

microRNAs in Vertebrate Physiology and Human Disease

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Abstract

Over the past five years, the importance of a diverse class of 18–24 nucleotide RNA molecules, known as microRNAs (miRNAs) has increasingly been recognized. These highly conserved RNAs regulate the stability and translational efficiency of complementary target messenger RNAs. The human genome is now predicted to encode nearly 1,000 miRNAs that likely regulate at least one third of all human transcripts. Despite rapid progress in miRNA discovery, the physiologic functions of only a small number have been definitively established. In this review, we discuss the principles of miRNA function that have emerged from the studies performed thus far in vertebrates. We also discuss known and potential roles for miRNAs in human disease states and discuss the influence of human genetic variation on miRNA-mediated regulation.

THE DISCOVERY OF microRNAs

MicroRNA (miRNA)-mediated regulation of gene expression was not discovered as a result of directed research aimed at better understanding regulatory RNAs. Rather, like many other fundamental cellular processes, our first glimpse at the importance of this mode of regulation came from forward genetic analyses of particular phenotypes in a model organism. Specifically, work from the laboratories of Ambros and Ruvkun in the early 1990s revealed that a miRNA controlled a specific step in developmental timing in *Caenorhabdi-*

tis elegans by downregulating a conventional protein-coding gene (89, 140). Only later was it appreciated that this regulatory mechanism is widely utilized to control diverse pathways in plants and animals.

Two key *C. elegans* mutants were central to the discovery of the first miRNA: *lin-4*, initially described by Horvitz & Sulston (60), and *lin-14*, also discovered in the Horvitz laboratory (48). *lin-4* loss of function results in failure of diverse cell lineages to differentiate properly as worms mature through larval development. Instead, early cell divisions are reiterated repeatedly. This is well illustrated by the seam-cell lineage that normally undergoes a defined series of divisions until adulthood (**Figure 1a**). At this time, seam cells normally stop dividing, terminally differentiate, and form part of the hypodermis. In *lin-4* mutants, the earliest division is repeated and proper seam-cell differentiation does not occur. A deletion in the 3' untranslated region (UTR) of the *lin-14* gene (a protein-coding transcript) results in a very similar phenotype. Both *lin-4* (loss of function) and the *lin-14* 3' UTR deletion mutants show inappropriately high expression of the *lin-14* protein after the first larval division. It was therefore proposed that *lin-4* downregulates *lin-14* by interacting with its 3' UTR. The real surprise came when causative mutations were identified in *lin-4* worms and found to result in deletion or mutation of a 22-nucleotide RNA molecule. Immediately thereafter, Ambros and Ruvkun recognized that the *lin-14* 3' UTR harbors multiple sites of imperfect complementarity to the *lin-4* small RNA. They proposed that *lin-4* binds to these sites and blocks *lin-14* translation, precisely foreshadowing our current understanding of animal miRNA function (**Figure 1b**).

Forward genetics also identified a second miRNA in *C. elegans*, known as *let-7*, that regulates developmental timing (118). This miRNA is highly conserved both in sequence and in its pattern of expression during development in various animal species (112). This observation prompted several laboratories to

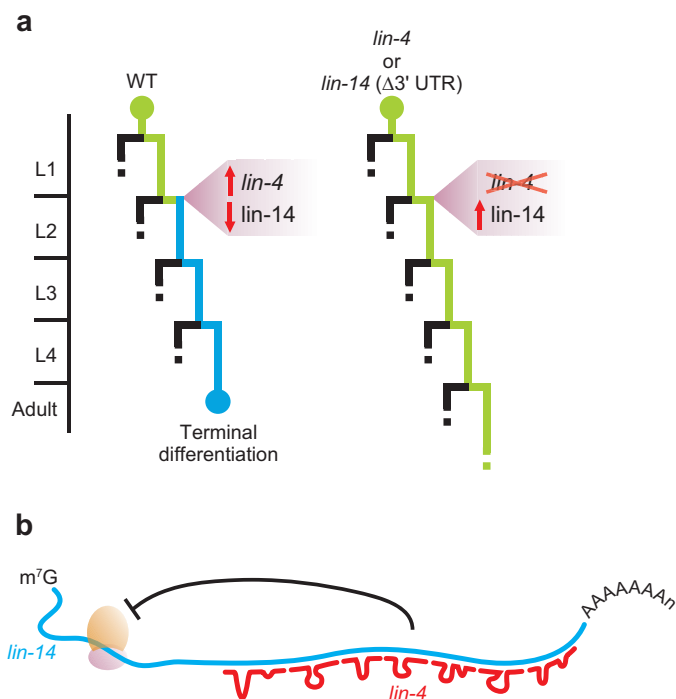


Figure 1

Discovery of the first microRNA (miRNA). (a) Schematic representation of the function of the *lin-4* miRNA in the *C. elegans* seam-cell lineage. In wild-type worms, *lin-4* is induced at the transition between the L1 and L2 larval stages. This correlates with a decrease in *lin-14* protein levels. In worms with mutations in *lin-4* or deletions in the *lin-14* 3' untranslated region (UTR), *lin-14* protein is not downregulated at this stage. As a consequence, cell divisions characteristic of L1 worms reiterate throughout development. (b) Cloning of the *lin-4* gene revealed that it encodes a small RNA that can base pair with imperfect complementarity to multiple sites in the 3' UTR of the *lin-14* transcript. This results in translational repression of *lin-14*.

hypothesize that *lin-4* and *let-7* may be founding members of a much more diverse class of small regulatory RNAs. This idea was also undoubtedly catalyzed by the elucidation of the RNA interference (RNAi) mechanism. RNAi refers to the potent inhibition of gene expression that occurs in most eukaryotic organisms when double-stranded RNA is introduced into a cell (49). A critical insight into the RNAi pathway was provided by studies demonstrating that long double-stranded RNA is first processed into small ~21-nucleotide RNA molecules known as short-interfering RNAs (siRNAs), which guide the RNAi machinery to target messages (44, 145). This mechanism in general, and siRNAs in particular, seemed reminiscent of *lin-4* and *let-7*, fostering the idea that these regulatory RNAs may be natural RNAi triggers. This idea was ultimately proven correct (64). Shortly after these ideas crystallized, several groups constructed and sequenced libraries of cloned small RNAs from diverse sources including *C. elegans*, *Drosophila*, and mammals, confirming that *lin-4* and *let-7* are members of an abundant class of small regulatory RNAs (81, 86, 88).

