microRNA Functions

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Abstract

microRNAs (miRNAs) are small noncoding RNAs that play important roles in posttranscriptional gene regulation. In animal cells, miRNAs regulate their targets by translational inhibition and mRNA destabilization. Here, we review recent work in animal models that provide insight into the diverse roles of miRNAs in vivo.

Contents

INTRODUCTION

Since the discovery of the founding members of the microRNA (miRNA) family, *lin-4* and *let-7* (Lee et al. 1993, Reinhart et al. 2000, Wightman et al. 1993), hundreds of miRNAs have been identified in plants, animals, and viruses by molecular cloning and bioinformatic approaches (Berezikov et al. 2006, Lagos-Quintana et al. 2001, Lau et al. 2001, Lee & Ambros 2001, Ruby et al. 2006). miRNAs were found to downregulate gene expression by base-pairing with the 3′ untranslated regions (3′UTRs) of target messenger RNAs (mRNAs) (Lee et al. 1993, Reinhart et al. 2000, Slack et al. 2000, Wightman et al. 1993). These discoveries indicated that this class of noncoding RNA molecules may constitute a new layer of regulatory control over gene expression programs in many organisms. Here, we review recent work, principally from animal models, that reveals how miRNAs are generated and act to silence gene expression, how targets of miRNAs can be identified, and how the biological functions of miRNAs can be illuminated by knowledge of gene expression patterns, by mutant phenotypes of miRNAs, and by overexpression of their targets.

microRNA (miRNA): ~22-nt noncoding RNA that serves as a posttranscriptional regulator

miRNA BIOGENESIS AND SILENCING MECHANISM

Most miRNA genes are transcribed by RNA polymerase II (Pol II) to generate a stemloop containing primary miRNA (pri-miRNA), which can range in size from hundreds of nucleotides to tens of kilobases (Cai et al. 2004, Y. Lee et al. 2004) (**Figure 1**). An exception are miRNAs lying within Alu-repetitive elements, which can be transcribed by RNA polymerase III (Borchert et al. 2006). Like mRNAs, Pol II–transcribed pri-miRNAs contain 5′ cap structures, are polyadenylated, and may be spliced (Bracht et al. 2004, Cai et al. 2004). A very recent report shows that most mammalian miRNAs are encoded in introns and that miRNA processing appears to occur before splicing (Kim et al. 2007). The pri-miRNA is processed within the nucleus by a multiprotein complex called the Microprocessor, of which the core components are the RNase III enzyme Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha (Denli et al. 2004, Gregory et al. 2004, Han et al. 2004, Landthaler et al. 2004, Lee et al. 2003). This complex cleaves the pri-miRNA

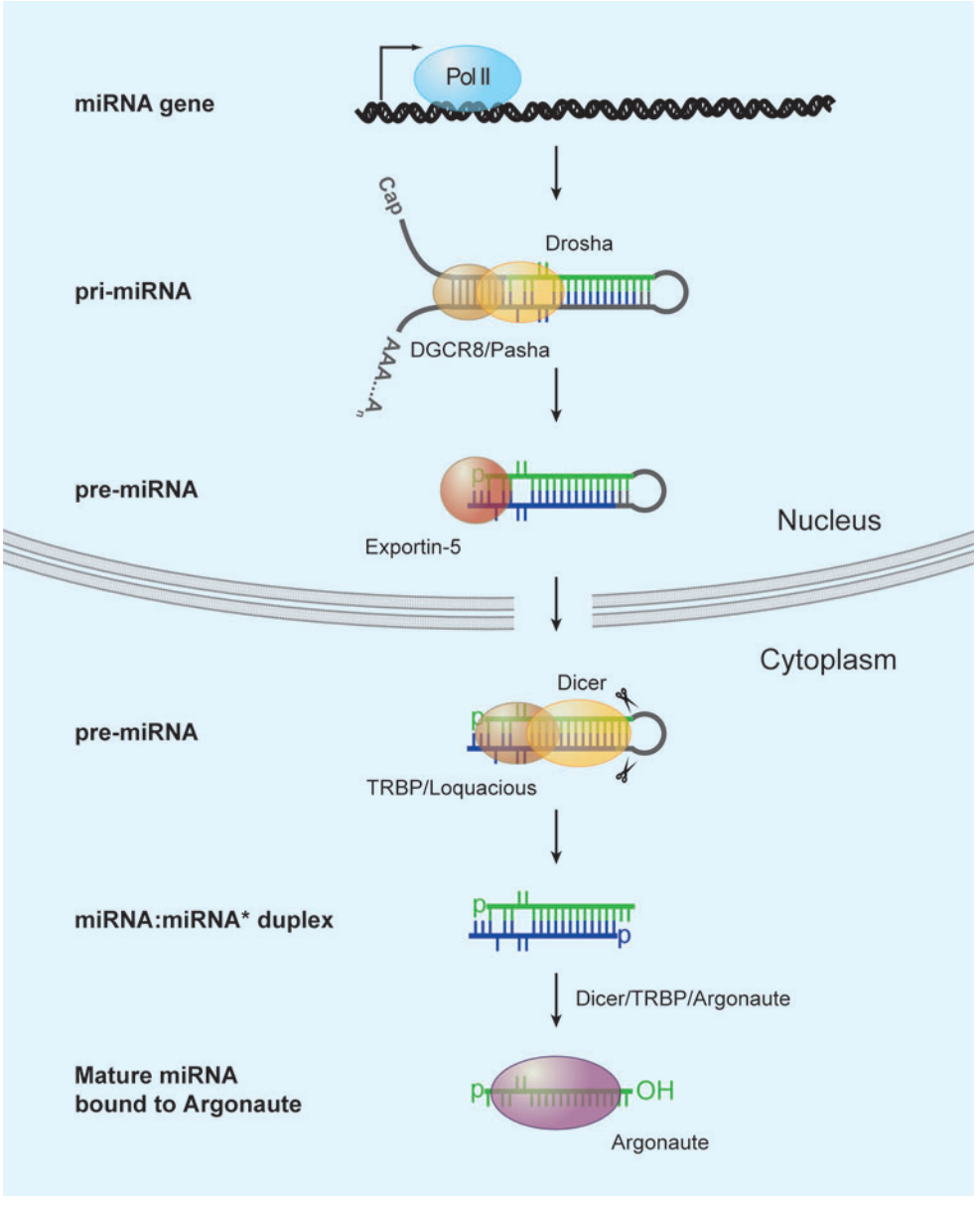

Figure 1

miRNA biogenesis. An miRNA gene is transcribed, generally by RNA polymerase II (Pol II), generating the primary miRNA (pri-miRNA). In the nucleus, the RNase III endonuclease Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha cleave the pri-miRNA to produce a 2-nt 3′ overhang containing the ~70-nt precursor miRNA (pre-miRNA). Exportin-5 transports the pre-miRNA into the cytoplasm, where it is cleaved by another RNase III endonuclease, Dicer, together with the dsRBD protein TRBP/Loquacious, releasing the 2-nt 3′ overhang containing a ~21-nt miRNA:miRNA* duplex. The miRNA strand is loaded into an Argonaute-containing RNA-induced silencing complex (RISC), whereas the miRNA* strand is typically degraded.

