1

MicroRNAs: A BRIEF INTRODUCTION

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ABBREVIATIONS

ADAR	adenosine deaminases that act on RNA
Ago	Argonaute
ALL	acute lymphoblastic leukemia
CLL	chronic lymphocytic leukemia
DGCR8	DiGeorge critical region 8
dsRNA	double-stranded RNA
Exp-5	exportin 5
HITS-CLIP	high-throughput sequencing of RNA isolated by cross-linking immunoprecipitation
IP	immunoprecipitation
lncRNA	long non-coding RNA
miRISC	miRNA RNA interference silencing complex
miRNA	microRNA
mRNA	messenger RNA
ncRNA	non-coding RNA
nt	nucleotide
PACT	protein activator of the interferon-induced protein kinase
PAR-CLIP	photoactivatable-ribonucleoside-enhanced cross-linking and immunoprecipitation
PASR	promoter-associated small RNAs
piRNA	piwi-interacting RNA
PROMPT	promoter upstream transcripts
RBP	RNA-binding protein
RNAi	RNA interference
snoRNA	small nucleolar RNA
ssRNA	single-stranded RNA
TF	transcription factor
tiRNA	tiny RNA
TRBP	HIV-1 TAR RNA binding protein
tRNA	transfer RNA
TSSa-RNA	TSS-associated RNA
T-UCR	transcribed ultraconserved regions
UTR	untranslated region

I. A SHORT HISTORY OF SMALL RNAs

The central dogma of molecular biology, first postulated by Francis Crick in 1958 and later refined in 1970, states that biological information flows unidirectionally from DNA to RNA to protein (Crick 1970). This view implies that non-coding RNA (ncRNA) has little or no intrinsic value, despite accounting for more than 90% of eukaryotic transcriptional output (Mattick 2001). Consequently, it is perhaps not surprising that microRNAs (miRNAs) were unknown to the scientific community until very recently. Indeed, it was only in 1993 when the first, what we now know to be a miRNA, was announced by the Ambros and Ruvkun laboratories simultaneously in the December edition of the journal *Cell* (Lee et al. 1993; Wightman et al. 1993). The Ambros group had identified and cloned a *Caenorhabditis elegans* developmental regulatory locus, *lin-4*, that did not contain con-

ventional start and stop codons. Furthermore, introducing mutations that disrupted the putative open reading frame in this 700-nt fragment did not affect function, suggesting that *lin-4* did not encode for a protein at all (Lee et al. 1993). At the same time, the Ruvkun lab were working on another temporal regulator of *C. elegans*, *lin-14*. They had found that *lin-14* was regulated posttranscriptionally via a repeat sequence in the 3′-UTR (untranslated region) of the gene (Wightman et al. 1993). The two labs shared their unpublished findings and realized that the small transcripts of *lin-4* (22 nt and 61 nt in length) contained complementary sequences to the 3′-UTR sequence of *lin-14*, and could regulate this gene via an entirely new regulatory mechanism involving non-coding RNA. However, as *lin-4* has no clear homologue outside of worms, the biological significance of this discovery was not realized until many years later.

Although RNA silencing had been known in plants since the beginning of the 1990s (Napoli et al. 1990), the connection with small RNAs was not made until 1999, when the Baulcombe laboratory identified small (25-nt) non-coding RNA species complementary to the target gene that were responsible for gene silencing (Hamilton and Baulcombe 1999). A few months later, it was demonstrated that dsRNA, the trigger for RNA interference (RNAi) (Fire et al. 1998), was sequentially processed into 21–23 nt ssRNA fragments (Zamore et al. 2000). Soon after, another publication from the Ruvkun laboratory described a heterochronic gene of *C. elegans*, *let-7*, that controls juvenile to adult transition in larval development (Reinhart et al. 2000). *let-7* shared many of the characteristics of *lin-4* as it encoded for a small (21 nt) ncRNA transcript that negatively regulated the mRNA of *lin* family members through complementary RNA-RNA interactions at the 3′-UTR of these genes. Unlike *lin-4,* however, the sequence of *let-7* was found to be conserved in most eukaryotic organisms (Pasquinelli et al. 2000; Lagos-Quintana et al. 2001). Together, these discoveries instigated the start of the miRNA revolution, a term first coined by Lee and Ambros in 2001 (Lee and Ambros 2001). Since this time, over 25,000 miRNAs (including more than 2000 human miRNAs) have been identified from a diverse range of more than 190 different species, including algae, plants, mycetozoa, arthropods, nematodes, protozoa, vertebrates, plants, and viruses (Griffiths-Jones et al. 2006). For a current list of annotated miRNAs, see the miRBase database (http://www.mirbase.org/).

MiRNAs primarily function as posttranscriptional (negative) regulators of gene expression via binding to complementary sequences located mainly within the UTRs of target genes. Because a single miRNA can target several hundred genes, it is believed ~60% of all human genes are a potential target for miRNA regulation (Friedman et al. 2009). In addition, a single target gene often contains binding sites for multiple miRNAs that can bind cooperatively (Lewis et al. 2003), allowing miRNAs to form complex regulatory control networks. Perhaps, unsurprisingly, miRNAs have been shown to play key regulatory roles in virtually every aspect of biology, including the many physiological and pathological processes described in the chapters of this book.

II. BIOGENESIS OF miRNAs

The majority of human miRNAs are encoded within introns of coding mRNAs, while others are located exgenically, in non-coding mRNAs or within the 3′-UTR sequence of coding mRNA (Rodriguez et al. 2004). MiRNAs are transcribed as 5′-capped large polyadenylated transcripts (pri-microRNA) primarily in a Pol II-dependent manner, although the involvement of Pol-III transcription has also been postulated for miRNAs encoded within Alu repeat sequences (Borchert et al. 2006).