Prior to the discovery of the first miRNAs and RNAi, it was generally believed that the most important components of the transcriptome were predominantly large RNA molecules. This way of thinking, rather than any technical limitation, was primarily responsible for the long delay in the appreciation that most eukaryotic genomes encode numerous small RNA molecules that function in diverse pathways. Once this mindset was overcome, identifying the first wave of new miRNAs through cloning was relatively rapid and straightforward. Small RNA cloning strategies generally involve purification of low-molecular-weight RNA, ligation of linkers to the 5' and 3' ends of these molecules, and amplification by polymerase chain reaction (PCR) (81, 86, 88). Newly identified miRNA candidates are then mapped to their positions in the genome and their sequence contexts are examined.

miRNAs are encoded by transcripts that fold back on themselves to form double-stranded hairpin structures (see microRNA Biogenesis and Mechanism, below). Thus, newly identified miRNA candidates are first screened by determining if they are surrounded by genomic sequence which can form this structure. Final validation that a candidate miRNA exists usually involves direct demonstration that the RNA is expressed using northern blotting, reverse transcriptase polymerase chain reaction (RT-PCR), or microarray approaches. These methods have now been applied to diverse tissues and cell types from several species, leading to the identification of several hundred miRNAs (6, 33, 61, 75, 103).

After the discovery of the first few hundred miRNAs by cloning, sufficient miRNA sequences were available to begin applying bioinformatic analyses to scan genomes for additional miRNA genes. Although numerous methods exist (11, 12, 84, 95, 137), they generally rely on three main principles for miRNA recognition. First, miRNAs are contained within regions that fold into characteristic hairpin structures. Second, miRNAs tend to be highly conserved in related species. Third, the specific pattern of conservation displayed by miRNAs is very distinctive, with extreme conservation of the hairpin stems and less conservation of the loop of the hairpin and flanking sequences. Applying these methods to scans of the human genome has revealed ~800–1000 potential human miRNAs, including those previously identified by cloning. Both direct sequencing and bioinformatics efforts to comprehensively identify miRNAs are ongoing at a rapid pace and, undoubtedly, the existence of many more miRNAs will be confirmed by the time this review is published.

The most carefully curated set of miRNA sequences is maintained by the Sanger Institute in a publicly available database termed miRBase (<http://microrna.sanger.ac.uk/>) (53). This resource provides a searchable interface for all known miRNAs supported by experimental evidence, supplying sequence

pri-miRNAs:

primary microRNA transcripts; generally several thousand nucleotides in length or longer

miRNA cluster:

one or more miRNAs that are located near one another in the genome and are co-transcribed as part of a single pri-miRNA

information, genomic coordinates, potential targets, and literature citations. miRBase currently has entries for 474 human miRNAs and 373 mouse miRNAs (release 9.0). Although miRNAs seem to be missing from lower eukaryotes such as *Saccharomyces cerevisiae*, all studied multicellular eukaryotes appear to possess miRNAs.

Despite these great advances in miRNA discovery, our understanding of the role of miRNAs in physiologic and pathophysiologic processes is at a very early stage. Here we provide examples that illuminate principles of miRNA function in normal developmental and cellular pathways. We also describe our current understanding of the links between miRNAs and human disease and the impact of natural genetic variation on miRNA function. It is important to keep in mind that we are just beginning to elucidate these connections and many basic principles of miRNA function remain to be described. Before delving into these areas, we first provide a description of miRNA biogenesis and molecular function.

miRNA BIOGENESIS AND MECHANISM

Despite the obvious differences between the biology of miRNAs and mRNAs, all available evidence suggests that these transcripts share common mechanisms of transcriptional regulation. In fact, at least 100 known miRNAs are located within introns of protein-coding genes and are believed to be processed from intron lariats liberated by splicing (120). miRNAs located in intergenic regions are also transcribed by RNA polymerase II (pol II). These noncoding primary miRNA transcripts, referred to as pri-miRNAs, have a 5' 7-methylguanosine cap, a poly(A) tail, and may be a few kilobases to several hundred kilobases in length (17, 91). The transcription factors that regulate pri-miRNA expression appear to overlap substantially with those that control protein-coding genes. For example, the highly studied mammalian transcription factors c-Myc, cAMP-response element binding

protein (CREB), and MyoD are now known to regulate specific miRNAs (108, 117, 134, 149). These findings illustrate the importance of considering miRNAs as potential targets to fully elucidate the functions of a given transcription factor.

Most noncoding pri-miRNAs are efficiently processed and therefore not easily detectable. Although this has hindered their characterization, a few common features have emerged from those that have been studied thus far (76, 120). First, a single pri-miRNA may contain one or more individual miRNAs. A group of co-transcribed miRNAs is usually referred to as a miRNA cluster. Surveys of tissue-specific miRNA expression patterns suggest that human miRNAs located within 50 kb of one another are likely co-transcribed (10). Second, noncoding pri-miRNAs are frequently spliced with the miRNA located in either introns or exons. Third, although most mature miRNA sequences are highly conserved among vertebrates, pri-miRNA sequences are not generally well conserved outside the vicinity of the mature miRNA. Moreover, the precise genomic organization of pri-miRNAs (e.g., the number of introns and exons) is also not a highly conserved feature. Nevertheless, if a miRNA is contained within an intron or exon, orthologous miRNAs in other vertebrates also tend to be intronic or exonic, respectively. This implies that the presence of a miRNA in an intron or exon, regardless of precise sequence context, may subject it to as-yet-undescribed conserved mechanisms of post-transcriptional regulation. Further characterization of pri-miRNA structure and regulation remains an important area for future research.

One other aspect of miRNA organization within animal genomes merits discussion. miRNAs frequently exist in multiple highly related or identical copies distributed throughout the genome of a given species. The precise expression patterns of these independently transcribed loci have not been systematically evaluated, so some of these related miRNAs may function in distinct

cell types. Nonetheless, this organization undoubtedly produces significant redundancy, as has been selectively demonstrated (2). This redundancy has likely contributed to the relative paucity of miRNAs identified through forward genetics, although their small size also makes them infrequent targets for mutation. Disease-causing mutations may also rarely occur in miRNAs for similar reasons. miRNA-binding sites in mRNAs, which are nonredundant, may still be important sites of mutation, as discussed below (see The Influence of Genetic Variation on miRNA Function).

miRNA processing has been reviewed extensively and we present only the basic scheme here (for further detail, see 76, 123). Mature miRNA sequences are contained within regions of pri-miRNAs that fold back on themselves to form approximately 60–80 nucleotide stem-loop structures (**Figure 2**). These hairpin structures are excised from pri-miRNAs in the nucleus by the microprocessor complex that includes the RNA endonuclease Drosha and its binding partner DGCR8 as core components (37, 52, 85, 90). The excised hairpins, referred to as pre-miRNAs, feature a short stem of ~22 base pairs and a two-nucleotide 3' overhang, which serve as structural requirements for recognition by the nuclear export factor exportin 5 (14, 99, 144, 146). Following export to the cytoplasm, pre-miRNAs undergo further processing by the RNA endonuclease Dicer, which removes the terminal loop, yielding a double-stranded 18–24 nucleotide RNA duplex (54, 62, 73). This fully processed miRNA duplex is subsequently incorporated into a multicomponent protein complex known as the RNA-induced silencing complex (RISC). During this process, one strand of the miRNA duplex is selected as the mature miRNA (referred to as the guide strand) and remains stably associated with RISC. The other strand, known as the passenger strand, or miRNA*, is rapidly removed and degraded. Selection of the appropriate strand is primarily determined by the strength of base pairing at the ends of

the miRNA/miRNA* duplex. The strand with less-stable base pairing at its 5' end (i.e., more A:U base pairs or mismatches) is usually destined to become the mature miRNA and is chosen for incorporation into RISC (74, 122). It is worth noting that fully processed miRNA duplexes are functionally equivalent to experimentally introduced siRNAs, which are commonly used to silence gene expression (43). siRNAs are loaded into RISC exactly as described for miRNAs and function in an identical manner. Consequently, current algorithms for choosing effective siRNAs usually incorporate asymmetric thermodynamic stability into the RNA duplexes to promote loading of the desired strand into RISC (74, 122).