3′ untranslated region (3′UTR): 3′ untranslated sequences that follow the protein-encoding open reading frame in a messenger RNA

Primary miRNA (pri-miRNA): the miRNA transcript

Precursor miRNA (pre-miRNA): a ~70-nt stemloop structure

Dicer: cytoplasmic RNase III enzyme that processes the pre-miRNA to produce the mature ~22-nt miRNA duplex

Argonaute: catalytic component of the RISC complex

RNA-induced silencing complex (RISC): the ribonucleoprotein complex required for small RNA-mediated gene silencing

Slicing: possible mode of action of the Ago2-containing RISC complex, in which the target mRNA is cleaved and subsequently degraded

stem by measuring the distance from the single-stranded/double-stranded RNA junction (Han et al. 2006), producing a ~70-nt hairpin precursor miRNA (pre-miRNA). The 2-nt 3′ overhang, characteristic of RNase III–mediated cleavage, is recognized by Exportin-5, which transports the pre-miRNA into the cytoplasm via a Ran-GTP-dependent mechanism (Bohnsack et al. 2004, Lund et al. 2004, Yi et al. 2003).

Next, the pre-miRNA is cleaved to produce the mature ~22-nt miRNA:miRNA* duplex by another RNase III enzyme, Dicer, which interacts with the dsRBD proteins TRBP/Loquacious and, in human cells, PACT (**Figure 1**; Chendrimada et al. 2005, Forstemann et al. 2005, Hutvagner et al. 2001, Jiang et al. 2005, Ketting et al. 2001, Lee et al. 2006, Saito et al. 2005). Subsequently, in human cells TRBP recruits the Argonaute protein Ago2 (and perhaps other Ago proteins), and together with Dicer they form a trimeric complex that initiates the assembly of the RNA-induced silencing complex (RISC), a ribonucleoprotein complex (Gregory et al. 2005, Maniataki & Mourelatos 2005). The miRNA strand with relatively lower stability of base-pairing at its 5′ end is incorporated into RISC, whereas the miRNA* strand is typically degraded (Schwarz et al. 2003, Du & Zamore 2005). Recent deep sequencing efforts indicate that the average ratio of miRNA to miRNA* is ~100:1 but can be much lower in cases in which both strands are functional (Ruby et al. 2006).

Once incorporated into RISC, the miRNA guides the complex to its RNA targets by base-pairing interactions. In cases of perfect or near-perfect complementarity to the miRNA, target mRNAs can be cleaved (sliced) and degraded; otherwise, their translation is repressed (Hutvagner & Zamore 2002, Martinez & Tuschl 2004). For slicing to occur, RISC must contain an Argonaute protein capable of endonucleolytic cleavage. Ago2 is the sole enzyme conferring this activity in mammals and is the major enzyme in flies (Liu et al. 2004, Meister et al. 2004, Okamura et al. 2004). Only one endogenous animal miRNA target has been reported to be sliced (Yekta et al. 2004). Most animal miRNAs base-pair imperfectly with their targets and promote translational repression rather than cleavage and degradation. In this mode of repression, target mRNAs are not actively degraded but can be destabilized owing to deadenylation and subsequent decapping (Behm-Ansmant et al. 2006, Giraldez et al. 2006, Jackson & Standart 2007, Wu et al. 2006). If slicing is so rare in animal miRNA-target interactions, it is curious that the mechanism has been so well conserved. Perhaps this mechanism is used more often than we are currently aware, or the conservation has some other basis.

The mechanism of translational repression by miRNAs remains unclear. Indeed, the step at which miRNAs block translation is controversial. There is evidence that miRNAs block translation initiation, whereas other studies suggest a block in elongation (Humphreys et al. 2005, Maroney et al. 2006, Nottrott et al. 2006, Petersen et al. 2006, Pillai et al. 2005). Argonaute proteins bound to miRNAs and their target mRNAs accumulate in processing bodies (P-bodies), cytoplasmic foci that are known sites of mRNA degradation (Liu et al. 2005a, Pillai et al. 2005, Sen & Blau 2005). P-bodies exclude ribosomal components and may therefore serve as sites in which mRNAs can be stored without translation. Several proteins found in P-bodies (GW182, the Dcp1/Dcp2 decapping complex, and the RCK/p54 helicase) can bind to Argonaute proteins, and this interaction mediates translational repression (Behm-Ansmant et al. 2006; Chu & Rana 2006; Liu et al. 2005a,b; Rehwinkel et al. 2005). Yet, doubts remain over the importance of P-bodies in target repression. Disrupting P-bodies does not affect the degree of translational repression, and therefore the P-body localization may be a consequence of repression rather than its cause (Chu & Rana 2006, Jackson & Standart 2007). Moreover, miRNA-mediated repression and P-body localization are reversible (Bhattacharyya

et al. 2006), indicating that the P-bodies may serve as sites of temporary storage of translationally repressed mRNAs.

Many human mRNAs that encode proteins whose levels are under tight control have AU-rich elements in their 3′UTRs. These elements are bound by an AU-rich element–binding protein, ARE. Intriguingly, a specific miRNA, *miR-16*, is required for the rapid turnover of mRNAs containing AU-rich elements in their 3′UTRs, to which *miR-16* binds (Jing et al. 2005). This does not seem to involve site-specific endonucleolytic cleavage because none of the decay intermediates expected from slicer activity were observed. Presumably, *miR-16* collaborates via RISC binding with TPP, which is required for ARE mRNA degradation. A very recent paper also suggests a new role for Argonaute proteins and AU elements in translational regulation (Vasudevan & Steitz 2007).

Although most attention to date has been paid to miRNA action in the cytoplasm, a recent report shows that mature *miR-29b* contains a 6-nt motif at its 3′ terminus, which directs import of the mature miRNA into the nucleus (Hwang et al. 2007), raising intriguing possibilities for other modes of miRNA function.

OTHER SMALL NONCODING RNAs

This review focuses on animal miRNAs and their biological functions. But other types of small noncoding RNA with distinct properties deserve mention. Small interfering RNAs (siRNAs) differ from miRNAs mainly in their origin: They derive from endogenous or exogenous double-stranded RNAs and are processed into siRNAs by Dicer. siRNAs usually induce cleavage of their targets when loaded onto an Ago2-containing RISC. However, siRNAs can also act as miRNAs on targets with imperfect complementarity and induce translational repression (Doench et al. 2003). Depending on the number and position of the mismatches, RISC-mediated cleavage of mismatched targets can also occur, albeit at a lower rate (Martinez & Tuschl 2004, Meister & Tuschl 2004).

