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Epigenetics and MicroRNAs

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ABSTRACT: MicroRNAs (miRNAs) regulate protein-coding genes post transcriptionally in higher eukaryotes. Argonaute proteins are important in miRNA regulation and are also implicated in epigenetic mechanisms such as histone modifications and DNA methylation. Here, we review miRNA regulation and outline its connections to epigenetics. **(*Pediatr Res* 61: 17R–23R, 2007)**

The discovery of RNA interference (RNAi)—a molecular process where double-stranded RNA can deplete mRNA *via* sequence-specific mechanisms—demonstrated both effective and specific RNA-mediated gene silencing (1). When, 3 years later, researchers identified a large class of nonprotein coding RNAs called microRNAs (miRNA), they confirmed the potential for large scale endogenous silencing (2–4). As subsequent research has unraveled these processes, we have seen that miRNAs have become increasingly important to our understanding of gene regulation. Also, miRNAs appear to be involved in many aspects of development, including the establishment and maintenance of tissue-specific expression profiles. Previously defined epigenetic mechanisms, such as DNA methylation and histone modification, are also important modifiers of gene expression. In some species, regulatory RNAs possess epigenetic-like properties, and *in vitro* experiments have linked RNAi to the classic epigenetic mechanisms, which further emphasize the need to view miRNAs as part of a larger apparatus of regulatory mechanisms.

This review will briefly describe epigenetic mechanisms before introducing important aspects of miRNA regulation. This includes transcription, biogenesis, and targeting, in addition to the relationship of miRNAs to other RNAs that associate with parts of the miRNA pathway. Also, we discuss the various epigenetic traits that RNA regulation possess, including possible links to the classical epigenetic mechanisms, and finally outline the clinical importance of miRNAs going forward.

EPIGENETICS AND THE EPIGENOME

Epigenetics is the study of heritable changes in gene function that cannot be attributed to changes in the DNA coding sequences. For example, the regulatory state of a cell is inherited by its daughter cells following cell division, but the cells' DNA may be identical to that of other cells that do not share the regulatory state. Epigenetic inheritance pertains to both mitotic and meiotic cell divisions. The classical epigenetic mechanisms include DNA methylation and histone modifications, but other mechanisms of gene regulation—especially those that involve nonprotein coding RNA—have become increasingly important. The epigenetic state of the cell, meaning the status of the various epigenetic mechanisms, is often referred to as the epigenome. Note, however, that the biologic end point, which is the regulatory state of the cell, is easier to observe directly than the actual epigenetic modifications.

In the following, we will briefly introduce the classical epigenetic mechanisms. Detailed reviews have been published elsewhere (5–7).

DNA methylation. As the name suggests, DNA methylation involves the addition of a methyl group to DNA residues. For example, the most extensively studied methylation is that of the fifth carbon of the cytosine's pyrimidine ring—a potentially mutagenic event that frequently causes C:G to T:A transitions. CpG islands—phosphodiester-linked cytosine and guanine pairs that span a region of at least 200 base pairs with more than 55% GC content—are found in approximately 40% of the promoters of mammalian genes. These islands are usually unmethylated when the genes are expressed and *vice versa*, which spurred the interest in DNA methylation as a general mechanism for transcriptional gene silencing.

In addition to maintaining regulatory roles that are important for the cell's function, DNA methylation plays a role in genome maintenance both in the defense against viral sequences and silencing of transposable elements. When the cell divides, the pattern of DNA methylation is maintained in the daughter cells. DNA methyltransferases—of which there are three types in mammals—are responsible for the DNA methylation. DNA methyltransferases 3a and 3b (Dnmt3a and Dnmt3b) initiate new methylation (8), whereas Dnmt1 is a maintenance enzyme (9). The function of Dnmt2 was undefined for a long time, but it was recently shown that Dnmt2 does not methylate DNA, but instead targets a specific position of aspartic acid tRNAs (10). Thus, Dnmt2 is in fact an RNA methyltransferase.