Once loaded with the guide strand miRNA, RISC accomplishes gene silencing through two major mechanisms: mRNA cleavage or translational repression. The choice of mechanism is governed by the degree of complementarity between a miRNA and its target (39, 64, 147). In plants, most miRNAs exhibit perfect or near-perfect complementarity to their target mRNAs (97, 119). This perfect base pairing leads to RISC-mediated endonucleolytic cleavage of the mRNA. In contrast, miRNAs in *C. elegans*, *Drosophila melanogaster*, and vertebrates usually recognize one or more imperfectly complementary binding sites in the target mRNA 3' UTR. This imperfect base pairing prevents RISC-mediated cleavage and instead results primarily in translational repression of the mRNA. To a lesser extent, this imperfect base pairing can also lead to decreased target mRNA abundance (8, 94), although this is probably a result of accelerated turnover of the mRNA through general cellular RNA decay pathways rather than RISC-mediated cleavage.

The core protein components of RISC that interact with miRNAs and carry out miRNA-mediated functions are the Argonaute proteins (22). Mammals possess four Argonaute family members (Ago1–4), which are widely coexpressed. Each Argonaute protein appears to be capable of binding any

pre-miRNAs:

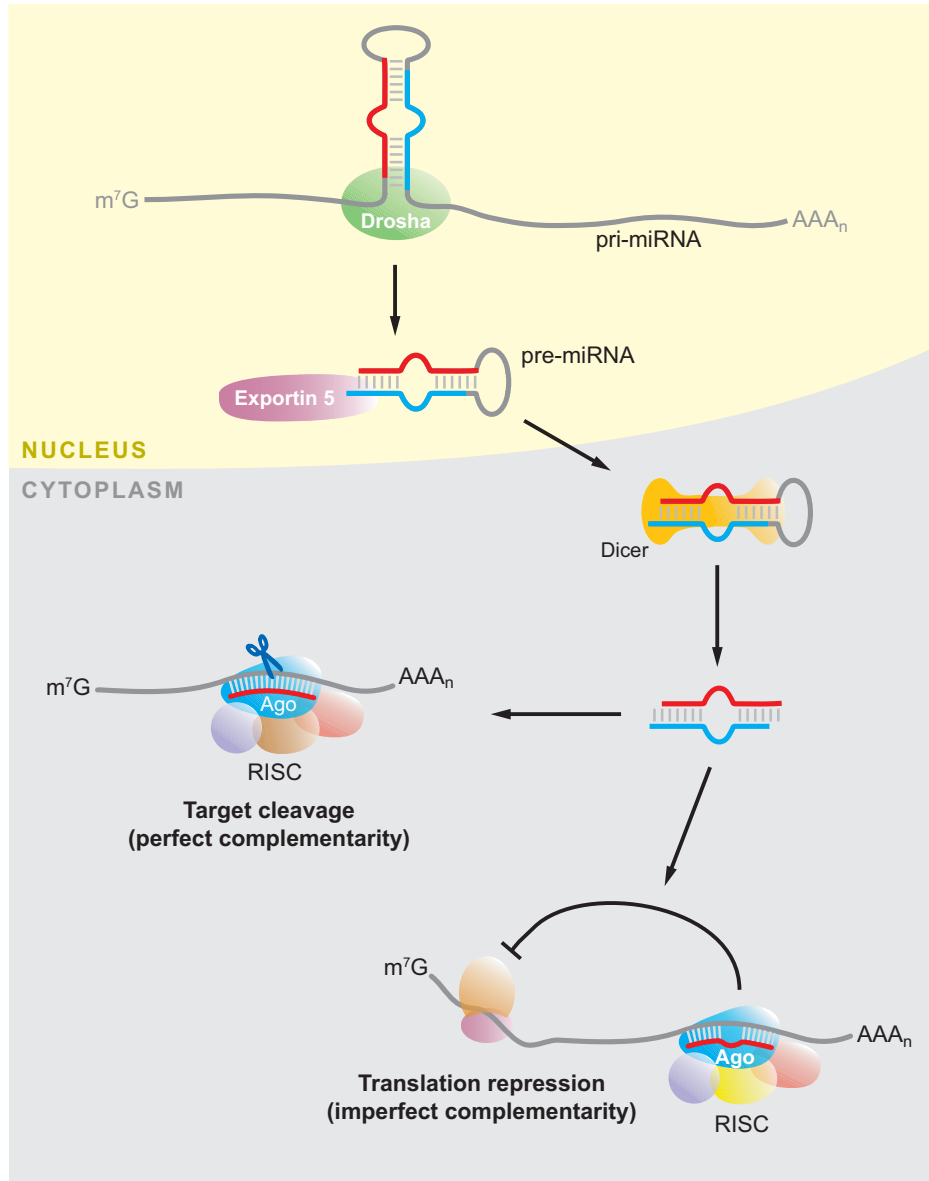
approximately 60–80 nucleotide stem-loop RNA structures containing the mature miRNA sequence; excised from pri-miRNAs

RISC:

RNA-induced silencing complex

Figure 2

miRNA biogenesis and function. miRNAs are initially transcribed as long primary transcripts (pri-miRNAs) that are capped and polyadenylated. The miRNA sequence is contained within a 60–80-nucleotide hairpin structure that is excised from the pri-miRNA by the endonuclease Drosha. This liberated stem-loop, or pre-miRNA, is transported to the cytoplasm by exportin 5, where it undergoes further processing by the Dicer endonuclease. One strand of the resulting duplex is selectively loaded into the RNA-induced silencing complex (RISC) and guides target transcript silencing through mRNA cleavage or translational repression.



miRNA, although only Ago2 can direct miRNA-mediated target mRNA cleavage (96, 102). A defining feature of Argonaute proteins is the PIWI domain, which structurally resembles RNase H. It is this domain of Ago2 that provides the RISC endonuclease activity (111, 128). Although the other mammalian Argonautes are catalytically inactive and can-

not cleave mRNA targets, all four Argonaute proteins can likely mediate translational repression through a mechanism that is still unresolved (96, 114). Evidence currently exists for miRNA-mediated inhibition of translation both at the level of translation initiation as well as at steps after initiation (109, 113, 115).

miRNA TARGET PREDICTION AND VALIDATION

Elucidation of the function of a miRNA requires identification of the mRNA targets that it regulates. As such, significant effort has been devoted to predicting and validating miRNA targets. In plants, these efforts have yielded rapid progress, due primarily to the perfect base pairing that typifies miRNA:target interactions in these species. This has dramatically simplified genome-wide searches for miRNA-binding sites (70, 148). Identifying potential miRNA targets is considerably more challenging in animal species because miRNAs are usually imperfectly complementary to their targets. In animals, the most consistent feature of miRNA:target interaction is base pairing at the 5' end of the miRNA. In particular, nucleotides 2–7 or 2–8 of the miRNA have been termed the seed sequence and complementarity to this region is considered most important, although not always essential, for target recognition (16, 83) (**Figure 3**).