Piwi-interacting RNAs (piRNAs) are a third group of small RNAs that has garnered considerable attention recently (Aravin et al. 2006, Girard et al. 2006, Grivna et al. 2006, Lau et al. 2006, Watanabe et al. 2006). They associate with members of the Piwi family, a subtype of Argonaute proteins, including the mouse proteins MILI and MIWI and perhaps MIWI2. piRNAs are 26–31 nt in length. Because piRNAs accumulate at the onset of male meiosis and sperm maturation arrests at different stages in *Mili* and *Miwi* knockout mice, piRNAs are thought to play an essential role during gametogenesis. Recent evidence indicates that the previously identified class of repeat-associated siRNAs (Aravin et al. 2003) is the major class of *Drosophila* piRNAs (Brennecke et al. 2007, Gunawardane et al. 2007, Saito et al. 2006, Vagin et al. 2006). Although the function and targets of mammalian piRNAs are unclear, *Drosophila* piRNAs are involved in silencing transposons in the male and female germ lines. Recently an intricate system for the control of transposons was identified; it involves discrete piRNA-generating loci composed of defective transposon copies and an amplification cycle to boost piRNAs against actively transcribed transposons (Brennecke et al. 2007, Gunawardane et al. 2007). piRNA biogenesis in flies is independent of Dicer and components of the miRNA and siRNA pathways (Vagin et al. 2006). Recent evidence indicates that the Piwi proteins themselves are directly involved in the biogenesis of piRNAs (Brennecke et al. 2007, Gunawardane et al. 2007).

Plant miRNAs differ from animal miRNAs in their biogenesis and in their mode of target regulation: Most plant miRNAs display perfect or near-perfect complementarity to their target mRNAs—sites occur in both coding regions and 3′UTRs—and therefore induce mRNA slicing (Vaucheret 2006). However, the plant miRNA family *miR-854*

miRNA target: a messenger RNA encoding a protein, containing target sites for and regulated by an miRNA

miRNA seed: seven to eight nucleotides at the 5′ end of an miRNA that serve as the primary determinant of target specificity

SNP: single-nucleotide polymorphism

was recently reported to act by translational repression via imperfect binding sites and to be conserved in animals (Arteaga-Vazquez et al. 2006). The observation of potential GU-rich binding sites in the animal ortholog of the plant *miR-854* target *UBP1b* is suggestive, but this target has not yet been validated. These observations are surprising because miRNAs are thought to have evolved independently in the plant and animal kingdoms (Chen & Rajewsky 2007). However, the recent discovery of miRNAs and genes involved in miRNA biogenesis in the unicellular green algae *Chlamydomonas* suggests an older, perhaps common origin for plant and animal miRNAs (D. Baulcombe, personal communication).

TARGET IDENTIFICATION

The first animal miRNA targets were identified by genetics in *Caenorhabditis elegans*. The heterochronic miRNA *lin-4* displays a mutant phenotype that can be suppressed by a second mutation in its target mRNA, *lin-14* (Lee et al. 1993, Wightman et al. 1993). These genetic interactions led to the identification of sequences complementary to *lin-4* in the 3′UTR of *lin-14* (**Figure 2**). Although powerful, this genetic approach can identify only those targets, such as *lin-14*, whose overexpression is directly responsible for the miRNA mutant phenotype, in this case *lin-4*. Few examples of this type have been found, and it is unclear whether this sort of relationship will be more the exception than the rule.

Subsequently, the criteria for miRNA-target interactions were deciphered by mutation of known miRNA-target sites and testing for function in miRNA misexpression assays (Brennecke et al. 2005b, Doench & Sharp 2004, Kiriakidou et al. 2004, Kloosterman et al. 2004, Lewis et al. 2003). These studies focused attention on the importance of pairing to the 5′ end of the miRNA, called the seed region (**Figure 2**). We have grouped target sites into two broad classes: (*a*) 5′ dominant sites, which base-pair precisely to the seed of the miRNA, with or without 3′ pairing support, and (*b*) 3′ compensatory sites, which have insufficient 5′ pairing compensated for by strong pairing to the miRNAs' 3′ region.

In addition to direct experimental tests for site function, further indication of the importance of the seed region in miRNA-target recognition has been inferred from computational studies that showed significant overrepresentation of conservation of matches to miRNA seeds or, in some cases, avoidance of miRNA seed matches (Brennecke et al. 2005b; Farh et al. 2005; Krek et al. 2005; Lewis et al. 2003, 2005; Stark et al. 2005; Xie et al. 2005). A recent analysis of single-nucleotide polymorphism (SNP) genotype data also showed that polymorphism density was significantly lower in conserved target site regions that are complementary to the 5′ portion of the miRNA (Chen & Rajewsky 2006).

miRNA-target prediction has gone through several iterations, with significant improvements to the original efforts (e.g.,

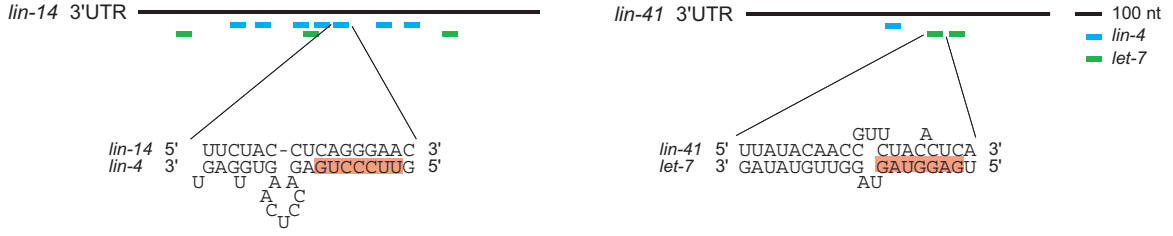

Figure 2

The microRNAs *lin-4* and *let-7* confer regulation to their targets via imprecise base-pairing with the target's 3′UTRs. The miRNA seed region is highlighted in red.

Enright et al. 2003, Lewis et al. 2003, Stark et al. 2003) made on the basis of rules derived from the experimental approaches described above and by the use of evolutionary conservation (Brennecke et al. 2005b, Grun et al. 2005, Krek et al. 2005, Lewis et al. 2005, Rajewsky 2006, Xie et al. 2005). The newer-generation methods have largely converged on a similar approach and have led to estimates that more than 30% of animal genes may be miRNA targets. Recently, a pattern-based algorithm that predicts miRNA-target sites without relying on cross-species conservation or miRNA sequence estimated even larger numbers of miRNA-regulated genes (Miranda et al. 2006). Whether this approach proves to be valid awaits verification.

One approach to assess target predictions uses miRNA misexpression and assays for target downregulation. Overexpression of miRNAs in tissue culture followed by expression profiling provides a global picture of target RNAs that are destabilized by miRNA binding (Lim et al. 2005). The converse approach, bulk depletion of miRNAs followed by mRNA profiling, demonstrates upregulation of many, but not all, predicted target mRNAs (Rehwinkel et al. 2006). Depletion of single miRNAs by antisense methods (Krutzfeldt et al. 2005) or by the use of miRNA mutants (N. Bushati, J. Karres, G. Easow & S.M. Cohen, unpublished results) shows that many RNAs are upregulated; among these are predicted targets but also many RNAs with potential target sites that were not predicted because they are not evolutionarily conserved. However, many potential targets are not affected on the RNA level, suggesting that these approaches will underestimate meaningful miRNA-target relationships. On the protein level, miRNA-target interactions are not amenable to high-throughput approaches and must be tested one by one in reporter assays. Although miRNA misexpression is useful for testing whether regulation is possible, such experiments are not sufficient to draw conclusions about miRNA-target relationships in vivo.