Histone modification. Histones are the main proteins of chromatin, which allows DNA to be very condensed in the cell's nucleus. Four histone classes—a H3-H4 tetramer and

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two H2A-H2B dimers—form the octamer that constitute the core histones around which a little less than two helical turns of DNA's double helix wrap (11). This situation is often schematically illustrated like beads on a string, and the structure folds into higher order chromatin, which is very compact. A gene can only be transcribed if the chromatin structure changes temporarily to allow regulatory proteins to bind the relevant portion of the DNA. Histone tails—short arms that are separate from the main structure—can be acetylated, methylated, phosphorylated, and ubiquitinated to change the histone structure and therefore enable or prevent access to the DNA. For example, acetylation most often marks active regions of transcription, whereas methylation can be present in both active and inactive regions. The vast number of combinations that exist of such histone tail modifications means that there may be a histone code that can be read by the transcriptional apparatus (6).

Potential for RNA-mediated effects. A number of nonprotein coding RNAs play important roles in modifying the sequence, structure, or expression of mRNAs and thereby also changes the protein expression from these genes. For example, small nuclear RNAs (snRNA) are involved in a range of processes, including gene splicing, telomere maintenance, and transcription factor regulation, whereas small nucleolar RNAs (snoRNA) guide chemical modifications to other RNA genes and guide RNAs (gRNA) are involved in RNA editing. One of the most interesting families of such nonprotein coding RNAs is the main topic of this review, namely the miRNAs that appear to be involved in gene regulation on multiple levels.

Whether regulatory changes are induced by transcription factors or mediated by RNAs, the cells can maintain the expression level long after the original factors that stimulated the effect has vanished. Cells differentiate into various types, but when their expression program has been established, their daughter cells remain of the same type even though the factors that triggered the changes may be gone. We currently have only limited knowledge about how nonprotein coding RNAs in general and miRNAs in particular play into this picture. Also, while it is unclear whether these RNA effects are inherited like epigenetic mechanisms, a recent paper demonstrated the potential for RNA-mediated inheritance (12).

MicroRNA REGULATION

miRNAs are nonprotein coding RNA genes that reside within longer transcripts as distinct hairpins that mature into 22 nucleotide (nt) long sequence-specific gene regulators. Since the initial discoveries that the small RNAs let-7 and lin-4 are crucial for *C. elegans*' correct transition from larvae into adult worm (13–16), researchers have identified miRNAs as an abundant and highly conserved gene class (2–4). Currently, the online repository of miRNA sequence data, miRBase, contains nearly 500 human miRNAs (474; miRBase release 9.0) (17), but recent reports estimate 1000 or more miRNAs in the human genome (18,19). Although some miRNAs, such as let-7, are conserved in most animals, many of the recently identified human miRNAs are specific to primates (18,20) and some are even human-specific (19). Curiously, the miRNAs identified in the initial small RNA cloning efforts are both highly expressed and widely conserved, whereas the more lineage-specific miRNAs appear to be less abundant—possibly because these newer miRNAs are very cell-specific or expressed at low levels in many different cells and tissues. Conservation of sequence does not, however, necessarily imply conservation of function, as expression patterns for some conserved miRNAs have diverged with evolutionary distance (21).

Transcription. Although little is known of miRNA regulation and transcription, the best characterized miRNAs originate from independent RNA polymerase II transcripts (22,23) or from introns or exons of protein-coding or nonprotein-coding genes (24). Intragenic miRNAs are relatively common, as 130 of the 464 human miRNAs from miRBase 8.1 map to protein-coding genes in UCSC's genome browser database (25). The majority of these intragenic miRNAs (107 of the 130) are sense transcripts that can be transcribed as part of the host gene, then spliced out, and further processed. Such intronic miRNAs rely on the established transcription and splicing of their host genes and are therefore only present when their host gene is transcribed. In effect, intronic miRNAs represent a simple mechanism for a protein-coding gene to down-regulate other protein-coding genes. Furthermore, mutations that lead to new miRNAs in introns could be an effective mechanism to establish a well-regulated miRNA and thereby be a driving force in animal evolution (26). However miRNAs are regulated and transcribed, they tend to cluster throughout the genome (3,27), and many of these clusters are likely transcribed as polycistrons (28).