In spite of the challenges, a diverse array of bioinformatic approaches have now been applied to identify the mRNAs regulated by animal miRNAs (125). Most of these prediction tools are available online for public use. In general, the different algorithms utilize at least some of the following criteria to identify and prioritize putative targets: (*a*) complementarity between the miRNA seed sequence and the 3' UTR of the target mRNA; (*b*) the overall stability of putative miRNA:target duplexes; (*c*) miRNA target site conservation between closely related species; (*d*) multiple binding sites for a single miRNA within a given target 3' UTR; and (*e*) weak or no secondary structure in the target at the miRNA-binding site. These programs predict hundreds of targets for each miRNA and estimate that between 30–90% of human mRNAs are subjected to miRNA-mediated regulatory control. The output from these diverse algorithms can be overwhelming to individual investigators interested in identifying likely targets for miRNAs of interest. Moreover,

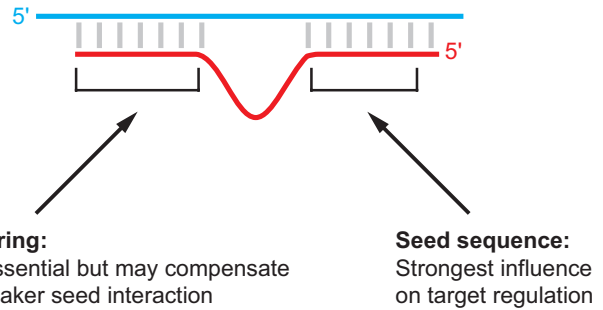


Figure 3

Anatomy of an animal miRNA:target mRNA interaction. Animal miRNAs base pair with their targets with imperfect complementarity. The miRNA is shown in red and the target is shown in blue. The seed sequence represents nucleotides 2–7 or 2–8 of the miRNA.

the true sensitivity and specificity of these methods is not clear. To address these issues, Sethupathy et al. recently evaluated the performance of several widely utilized algorithms in predicting a set of experimentally verified targets (125). In general, they found that no single program is sufficient to accurately predict all targets, yet combining predictions from multiple algorithms produces an exhaustive list with probable high false positive rates. One approach is to first focus on a high-yield set derived from the intersection of multiple predictions (assembled by these authors at <http://www.diana.pcbi.upenn.edu/cgi-bin/TargetCombo.cgi>). This, of course, will miss many bona fide targets. To expand this list, we suggest careful examination of all predictions to identify those targets that may fit with the known biology of the miRNA under study.

Experimental identification of novel miRNA targets is also effective. These strategies generally involve expressing or inhibiting a miRNA of interest in a cell line or animal and examining changes in global mRNA expression profiles using microarrays. This approach relies on the detection of the relatively small-magnitude changes in mRNA abundance that result from miRNA-mediated regulation (8, 94). Once a set of mRNAs with altered expression is identified, the 3' UTRs can be directly searched for sites

miRNA seed sequence:

nucleotides 2–7 or 2–8 of a mature miRNA. This region has the strongest influence on target selection

complementary to the seed sequence of the miRNA of interest. Although this method has been quite successful (79, 136), it is not comprehensive because some targets may change dramatically at the protein level but fail to show detectable changes in mRNA abundance.

Despite successes in computational or microarray-based identification of putative targets, subsequent experimental verification of these targets has advanced much more slowly. A recent compilation of experimentally supported human miRNA targets reports fewer than 100, despite thousands of published predictions (124). Further progress in this area is critical to refine and improve the accuracy of computational methods. Two major approaches are utilized to verify targets. The most common method involves placing a putative miRNA-binding site in the 3' UTR of a reporter transcript such as luciferase or green fluorescent protein (GFP) (16, 93). The behavior of the reporter in cell lines or animals is examined with or without mutations in the binding site or with ectopic expression of the miRNA. Although this approach may demonstrate the potential for direct interaction between a miRNA and target of interest, it is important to consider that these assays rarely recapitulate the expression levels of the endogenous RNAs. Thus, the magnitude of effects observed may be dramatically different than that which occurs *in vivo*. Ideally, reporter studies should be coupled with loss-of-function studies to determine whether inhibition of the miRNA leads to upregulation of the target mRNA or protein. Inhibition of miRNAs in cell lines may be accomplished by introducing 2'-*O*-methyl oligoribonucleotides complementary to the miRNA (63, 101). Recently, similar antisense oligos were developed for systemic delivery to whole animals (79). Although somewhat less reliable than loss of function, overexpression of a miRNA in cell lines or animals may also be informative for target validation. Combined approaches incorporating reporter assays and *in vivo* expression studies are recommended.

PHYSIOLOGIC FUNCTIONS OF VERTEBRATE microRNAs

The importance of miRNAs in select developmental pathways in model organisms is clear from the *lin-4* and *let-7* examples described above. But how generalizable are these observations? Do miRNAs play widespread, important roles in normal physiology in vertebrates? One way to address this question is to mutate or delete *Dicer*, thus removing all miRNAs. Although this experimental approach will likely reveal the consequences of a block in miRNA biogenesis, an important caveat that must always be considered when interpreting these data is that *Dicer* may be performing additional functions unrelated to miRNA biology. Thus, these results must be interpreted with caution.

Dicer loss of function results in profound developmental defects in both zebrafish and mice (13, 139). Zebrafish embryos lacking *Dicer* die approximately two weeks postfertilization due to what appears to be a general growth arrest rather than a failure in the development of one or more specific organ systems. The survival of *Dicer*^{-/-} embryos to this stage of development likely reflects the activity of maternal *Dicer*. To interrogate the functions of *Dicer* at earlier stages of development, when the major steps of morphogenesis and organogenesis occur, Schier and colleagues used a germ-line replacement technique to produce wild-type zebrafish with a *Dicer*^{-/-} germ line (51). Intercrosses of such fish produced offspring lacking both maternal and zygotic *Dicer*. These embryos exhibited severe defects most prominently in gastrulation, brain morphogenesis, and cardiac development. Remarkably, restoring expression of a single miRNA (miR-430) by injecting a fully processed synthetic miRNA duplex into early *Dicer*^{-/-} embryos was sufficient to largely rescue the defects in brain morphogenesis. These results definitely establish an important role for miRNAs in neural development in zebrafish and likely indicate that these molecules provide

critical functions in other pathways during embryogenesis.