Adenosine deaminases that act on RNA (ADARs) can alter the specificity and binding capacity of miRNA transcripts by changing adenosine bases to inosine post-transcriptionally. For example, ADAR-mediated changes to the *pre-miR-151* sequence cause accumulation of the pre-miRNA by blocking Dicer processing (Kawahara et al. 2007a). A selective change to the seed sequence of *miR-376* by ADARs causes it to additionally target *PRPS1* (Kawahara et al. 2007b). Deep sequencing of mouse brain tissue has identified a number of "edited" miRNAs, increasing the potential repertoire of miRNA targets available for regulation (Chiang et al. 2010).

Approximately 40% of human miRNAs are cotranscribed as clusters encoding multiple miRNA sequences in a single pri-microRNA transcript (Altuvia et al. 2005; Hertel et al. 2006). Pri-miRNAs are cleaved within the nucleus by Drosha, an RNaseIII-type nuclease, to form 60–110 nucleotide hairpin structures (pre-microRNA) (Figure 1.1). Drosha by itself possesses little enzymatic activity and requires the cofactor DiGeorge syndrome critical region 8 gene (DGCR8) in humans (Pasha in *Drosophila*) to form the microprocessor complex (Yeom et al. 2006).

Once produced, pre-miRNAs are exported from the nucleus to the cytoplasm by Exportin-5 (Exp-5) in a Ran-GTP dependent manner (Zeng 2006). The cytoplasmic pre-miRNA is further cleaved by Dicer, another RNaseIII-type enzyme, to form an asymmetric duplex intermediate (miRNA:miRNA*), consisting of the mature miRNA sequence and the antisense miRNA passenger strand (miRNA*). Similar to Drosha, cofactors, such as

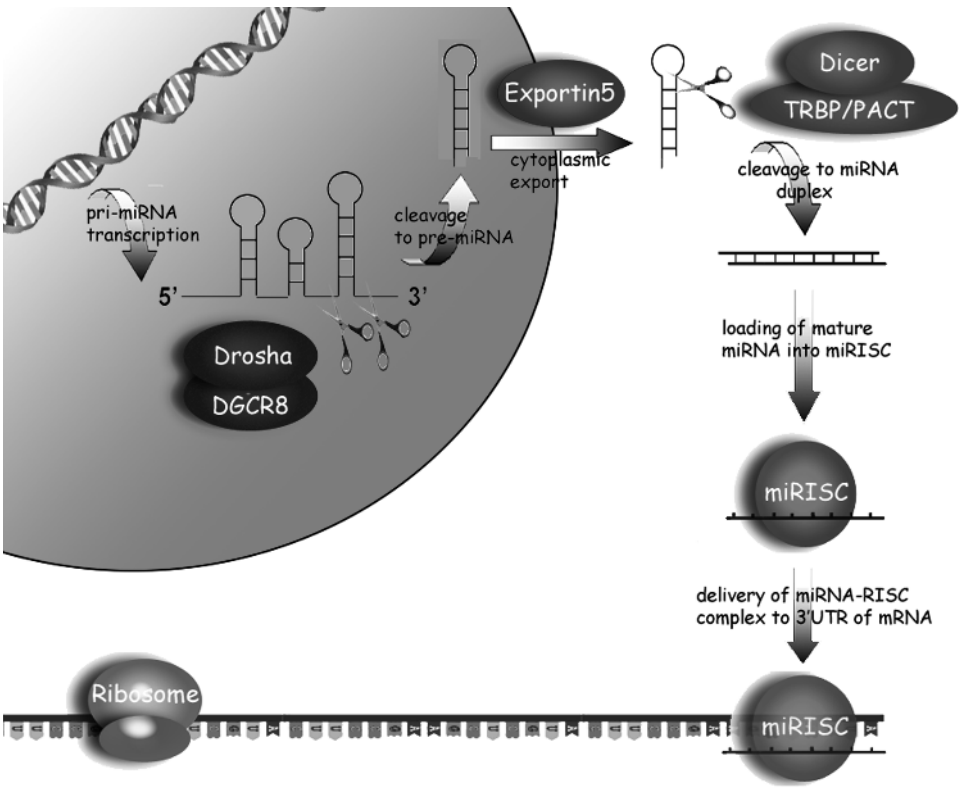

Figure 1.1. Schematic diagram of the canonical miRNA biosynthetic pathway. Reproduced from Lawrie, C.H. (2007) *Br J Haematol* **136** (6):503–512. See color insert.

TRBP and PACT (in humans), are necessary for Dicer activity (Lee et al. 2006). The miRNA:miRNA* duplex is, in turn, loaded into the miRISC complex in which Argonaut (Ago) proteins are the key effector molecules. The strand that becomes the active mature miRNA appears to be dependent upon which has the lowest free energy 5′ end and is retained by the miRISC complex, while the passenger strand is generally believed to be degraded by an unknown nuclease (Khvorova et al. 2003; Schwarz et al. 2003). It should be noted, however, that many miRNA passenger strands are also capable of silencing target transcripts and probably play a more important biological role than was previously realized (Okamura et al. 2008; Ghildiyal et al. 2010).

The loaded miRISC is guided by the mature miRNA sequence (19–24 nucleotide) to complementary sequences located primarily within the 3′-UTR of the target gene mRNA, although binding sites have additionally been identified in both 5′-UTR (Lytle et al. 2007) and coding regions of genes (Tay et al. 2008). In contrast to plant miRNAs that contain extensive regions of complementarity with their target genes, animal miRNAs are only partially complementary and have a propensity to recognize targets via 6–8 nt "seed" sequences, usually located at nt position 2–8 of the 5′-end of the miRNA (Bartel 2009), although sometimes also in the center of the miRNA sequence (Shin et al. 2010). There are rare examples of animal miRNAs (e.g., *miR-196* and *HOXB8*) that do share near-perfect complementarity, resulting in direct cleavage of the mRNA (Yekta et al. 2004).