Biogenesis. Post transcription—and splicing for intronic miRNAs—miRNAs rely on at least three different protein complexes to mature into the short ~22 nt single-stranded gene regulators. First, the nuclear Microprocessor complex, which consists of DGCR8 and the ribonuclease (RNase) III Drosha, processes the primary transcript into miRNA precursors (pre-miRNAs) (29–32). Most known animal miRNAs have a hairpin with a ~33 base pair stem flanked by a single-stranded region, and current models suggest that DGCR8 recognize this characteristic structure and guides Drosha to cleave the hairpin about 11 nts from the single-stranded region (33). Drosha or other unknown co-factors may, however, also recognize some of the miRNA hairpin's key features and thereby contribute to cleavage specificity (34,35). Second, the carrier protein Exportin-5 and Ran GTPase transport pre-miRNA from the nucleus to the cytoplasm (36–38). Exportin-5 recognizes the precursor stem and the characteristic RNase III 3′ overhang (39) and may represent a rate-limiting step in miRNA biogenesis (40,41). Third, the cytoplasmic RNase III Dicer cleaves the pre-miRNA about 22 nts from its end (42–44). Dicer and the Tar RNA binding protein (TRBP) hands the duplex over to Argonaute 2 (Ago2), which then incorporates one of the duplex strands and cleaves or dissociates the other strand (45–48). In *Drosophila*, Ago2, with the aid of R2D2 (49), preferentially incorporates the strand that has the least stable 5′ end (50,51), which explains why one of the duplex strands predominates as the mature miRNA. Disruptions in any of these three processing steps can

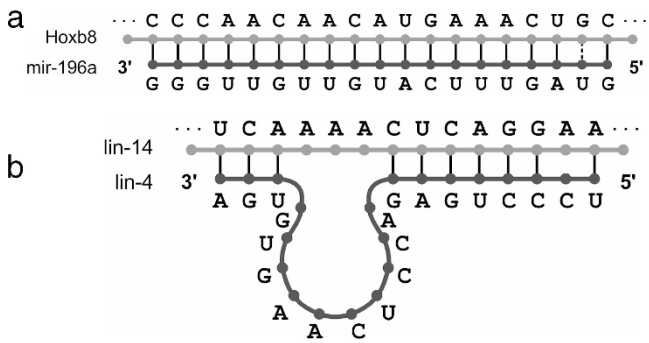

Figure 1. Two types of miRNA target sites. (*a*) Near perfect complementarity between miRNA and mRNA gives mRNA cleavage, whereas (*b*) less complementarity gives mRNA degradation and translational suppression.

alter miRNA expression patterns (52,53). The mechanism by which the antisense strand is appropriately handed off to Ago 2 in mammals is not clearly defined as of yet.

Targeting. The mature miRNA, bound to Ago2 (54,55), forms the core of the RNA-induced silencing complex (RISC) (56). The miRNA guides RISC to mRNAs that have miRNA-complementary sites and RISC then cleaves (57,58), degrades (59–61), or suppresses translation (14,15) of the target mRNA, depending on the degree of complementarity between miRNA and mRNA (Fig. 1). Cleavage is the strongest mechanism and is also the most specific, as the miRNA and mRNA must form a near-perfect duplex to induce cleavage (62–65). Degradation and translational suppression require less complementarity; seven consecutive base pairs between the mRNA and nucleotides 2–8 from the miRNA's 5′ end are enough to reduce the protein levels of the target (66). Consecutive matches between mRNA and miRNA nucleotides 2–8 or 2–7 are commonly referred to as seed sites, and analyses of conserved seed sites in 3′ untranslated regions (UTR) have shown that a single miRNA may regulate hundreds of genes (66–70). An experiment that over-expressed miR-1 and miR-124 confirmed this potential, as microarray analyses showed that each miRNA down-regulated more than 100 mRNAs (71). Seed sites are not necessary for miRNA targeting, however, as target sites can have mismatches or GU-wobble base pairs within the seed region and still be functional (72,73). To be functional, however, these sites do require more extensive base-pairing between the mRNA and the miRNA 3′ end than do the seed sites (66). Furthermore, even if conserved seed sites are the best current method for predicting miRNA targets, a conserved seed site is not sufficient for down-regulation (74). Instead, miRNA targeting likely relies on the target site's sequence context (72).

OTHER RNAs THAT ASSOCIATE WITH THE MIRNA PATHWAY

As previously mentioned, animal miRNAs are only one of several types of small regulatory RNAs (75). Some of these are similar to miRNAs and associate with several of the protein complexes that are used by animal miRNAs. Plant miRNAs and exogenous and endogenous small interfering RNAs (siRNAs) are the best characterized small RNAs that associate with Dicer, Ago1, and their homologues, but cloning studies have identified several other small RNA that may rely on one or more of these proteins for biogenesis or function. The different classes of small RNAs have been implicated in many different mechanisms (Table 1).

