregulated, or by inhibiting oncogenic miRNAs that are overexpressed (Li and Rana, 2014).

Other anticancer medications include highly toxic chemotherapeutic agents and highly specific protein-targeting agents, either antibody based or small molecules, which are difficult and expensive to develop. In contrast, miRNAs operate by highly specific, Watson–Crick base pairing. In the case of oncogenic miRNAs, base pairing can be blocked with anti-miRNA oligonucleotides, more frequently referred to as “antagomiRs,” which can bind with high affinity to miRISCs, disrupting their inhibitory function. In the case of tumor-suppressive miRNAs, miRNA mimetics can restore miRNA function. While strategies taking advantage of this sequence specificity are the most aggressively pursued, other therapeutic strategies include miRNA antagonism through the expression of sponge constructs, and the development of small molecules that target the transcriptional regulation of certain miRNAs. The primary concerns with miRNA-based therapies are tissue-specific delivery and, in the case of systemic administration, off-target effects of the administered antagomiRs or mimetics.

5.1 Modified Nucleic Acids Are Good Candidates for miRNA-Based Therapeutics

Unmodified RNA oligonucleotides are unsuitable for therapeutic applications because they are sensitive to serum nucleases (Hutvagner et al., 2004; Meister et al., 2004a). 2′-*O*-Methyl (2′-OMe)-modified nucleotides were the first modified nucleotides used in for miRNA antagonism due to their resistance to nucleases and their ability to stably base pair with ssRNAs (Majlessi et al., 1998), such as miRNAs in miRISCs in *Drosophila melanogaster* and in human cell lines (Hutvagner et al., 2004; Meister et al., 2004a) (Table 2). But still, 2′-OMe-modified nucleotides are not invulnerable to serum exonucleases and are therefore not suitable for human therapeutic applications. A number of other nucleotide modifications have been assessed for their resistance to nuclease degradation, their ability to permeate tissues and cell membranes, and their ability to bind and inhibit miRISCs. For example, substitution of some of the phosphodiester bonds with phosphorothioate bonds in oligonucleotides improves their absorption and makes them resistant to degradation, though oligonucleotides fully substituted with phosphorothioate-modified nucleotides do not exhibit any effect on miRISCs (Geary, 2009; Geary et al., 1997; Krutzfeldt et al., 2005; Lennox and Behlke, 2011). More recently, attempts to modify these therapeutic oligonucleotides have

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focused on utilizing LNAs, which utilize a bicyclic nucleic acid that is resistant to nuclease degradation, and bind anti-sense oligonucleotides with high strength (Singh et al., 1998). Several LNA design and modification strategies have been employed in mammalian in vivo studies, demonstrating their therapeutic potential and providing instruction for investigators who would wish to design their own (Elmen et al., 2008a,b; Lennox et al., 2013; Obad et al., 2011).