Given the large numbers of predicted miRNA-target genes and the paucity of genetic evidence for miRNA-target relationships, the significance of the predicted targets remains an open question. Computational studies suggest that, on the basis of the occurrence of SNPs within conserved miRNA sites, ~85% of conserved miRNA sites are functionally important (Chen & Rajewsky 2006). The regulation of such sites by miRNAs presumably improves the fitness of the organism in some way, unless the sequences are conserved for reasons unrelated to miRNAs. However, 16 predicted conserved target sites of *lsy-6* were not regulated by endogenous levels of miRNA (Didiano & Hobert 2006). A possible caveat is that target-containing UTRs were expressed at potentially unphysiological levels, perhaps to levels beyond the capacity of the endogenous miRNA to regulate, through the use of multicopy transgenes. Whether this provides a firm basis to doubt the significance of the many evolutionarily conserved seed matches is open to debate.

Accumulating evidence suggests that miRNA-target regulation can be context dependent. For example, (*a*) zebrafish *miR-430* directly contributes to the repression of residual maternal *nanos1* in somatic cells (Mishima et al. 2006). Although both *miR-430* and *nanos1* are also present in primordial germ cells, *nanos1* can overcome the regulation conferred by the miRNA and is robustly expressed. (*b*) In mammalian hepatocarcinoma cells, stress-induced derepression of the *miR-122* target CAT-1 depends on the presence of a binding site for the HuR protein in the UTR, which is distinct from the miRNA sites (Bhattacharyya et al. 2006). Derepression did not occur if the HuR sites were removed in a reporter construct, implying context dependence. (*c*) Didiano & Hobert (2006) have reported that a minimal *lsy-6* target site can be functional when embedded in the 3′UTR of its endogenous target but not when embedded in a heterologous 3′UTR. They took this as evidence that target site function may depend on the specific 3′UTR context.

However, in this case the conclusion is weakened because the presence of a second site in the endogenous 3′UTR that may contribute to its regulation was not considered.

miRNA EXPRESSION PATTERNS

miRNA-target prediction programs do not take into account whether an miRNA is ever expressed in the same cell as its predicted target. Information about miRNA and target expression patterns can help to assess the likelihood that a predicted miRNA-target relationship is relevant in vivo.

miRNA expression profiles were first generated by small RNA cloning and Northern blotting (Aravin et al. 2003, Berezikov et al. 2006, Chen et al. 2005, Lagos-Quintana et al. 2001, Lau et al. 2001, Lee & Ambros 2001, Ruby et al. 2006). Cloning can identify new miRNAs and provide quantitative information about their expression levels. miRNA microarrays (Baskerville & Bartel 2005, Miska et al. 2004, Nelson et al. 2004, Thomson et al. 2004), quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) methods, and in situ hybridization have provided further insight into the tissue-specific expression of pri- and mature miRNAs (Aboobaker et al. 2005, Ason et al. 2006, Kloosterman et al. 2006, Wienholds et al. 2005). Some miRNAs with deeply conserved sequences display striking conservation of their spatial expression patterns, providing further evidence for the conservation of their functions. Prominent examples are the muscle-specific *miR-1* and the nervous system–specific *miR-124*, which are conserved in flies, fish, and mammals. Other miRNAs, e.g., *miR-279* and *miR-9a* in *Drosophila*, are expressed in highly dynamic patterns during development of the embryo (Stark et al. 2005). It should be noted that the expression pattern of the pri-miRNA does not necessarily reflect that of the mature miRNA because some miRNAs are regulated post-transcriptionally (Obernosterer et al. 2006, Thomson et al. 2006).

FUNCTIONS IN ANIMAL DEVELOPMENT

Functions Inferred from Bioinformatic Approaches

Transfection of the tissue-specific miRNAs *miR-1* and *miR-124* into HeLa cells shifted the expression profiles toward those of the respective miRNA expressing tissues, affecting 100–200 transcripts (Lim et al. 2005). The interesting insight that emerged from this study was that the transcripts targeted were those that would normally be expressed at a low level in the cell type in which the miRNA is expressed. For example, RNAs targeted by the muscle-specific *miR-1* upon its transfection into nonmuscle cells were RNAs that are normally at low or undetectable levels in muscle. Global analyses of the relations of miRNA expression patterns to those of their conserved targets arrived at a similar conclusion (Farh et al. 2005, Sood et al. 2006, Stark et al. 2005). These studies provided evidence that (*a*) the average miRNA has target sites in hundreds of genes; (*b*) few predicted targets contained multiple conserved binding sites for a single miRNA, suggesting that the large-magnitude regulation conferred by multiple sites for a single miRNA is exceptional; (*c*) abundant mRNAs are under selective pressure to avoid regulation by coexpressed miRNAs; and (*d*) genes involved in basic cellular processes have been selected for loss of sites and for short 3′UTRs to limit the occurrence of potentially detrimental miRNA sites, whereas genes involved in developmental processes show evidence of enrichment for miRNA sites.

An additional observation made primarily in *Drosophila* is that many miRNAs and their targets appear to be expressed in a largely nonoverlapping manner, either temporally or spatially (Stark et al. 2005) (**Figure 3**). In the latter case, targets are typically present in domains adjacent to the miRNA-expressing tissue. Expression profiling of mammalian cells indicated that many conserved targets are present in the tissue expressing the miRNA,

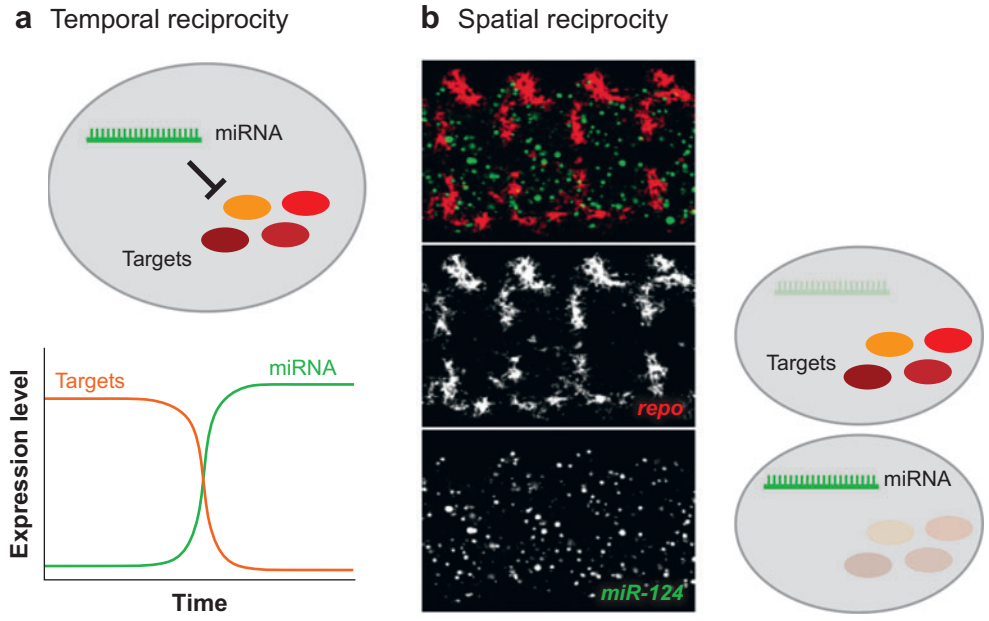

Figure 3

Mutually exclusive expression of miRNAs and their targets. (*a*) miRNAs and their targets can be expressed in a temporally reciprocal manner: Target transcription may be turned off, concurrent with the turning on of miRNA expression. miRNA expression may also actively contribute to target repression, without an underlying change in target transcription. (*b*) In the case of spatial reciprocity, the miRNA and its targets, e.g., *miR-124* (*green*) and its target *repo* (*red*) in the *Drosophila* central nervous system, are often expressed in adjacent domains (from Stark et al. 2005).