Table 1. *Known functions of small regulatory RNAs*

Regulatory function	Small RNAs
mRNA cleavage	Animal miRNA (minority)
	Plant miRNA (majority)
	Exogenous siRNA
	tasiRNA
	nat-siRNA
mRNA degradation/translational suppression	Animal miRNA (majority)
	Plant miRNA (minority)
	Exogenous siRNA (off-target effects)
Chromatin structure	rasiRNA
Transcriptional silencing/ methylation	Plant miRNA
	Exogenous siRNA
	rasiRNA

Plants, like animals, have 22 nt RNAs that mature from hairpins within longer primary transcripts in a series of distinct processing steps (76–78). Because of the similarities to animal miRNAs, these RNAs were also named miRNAs, but the processing pathways and dominant targeting mechanisms for animal and plant miRNAs are so different that animal and plant miRNAs can be considered two different gene classes. The differences between animal and plant miRNAs can be summarized as follows. First, plants lack a Drosha homologue, and so a Dicer homologue processes the primary transcript into a duplex form in the nucleus (79). This lack of Drosha processing likely explains why plant miRNA hairpin stems do not have the animal miRNAs' well-defined length of about three helical turns, but vary from 60 to more than 400 nucleotides (miRBase 8.2). Second, the ends of plant miRNAs are methylated, whereas animal miRNAs end with free 2′, 3′ hydroxyl groups (80). Third, most plant miRNAs target mRNAs with near-perfect complementarity thereby inducing mRNA cleavage (81). Most animal miRNA targets have less complementarity thereby inducing translational suppression and sometimes degradation. Furthermore, there are examples of plant miRNAs that can direct methylation and transcriptional gene silencing (82), but to date, there are no examples of encoded animal miRNAs that do this.

Small interfering RNAs are processed from long double-stranded RNAs (dsRNAs) by Dicer (42). The resulting 21 nt long duplexes then enter the miRNA pathway and can induce mRNA cleavage, degradation, and translational suppression (83). Exogenous siRNAs have become a popular tool for gene knockdown, as researchers can design siRNAs for specific and effective knockdown of target genes (84,85). In mammals, long dsRNAs induce a strong interferon response, which usually leads to cell-death, but exogenous siRNAs can be introduced as 21 nt duplexes that mimic the Dicer product or as short (<30 nt) Dicer substrates (86–88). Exogenous siRNAs will usually only induce transient knockdown, but to create stable knockdown, one can instead express siRNAs as miRNA-mimicking hairpins (89–91).

Current literature describes few endogenous siRNAs, with some notable exceptions. Repeat-associated siRNAs (rasiRNAs)

presumably arise from overlapping sense and antisense repeat-associated transcripts, regulate transposable elements pre and post transcription, and are involved in establishing and maintaining heterochromatin structure (92). Repeat-associated siRNAs may be crucial for protecting the germ line from transposable elements (93–95). Trans-acting siRNAs (tasiRNAs) arise from plant miRNAs that cleave nonprotein coding transcripts. The plant RdRp then uses the cleavage product as template for dsRNA generation, which leads to subsequent Dicer cleavage and siRNA generation (96). Small interfering RNAs can also arise from natural antisense transcripts (nat-siRNAs) (97).

Recently, a new class of small RNAs were identified in murine testes (95,98–101). These RNAs, which are located in strand-specific, nonoverlapping clusters throughout the genome, interact with the Piwi-subclass of Argonaute proteins—hence the name PIWI-interacting RNAs (piRNAs). The function of piRNAs is not clear, but as Piwi-family proteins associate with heterochromatic proteins and piRNA clusters are conserved in location and structure but not sequence, one could speculate that the piRNAs regulate or silence their own genomic regions.

MicroRNAs' RELATION TO EPIGENETICS

In the past few years, miRNAs have been established as enormously important mediators of gene regulation. Endogenous miRNAs are important in developmental processes, including differentiation, proliferation, and apoptosis (102). While classical epigenetic mechanisms, such as histone modification and DNA methylation, regulate expression at the transcriptional level, miRNAs putatively function mainly at the posttranscriptional level.