Reminiscent of the zebrafish *Dicer*-null phenotype, mouse embryos lacking this protein die at embryonic day 8.5 (13). One prominent defect in these embryos is a lack of pluripotent stem cells. To further explore the consequences of *Dicer* deletion, several laboratories have generated mice harboring conditional *Dicer* alleles (30, 55, 71, 105). Such mice have been used to demonstrate essential functions for *Dicer* in T-cell differentiation (30, 104) as well as for morphogenesis of the lung (56), limb (55), skin, and hair follicles (5, 143). Taken together with the data from zebrafish, it appears that the miRNA pathway is not generally required for cellular viability but plays a prominent role in the differentiation of various tissue-specific cell types and the morphogenesis of embryonic structures.

Although these studies emphasize the importance of the miRNA pathway in vertebrate development, most do not provide information regarding the functions of specific miRNAs. Studies aimed at elucidating the role of individual miRNAs in various biologic processes are increasingly being performed and a few examples that illustrate principles of miRNA function are presented here. The diverse functions of vertebrate miRNAs are reflected in their expression patterns. The Plasterk laboratory recently used *in situ* hybridization to study the expression of more than 100 zebrafish miRNAs during embryogenesis (138). Despite their small size, miRNAs can be detected with high sensitivity and specificity using modified oligonucleotides known as locked-nucleic-acid (LNA) probes. These studies revealed that most miRNAs are expressed with precise tissue specificity late in development, again pointing to an important role for these RNAs in differentiation or maintenance of tissue-specific cell types.

There are now several known vertebrate miRNAs that participate in such tissue-specific functions. For example, one of the first mammalian miRNAs to be carefully stud-

ied was miR-181. Expression of this miRNA is highly enriched in B lymphocytes and ectopic expression of miR-181 in hematopoietic progenitor cells skews lymphocyte differentiation toward the B-cell lineage (25). The critical targets underlying the functions of miR-181 in lymphocyte development have yet to be identified. miR-181 was also identified in a screen for miRNAs that are upregulated during muscle cell differentiation and regeneration (106). This miRNA promotes muscle differentiation in part by downregulating *Hox-A11* (an inhibitor of differentiation). These studies emphasize the potential for a single miRNA to participate in distinct pathways in different tissues. Given that the function of a miRNA is dictated by the milieu of targets that are co-expressed, distinct tissue-specific roles for individual miRNAs may emerge as a common theme.

Another miRNA that is critical for normal muscle development is miR-1, one of the most highly conserved miRNAs studied to date. This miRNA is nearly identical in sequence in *C. elegans*, *Drosophila*, and vertebrates and shows skeletal muscle and heart-specific expression in these species (82, 88, 138). Two groups have independently studied the consequences of *Drosophila* miR-1 loss of function. Sokol & Ambros (127) observed the death of second instar *Drosophila* larvae lacking this miRNA, due predominantly to a failure in postmitotic muscle growth. Kwon et al. (80) described a somewhat more severe phenotype resulting from miR-1 deletion with a higher prevalence of embryonic lethality and a more pronounced muscle differentiation phenotype. miR-1 also plays an important role in muscle physiology in mammals. miR-1 is directly transcriptionally upregulated by the mammalian MyoD and myogenin transcription factors, which are important positive regulators of myogenic differentiation (117). Overexpression or inhibition of miR-1 promotes or inhibits mammalian *in vitro* muscle cell differentiation, respectively (26, 107). These effects appear to be due in part to miR-1-mediated downregulation of the inhibitor

of myogenic differentiation HDAC4. Thus, miR-1 provides highly conserved functions essential for normal muscle development in diverse animal species. Other miRNAs with roles in tissue-specific differentiation pathways have been studied and are reviewed elsewhere (4, 9, 78).

These examples illustrate the ability of miRNAs to dramatically influence the generation and behavior of tissue-specific cell types. As described above, studies to date have identified a few key targets that seem to be important in mediating these functions. However, most miRNAs are predicted to regulate hundreds of targets each (68, 77, 92), so the target transcript networks that underlie the phenotypes under miRNA control are likely much more complex. A fascinating study by Lim and colleagues (94) sheds light on the potential roles for miRNAs in regulating large sets of transcripts. In this series of experiments, the human muscle-specific miRNA miR-1 and the brain-specific miRNA miR-124 were expressed ectopically in HeLa cells. This resulted in the downregulation of ~100–200 transcripts for each miRNA, as revealed by microarray-based expression profiling. These downregulated transcripts, many of which appear to be direct targets of these miRNAs, were greatly enriched for those that are expressed poorly in muscle (for miR-1) and brain (for miR-124). Thus, expression of these miRNAs skewed the expression profiles of HeLa cells toward that of the tissue in which the miRNA is normally expressed. These data point to an important role for miRNAs in establishing and/or maintaining gene expression patterns characteristic of specific tissues. This activity of miR-1 and miR-124 does not appear to be unique as examination of large-scale gene expression data sets across various mammalian tissues reveals that many miRNAs and their predicted targets are reciprocally expressed (47, 129).

Further insight into the mechanisms through which miRNAs establish tissue-specific expression patterns was provided by studies focused on the RE1 silencing tran-

scription factor (REST). This protein inhibits expression of miR-124 in neuronal precursor cells and non-neuronal cells (31). Upon commitment of precursor cells to a neuronal differentiation pathway, repression of miR-124 by REST is relieved. These observations suggest a model whereby neuronal differentiation is coupled to the induction of miR-124, which globally tunes the transcriptome toward that of the terminally differentiated cell type.

Before concluding this section, it is important to emphasize that although a prominent role for miRNAs in tissue development and identity is clear, there are several known examples of vertebrate miRNAs that participate in cell-autonomous functions not related to development. For example, human miR-375 is expressed specifically in pancreatic islet β -cells and regulates insulin secretion (116). The liver-specific miRNA miR-122 regulates cholesterol homeostasis (45, 79). miRNAs have also been implicated in the regulation of neuronal synaptic function and neurite outgrowth (121, 134). Finally, specific miRNAs control cellular proliferation and apoptosis and accordingly play important roles in cancer. miRNAs with these latter properties are discussed in detail below. In sum, the relatively few miRNAs that have been studied in detail thus far suggest that these molecules participate broadly in normal physiologic processes. The logical extension of this conclusion is that abnormalities in miRNA function should influence human disease phenotypes. Accumulating evidence suggests that this is the case.

miRNAs IN CANCER

Although studies linking miRNA dysfunction to human disease are in their infancy, a great deal of data already exists establishing an important role for miRNAs in the pathogenesis of cancer. In retrospect, a role for miRNAs in this group of disorders may not have been surprising given the phenotype of the founding miRNA, *lin-4*. In the absence of this miRNA, the *C. elegans* seam-cell lineage fails to

differentiate properly and instead these cells reiterate early larval divisions throughout development and into adulthood (89). This phenotype is reminiscent of many human malignancies, such as several derived from hematopoietic lineages, which result from the expansion of a population of precursor cells that have failed to execute a program of differentiation. Several miRNAs that regulate proliferation and apoptosis in *Drosophila* have also been described (15, 87, 141), suggesting widespread regulation of cancer-relevant pathways by miRNAs in model organisms.