While the vast majority of animal miRNAs are generated by the canonical miRNA biosynthetic pathway described above (Ghildiyal and Zamore 2009) (Figure 1.1), alternative Drosha-independent and Dicer-independent pathways do exist (for detailed review, see Yang and Lai 2011). For example, mirtrons are short RNA duplexes derived from the splice acceptor and donor sites of introns (Okamura et al. 2007). Splicing occurs independently of Drosha cleavage, and mirtrons can enter the canonical pathway via Exp-5 export and can regulate typical seed-matching targets. While mirtrons appear to be prevalent in both *D. melanogaster* and *C. elegans* genomes (Chung et al. 2011), they are little studied in vertebrates, although *drosha* and *dgcr8* murine knockouts maintain expression of mirtrons, suggesting that a similar mechanism does exist in mammals (Berezikov et al. 2007; Chan and Slack 2007; Babiarz et al. 2011; Ladewig et al. 2012). In addition, functional miRNAs can also be derived from larger ncRNA molecules, such as snoRNAs and tRNAs (Babiarz et al. 2008), and tRNaseZ (Bogerd et al. 2010), in a Drosha-independent Dicer-dependent manner. In contrast, *miR-451* is processed independently of Dicer but requires Drosha activity (Cheloufi et al. 2010; Cifuentes et al. 2010).

A. miRNA Nomenclature: What's in a Name?

The need for a rationalized system of nomenclature for miRNAs was realized soon after their discovery (Ambros et al. 2003). While the first miRNAs were named on the basis of position or function (i.e., *let-7* is "*lethal phenotype-7*" and *lin-4* represents a previously defined *C. elegans* genetic locus), it became apparent that such an approach would quickly become unmanageable. Novel miRNA sequences are therefore assigned a number that reflects the order of their discovery by the central miRNA database, miRBase (http://www.mirbase.org/) (Griffiths-Jones et al. 2006). Closely related miRNA sequences are followed by a letter (e.g., *miR-34a, miR-34b*, and *miR-34c* [*miR-34* was the 34th discovered miRNA]). The same sequence may be encoded at multiple genomic locations, in which case a further suffix is added (e.g., *hsa-mir-16-1* is encoded at chromosome 13 and *hsa-mir-16-2* at chromosome 3). The first prefix in the earlier example indicates species (e.g., *hsa-* is *Homo sapiens*) and is followed by either "*mir*" or "*miR*," the former

indicating a pri-miRNA, pre-miRNA, or genomic locus, and the latter (with a capitalized R), the mature miRNA sequence. Each pre-miRNA encodes for two possible mature miRNA forms derived from either the 3′ or 5′ arm of the hairpin sequence (Figure 1.1). Previously, these were designated the "miR" sequence, for the major mature product and the "miR*" sequence, for the minor mature product (e.g., *miR-155* and *miR-155**). This has recently been replaced in miRBase by the use of the -5p or -3p suffix to reflect their origin (e.g., *miR-17-3p* and *miR-17-5p*). This change also acknowledges increasing evidence that both strands can be functionally important (Czech and Hannon 2011), and that the frequency of a particular form may differ between different cell types (Griffiths-Jones et al. 2011).

III. miRNA FUNCTION: CONTROLLING mRNA STABILITY, DEGRADATION, AND/OR TRANSLATION

Although repression of translation without mRNA degradation was originally believed to be the *modus operandi* of animal miRNAs, the situation appears to be more complex than previously thought, and there is now compelling evidence that miRNAs can also affect transcriptional levels through deadenylation, degradation, and/or destabilization of target mRNAs (Giraldez et al. 2006). Indeed, it has been suggested that translational inhibition has only a modest role to play in miRNA function, and that mRNA destabilization is the predominant mechanism used to inhibit target genes in mammals (Guo et al. 2010). MiRNAs appear capable of utilizing a wide range of methods to regulate gene expression; however, the relative contribution of each of these in mammalian cells remains unclear, not least of all because most studies have been carried out in invertebrates.

In perhaps the simplest mechanism, near-perfect pairing of miRNA and target gene sequences, a common occurrence in plants (Llave et al. 2002) but exceedingly rare in animals (Yekta et al. 2004), allows Ago-mediated endonucleotic cleavage of the target mRNA to take place (Figure 1.2A). More commonly in animals, miRNA binding results in destabilization of the target mRNA via recruitment of deadenylation factors, making the mRNA more susceptible to degradation (Figure 1.2B). Deadenylation is mediated by GW182 proteins that form part of the miRISC complex. The carboxy-terminus of GW182 interacts with poly(A)-binding protein (PABP) and recruits CCR4 and CAF1 deadenylases (Wu et al. 2006; Huntzinger and Izaurralde 2011).

How translational repression in the absence of mRNA degradation operates remains controversial, and several mechanisms have been proposed. There is evidence for inhibition of translation occurring at the stage of initiation via repression of eukaryotic initiation factors (eIF) (Humphreys et al. 2005) (Figure 1.2C). Alternatively, other studies demonstrate that miRNAs can inhibit translation after initiation during elongation of the nascent peptide (Olsen and Ambros 1999) (Figure 1.2D). It has also been suggested that miRNA-bound mRNA can be sequestered away from the translational machinery in P-bodies that additionally act in concert with enzymes to remove the 5′ cap, hence preventing translation (Liu et al. 2005; Sen and Blau 2005) (Figure 1.2E), or may prevent recognition of the 5′ cap by translation factors (Pillai et al. 2005).

In addition to negative regulation, miRNAs are also able to function as translational activators of some genes (Vasudevan et al. 2007) (Figure 1.2F). To complicate matters further, miRNAs can also possess decoy activity that interferes with the function of regulatory proteins. For example, *miR-328* can bind directly to hnERP E2, preventing its interaction with *CEBP* mRNA and therefore function (Eiring et al. 2010).