5.2 Tissue-Specific Delivery of miRNA-Based Therapeutics Remains a Challenge

The great challenge in the application of miRNA-based therapeutics comes in their tissue-specific delivery (Li and Rana, 2014). Oligonucleotides that achieve resistance to nuclease degradation and efficient antisense binding exhibit limited tissue distribution—they are quickly taken up by the liver and kidneys and excreted in the urine, which necessitates administration of high doses, increasing the risk of dangerous off-target effects. A number of methods have been applied to try and improve the delivery and tissue specificity of small oligonucleotides in vivo: oligonucleotide conjugation, liposomes, nanoparticle-based methods, and antibody-based methods. The earliest conjugation method sought, simply, to improve the distribution of small oligonucleotides in tissue, generally, which was achieved by conjugating an antagomiR with cholesterol at its 3′-end (Krutzfeldt et al., 2005). Further studies demonstrated that small oligonucleotides could be assembled into either high- or low-density lipoproteins, or vitamin E for preferential accumulation at different organs, such as the liver, digestive tract, endocrine organs, and the kidneys (Nishina et al., 2008; Wolfrum et al., 2007). Additionally, small oligonucleotides conjugated to unmethylated CpG oligonucleotides can target cells expressing Toll-like receptors 9 (TLR9), such as myeloid and B cells (Kortylewski et al., 2009). Liposome-based delivery strategies have been very effective at improving delivery to the liver, greatly reducing the dose of small oligonucleotides needed to achieve an effective dose, with a library of effective lipidoids available for liposome construction (Akinc et al., 2008; Morrissey et al., 2005). Though systemic administration of liposome-based therapies leads to their accumulation in the liver, studies have demonstrated that tissue-specific administration can be achieved by localized delivery, such as to the vagina for the prevention of herpes simplex 2 infection (Palliser et al., 2006), and intracranial injection to prevent infection by the Japanese encephalitis and West Nile viruses (Kumar et al., 2006). Still, nanoparticle-based delivery methods may yield improvements over liposome-based methods because of the ability to engineer them to uniform size with more easily customizable conjugations. Strong support for nanoparticle-based delivery systems came from a Phase I clinical trial in patients suffering from Ewing's sarcoma. A systemically administered siRNA targeting the *EWS–FLI1* fusion oncogene, encapsulated in a transferrin-containing nanoparticle, meant to target tumor cells that have upregulated transferrin receptors, was able to accumulate in the solid tumors of Ewing's sarcoma and reduce the expression of the target transcript (Davis et al., 2010). More recent advances in nanoparticle assembly and size control make this a promising method for small oligonucleotide delivery, going forward (Lee et al., 2012). Of course, as it concerns tissue- and cell type specificity, the most exciting and promising delivery method is antibody based. RNA binding proteins fused to an antigen binding fragment can carry a small oligonucleotide to a given target. For example, antibodies against gp160, the human immunodeficiency virus 1 (HIV-1) envelope protein, has been used to target siRNAs to HIV-

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infected cells in mice (Song et al., 2005). Similarly, antibodies against the human epidermal growth factor receptor 2 (HER2) have been able to deliver siRNAs to $HER2^+$ breast cancer cells in mice, resulting in significant therapeutic effect (Yao et al., 2012), suggesting that antibody-based oligonucleotide delivery has a lot of potential in miRNA-based anticancer therapeutics.

5.3 Off-Target Effects of miRNA-Based Therapeutics Remain Another Challenge

miRNA-based therapeutics are definitely not without challenges. We have already touched upon issues related to tissue-specific delivery—this is of real concern because a miRNA that is oncogenic in one cell type may be physiologically critical in another. Additionally, off-target effects, which can be related to oligonucleotide hybridization, or can be independent of sequence-specific hybridization, must also be considered. For the most part, the sequence specificity with which anti-miRs can be designed is a benefit that precludes significant off-target effects. However, we must consider that many miRNAs, particularly within the same family, share similar seed sequences. Under physiological conditions, these other miRNAs could be targeted by a synthesized antagomiR—such promiscuity has been previously reported (Li et al., 2011). One strategy that might allow for even greater specificity would be to target the pre-miRNAs, which are longer, and therefore targeting could be less dependent on the seed sequence, though this strategy has not yet been applied in mammals (Kloosterman et al., 2007).

Hybridization-independent off-target effects are largely related to the immunogenicity of oligonucleotides and their delivery systems, or any toxicity associated with them. The most ancient and evolutionary conserved arm of the innate immune system includes TLRs, some of which are capable of recognizing single-strand and double-strand RNAs, though the immunostimulatory effect can be minimized by using short, seed sequence-specific, oligonucleotides, as previous studies have demonstrated that oligos >12 nucleotides are necessary for TLR activation. Toxicity of synthetic oligonucleotides is also of some concern—phosphorothiate-modified nucleotides have been shown to transiently disrupt coagulation, phosphorothiate-modified nucleotides and LNAs can induce the complement cascade, and LNAs have been shown to have some liver toxicity (Li and Rana, 2014).