Genome-wide miRNA expression profiling using specialized microarrays and other high-throughput technologies has now been broadly applied to the study of diverse cancer subtypes. It is clear from these surveys that abnormal patterns of miRNA expression are a typical, if not ubiquitous, feature of cancer cells (98, 135). Although these studies have illustrated the potential for miRNAs to act as novel diagnostic and prognostic markers for several types of cancer, a major question is whether these expression changes directly contribute to tumorigenesis. Indeed, given that miRNA expression is tightly regulated during development and cellular differentiation, abnormal miRNA expression in tumors may simply reflect the loss of normal cellular identity that accompanies malignant transformation. Nevertheless, several observations suggest that the dysfunction of select miRNAs may be a causative event in cancer pathogenesis. For example, miRNAs are greatly enriched at genomic loci known to undergo amplification, deletion, or rearrangement in human cancers (20). Additionally, a number of studies using human cancer cells and animal models have convincingly established that specific miRNAs possess tumor-suppressor or oncogenic activity. Select examples of likely tumor-promoting and tumor-suppressing miRNAs and the evidence supporting these activities are presented below. For further details and additional examples, we refer the reader to other reviews on this topic (46, 72).

miRNAs WITH TUMOR-SUPPRESSOR ACTIVITY

miR-15a and miR-16-1

The first miRNAs causatively linked to cancer development were miR-15a and miR-16-1, which are clustered together on human chromosome 13q14. This locus is deleted in over half of B-cell chronic lymphocytic leukemia (CLL) cases as well as in other malignancies and there has been significant effort to identify the putative tumor-suppressor gene located within this interval (40, 41). After refining the critical region of the deletion to an approximately 30-kb interval, Croce and colleagues (18) recognized that this region contained only miR-15a and miR-16-1 and no other identifiable genes. They subsequently demonstrated reduced expression of these miRNAs in over two thirds of CLL cases. Sequencing these miRNAs in 75 CLL patients later identified two individuals with the identical germ-line mutation near miR-16-1 that was not present in 160 control subjects (19). This mutation is associated with reduced expression of this miRNA, although the underlying mechanism of this effect is unclear. Insight into the mechanism through which miR-15a and miR-16-1 participate in tumorigenesis was provided by the demonstration that at least one target of these miRNAs is the antiapoptotic gene *BCL2* (28). This suggests that loss of function of miR-15a and miR-16-1 promotes high expression of Bcl2 and abnormal survival of CLL cells. Accordingly, overexpression of Bcl2 occurs frequently in CLL. Further studies are necessary to identify additional targets of these miRNAs that may be important in the pathogenesis of CLL and other cancers.

The let-7 Family

A variety of studies have now implicated human homologs of the *C. elegans* *let-7* miRNA as potential tumor suppressors. During mammalian evolution, the family of miRNAs

related in sequence to *let-7* has dramatically expanded such that humans possess twelve *let-7* homologs organized in eight distinct clusters. At least four of these clusters are located in genomic regions known to be deleted in cancer (20). Reduced expression of human *let-7* family members has also been observed in lung cancer and is associated with poor prognosis (131, 142). Consistent with these observations, expression of *let-7* in lung adenocarcinoma cell lines reduces clonogenicity (131). Slack and colleagues (69) convincingly demonstrated that the highly studied oncogene *RAS* is a target of human *let-7* family members. Low expression of *let-7* was shown to correlate with high expression of Ras in a small set of lung tumors, providing a potential mechanism through which loss of function of these miRNAs promotes tumorigenesis. Of note, the high degree of nucleotide similarity between the members of the expanded human *let-7* family has imposed unique challenges on studying these miRNAs. Microarrays, northern blotting, PCR approaches, and other techniques currently used to analyze miRNA expression do not readily discriminate between these related miRNAs. Consequently, the extent of redundancy between the *let-7* clusters, their precise expression patterns, and the mechanisms that regulate their individual expression are not yet clear. Unraveling these issues will be critical to fully understand the role of *let-7* in human cancers.

ONCOGENIC miRNAs

BIC/miR-155

Before the discovery of miRNAs, the B-cell integration cluster (BIC) noncoding RNA was shown to accelerate Myc-mediated lymphomagenesis in chicken (132, 133). It was later recognized that BIC is the pri-miRNA for miR-155 (42). Overexpression of BIC/miR-155 occurs frequently in diverse cancers including B-cell lymphoma and tumors of the breast, lung, colon, and thyroid (42, 58, 135). Strikingly, transgenic mice

expressing miR-155 in B cells rapidly develop polyclonal B-cell malignancies, suggesting that the expression of this miRNA alone or in combination with minimal additional genetic “hits” is sufficient to induce lymphomagenesis (32). The available evidence therefore strongly implicates miR-155 as an oncogene.

The *mir-17* Cluster

One of most extensively studied groups of miRNAs is the *mir-17* cluster, which includes miR-17-5p, miR-18a, miR-19a, miR-20a, miR-19b-1, and miR-92. The genomic locus encompassing these miRNAs (13q31-32) is frequently amplified in several types of lymphoma and solid tumors (110) and widespread overexpression of these miRNAs has been observed in diverse types of cancer (57, 59, 110, 135). Direct evidence for *in vivo* oncogenic activity of the *mir-17* cluster was obtained using *E μ -myc* transgenic mice, a model of B-cell lymphoma (59). Introduction of the *mir-17* cluster into hematopoietic stem cells from *E μ -myc* animals significantly accelerated lymphomagenesis and death of recipient animals. Moreover, it was recently demonstrated that expression of these miRNAs in a mouse model of colon cancer dramatically promotes tumor angiogenesis (38). Studies from O'Donnell et al. (108) provide further insight into the molecular mechanisms underlying overexpression of these oncogenic miRNAs in cancer cells. Using both model cell lines and primary cells, these authors demonstrated that the *mir-17* cluster is directly upregulated by the oncogenic transcription factor c-Myc. Together with the data obtained in animal models, these observations suggest that the *mir-17* cluster is an important downstream effector of the Myc oncogene, a protein that is pathologically activated in a large fraction of human malignancies (34).

miR-21

A potential role for miR-21 as an oncogene was first uncovered in a screen for

altered miRNA expression in glioblastoma (24). These studies showed that miR-21 expression was highly elevated in human glioblastoma tumor tissues, primary tumor cultures, and established glioblastoma cell lines relative to normal fetal and adult brain tissue and primary cultured neurons and astrocytes. More recently, miR-21 was demonstrated to be widely overexpressed in an array of tumors including those derived from breast, colon, lung, pancreas, stomach, and prostate (135). Inhibition of miR-21 in glioblastoma or breast cancer cell lines using antisense oligonucleotides results in caspase activation, increased apoptotic cell death, and decreased tumor growth in a xenograft model (24, 126). These data provide strong evidence that overexpression of miR-21 promotes tumorigenesis by suppressing apoptosis.