albeit at significantly lower levels than in most other tissues (Farh et al. 2005, Sood et al. 2006). In *Drosophila* this possibility is not excluded, although in situ hybridization failed to detect the targets in the miRNA-expressing cell. This probably reflects differences in sensitivity of the methodology because selection for miRNA sites would not emerge if the targets were not present at some level. In some cases, the apparent reciprocity of miRNAs and targets may reflect the action of the miRNA on target-RNA levels. This can be seen when the target-RNA level changes in an miRNA mutant (e.g. Teleman et al. 2006). In other cases, comparison of primary transcript and mature target-RNA patterns has suggested no effect of the miRNA (Stark et al. 2005).

On the basis of these studies, it was suggested that some miRNAs may help maintain and define cell types by dampening the expression of unwanted transcripts, whether from preexisting mRNAs during developmental transitions or simply from leaky transcription. As a cell changes fate, transcription of a target may be turned off while miRNA transcription is being turned on, producing what appear as mutually exclusive patterns (**Figure 3**). Thus, transcriptional regulation is primary, and miRNA regulation is the second level that reinforces the transition as the targets are on their way out (or present only at low levels as leaky transcripts). In this way, miRNAs would provide a fail-safe mechanism to ensure accuracy and confer robustness to underlying gene expression programs.

Although the trend of anticorrelation between miRNA and target expression is common enough to emerge from a global computational study of miRNA and target expression patterns with high statistical

Robustness: resistance of a biological system to perturbation; in genetic terms, invariance of phenotype in response to changing conditions or altered gene dosage

significance, it does not follow that all miRNA-target relationships will be of this type. Further in-depth analysis of miRNAs and their targets as well as of miRNA mutants will be needed to reveal how prevalent this mode of regulation is. Mutants lacking miRNAs for this class of regulation would be expected to suffer subtle changes in the expression of many targets. We consider it likely that the accumulated consequences of many small misregulations may lead to relatively mild and difficult-to-characterize defects in such mutants rather than to obvious phenotypes.

DEPLETION OF ALL miRNAs

An approach to examine the spectrum of possible miRNA functions in development is to eliminate the production of all miRNAs. Dicer mutants have proven informative in the study of the miRNAs expressed earliest during development. Moreover, conditional inactivation of Dicer in specific mouse tissues has shed some light on the functions of some differentially expressed miRNAs. This approach is useful in asking if any miRNA might be involved in a particular biological process.

Animals that are unable to produce mature miRNAs do not survive or reproduce (Bernstein et al. 2003, Forstemann et al. 2005, Ketting et al. 2001, Wienholds et al. 2003). However, the degree to which development is impaired varies. In *C. elegans*, *dicer-1* mutants display defects in germ-line development and a burst vulva phenotype reminiscent of the *let*-7 mutant (Ketting et al. 2001, Knight & Bass 2001). Simultaneous removal of maternal and zygotic *dicer-1* is embryonic lethal, suggesting an essential role for miRNAs during embryogenesis (Grishok et al. 2001). In *Drosophila*, depletion of Loquacious, the partner of Dicer-1, causes female sterility (Forstemann et al. 2005), and *dicer-1*-mutant germ-line stem cells display cell division defects (Hatfield et al. 2005). Unfortunately, the full phenotype of *dicer-1*-mutant flies has not been described, but mutant eyes are small, with disorganized ommatidial arrays and missing interommatidial bristles (Y.S. Lee et al. 2004). Unlike their single mutants, *ago1*, *dicer-1* double mutants exhibit strong segmentation defects (Meyer et al. 2006), suggesting that miRNAs may regulate patterning of the *Drosophila* embryo.

miRNAs have also been implicated in memory formation in *Drosophila*. The putative RNA helicase Armitage, which is involved in the maturation of RISC (Tomari et al. 2004), is colocalized at synapses with calcium/calmodulin-dependent protein kinase II (CaMKII), a kinase required for memory (Ashraf et al. 2006). Upon neural stimulation in olfactory-avoidance learning, Armitage is degraded, and CaMKII translation increases, leading to the establishment of a stable memory. The CaMKII 3′UTR contains some miRNA-binding sites and is required for this regulation. In *armitage* and *dicer-2* mutants, CaMKII expression is significantly higher. However, because *Drosophila dicer-2* has been implicated in the biogenesis of siRNAs rather than of miRNAs (Y.S. Lee et al. 2004), it remains unclear if the canonical miRNA pathway will have a role in the establishment of long-term memory.

dicer-1-mutant zebrafish die by 2–3 weeks of age, without obvious defects except for a general growth arrest (Wienholds et al. 2003). miRNAs do not have a function in the zebrafish germ line, so the germ line can be transferred from zygotic mutant fish into a normal host. When mated to mutant males, the resulting females can produce eggs lacking maternal and zygotic *dicer-1* (Giraldez et al. 2005). Although these embryos cannot produce any mature miRNAs, their early development is not severely perturbed. Abnormalities have been seen in morphogenetic processes during gastrulation, somitogenesis, and heart and brain development, ultimately leading to late-embryonic lethality. These comparatively mild defects contrast sharply with the case of *Dicer*-mutant mice, which arrest development and die during gastrulation, before axis formation (Bernstein et al. 2003).

In studies using conditional mouse *Dicer*, embryonic stem cells were impaired in their ability to proliferate (Murchison et al. 2005), and those selected for survival failed to differentiate (Kanellopoulou et al. 2005). In contrast to the zebrafish situation, recent reports indicate an essential role for dicer-dependent miRNA biogenesis in the oocyte to support normal development of the mouse embryo (Murchison et al. 2007, Tang et al. 2007). Other *Dicer*-depleted tissues were able to differentiate and pattern properly, yet the morphogenesis of hair follicles, the lung epithelium, and limbs was perturbed (Andl et al. 2006, Harfe et al. 2005, Harris et al. 2006, Yi et al. 2006), and apoptosis was induced. Specific deletion of *Dicer* in the mouse thymus early in T cell development reduced the proliferation and survival of αβ T cells, but the CD4/CD8 lineage choice was not affected (Cobb et al. 2005). These studies suggest that some miRNAs play important roles in these tissues, but leave open the question of what aspect of their biology is being regulated. The identification of which miRNAs act and on which targets will be needed for a deeper understanding of their roles in development.