5.4 Drug Development and Clinical Trials

A number of miRNA-targeting and miRNA-replacement therapies are under development by pharmaceutical companies. Though not an anticancer therapy, one of the most successful antagomiR-based therapies is for the targeting of miR-122 and deserves mention here. miR-122 is highly expressed in the liver—observations that HCV only infects hepatocyte-derived cell lines that express miR-122 led researchers to identify miR-122 as an essential cofactor in HCV replication. Treatment of cell lines with 2′-OMe antisense RNA with exact complementarity to miR-122 abrogated HCV replication (Jopling et al., 2005). With the ease with which miRNA-based therapies can be delivered to the liver, a number of pharmaceutical companies have developed miR-122-based therapies. One such therapy, Miravirsen, which has been developed by Santaris Pharma and Hoffman-La Roche, has entered clinical trials—it is well tolerated in nonhuman primates and greatly reduces HCV burden (Hildebrandt-Eriksen et al., 2012; Lanford et al., 2010). In humans, HCV RNA is

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undetectable after five subcutaneous doses of miravirsen, with no reported adverse effects (Lindow and Kauppinen, 2012). Trials continue for miravirsen, and it may well become the first miRNA-based therapeutic to come to market.

As we have discussed previously, miR-21 overexpression has been associated with the pathogenesis of several types of cancers. In vitro inhibition of miR-21 leads to apoptotic cell death in glioblastoma, breast cancer, and liver cancer (Chan et al., 2005; Frankel et al., 2008; Meng et al., 2007). Regulus Pharmaceuticals has initiated studies for the investigation of anti-miR-21 for HCC and Alport syndrome (Wagenaar et al., 2015). Regulus Pharmaceuticals has also initiated preclinical trials into the treatment of HCC with an anti-miR-221-based therapy (Park et al., 2011).

In contrast to antagonistic miRNA-based therapies, such as in miR-21 and miR-221, Mirna Therapeutics has been developing several miRNA-replacement therapeutics for tumor-suppressive miRNAs, namely miR-34 and let-7. MRX34, a miR-34-replacement therapy formulated in liposomal nanoparticles, is currently undergoing Phase 1 clinical trials for safety and is likely to be the first miRNA-replacement therapy tested for efficacy in human cancers, particularly for its efficacy in treating melanoma. miR-34 is a proapoptotic miRNA that has been associated with metastatic potential in malignant metastatic melanoma (Hermeking, 2010; Lujambio et al., 2008). let-7, the miRNA whose homology in *C. elegans* and humans led to the identification of miRNAs in higher organisms, is frequently downregulated in human cancers (Chiu et al., 2014). Replacement therapy for let-7, as well as for miR-215, miR-101, and miR-16, is also being developed by Mirna Therapeutics, but is still in preclinical investigation.

There is still much to learn about miRNA-based therapeutics. As these drugs enter clinical trials and enter clinical practice, it is important to consider potential mechanisms of resistance against these therapies. In the case of miRNA antagonism, targeted oncogenic miRNAs may be upregulated, overcoming the antagonistic therapy. In the case of miRNA replacement for tumor-suppressive miRNAs, the seed sequence in targeted oncogenes may become mutated, registering miRNA replacement useless. Further, upregulation of nucleases and pumps might interfere with the delivery of miRNA-based therapies to affected tissues and cells. Of course, as identification of miRNAs involved in disease continues, and as therapy development progresses, the need for sophisticated delivery systems for cell and tissue specification will remain an ongoing challenge. Finally, it will become increasingly important to investigate how these therapies interact with other first-line treatments, and how they can be used together to treat disease most effectively. It is likely that miRNA-based therapies will be most effective when they are paired with other disease-specific therapies, including protein-targeting therapies.

5.5 miRNAs as Biomarkers in Diagnosis and in Determining Prognosis

Biomarkers are measured to aid in diagnosis, and good biomarkers should be specific, sensitive, noninvasive, consistent across epidemiological groups, easily quantifiable, and cost effective. Great biomarkers should also be modifiable with the progression of the disease or treatment for easy monitoring (Faruq and Vecchione, 2015). MicroRNAs have been detected in body fluids, including blood plasma, saliva, milk, and cerebrospinal fluid

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(Chen et al., 2008b; Mitchell et al., 2008). miRNAs are easily measured by micro-array, next-generation sequencing, and quantitative PCR, making them ideal candidates to serve as biomarkers.