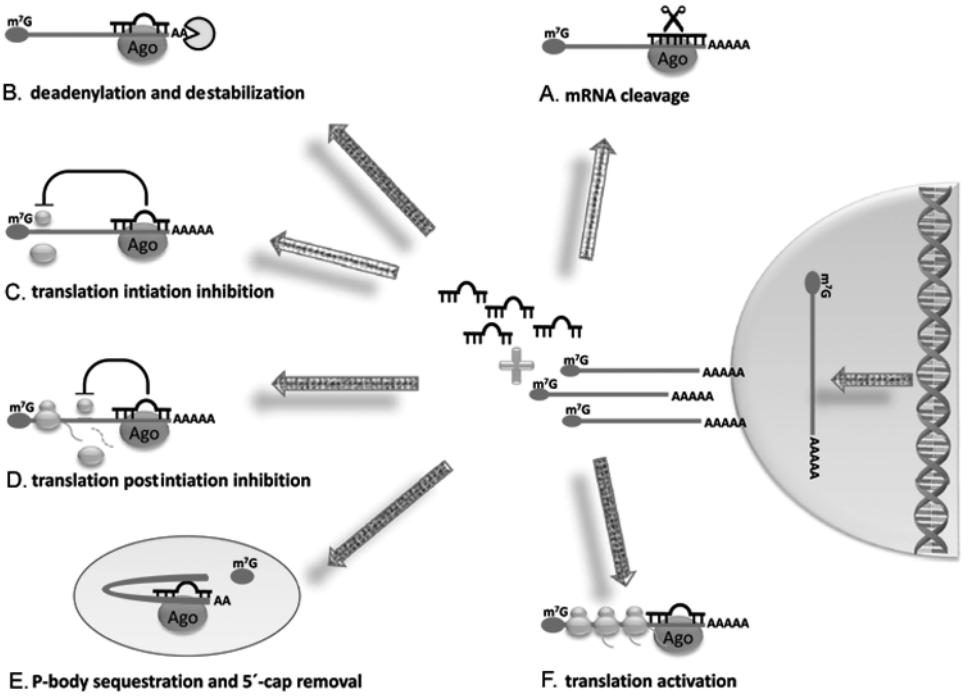

Figure 1.2. Schematic diagram of proposed mechanisms for miRNA function. (A) Ago-mediated cleavage of mRNA can occur when the miRNA sequence is complementary to the target gene-binding site. (B) Removal of poly(A) tail by deadenylases causes destabilization and degradation of mRNA. (C) Translation initiation inhibited by miRISC interactions with eukaryotic translation initiation factors (eIFs). (D) Inhibition of translation postinitiation. (E) Sequestration of mRNA in P-bodies. (F) miRNA-mediated translational activation. See color insert.

IV. REGULATING THE REGULATORS: miRNA CONTROL AND DYSFUNCTION IN DISEASE

Under physiological conditions the range of mechanisms available to the cell to control miRNA expression and function are every bit as varied as those involved in regulating protein-encoding genes, and can occur at the level of miRNA expression or posttranscriptionally via modulation of miRNA processing or miRNA function. As with protein-encoding genes, dysfunction of these regulatory mechanisms is often associated with disease, as are genetic alterations that result in dysregulated expression of miRNA-associated regions.

A. Genetic Dysregulation of miRNA Expression

Changes to the genetic structure of miRNA-associated regions have been linked with many different pathologies, most notably cancer. Such chromosomal aberrations include amplifications (e.g., *miR-26a* in glioma [Huse et al. 2009] [Figure 1.3A]), deletions (e.g., *miR-15a/16-1* in chronic lymphocytic leukemia [CLL] [Calin et al. 2002] [Figure 1.3B]), mutations (e.g., *miR-125a* in breast cancer [Li et al. 2009] [Figure 1.3C]), translocations

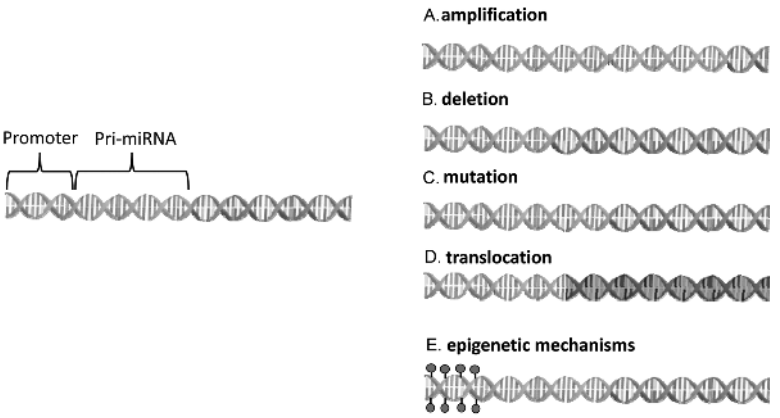

Figure 1.3. The various genetic and epigenetic alterations that can result in aberrant expression of miRNAs. (A) Amplification of miRNA-encoding regions. (B) Deletion of miRNA-encoding regions. (C) Mutations in the miRNA sequence (including SNPs). (D) Translocation occurring between distal, usually gene promoter regions, and miRNA-encoding regions. (E) Epigenetic mechanisms, such as histone modification and methylation of promoter regions of miRNAs, can silence miRNA expression. See color insert.

(e.g., *miR-125b* in leukemia [Bousquet et al. 2008] [Figure 1.3D]), single nucleotide polymorphisms (e.g., *miR-608* in lung cancer [Lin et al. 2012]), and loss of heterozygosity (e.g., 14q32 cluster in acute lymphoblastic leukemia [ALL] [Agueli et al. 2010]). These alterations can occur not only in the sequence of the mature miRNA itself, but also in the promoter region/pri-miRNA sequence (Calin et al. 2002, 2005) or at the miRNA-binding sites of target genes (Abelson et al. 2005), and can occur somatically or hereditarily.

B. Epigenetic Regulation

In addition to genetic alterations, aberrant miRNA expression can also result from epigenetic mechanisms, such as DNA methylation and histone modification (Figure 1.3E). While the importance of these mechanisms in disease is now apparent, the role of epigenetic regulation of miRNA expression under physiological conditions is at present much less clear.