These approaches suggest that miRNAs are likely required in a broad range of biological processes during animal development. Surprisingly, at least in vertebrates, basic patterning events and differentiation do not seem to rely heavily on miRNA-mediated regulation, whereas physiological processes may do so.

SPECIFIC miRNA FUNCTIONS

If one considers the abundance of miRNA genes, it may seem surprising that so few miRNA mutants have been recovered in the multitude of genetic screens done in *Drosophila* or *C. elegans*. Why have all these miRNA genes been missed?

An obvious reason might be the small size of miRNAs. In most cases, the seven nucleotides that constitute the seed must be affected to eliminate miRNA function, making miRNAs difficult-to-hit targets in chemical mutagenesis. However, this explanation does not hold true for screens based on transposon insertion for loss- or gain-of-function. Aside from the trivial possibility that miRNA genes may have been overlooked historically because they lacked protein-coding regions, redundancy between miRNAs that share the same seed sequence and that are coexpressed, may mask the effects of loss of single miRNAs (Abbott et al. 2005). Another possibility is that many miRNA mutants show subtle defects or low-penetrance defects that may be difficult to identify in high-throughput genetic screens.

To address these issues, a large-scale mutagenesis project was undertaken to identify mutants for all miRNAs in *C. elegans* (E.A. Miska, E. Alvarez-Saavedra, A.L. Abbott, N.P. Lau, A. Helmann, S. McGonagle, D.P. Bartel, V. Ambros & H.R. Horvitz, personal communication). Relatively few miRNA genes individually generate robust abnormal phenotypes. In some cases, this can be attributed to overlapping functions; multiple mutants that remove related miRNAs reveal stronger defects than do the single mutants. However, some miRNA multiple mutants still produce few or not discernible defects. This group may reflect those miRNAs that confer precision to developmental processes rather than making developmental decisions.

Misexpression and Overexpression

When an miRNA is misexpressed, it has the potential to regulate many targets that it might never encounter in its endogenous expression domain (Farh et al. 2005, Lim et al. 2005, Sood et al. 2006, Stark et al. 2005). Shutting down such targets can be expected to cause strong phenotypes. But because the miRNA and target may not normally be significantly coexpressed, it is difficult to extrapolate from the misexpression effect to the normal function of the miRNA. miRNA misexpression can produce intriguing defects that have limited relevance to what was learned from mutants removing the miRNA (Teleman

et al. 2006), although both defects may result from misregulation of the same target in different contexts. Our unpublished observations suggest that this may often be the case (N. Bushati, J. Karres, J. Varghese & S.M. Cohen). We suggest that reciprocity of outcome should be considered an essential criterion in assessing miRNA function.

In *Drosophila*, two families of Notch target genes, the Enhancer of split-Complex and the Bearded-Complex genes, contain conserved motifs in their 3′UTRs complementary to the seed sequences of a family of related miRNAs (Lai 2002, Stark et al. 2003). Some of these miRNAs, when misexpressed, can induce phenotypes reminiscent of loss of Notch pathway function (Stark et al. 2003, Lai et al. 2005). Similarly, misexpression of *miR-iab-5p* can repress Ubx and induce a homeotic phenotype (Ronshaugen et al. 2005). But it remains to be determined if mutants removing these miRNAs impact Notch signaling or Ubx function in vivo. However tantalizing the misexpression results, we consider studies based solely on miRNA misexpression to have limited predictive value. In some cases, they may help to identify the correct target gene (e.g., Teleman et al. 2006), but this cannot be taken for granted. Below we outline several examples in which misexpression and loss-of-function approaches have provided consistent results, giving us confidence in their insights into miRNA functions.

When overexpressed, the pancreatic islet–specific *miR-375* inhibits glucose-induced insulin secretion. This can be mimicked by knockdown of its target, *myotrophin* (Poy et al. 2004). Importantly, depletion of *miR-375* increases *myotrophin* levels and enhances glucose-stimulated insulin secretion, indicating that *miR-375* is an inhibitor of glucose-stimulated insulin secretion. Using a similar approach in cultured hippocampal neurons, Schratt et al. (2006) found that *miR-134* regulates dendritic spine size by inhibiting translation of *Limk1*.

In hematopoietic stem cells, enforced expression of the B cell–specific miRNA *miR-181* stimulates their differentiation to B-lineage cells (Chen et al. 2004), but it remains to be determined if loss of *miR-181* impedes differentiation. A very recent report (Li et al. 2007) provides evidence that *miR-181* overexpression and depletion produce opposing effects on antigen sensitivity in T cells.

miR-1 and *miR-133* are absent from undifferentiated myoblasts and strongly upregulated upon differentiation into myotubes (Boutz et al. 2007). Tissue-specific overexpression of *miR-1* in the developing mouse heart induces premature differentiation of myocytes (Zhao et al. 2005). *miR-1* misexpression can accelerate myoblast differentiation by the targeting of histone deacetylase 4, a repressor of muscle differentiation, whereas depletion of *miR-1* impedes differentiation, indicated by a decrease in myogenic markers (Chen et al. 2006). *miR-1* and *miR-133* form one genomic cluster and are coexpressed in heart and skeletal muscle. *miR-133* promotes myoblast proliferation by targeting serum response factor (Chen et al. 2006), but in a different experimental setup myoblast differentiation was not observed (Boutz et al. 2007). *miR-133* was required during differentiation to downregulate nPTB protein, a repressor of alternative splicing, resulting in splicing of a group of silenced exons in mature myotubes. *miR-1* and *miR-133* are seemingly required to define and maintain the properties of differentiated muscle cells, in agreement with the conclusions drawn from *miR-1* misexpression (Lim et al. 2005). In a very recent report, a mouse lacking one of the *miR-1* genes had defects in heart development and function, providing robust genetic evidence for the importance of this miRNA family in cardiogenesis (Zhao et al. 2007).

In *C. elegans*, *let-60/RAS* contains several putative *miR-84/let-7*-binding sites and can be downregulated by *miR-84* and *let-7*. *let-7* mutants display a burst vulva phenotype that can be suppressed by RNAi of *let-60/RAS* (Johnson et al. 2005), suggesting that excess *let-60/RAS* activity contributes to the defect. Overexpression of *miR-84*, a member of the

let-7 family, can rescue the multivulva phenotype caused by *let-60/RAS* gain-of-function alleles (Johnson et al. 2005). These results implicate *let-7* as an important in vivo regulator of *let-60/RAS*, but final conclusions about the relevance of *miR-84* await mutant analysis.