One such example is miR-21, which we have reviewed as an oncomiR in Section 4.1.3. miR-21, miR-155, and miR-210 were found to be upregulated in serum samples from 60 DLBCL patients as compared to 43 healthy controls (Lawrie et al., 2008). Moreover, elevated serum miR-21 was associated with an improved prognosis, with extended relapse-free survival times, which is consistent with findings that elevated miR-21 in DLBCL tumor biopsies is associated with better prognosis (Lawrie et al., 2007).

miR-155, which we reviewed for its role in B-cell maturation in Section 3.2, has been studied as a biomarker in B-CLL (Ferrajoli et al., 2013). Elevated miR-155, detected in B cells and in plasma, was diagnostic for B-CLL, as compared to normal controls. Further, elevated serum miR-155 among B-CLL patients was associated with poor response to therapy. miR-150 and miR-342 were detected at high levels in serum from AML patients, as compared to healthy controls (Fayyad-Kazan et al., 2013). Importantly, miR-150 and miR-342 levels were shown to return to normal levels in AML patients in complete remission, suggesting that these two miRNAs are biomarkers that can be measured to monitor the efficacy of treatment. Circulating miRNAs have also been identified as biomarkers in a number of solid tumors and other disease processes, such as cardiovascular disease (Faruq and Vecchione, 2015).

Previous and ongoing studies into miRNA signatures in hematologic disease may aid in the development of biomarkers for the discovery and prognostic classification of leukemias. miRNA signatures have been established from CLL—miRNA profiling can distinguish between CLL with variable Zeta-chain-associated protein kinase 70 (ZAP70) expression, and between CLL with chromosome 13q14 deletion and expression of mutated IgV_H genes (Calin et al., 2004a, 2005). A more recent study was able to use miRNA signatures to distinguish between five different karyotypic subtypes of CLL: normal karyotype, 11q deletion, 17p deletion, trisomy 12, and 13q deletion (Visone et al., 2009).

Though AML and acute lymphocytic leukemia (ALL) can be distinguished through clinical presentation, cell morphology, and immunohistochemistry, researchers sought after a single test that could easily distinguish between AML and ALL (Mi et al., 2007). The miRNA signatures between ALL and AML were easily distinguished. Among the many miRNAs with differential expression between AML and ALL, the researchers identified four miRNAs, miR-128a, miR-128b, let-7b, and miR-223, that were minimally capable of distinguishing between the two diseases with 99% accuracy, demonstrating the power of miRNA signatures in providing diagnostic criteria that can be clinically applied. Other miRNA signatures for AML have been established. *Nucleophosmin* (*NPM1*)-mutated AML ($NPMc^+$ AML) can be distinguished from *NPM1*-unmutated AML by elevated miR-10, let-7, and miR-29 and downregulated miR-204 and miR-128 (Garzon et al., 2008a). Another group established minimal diagnostic expression signatures for several distinct karyotypes of AML, including mixed lineage leukemia 1 (MLL1)-rearranged, t(15;17), t(8;21), inv(16), and t(8;21) plus inv(16) as compared to normal controls (Li et al., 2008). Additional

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profiling has led to the identification of overexpression of miR-191 and miR-199a as prognostic of lower rates of disease-free survival in AML (Garzon et al., 2008b).

Recently, John Dick's group established miRNA signatures to differentiate between leukemia stem cells (LSCs) and normal HSCs in AML, in which they identified a number of miRNAs that were downregulated in LSCs or upregulated in LSCs, including miR-155, miR-125b, and miR-126 (Lechman et al., 2016). The study also established a minimal LSC signature, which includes miR-155, miR-15b, miR-126, and miR-22, that is significantly prognostic for overall survival. Though the remaining experiments described in this publication deal with miR-126, specifically, we can learn from the strategy of establishing a miRNA signature from prospectively identified cancer stem cells. Examination of differentially expressed miRNAs in cancer stem cells, as compared to the cancer bulk or healthy cell types, can help us to identify strongly prognostic miRNAs, miRNAs that may be useful in early diagnosis, or miRNAs that can be therapeutically targeted to specifically eliminate the cancer stem cell population.

The continued development of miRNA signatures in malignancy and in other diseases is an ongoing challenge for miRNA researchers, but one that is likely to yield critically important results in the identification of pathology-associated miRNAs that can be therapeutically targeted and biomarkers that can be assessed for their diagnostic and prognostic potential.