THE ROLE OF miRNAs IN FRAGILE X SYNDROME AND SYNAPTIC FUNCTION

Although most attention has been focused on cancer, miRNA dysfunction may contribute to other human diseases. For example, recent findings have uncovered an unexpected link between Fragile X Syndrome and the miRNA pathway. Fragile X Syndrome represents one of the most common inherited forms of mental retardation and is caused by loss of function of the Fragile X Mental Retardation 1 (*FMR1*) gene. In the vast majority of cases, the causative mutation is a triplet repeat expansion in the *FMR1* 5' UTR that leads to hypermethylation of the *FMR1* promoter and subsequent transcriptional silencing (50). Significant evidence points to an important role for the product of this gene, known as FMRP, as a negative regulator of local translation within dendrites (66). One critical aspect of FMRP function that has been extensively investigated is the mechanism through which this protein selects mRNA targets for translational repression. FMRP appears to recognize at least two RNA structures within its targets: a G-quartet, which is a special stem-

loop structure formed by hydrogen bonding between four guanine residues, and a more complex tertiary structure referred to as a FMRP-kissing complex (35, 36). New data suggest that miRNAs are intimately involved in FMRP-mediated translational repression, perhaps in the selection of target mRNAs.

The first indication of mechanistic linkage between Fragile X and miRNAs came from biochemical studies of the RISC protein complex, the molecular machinery that accomplishes miRNA- and siRNA-mediated regulation. Purification of this complex from *Drosophila* cells revealed that an abundant component is the fly ortholog of FMRP, dFMR1 (23, 65). These findings were extended to human cells where FMRP was also demonstrated to interact with RISC components and miRNAs (67). Compelling genetic evidence from fly also links the miRNA pathway to functions mediated by dFMR1. Overexpression of dFMR1 in the *Drosophila* eye leads to apoptosis, which can be largely suppressed by heterozygous loss of function of AGO1, a RISC component that is essential for miRNA function (67). Perhaps more relevant to the human phenotype, loss of dFMR1 leads to synaptic overgrowth at neuromuscular junctions, which is exacerbated by heterozygous loss of AGO1. The results suggest that functions mediated by dFMR1 or FMRP require RISC and possibly miRNAs. Warren and colleagues have therefore proposed a model whereby FMRP initially selects targets through low-affinity interactions with G-quartets and/or kissing complexes. FMRP-associated miRNAs may then provide additional specificity during translational silencing. These interactions may also indicate that FMRP and miRNAs share a common mechanism of translational repression. While this is an elegant hypothesis, a definitive link between human miRNAs, FMRP-mediated translational silencing, and the Fragile X phenotype remains to be established.

Although not explicitly related to a disease phenotype, new data from *Drosophila*

SNP: single nucleotide polymorphism

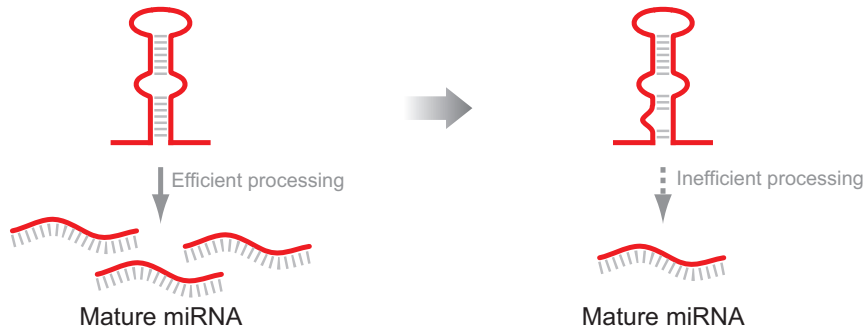
provide additional evidence that the miRNA pathway may be important in the control of local protein synthesis at synapses. To study these connections, Ashraf and colleagues (7) used a classic olfactory conditioning model whereby flies are trained to avoid certain odorants by exposing them to electric shock. Specific regions of the brain in flies trained in this manner show increased transport of *Calcium-calmodulin-dependent kinase II (CamKII)* mRNA to dendrites and increased local production of CamKII protein. Interestingly, this localization and regulated translation of *CamKII* is mediated by its 3' UTR, which harbors predicted binding sites for multiple miRNAs. These sites are likely functional because flies with mutations in multiple components of RISC show dramatically increased CamKII protein expression. Loss of RISC activity also leads to increased transport of *CamKII* message to synapses. The authors further demonstrate that synaptic activity leads to proteasome-mediated destruction of RISC. These results support a compelling model in which miRNA-programmed RISC maintains *CamKII* and other localized messages in a translationally silenced state. Synaptic activity provides a signal to destroy RISC and these mRNAs are subsequently used as templates for local protein synthesis. A similar mechanism has been demonstrated to be operating in mammalian neurons, at least for some select miRNAs and their targets. For example, miR-134 has been demonstrated to maintain the *Lim-domain-containing protein kinase 1 (LimK1)* mRNA, which encodes a positive regulator of dendritic growth, in a translationally silenced state prior to synaptic activity (121). Reminiscent of *Drosophila CamKII*, incoming synaptic stimuli somehow inactivate miR-134, releasing *LimK1* for local protein synthesis and leading to dendritic growth. An extremely abundant and diverse population of miRNAs are expressed in the mammalian brain (21), suggesting that these mechanisms are likely widespread.

THE INFLUENCE OF GENETIC VARIATION ON miRNA FUNCTION

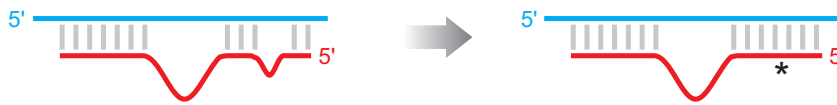
The relationship between genetic variation in miRNAs or miRNA-binding sites and human phenotypes is only just beginning to be investigated. In theory, a single nucleotide polymorphism (SNP) in a miRNA could influence its function by affecting the efficiency of processing or by altering target specificity. Alternatively, SNPs in mRNA 3' UTRs could strengthen or weaken miRNA:target interactions. These potential mechanisms are summarized in **Figure 4**. Although genetic variation in miRNAs appears to be very rare, SNPs in potential target sites have now been shown to influence human and animal phenotypes. For example, the neuropsychiatric disorder Tourette's syndrome (TS) was one of the first human diseases linked to alterations in a miRNA-binding site (3). Abelson and colleagues focused on *Slit and Trk-like 1 (SLITRK1)* as a candidate gene for this disease because it is located near a chromosomal breakpoint found in a TS patient harboring an inversion on chromosome 13q31. Subsequent sequencing of the *SLITRK1* gene in 174 unrelated TS patients identified a frameshift mutation and two independent occurrences of the identical mutation in the 3' UTR. These variants were absent in thousands of screened control chromosomes. Interestingly, the 3'UTR variants strengthen a predicted binding site for miR-189, a miRNA that is expressed in a pattern that overlaps with *SLITRK1*. Luciferase reporter assays provided evidence that this is indeed a functional miR-189 site and, although the magnitude of effects were quite small, the rare variant seems to strengthen this miRNA:target interaction. Although further investigation of this hypothesis is necessary, these data are consistent with the TS phenotype being influenced by inappropriate downregulation of *SLITRK1* by miR-189.