Link to classical mechanisms. We have described the classical epigenetic mechanisms, namely DNA methylation and histone modification, as distinct mechanisms, but there is overwhelming evidence that this is not the case. Epigenetics mechanisms appear to be interconnected on multiple levels, and reports have shown three possible models [evidence summarized and models depicted in (7)]. The models propose that DNA methylation may direct histone methylation or *vice versa*—alternatively that chromatin remodeling drives DNA methylation. Of course, since the mechanisms appear to be interconnected, both models may be correct.

Whether miRNA regulation is an epigenetic mechanism in its own right is unclear, but several papers have described how miRNA expression is tissue-specific during development, which may implicate that miRNAs are crucial to establish and maintain cell type and tissue identity (60,103). Overexpression of tissue-specific miRNAs has also confirmed a single miRNA's potential to change the gene expression profile of a cell (71). Even though there have been speculations that animal miRNAs may be involved in the classical epigenetic mechanisms, as some plant miRNAs can direct methylation, the literature currently does not contain any evidence for direct endogenous miRNA involvement in transcriptional gene silencing. It has, however, been shown that RNA interference seems to be capable of inducing transcriptional gene silencing in cultured cells. In plants, small interfering RNAs (siRNA) are able to induce DNA methylation, which in turn gives transcriptional gene silencing (104,105). Whether this translates to animals has been and still is debated, and while early reports indicated that DNA methylation was involved in transcriptional gene silencing in mammalian cells (106), more recent evidence suggest that siRNA-induced transcriptional gene silencing is independent of DNA methylation (107).

A link between siRNA-induced transcriptional gene silencing and histone modifications has, however, been established. For example, Kim *et al.* (108) showed that Ago1 is required for histone H3 Lys9 dimethylation and transcriptional gene silencing. Furthermore, Janowsky *et al.* (109) found that Ago2 is also involved in addition to Ago1 (Fig. 2). The details on how the RNAi apparatus is involved in transcriptional gene silencing and the differences between organisms remain to be seen. Also, it will be interesting to see whether endogenous miRNAs or other RNAs are involved in transcriptional silencing *in vivo* or if the examples shown so far pertains strictly to artificial situations *in vitro*.

Differences between organisms. As mentioned previously, there are important differences in how miRNAs function in various species. In *C. elegans*, the effect of a miRNA can be maintained for a long time after the original stimuli for its expression has vanished. Single-stranded RNA that is complementary to the mRNA can serve as a primer for an RNA-directed RNA polymerase that can make long double-stranded RNA that can give rise to a wide range of siRNA species (110). Thus, silencing can be maintained, amplified, and even carried over to other genes without *de novo* expression of additional RNAs. Plants are also able to maintain silencing much the same way as worms do (111). Mammals, however, seem to lack an RNA-dependent RNA polymerase and are therefore unable to amplify and maintain RNA-mediated silencing without continuous expression of the RNA that mediates the effect. Consequently, siRNA-mediated RNAi is transient and the effect will usually last only through a few cell cycles without continuous expression of the RNA that mediates the effect (112).

In addition to the ability to maintain silencing, worms also inherit the effect from parent to offspring (113). Females that receive double-stranded RNA can transfer the effect to their offspring, and males can also transfer it to the subsequent

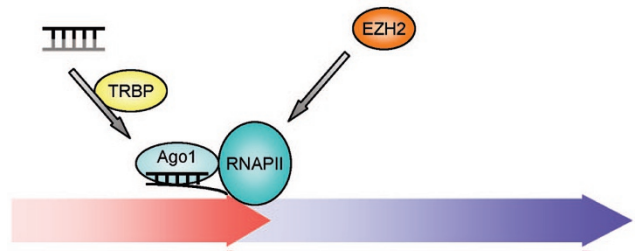

Figure 2. Proposed mechanism for small RNA triggered transcriptional gene silencing in mammals. SiRNAs complex with one or more of the Argonaute proteins, which direct the siRNA to a promoter region. There is evidence that promoter region transcripts may be required for this targeting, allowing sequence specific triggering of histone methylation and polycomb protein based remodeling of the chromatin, ultimately resulting in a heterochromatic status. The possible recruitment of DNA methyl transferases may follow the histone modifications.

generation. Note that this can happen even in the absence of the endogenous gene that is targeted, which means that RNAi can be inherited epigenetically in worms. Again, this has not been demonstrated to happen in mammals, which perhaps reflects the differences between RNA-mediated silencing in the various organisms.