DNA methylation occurs primarily at cytosine residues (changing it to 5-methylcytosine) that form part of the CpG dinucleotide motif, which is most commonly found at the proximal end of the promoter region of genes. Under physiological conditions, most genes are unmethylated; however, in tumor cells, the majority of genes are hypermethylated leading to aberrant silencing of multiple tumor suppressor genes (Esteller 2008). As with protein-encoding genes, approximately half of miRNA genes are associated with CpG islands (Weber et al. 2007). It has been shown that malignancy-associated changes in the methylation status of miRNAs can specifically regulate the expression of genes directly implicated in tumorigenesis (Bueno et al. 2008; Agirre et al. 2009). To complicate matters further, miRNAs can themselves regulate the expression of important components of the epigenetic machinery, including DNA methyltransferases (Benetti et al. 2008) and histone deacetylases (Roccaro et al. 2010).

Epigenetic gene silencing can also occur via histone modification. The amino acids of histone proteins (particularly their N-terminal "tails") can be posttranslationally modified in a number of ways that can repress transcriptional activity, including an increase in closed chromatin marks, such as trimethylation of 3mK9H3 and 3mK27H3, and a decrease in marks of open chromatin, for example, acetylation of AcH3 and AcH4 or trimethylation of 3mK4H3. The importance of histone regulation for miRNA expression is implied by the fact that histone modulators, such as HDAC-inhibitors, fundamentally alter the miRNA profile of treated cells (Scott et al. 2006; Barski et al. 2009). Indeed, the use of epigenetic drugs, such as DNA-demethylating agents (e.g., 5-aza-2'-deoxycytidine and zebularine) and histone deacetylase inhibitors (e.g., trichostatin A), may be of potential therapeutic use in reexpressing epigenetically silenced miRNAs, such as *miR-124a*, which is associated with poor prognosis in acute lymphoblastic leukemia (ALL) (Agirre et al. 2009). For a much more detailed description of the role of epigenetic regulation of miRNAs in cancer, the reader is directed to Chapter 22.

C. Transcription Factors and miRNA Regulatory Networks

Like protein-encoding genes, a myriad of transcription factors (TFs) can influence miRNA expression levels, and this form of regulation appears to be particularly important in controlling tissue specificity and developmental stage. The promoter regions of autonomously expressed miRNA genes are very similar to that of protein-encoding genes and can contain initiation and response elements, TATA boxes, and CpG islands, and so on (Corcoran et al. 2009). TFs can regulate miRNA expression by direct binding to promoter regions either positively (e.g., p53 stimulates *miR-34* expression [Christoffersen et al. 2010]) or negatively (e.g., ERα inhibits *miR-221* and *miR-222* expression [Di Leva et al. 2010]), or even both positively and negatively. MYC, for example, an important oncogene, up-regulates the *miR-17~92* cluster (O'Donnell et al. 2005), but can also down-regulate *miR-15a/16-1* (Chang et al. 2008) (Figure 1.4A).

In addition to direct control of miRNA expression, TFs are commonly involved in regulatory feedforward and feedback loops, whereby miRNAs autoregulate their expression in a negative (type I) or positive (type II) manner (Tsang et al. 2007). For example, *miR-27a* inhibits expression of *RUNX1*, which, in turn, stimulates expression of *miR-27a* in a simple unilateral negative feedback loop (Ben-Ami et al. 2009) (Figure 1.4B). *miR-15a* and *MYB* operate in a similar manner in hematopoietic cells (Zhao et al. 2009). Alternatively, a reciprocal-negative (positive) feedback loop can exist when the TF inhibits the miRNA, which, in turn, inhibits the TF. This, for example, is the relationship between *HBL1* and *let-7* (Roush and Slack 2009), and *ZEB1* and *miR-200* (Bracken et al. 2008) (Figure 1.4C).

Further levels of control can be achieved by adding extra components to the loops. In its simplest form, this results in a double-negative (or positive) loop, such as what occurs in the establishment of left-right asymmetry for the ASE chemosensory neurons of *C. elegans*. In this system, COG-1 inhibits the left ASE while stimulating expression of *miR-273* in the right ASE, which, in turn, inhibits *DIE-1* that up-regulates lys-6 expression promoting the left ASE-specific pathway. In the left, ASE *COG-1* is blocked by lys-6 (Johnston et al. 2005).

In reality, such loops represent the basic network motifs that make up much more complicated TF/miRNA regulatory networks that are a common feature of mammalian cell physiology (Shalgi et al. 2007; Tsang et al. 2007). These networks allow for

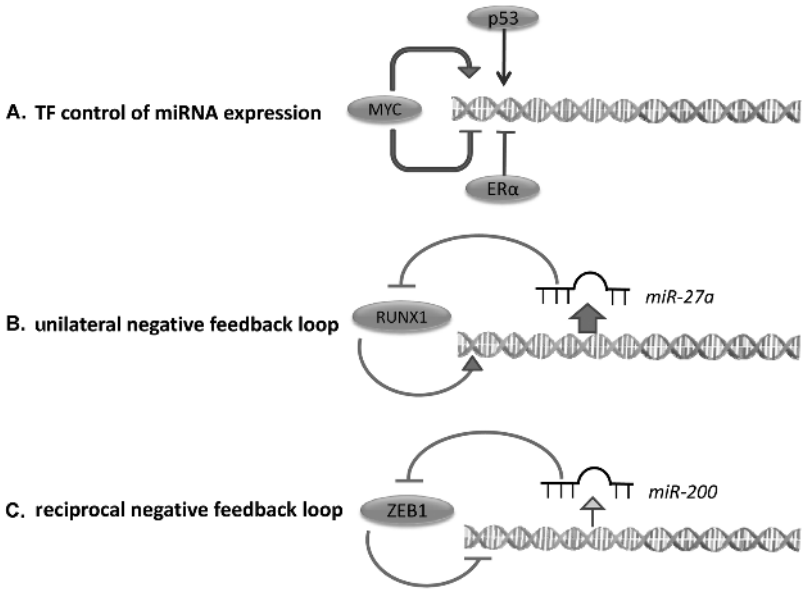

Figure 1.4. Examples of simple regulatory loop motifs involving miRNAs and transcription factors (TFs). (A) TFs can bind directly to the promoter region of miRNAs, either up-regulating or down-regulating expression, or a single TF (e.g., MYC) can up-regulate expression of one miRNA but down-regulate another. (B) When the TF that regulates the miRNA is itself regulated by that miRNA, a simple loop motif results. Up-regulation of the miRNA by the TF decreases its own expression in a unilateral negative feedback loop. (C) When the TF down-regulates the miRNA, this can increase TF expression in a reciprocal (positive) feedback loop. See color insert.

acceleration of transcriptional response times and dampening of fluctuating protein/miRNA levels within the cellular environment (Becskei and Serrano 2000; Rosenfeld et al. 2002).