6. CONCLUDING REMARKS

Though miRNAs were only discovered within the last 25 years, we now understand how they are encoded in the genome, how they are transcribed and processed, and the mechanisms through which they inhibit protein translation. We also have begun to appreciate the physiological importance of these posttranscriptional regulators, in large part due to the observation of malignancies that can develop when miRNAs are dysregulated. miRNAs have been demonstrated to play a critical role in the hematopoietic system, both in HSC maintenance and in lineage-specific differentiation. Our understanding of stepwise hematopoietic differentiation and our ability to prospectively isolate various cell types in the hematopoietic system by flow cytometry have enabled us to identify a number of specific miRNAs that are key regulators for certain differentiation decisions. Single miRNAs can target many different transcripts, often within similar or related pathways. Likewise, transcripts can be targeted by multiple different miRNAs. Studies aimed at understanding the "targetome," the interactions of miRNAs and their targets will become increasingly important in our understanding of how miRNAs regulate cellular processes. Techniques capable of directly identifying miRNA:target interactions, such as PAR-CLIP, must be applied more broadly to cell lines with different tissues of origin. The continued development of animal models of miRNA dysregulation will provide great insights into the contribution of miRNAs to pathogenesis. Further, animal models provide a platform in which miRNA-based therapies can be tested, which is of particular importance in the design of new tissue-targeting strategies.

While this review has discussed miRNAs contribution to physiology and disease through their capacity to inhibit translation as part of the miRISC, other roles for miRNAs in the

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regulation of various cellular processes have recently begun to emerge. miRNAs have been implicated in modulating epigenetic effectors, such as histone modifications and DNA methylation, and in regulating alternate splicing and gene expression by transactivation. Clearly, there is more work to be done, and it is an exciting time for those researchers who choose to study the effect of miRNAs in their model systems.

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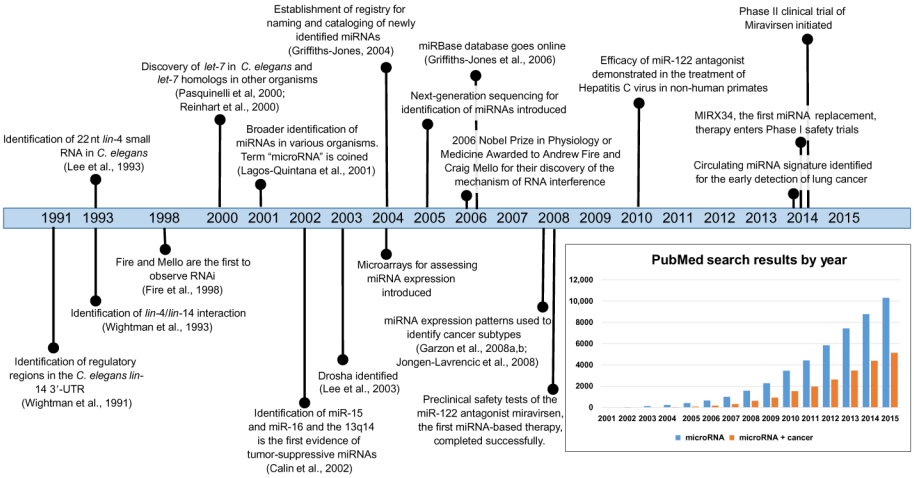

Fig. 1.

Timeline for the discovery and understanding of microRNAs. Over the last two decades, research into miRNAs has proceeded at a remarkable pace. This timeline takes us through the discovery of miRNAs, to the appreciation that they represent a broadly conserved regulatory mechanism, to the appreciation of the contribution of dysregulated miRNAs to disease, and, finally, to the initiation of clinical trials for miRNA-based therapeutics (*main*). This miRNA revolution can be easily tracked by examining PubMed search results for either "microRNA" or "microRNA+ cancer," both of which show a year-after-year increase in the number of publications (*inset*).