A similar mechanism was revealed by studies aimed at mapping the quantitative trait loci

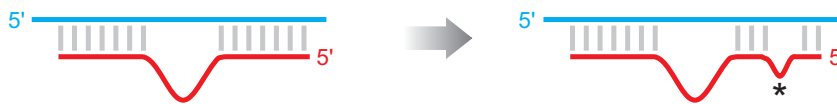
1 SNP affects miRNA processing



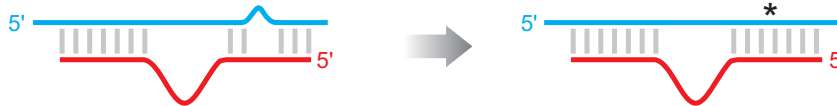
2 SNP in miRNA creates stronger target interaction



3 SNP in miRNA creates weaker target interaction



4 SNP in target strengthens binding site



5 SNP in target weakens binding site

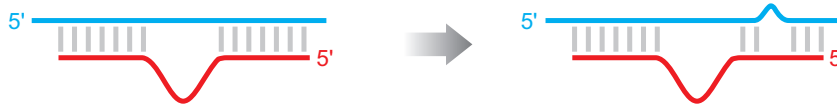


Figure 4

Potential effects of single nucleotide polymorphisms (SNPs) on microRNA (miRNA) biogenesis and function. Target sequences are shown in blue and miRNAs are shown in red. Asterisks indicate the position of sequence variants.

(QTLs) that regulate muscle mass in sheep (29). One locus that has a pronounced effect on musculature contains the well-known negative regulator of muscle growth myostatin (also known as *growth and differentiation factor 8*) (100). A naturally occurring variant in the 3' UTR of the myostatin transcript is highly

enriched in a muscular breed of sheep and appears to result in lower myostatin protein levels. This variant creates a potential binding site for two muscle-specific miRNAs, miR-1 and miR-206. Luciferase assays were again used to demonstrate that these miRNAs can regulate reporter transcripts harboring this

newly created site. Based on these data, the authors formulated a model very similar to that proposed for *SLITRK1*, whereby fortuitous creation of a miRNA-binding site leads to downregulation of the myostatin protein and greater muscle mass.

These examples have led to speculation that polymorphic sequence variants in human populations that create or destroy miRNA-binding sites may have significant effects on phenotypic variation. Consistent with this possibility, ~2500 human SNPs that create potential miRNA-binding sites and ~2500 human SNPs that destroy potential miRNA-binding sites have been identified (29). These SNPs were deposited in a publicly available database that can be accessed at <http://www.patrocles.org>. Using SNP genotyping data provided by the International HapMap Project (1), Chen & Rajewsky (27) provided strong evidence for natural selection acting on a significant fraction of both conserved and nonconserved predicted miRNA-binding sites. Negative selection operating on these sites during human evolution has resulted in a relative decrease in SNP density. Nevertheless, these findings underscore the potential for variants in miRNA-binding sites to influence human phenotypes in a manner that clearly affects Darwinian fitness and likely human disease. The prevalence of miRNA-binding sites in human transcripts requires that these motifs be considered potential reservoirs of causative genetic variation when

performing association studies or mutation screening.

CONCLUSIONS AND FUTURE DIRECTIONS

Over the past six years, the identification of miRNAs and our understanding of their biogenesis pathway and mechanisms of action have advanced at a remarkable pace. However, the phase of rapid miRNA discovery is likely reaching its pinnacle. Progress in elucidating the physiologic roles for these newly defined RNAs has advanced much more slowly. Undoubtedly, closing this knowledge gap between miRNA discovery and miRNA functional annotation represents a major priority and challenge for future research. These efforts must include improved methods for high-throughput miRNA target validation and loss-of-function studies in knockout mice. In parallel, continued exploration of the roles of miRNAs in human disease states and the influence of genetic variation in miRNAs on human phenotypes is necessary. This is particularly important given the recent development of technologies that allow systemic delivery of miRNAs or miRNA inhibitors to whole animals (79, 130). Application of these methods, in the context of improved understanding of physiologic and pathophysiologic roles for miRNAs, may enable the development of an entirely new class of miRNA-based therapeutics.

SUMMARY POINTS

1. miRNAs were originally discovered through genetic screens in *C. elegans* in the early 1990s. Since then, both experimental and bioinformatic approaches have led to the identification of thousands of miRNAs in diverse multicellular eukaryotes. The human genome probably encodes as many as 1000 miRNAs, if not more.
2. miRNAs are transcriptionally regulated in a manner identical to typical messenger RNAs. Mature miRNAs, which are 18–24-nucleotide single-stranded RNAs, are processed from long primary transcripts in a stepwise process involving a series of endonucleolytic cleavages. The mature miRNAs are loaded in a large protein complex known as the RNA-induced silencing complex (RISC). miRNAs guide RISC to complementary target messenger RNAs, which are cleaved or translationally repressed.

3. Animal miRNAs are usually imperfectly complementary to their targets, which has made bioinformatic identification of targets challenging. Many publicly available target prediction algorithms are available and should be used with caution.
4. miRNAs are involved in diverse physiologic processes. For example, they provide essential functions during animal development, regulate cellular differentiation, and help establish tissue-specific gene expression programs.
5. Aberrant miRNA function influences human disease. In particular, miRNAs are now known to participate in human malignancies by acting as novel oncogenes and tumor suppressors. miRNAs may also be involved in neuronal phenotypes, including Fragile X Syndrome.
6. Genetic variation in miRNA-binding sites in target mRNAs has been linked to dramatic phenotypes in humans and animals. A large number of SNPs that create or destroy potential miRNA-binding sites have been identified.

FUTURE ISSUES

1. Further characterization of primary miRNA transcripts is necessary to improve our understanding of the transcriptional and post-transcriptional mechanisms that govern miRNA expression.
2. More emphasis on loss-of-function studies of miRNAs in animal models is necessary to better establish the physiologic functions of these transcripts. These studies may be hindered by the redundancy that typifies miRNA organization in mammalian genomes.
3. High-throughput methods for miRNA target validation would not only expand our understanding of miRNA functions but also would allow refinement of target prediction algorithms to improve their sensitivity and specificity.
4. Efforts to investigate noncanonical roles for miRNAs in pathways distinct from post-transcriptional regulation of gene expression should be increased.

DISCLOSURE STATEMENT

The authors are not aware of any biases that might be perceived as affecting the objectivity of this review.

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