In addition to employing TFs to regulate miRNA expression, it has recently been shown that miRNAs can autoregulate their own expression. *Let-7* bound to miRISC was found to directly bind and regulate its own primary transcript in a feedforward loop (Zisoulis et al. 2012).

D. Regulating miRNA Synthesis and Processing

The importance of correct functioning of the miRNA biosynthetic canonical pathway to mammalian biology is implied by the severity of phenotypes obtained when components of this pathway are deleted. Indeed, when the first mouse models were created using constitutive deletion of *Dicer* (Bernstein et al. 2003), *DGCR8* (Wang et al. 2007), *Drosha* (Fukuda et al. 2007), or *Ago2* (Morita et al. 2007), all resulting progeny were nonviable and died during early gestation with severe developmental defects. Needless to say subsequent studies utilized Cre-inducible conditional knockout mice in a more targeted approach. For example, targeted deletion of *Dicer* activity in astroglial cells (Tao et al. 2011) or oligodendrocytes (Shin et al. 2009) resulted in severe neuronal dysfunction. When *Dicer* was deleted specifically in the myogenic compartment this caused severe disruption to early skeletal muscle development (O'Rourke et al. 2007), while when deleted in the

female reproductive tract resulted in a loss of fertility and adenomyosis (Gonzalez and Behringer 2009). Further studies have demonstrated the essential nature of Dicer to the development of the heart (da Costa Martins et al. 2008) and the lung (Harris et al. 2006). In a similar manner, conditional knockouts of *DGCR8* in vascular smooth muscle cells resulted in severe liver hemorrhage (Chen et al. 2012), while deletion in cardiac neural crest cells lead to severe cardiac malformations (Chapnik et al. 2012). Interestingly, when *Dicer* was conditionally mutated in the lung tissue of K-ras mice, this resulted in an enhancement of lung tumor development (Kumar et al. 2007).

The levels and activities of the components of the miRNA biosynthetic pathway are subject to careful regulation. For example, Drosha is stabilized by its co-factor DGCR8, and conversely *DGCR8* mRNA levels are controlled by Drosha-mediated degradation (Han et al. 2009). Similarly, a decrease in levels of co-factor TRBP can destabilize Dicer (Chendrimada et al. 2005). In addition, a multitude of factors interacting with Drosha, Dicer or miRNA precursors can control the kinetics of miRNA biogenesis. For example, *LIN-28* can bind to the terminal loop of *pri-let-7*, interfering with Drosha cleavage and also Dicer processing (Viswanathan and Daley 2010). In contrast, SF2/ASF promotes cleavage of *pri-let-7* by Drosha (Winter et al. 2009).

Defects in the functioning of the miRNA biosynthetic pathway have frequently been associated with disease and cancer in particular. For example, low levels of Dicer and/or Drosha have been linked with poor clinical outcome for patients with ovarian cancer (Merritt et al. 2008), nasopharyngeal cancer (Guo et al. 2012), neuroblastoma (Lin et al. 2010), breast cancer (Khoshnaw et al. 2012), and lung cancer (Karube et al. 2005). In contrast, high levels of Dicer and/or Drosha have been associated with poor prognosis for esophageal cancer (Sugito et al. 2006), colorectal carcinoma (Faber et al. 2011), and prostate cancer (Chiosea et al. 2006). Whether these apparently contradictory findings represent true biological differences between the cancer types or are artifactual remains to be determined. Nevertheless, such studies underline the fundamental importance of the global miRNA machinery to both physiological and pathological processes, and suggest intriguing therapeutic possibilities based upon restoration or inhibition of components of this pathway.

E. Control of miRNA Function

The miRNA pathway downstream of synthesis of the mature miRNA is also subject to extensive regulation. For example, members of the TRIM-NHL family of proteins can regulate the ability of miRISC to repress target gene expression by directly associating with Ago proteins. TRIM71 can ubiquitinate Ago proteins, targeting them for proteasome-mediated degradation, which, in turn, regulates miRNA stability and decreases miRNA-mediated repression (Chatterjee and Grosshans 2009; Rybak et al. 2009). Mei-26 has also been demonstrated to repress miRISC function, although the exact mechanism involved is currently unclear (Neumuller et al. 2008). In contrast, NHL2 and TRIM32 can positively regulate miRISC function (Hammell et al. 2009; Schwamborn et al. 2009).

RBPs (RNA-binding proteins), such as HuR and Deadend1 (DND1), can bind target mRNA and therefore interfere with miRNA functioning. For example, HuR binding to the mRNA of *CAT1* can inhibit repression of this gene by *miR-122* (Bhattacharyya et al. 2006). In contrast, HuR binding to the 3′-UTR of *MYC* is necessary for *let-7*-mediated repression (Kim et al. 2009). HuR mediated control of miRNA repression may be a widespread phenomena, as 75% of 3′-UTRs with miRNA-binding sites also contain binding sites for HuR (Mukherjee et al. 2011). DND1 can also bind in the vicinity of

miRNA-binding sites apparently functioning by reducing the accessibility of the mRNA to miRISC (Kedde et al. 2007). *DND1*, in turn, can be regulated by *miR-24*-mediated inhibition (Liu et al. 2010).

Besides the regulatory mechanisms described earlier, other levels of control of miRNA function exist. For example, control of intracellular compartmentalization occurs when miRISC-bound mRNA is sequestered in P-bodies and stress granules (Liu et al. 2005). Regulation of the kinetics of miRNA decay have also been observed (Hwang et al. 2007).