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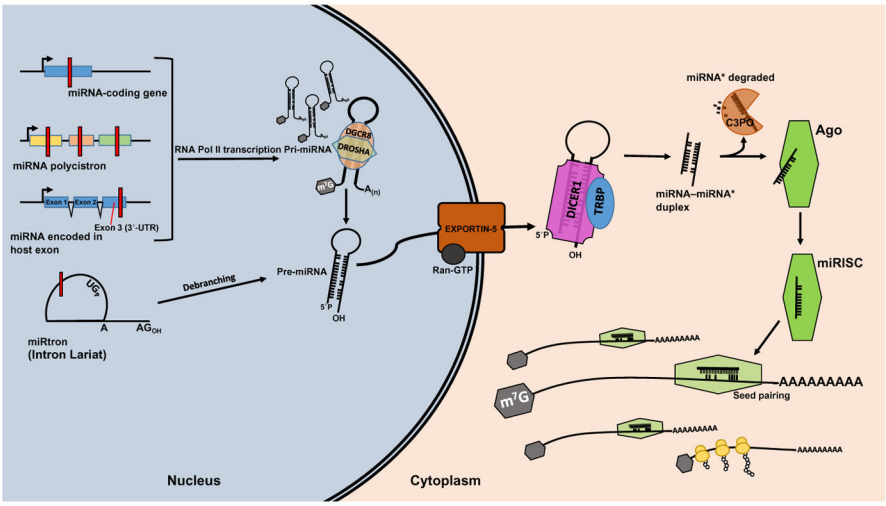

Fig. 2.

miRNA biogenesis. miRNA biogenesis begins in the nucleus, where miRNA-encoding genes are transcribed. For miRNAs that are not derived from an intron, i.e., miRNAs encoded in their own genes (*left top*), in polycistrons, or in the nonprotein-coding regions of host genes (*left middle*), hairpin primary-miRNAs (pri-miRNAs) are recognized by DROSHA, which utilizes RNAse III activity to trim the pri-miRNAs to precursor miRNAs (pre-miRNAs). miRNAs generated from introns and miRtrons bypass DROSHA cleavage and proceed as pre-miRNAs and normal processing (*left bottom*). Pre-miRNAs are exported from the nucleus by EXPORTIN-5 and they meet DICER in the cytoplasm. DICER further cleaves the pre-miRNA to a miRNA duplex and selects the miRNA with the least stable 5′-end for loading into the RNA-induced silencing complex (RISC), whose primary constituent is an Argonaute (Ago) protein (*right top*). The miRNA-RISC (miRISC) is then capable of targeting mRNA transcripts for repression through a variety of mechanisms (*right bottom*). *C3PO*, component 3 promoter of RISC; *TRBP*, TAR RNA binding protein.

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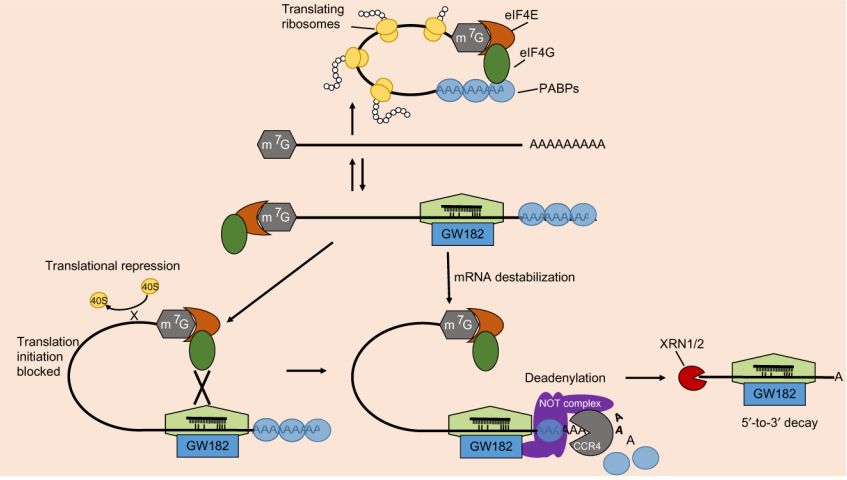

Fig. 3.