MODES OF miRNA FUNCTION: miRNA MUTANTS

How essential are the contributions of specific miRNAs to developmental programs? Because many miRNAs are highly conserved, their functions must be advantageous, even if they appear vanishingly subtle to us in the lab. As for other regulatory molecules, individual miRNAs can be expected to influence development to different degrees. Whether a general mode of action is attributable to most miRNAs is still a subject of discussion (Bartel & Chen 2004, Hornstein & Shomron 2006, Stark et al. 2005). Most likely one size won't fit all. Comparison of the insights gained from experimental analysis of miRNAs and computational studies suggests that there may be several conceptually distinct modes in which miRNAs act. Here we present evidence for several such modes of action and discuss what sort of evidence would be required to support the case for others.

miRNAs Acting as Developmental Switches

When deleted, miRNAs required for developmental decision-making can be expected to cause strong phenotypes. The first miRNAs identified, *C. elegans lin-4* and *let-7*, are of this kind. Indeed, it could hardly be otherwise, given that they were found in forward genetic screens for mutant phenotypes (Chalfie et al. 1981, Lee et al. 1993, Reinhart et al. 2000, Wightman et al. 1993). Without *lin-4*, the animal is unable to make the transition from the first to the second larval stage owing to a differentiation defect. This developmentally retarded phenotype is caused by a failure to posttranscriptionally downregulate the *lin-14* gene, which harbors seven *lin-4* target sites in its 3′UTR (**Figure 2**) (Lee et al. 1993, Wightman et al. 1993). In the adult worm, *lin-4*-mediated downregulation of *lin-14* has been implicated in life-span regulation (Boehm & Slack 2005).

Depletion of *let-7*, a highly conserved miRNA, also leads to a heterochronic defect, failure of larval-to-adult transition (Reinhart et al. 2000). Several genes are direct targets of *let-7* during this transition: *lin-41*, *hbl-1*, and *daf-12* and the forkhead transcription factor *pha-4* (Abrahante et al. 2003, Grosshans et al. 2005, Lin et al. 2003, Slack et al. 2000). *let-7* is a member of a family of miRNAs including *miR-48*, *miR-84*, and *miR-241*, which function in a cooperative manner to control the earlier L2-to-L3 transition, probably by targeting *hbl-1* (Abbott et al. 2005, Li et al. 2005). Additionally, in adult worms *miR-48* and *miR-84* are coordinately required to bring the larval molting cycle to an end (Abbott et al. 2005). These *C. elegans* miRNAs act as key regulators of developmental timing. The temporal regulation of *let-7* during development and the presence of a target site in *lin-41* (**Figure 2**) are conserved (Pasquinelli et al. 2000), raising the possibility that this regulatory relationship controls some aspect of developmental timing in other organisms.

Loss of *lsy-6* in *C. elegans* induces a cell-fate switch: Instead of having two morphologically distinct gustatory neurons on left and right sides, known as ASEL and ASER, respectively, two ASER neurons are specified (Johnston 2003). Asymmetry is lost owing to the failure of *lsy-6* to repress cog-1 expression in ASEL. die-1, present only in ASEL, is required for *lsy-6* expression (Chang et al. 2004). die-1 in turn is downregulated in ASER by *miR-273*, which is expressed predominantly in the ASER because it is activated there by the *lsy-6* target cog-1 (Johnston et al. 2005). Therefore, the cell-fate decision and stabilization of the gustatory ASE neurons are controlled via a double negative-feedback loop in which two transcription factors use miRNAs to repress each other (**Figure 4**).

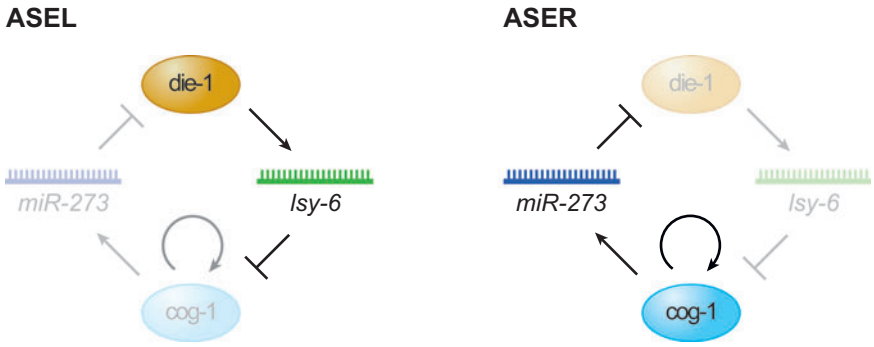

Figure 4

miRNAs acting in a double negative-feedback loop control neuronal asymmetry in *C. elegans*. In *C. elegans*, *lsy-6*, induced by the transcription factor die-1, determines adoption of the ASEL fate by repressing cog-1 expression. In the ASER neuron, cog-1 positively regulates its own expression and induces *miR-273*, which suppresses die-1 and, consequently, *lsy-6*.

miR-181 expression is upregulated during terminal differentiation of myoblasts, and the depletion of *miR-181* impedes their differentiation, partly owing to the upregulation of one of its targets, Hox-A11, an inhibitor of differentiation (Naguibneva et al. 2006). However, *miR-181* overexpression is not sufficient to induce differentiation. In adult muscle, *miR-181* is expressed at very low levels, suggesting that it is involved in muscle establishment, not maintenance. Whether or not this miRNA acts as a developmental switch in vivo awaits analysis of a knockout mouse.

Fine-Tuning Developmental Programs: Robustness

On the basis of the computational studies described above, many miRNAs are expected to regulate a large set of targets that may be expressed at low levels in the miRNA-expressing cells. This may provide a second layer of regulation to reinforce transcriptional controls. miRNA mutants of this class may be expected to have subtle phenotypes and, perhaps, phenotypes that are difficult to study, owing to the heterogeneous nature of their targets.

Members of the *miR-430* multigene family are expressed at high levels at the onset of zygotic transcription in zebrafish embryos. Supplying miRNA-depleted embryos with *miR-430* by injection rescues the brain morphogenesis defects seen in these embryos (Giraldez et al. 2005). Comparing expression profiles of miRNA-depleted embryos with those of embryos resupplied with *miR-430* revealed that several hundred transcripts, likely direct *miR-430* targets based on miRNA seed matches, are misregulated in the absence of *miR-430* (Giraldez et al. 2006). Approximately 40% are maternally deposited RNAs, suggesting that *miR-430* is required to accelerate the clearance of preexisting maternal mRNAs in the embryo when zygotic transcription starts (**Figure 5**). This study provides experimental evidence that an endogenous miRNA targets hundreds of transcripts in vivo and that their regulation by the miRNA serves to ensure robustness of the developmental program. A similar situation exists in *Drosophila*, in which the six miRNAs encoded of the *miR-309* miRNA gene cluster contribute to the clearance of maternally encoded mRNAs at the onset of zygotic transcription (N. Bushati, A. Stark, J. Brennecke & S.M. Cohen, unpublished observations).