CLINICAL RELEVANCE OF MIRNA

Like epigenetic mechanisms, which have been shown to play a role in disorders ranging from various forms of cancer to syndromes involving behavioral disabilities, chromosome instability, organ overgrowth, and anemia (114), miRNAs appear to be important in the onset and development of several diseases. In humans, miRNAs' involvement in cancer has spurred a lot of interest (115). Curiously, many miRNAs are located at or close to genomic sites that are commonly deleted or amplified in various cancers (116). Also, expression of miRNAs is frequently decreased or increased in cancerous tissues. For example, miR-15 and miR-16 are often deleted or otherwise down-regulated in chronic lymphocytic leukemia (117), whereas miR-155 expression levels are elevated in human B-cell lymphomas (118). The interactions are likely context specific, and to illustrate the difficulties involved when assigning a cancer-related function to a miRNA, the *mir-17-92* polycistron can function both as an oncogene and as a tumor suppressor (119,120). In addition to various cancers, miRNAs have also been implicated in genetic disorders, such as DiGeorge syndrome, and likely play a role in virus infection and defense.

Short hairpin RNA (shRNA) and small interfering RNA (siRNA)—the molecules that trigger stable and transient RNAi—are similar to the intermediates of miRNA biogenesis before and after Dicer processing. Consequently, an understanding of miRNA transcription, biogenesis, and function is important for therapeutic RNAi initiatives (121). Several side effects are known for RNAi. First, immunostimulatory effects, such as the interferon response, may be triggered by both shRNAs and siRNAs (122–124). Second, down-regulation of genes other than the intended target, so called off-target effects, are probably due to miRNA-like targeting and may cause widespread phenotypic effects (125–129). Third, since RNAi uses the same cellular factors as miRNAs, shRNAs and siRNAs may compete for and saturate critical enzymes and protein complexes needed for biogenesis and targeting. As previously mentioned, Exportin-5 may be rate limiting for miRNA biogenesis (40,41), which may explain why siRNAs appear not to saturate the pathway *in vivo* (130,131). Note that all of these problems can be mitigated by working at the lowest possible concentration, which means that more research into the miRNA pathway is needed to understand the consequences of therapeutic intervention using miRNA-like molecules; see (1,132) for reviews.

In addition to the therapeutic potential, miRNAs contain a lot of information about the regulatory state of the cells. In attempts to classify various forms of cancer with microarray expression profiles, miRNAs have been shown to harbor more information about disease states than do mRNA profiles (133). MicroRNA expression profiles therefore have the potential to become important diagnostic tools.

OUTLOOK

An impressive number of papers have been published on miRNAs since the discovery of these regulatory RNAs in 2001. But as the importance of miRNAs has grown, the number of scientific challenges has increased. Perhaps the most important task is to establish reliable models for how miRNAs target endogenous mRNAs. While a perfect model is unlikely due to the complexity of the problem, even significantly improved models will have a daunting effect on miRNA research. Coupled with data on miRNA expression in various tissues, reliable target prediction may allow simulations that may reveal important regulatory networks. Studies of how cells would react to aberrant miRNA expression may even become feasible if the target prediction methods improve sufficiently.

RNAi appears to be interconnected with DNA methylation and histone modifications. In some species, the link between miRNAs and epigenetics is strong. While the potential may be there, as shown *in vitro* by transfection of synthetic siRNAs, it remains to be seen whether these effects take place naturally. It will be interesting to see whether endogenous miRNAs or other types of nonprotein coding RNAs can be linked to epigenetic mechanisms *in vivo*. Also, an important question going forward is how miRNAs are involved in the establishment and maintenance of tissue-specific expression profiles. When the details on this become clearer, it is likely to reveal additional links to epigenetic mechanisms if they exist.

Finally, it is necessary to continue to research other nonprotein coding RNAs. The recent discovery of piRNAs—an abundant class of RNAs that were discovered in murine testes—shows that although miRNAs constitute an important part of the puzzle, we should be open to the possibility that additional classes of regulatory RNA may exist. Insight into the function of these additional classes of regulatory RNA and how they may involve all or some of the proteins in the miRNA pathway will be important to unravel all aspects of gene regulation. Naturally, all classes of regulatory RNA may be important players in what constitutes the epigenome.

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