Mechanism of action of miRNA in the RISC. mRNAs are bound at the 5′-m^{7}G cap by translation initiation factors, and at the polyA tail by poly(A) binding proteins (PABPs). Interaction between the initiation factors and the PABPs is required for ribosome scanning, assembly, and translation initiation (*top*). miRNAs disrupt translation by two mechanisms: by translational repression and through mRNA destabilization. On a short timescale, the miRISC, including GW182, blocks association of initiation factors and PABPs, disrupting ribosome assembly and translation (*left bottom*). On a longer time-scale, the CCR4–NOT complex is recruited to the miRISC and GW182 and deadenylation is initiated. After deadenylation, the mRNA is degraded by 5′-to-3′ decay (*right bottom*). *eiF*, elongation initiation factor; *CCR4*, C–C motif chemokine receptor; *GW182*, glycine-tryptophan 182; *NOT*, negative on TATA; *XRN1/2*, 5′-to-3′ exoribonuclease 1/2.

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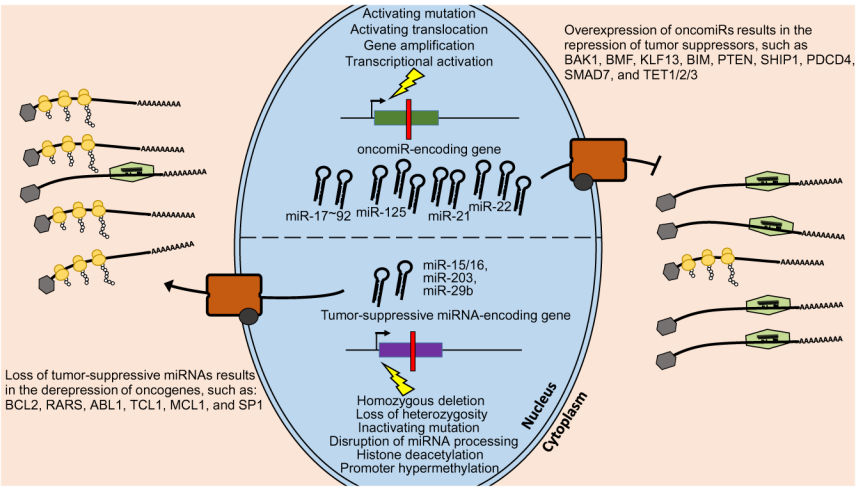

Fig. 4.

miRNAs can serve as oncogenes and tumor suppressors. miRNAs that normally repress the expression of tumor suppressors can serve as oncogenes, also called oncomiRs (*top* and *right*). Mutations and other genetic aberrations result in the increased expression of oncomiRs, which repress their tumor-suppressive targets and contribute to the development of malignancy. On the flip side, tumor-suppressive miRNAs normally repress the expression of oncogenes (*bottom* and *left*). When mutations or other defects result in decreased expression of tumor-suppressive miRNAs, expression of oncogenes is increased and contributes to the development of malignancy.

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Table 1

miRNAs That Contribute to Hematopoiesis and Hematologic Malignancies

MicroRNA	Role in Hematopoiesis	Role in Hematologic Malignancy	Known Targets
lin-4/miR-125	Myelopoiesis	AML, MDS, ALL, DLBCL	BAK1, ST18, DICER1, BMF, KLF13
let-7	Megakaryopoiesis Granulopoiesis HSC differentiation		LIN-28, HMGA2, RAS
miR-15/miR-16	Late erythroid differentiation	B-ALL, B-CLL, MCL, MM	BCL2, RARS, CCDN1, MCL1, CDK6
miR-181	B- and T-cell development Megakaryopoiesis Erythropoiesis	AML, CLL	LIN-28, AID, BCL2
miR-223	Myelopoiesis Erythropoiesis Granulopoiesis B-Cell development	CLL, ALL, AML, MALT	NFI-A, CEBPA, MEF2C, E2F1
miR-155	B- and T-cell development	DLBCL, BCL, AML	AID, SMAD5, INPP5D, HGAL, PU.1
miR-146a	HSC maintenance Represses megakaryopoiesis and granulopoiesis	MDS	CXCR4, TRAF6, IRAK1/2
miR-17~92	B-Cell development	BCL, CML, CLL, MCL, MM, FL	BIM, PTEN, E2F1, CCND1
miR-21	Myelopoiesis	CML, CLL, B-CLL, MDS, NK/T lymphoma	PTEN, PDCD4, SMAD7, MSH2, STAT3
miR-22	HSC maintenance	MDS, AML	TET1/2/3, PTEN
miR-203		AML, CML, ALL, CLL, TCL	ABL1
miR-29	HSC development and maintenance	B-CLL, CLL, AML, MCL	TCL1, MCL1, SP1, DNMT3B, HBP1