V. PRESENT AND FUTURE PERSPECTIVES FOR miRNAs IN MEDICINE

The aim of the following section is to cover some of the current controversies in the miRNA field and how they are being addressed, and additionally speculate about future developments for miRNAs, in particular their potential usefulness to medicine.

A. Deciphering the miRNA Targetome: Understanding the Functional Consequences of miRNA Dysregulation in Disease

The first indication that miRNA dysfunction was directly associated with disease originated from the laboratory of Carlo Croce in 2002, whose seminal publication by Calin et al., made the connection between 13q14, a frequently deleted locus in CLL, and downregulation of the *mir-15a/16-1* cluster that is encoded within this region (Calin et al. 2002) (see Chapter 23 for more details). The same group later highlighted the potential importance of miRNAs in cancer, with the somewhat surprising finding that the majority of human miRNAs are in fact located at cancer-associated genomic regions (Calin et al. 2004). There is now overwhelming evidence that dysfunctional expression of miRNAs is a major contributor to the pathogenesis of most, if not all, human malignancies (Croce 2009; Iorio and Croce 2012). Besides cancer, evidence is rapidly accumulating that the dysregulation of miRNAs is fundamental to the pathogenesis of many non-neoplastic diseases as well, including infectious diseases, autoimmune conditions, cardiovascular, neuropathologies, hereditary, and inflammatory diseases, many of which are discussed in detail in subsequent chapters of this book.

However, while great effort has been put into identifying and cataloging aberrantly expressed miRNAs in disease, very little is known about the functional consequences of this dysregulation, and understanding the biological function of identified miRNAs is perhaps the biggest challenge facing the miRNA field at the moment. The primary reason for this is a paucity of knowledge about which genes are actually targeted by individual miRNAs and which of these genes are functionally important in specific cellular settings.

With very few functionally annotated exceptions, current approaches to this problem primarily rely upon the use of the many predictive computational algorithms available (Krek et al. 2005; Lewis et al. 2005; Miranda et al. 2006; Grimson et al. 2007; Kertesz et al. 2007). However, these algorithms typically predict hundreds or even thousands of target genes for each miRNA, and in reality perform very poorly. When the most widely used algorithms were tested against experimentally validated miRNA–target gene interactions, sensitivity ranged from just 1.3% to 48.8% (Sethupathy et al. 2006). Additionally, the degree of overlap between predictions (three algorithms) was found to range from 3.6% to 28.6%, and surprisingly, no commonly predicted genes were identified at all when the five most commonly used algorithms were compared. Importantly, this study showed that even when all five algorithms were used in union, only 72% of experimentally vali-

dated miRNA–target gene interactions were predicted. For example, *KRAS* and *HRAS* targeting by let-7 (Johnson et al. 2005), or *E2F2* and *MYC* targeting by miR-24 (Lal et al. 2009) are not predicted targets of these algorithms. To compound matters further, the function of a particular miRNA is dependent upon cellular context. Indeed, the same miRNA can act as both tumor suppressor and oncogene depending upon the cell type. For example, *miR-222* is overexpressed in hepatocarcinoma, where it targets tumor suppressor *PTEN* (Garofalo et al. 2009), but is down-regulated in erythroblastic leukemias, where it targets the *KIT* oncogene (Felli et al. 2005).

Consequently, much effort has been expended to resolve this issue. Particularly hopeful is the development of techniques to directly measure the miRNA:target gene interface, the so-called *targetome*, in cells under physiologically relevant conditions. A strategy that has frequently been employed to this end, is the use of gene expression arrays to elucidate which genes change in response to permutations of individual microRNAs (Lim et al. 2005; Johnson et al. 2007). A similar approach has been used to measure differences in protein levels using state-of-the-art proteomic techniques, such as stable isotope labeling by amino acids in culture (SILAC) (Yang et al. 2010; Kaller et al. 2011). A major drawback of these techniques, however, is their inability to distinguish between direct and indirect targets of miRNAs.

A more promising tactic is the use of immunoprecipitation (IP)-based techniques that allow for recovery of RNA that is directly bound to miRISC using antibodies against Ago proteins (Karginov et al. 2007). However, in the first incarnations of this approach, only the mRNA fraction could be recovered, making subsequent target gene identification difficult. Development of the HITS-CLIP technique overcame this problem by adding a UV cross-linking step to the IP procedure so that the miRNA fraction is also retained (Chi et al. 2009). Additionally, RNAse degradation of non-miRISC bound RNA, as well as the use of next generation sequencing to elucidate the recovered miRNA:target mRNA population, helped to increase the specificity of this approach. Further refinement has been obtained with the PAR-CLIP technique that utilizes incorporation of photoreactive ribonucleoside analogues, such as 4-thiouridine or 6-thioguanosine, into living cells, and allows direct identification of miRISC-bound miRNA:mRNA by mapping characteristic T-to-C mutations (Hafner et al. 2010). There are still limitations to this approach, however, as it does not address whether or not changes to protein levels occur, and because a target gene is bound to miRISC does not necessarily mean it is regulated. In addition, the choice of a particular antibody can greatly influence the results obtained. When IP experiments were compared using either Ago1 or Ago2 antibodies, there was only a partial overlap in the resultant targetome profiles (Karginov et al. 2007).

Nevertheless, techniques such as PAR-CLIP can provide a potential means to decipher the targetome of cells under physiologically relevant conditions and hence determine the true sphere of influence of dysregulated miRNAs in disease. However, these experiments are currently difficult and expensive to perform and consequently remain little used outside of specialist laboratories. In order to progress the miRNA field beyond the "stamp-collecting" phase and truly understand the function of these molecules in disease, we must apply such techniques in a much more concerted and systematic manner similar to that currently employed by the genomic, transcriptomic, and epigenomic fields.