This table summarizes many of the miRNAs discussed in this review, their known roles in hematopoiesis and in hematologic malignancy, and a selection of their known targets. *ALL*, acute lymphoblastic leukemia; *AML*, acute myeloid leukemia; *BCL*, B-cell lymphoma; *CLL*, chronic lymphoblastic leukemia; *CML*, chronic myeloid leukemia; *DLBCL*, diffuse large B-cell lymphoma; *FL*, follicular lymphoma; *MALT*, mucosa-associated lymphoid tissue lymphoma; *MCL*, mantle cell lymphoma; *MDS*, myelodysplastic syndrome; *MM*, multiple myeloma; *TCL*, T-cell lymphoma.

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Table 2

Types of miRNA-Based Therapeutics

Therapeutic	Description	Mechanism of Action	Clinical Development	Example
miRNA antagonism				
miRNA sponges	miRNA sponges are transcribed from virally introduced or transfected constructs and contain many miRNA recognition elements (MREs)	miRNA sponges block miRNA activity by competing with miRNAs, which bind their MREs. This sponge effect prevents miRNAs from exerting their function	Preclinical studies	miRNA sponges are frequently used in preclinical studies to antagonize miRNA activity. Philip Sharp's lab first developed miRNA sponges in 2007
Antisense oligonucleotides (ASOs)	ASOs are short, chemically modified, single-stranded, DNA-like, antisense oligonucleotides that are capable of binding to miRNAs, disrupting their action, or mRNAs, disrupting their transcription. LNAs and other antagomiRs are included within the ASO category	ASOs bind their RNA targets by Watson–Crick base pairing. When ASOs target mRNAs, the resulting DNA–RNA hybrids are cleaved by RNAse H. ASOs disrupt miRNA function by binding them in miRISCs	Preclinical studies	2′-OMe-modified oligonucleotides were used to study miRNAs and RNAi activity in *D. melanogaster* and in human cell lines
Locked nucleic acid (LNA) antagomiRs	LNAs are locked because they are synthesized with some RNA nucleotides modified with a bridge connecting the 2′O to the 4′C. This locked conformation makes them resistant to nucleases. LNAs are usually designed to target the functional 5′-seed region of miRNAs	LNA antagomiRs bind miRNAs by Watson–Crick base pairing, blocking miRNA action by binding them in miRISCs. miRNAs are degraded and the antagomiR is recycled	Preclinical Studies Phase I trials Phase IIa trials	Miravirsen, an LNA antagomiR against miR-122 in the treatment of HCV
Other antagomiRs	Oligonucleotides partially modified with phosphorothioate bonds can also be used to inhibit miRNAs and are resistant to serum nucleases	Watson–Crick base pairing blocks miRNAs in the miRISC and leads to their degradation. The antagomiR is recycled	Preclinical studies	Early studies in the targeting of miR-122 in the liver
miRNA replacement				
miRNA mimics	Synthetic double-stranded oligonucleotides that are processed to single-stranded miRNA-like molecules	miRNA mimics are incorporated into miRISCs and act like endogenous miRNAs. miRNA mimics can be used to restore miRNA levels, as in the case of lost tumor-suppressive miRNAs	Preclinical studies Phase I trials	MRX34 for the restoration of the tumor-suppressive miR-34 in the treatment of various malignant cancers Restoration of miR-26a in the treatment of hepatocellular carcinoma (HCC) in mice
miRNA expression vectors	miRNA expression vectors can be introduced virally or by transfection	miRNAs are transcribed from expression vectors downstream of constitutive or cell type-specific promoters	Preclinical studies	miRNA expression vectors are commonly used in preclinical studies, but are not well suited for clinical use due to concerns with virally introduced gene therapy