B. Tip of the Non-Coding RNA Iceberg

One of the major shocks that arose from completion of the human genome project was that there are far fewer protein-encoding genes (~20,000) than was first envisaged (Schmutz

et al. 2004). Indeed, although ~75% of the human genome is transcribed (Djebali et al. 2012), the protein-encoding portion of the genome only accounts for 1.5% (Alexander et al. 2010). Despite the level of excitement generated in the scientific/medical world, it is worth remembering that miRNAs only represent a small proportion of the so-called dark matter of the genome, the non-coding transcriptome (Yamada et al. 2003). The most recent data from the Encyclopedia of DNA Elements (ENCODE) project annotated 1756 miRNAs in the human genome, representing just 1.8% of the transcriptional output (Djebali et al. 2012). So what about the other >95% of the transcriptome? While we cannot expect all of the remaining ncRNA to be functional, particularly because transcripts are present at very low levels and permissive transcription appears to be a common feature in eukaryotes (Ebisuya et al. 2008), there is emerging evidence that ncRNA other than miRNA is essential for both physiological function and development, as well as playing a fundamental role in disease (Mercer et al. 2009; Esteller 2011). Although relative to miRNAs, the study of other ncRNA molecules is very much in its infancy, many classes of ncRNAs are now recognized, including short ncRNAs, such as miRNAs, piRNAs, and tiRNAs; mid-size ncRNAs, such as snoRNAs, PASRs, TSSa-RNAs, and PROMPTs; and long ncRNAs (lncRNAs) (Esteller 2011; Harries 2012). For a much more detailed description of ncRNAs, and in particular lncRNAs and their role in cancer, please see Chapter 15.

The current emphasis of this field is on understanding the contribution and function of ncRNA to disease. In terms of this first goal, the development of next-generation sequencing technology and large concerted efforts, such as the ENCODE project, have made great strides over the last couple of years that will surely only accelerate in the future. In parallel, many of the approaches and techniques developed by the miRNA field are applicable to other classes of ncRNA and in all probability will uncover new insights concerning disease in the next couple of years.

C. Are miRNAs Clinically Useful Molecules?

While there is now overwhelming evidence that miRNAs play a fundamental role in the pathogenesis of many, if not all, diseases, the obvious question remains: What practical use are miRNAs likely to have for future clinical practice? MiRNAs show perhaps their greatest, and certainly most immediate potential, as novel biomarkers of diagnosis and prognosis, and as predictors of treatment response. MiRNA expression profiling can distinguish cancers according to diagnosis and developmental stage of the tumor to a greater degree of accuracy than traditional gene expression analysis (Lu et al. 2005). The use of miRNAs as novel classifiers of disease is extensively discussed within the chapters of this book. A particularly attractive characteristic of miRNAs in this regard is their stability to chemical and enzymatic degradation. This means they can be purified and robustly measured from routinely prepared formalin-fixed paraffin embedded (FFPE) biopsy material (Lawrie et al. 2007). Consequently, miRNA expression studies can be carried out (albeit retrospectively) on the vast resources of the world's pathology departments in a way that is simply not feasible for traditional gene expression studies, and miRNA expression studies have now been carried out for all except the rarest of pathologies.

A further manifestation of the stability of miRNAs, as well as their potential clinical usefulness, is their presence in extracellular biological fluids including blood. Tumor-associated miRNAs have been found at higher levels in sera and plasma of cancer patients than healthy controls (Lawrie et al. 2008). Subsequently, there has been a great deal of interest in the use of miRNAs as non-invasive biomarkers of disease, and miRNAs have now been detected in many biological fluids, including plasma, serum, tears, urine,

cerebral spinal fluid, breast milk, and saliva (Weber et al. 2010). This has particular clinical potential for cancer, where typically the diagnostic gold standards are invasive biopsy procedures that are expensive, uncomfortable, and sometimes risky for patients. Furthermore, a reliable blood test for cancer could pave the way for screening programs, leading to better detection rates and helping to increase cancer prevention. Further discussion on the use of miRNAs as noninvasive biomarkers can be found in Chapter 35.

Remarkably, it has even been suggested that exogenous miRNAs can be acquired in the blood as a result of the food that we eat (Zhang et al. 2012). Furthermore, these food-derived plant-specific miRNAs were demonstrated to be functional in human cells. This leads to the intriguing possibility that therapeutic miRNAs could be administered by incorporating them in food directly, or even that GM (genetically modified) crops could be engineered to express miRNAs (or antimiRs) with, for example, anti-cancer properties.

Of course, perhaps the most promising clinical aspect of miRNAs is their potential as novel therapeutic molecules, either as a tool to modulate target genes associated with disease or by correcting dysfunctional expression of the miRNAs themselves. The former approach is particularly attractive in that a single agent (i.e., a miRNA) can be used against multiple targets in a disease pathway or even against the whole pathway (Bui and Mendell 2010). There are two major strategies to therapeutically modulate dysregulated miRNAs in disease: using miRNA mimics to restore physiological levels of miRNAs that are downregulated (e.g., tumor suppressor miRNAs, such as *let-7* or *miR-34*), or the use of miRNA inhibitors targeted against overexpressed miRNAs (e.g., oncomirs, such as *miR-21* or *miR-155*). There is now a wealth of *in vivo* animal experiments that have established the proof-of-principle for the therapeutic efficacy of miRNAs in disease; however, at present, all bar a couple of these studies are still at the preclinical stage. The major hurdles to be overcome in order to translate these results into the clinic include the effective targeting of therapy (e.g., tissue-specific delivery, dosage, and pharmacodynamics) and safety concerns (e.g., off-target effects, RNA-mediated immunostimulation, and the use of viral vectors). That said, this is an area very much still in its infancy that is almost certain to flourish in the near future as the field matures, and promises to add to the current arsenal of therapies available to the clinician in the continual fight against disease. The use of miRNAs as therapeutics are covered in greater detail in Chapters 38–40 of this book.

In summary, although it is clear that the functional importance of microRNAs in medicine is gaining momentum rapidly, it is equally obvious that we still have much to learn from these tiny molecules. To answer the question, "Are miRNAs *really* clinically useful molecules?," readers are encouraged to explore further chapters of this book, which includes contributions from many of the most respected pioneers and experts in the miRNA field, and make up their own mind.

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