Surprisingly, most *Drosophila* larvae lacking the highly conserved, muscle-specific miRNA *miR-1* develop apparently normal, functional muscles (Sokol & Ambros 2005). Twenty percent of the mutant embryos do not hatch, but the lethality is not due to obvious

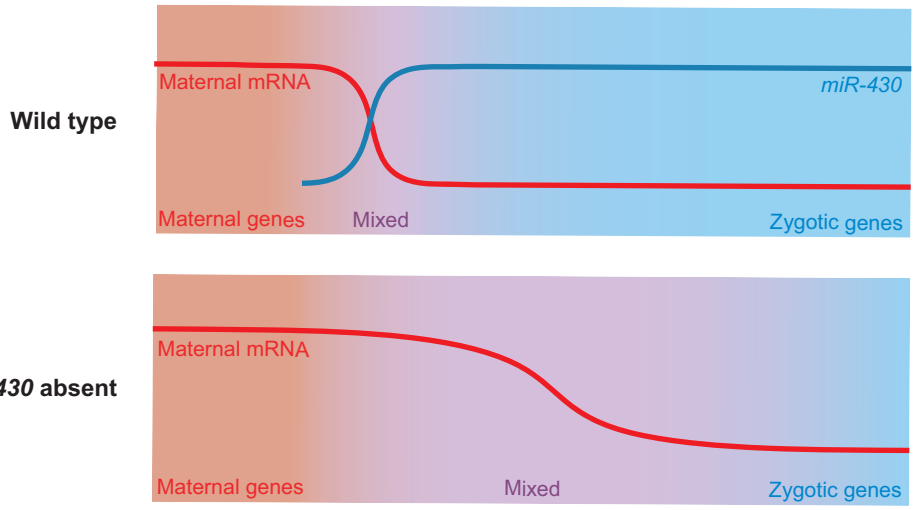

Figure 5

Fine-tuning embryonic development. In early zebrafish embryogenesis, the miRNA *miR-430* regulates the transition from maternal to zygotic mRNA transcription by targeting maternal mRNAs for degradation. In the absence of *miR-430*, maternal mRNA expression overlaps with zygotic gene expression and interferes with morphogenesis.

muscle defects (Brennecke et al. 2005a). The remaining 80% die with massively disrupted muscles when the mutants begin the rapid phase of larval growth upon feeding. However, if the mutant larvae are fed on sugar, conditions under which they live but do not grow, muscle function is normal, and the animals survive. It is unclear which target(s) is misregulated in the *miR-1* mutant or how growth causes muscle degeneration. Perhaps this defect is the consequence of many subtle errors earlier during development, when *miR-1* may be required to confer robustness to the identity and/or physiology of muscle cells. But a specific role during muscle growth is also possible.

Zebrafish *miR-214* is expressed in mesoderm during early somitogenesis and throughout embryogenesis. Antisense oligonucleotide–mediated depletion of *miR-214* reduced Hedgehog signaling in the presomitic mesodermal cells adjacent to the notochord and therefore reduced the number of slow-muscle cells (Flynt et al. 2007). During normal development, these cells experience high Hedgehog levels, whereas their more lateral neighbors are exposed to less Hedgehog. Su(fu), a negative regulator of Hedgehog signaling, is upregulated in *miR-214*-depleted embryos, probably owing to the lack of regulation by *miR-214*. Su(fu) participates in Hedgehog signaling by retaining both the activator and repressor forms of the Gli transcription factors in the cytoplasm. miRNA-mediated downregulation of Su(fu) may maximize the response to different levels of Hedgehog signaling.

Antisense-mediated silencing of the abundant liver-specific *miR-122* in the mouse leads to significant upregulation of >100 mRNAs containing *miR-122* seed matches in their 3′UTRs. These mice are healthy and show substantially reduced levels of circulating cholesterol and triglycerides. *miR-122* therefore has a function in hepatic lipid metabolism and may be required to fine-tune this process (Esau et al. 2006, Krutzfeldt et al. 2005). Studies such as this suggest the therapeutic potential of miRNA depletion.

In mouse embryos, an *miR-196* gene is located in all four mammalian HOX clusters. *miR-196* is expressed in the hindlimb but not

Antisense oligonucleotide–mediated depletion: an approach to eliminate miRNA function by the injection/transfection of complementary modified RNA, which binds and sequesters the miRNA and so prevents it from binding its targets

in the forelimb. Its targets, *Hoxa7* and *Hoxb8*, are induced by retinoic acid in the vertebrate forelimb but not in the hindlimb. *miR-196* cleaves its target HOXB8 (Yekta et al. 2004) and may block translation of *Hoxa7* when these RNAs are present in the hindlimb (Hornstein et al. 2005). Thus, *miR-196* suppresses unwanted transcripts in a domain in which they are normally transcriptionally repressed, supporting a primary transcriptional level of regulation. This provides an in vivo example of how apparently spatially reciprocal expression domains of miRNAs and targets can reflect an important regulatory relationship.

A similar relationship exists between *miR-278* and its target, *expanded*, in adipose tissue in *Drosophila*. *miR-278*-mutant flies are lean owing to insulin insensitivity in the adipose tissue, in which *miR-278* is most strongly expressed (Teleman et al. 2006). A similar defect results in mice with adipose-tissue-specific knockout of insulin responsiveness (Teleman et al. 2006). *expanded* mRNA and protein levels are normally very low in adipose tissue but increase considerably in the *miR-278* mutant. Genetic analysis has shown that *expanded* overexpression causes the lean phenotype. In this case, the miRNA contributes strongly to downregulation of its target's transcript levels, thereby helping to shape its expression domain.

Proliferation and Apoptosis

The *Drosophila* miRNA *bantam* was identified in a screen for regulators of tissue growth in which its overexpression induced overgrowth. *bantam* mutants are small and die as early pupae (Brennecke et al. 2003). To allow net tissue growth, apoptosis must be overcome. The 3′UTR of the proapoptotic gene *bid* contains five *bantam* target sites, and part of the ability of *bantam* to promote growth can be explained by this regulatory relationship. But this is not sufficient to explain the mutant phenotype because blocking apoptosis is not enough to cause growth. The targets involved in the generation of a positive-growth-regulatory output by *bantam*, e.g., by inhibition of a negative growth regulator, have not yet been identified. Recently, the Hippo signaling pathway, which coordinately controls cell proliferation and apoptosis, has been shown to regulate *bantam* expression (Nolo et al. 2006, Thompson & Cohen 2006).

Feedback Loops

Several miRNAs are part of regulatory feedback loops. The roles they play can be switch-like, as described above for *C. elegans lsy-6* and *miR-273*, which are thought to act in a double negative-feedback loop to specify left-right asymmetry of gustatory neurons (Chang et al. 2004, Johnston & Hobert 2003) (**Figure 4**).

In other cases, the role of the miRNA in the feedback loop can be quite subtle, reinforcing or stabilizing decisions made by other factors. In the *Drosophila* eye, *miR-7* reinforces a developmental decision via a reciprocal negative-feedback loop (Li & Carthew 2005) (**Figure 6**). During photoreceptor differentiation, a transient EGF signal leads to degradation of the protein Yan and, by the release of Yan-mediated repression of *miR-7* transcription, to elevated *miR-7* expression. Subsequently, *miR-7* represses Yan. A stable change in cell fate from progenitor to photoreceptor is thereby achieved, and the differentiated fate is maintained by the presence of the miRNA. The overall eye development of *miR-7*-mutant flies appears normal. Only when the system was sensitized by the introduction of a *Yan* allele, which does not respond normally to epidermal growth factor receptor (EGFR), were defects observed, demonstrating that the miRNA does not control the feedback loop but reinforces a decision made in response to an extracellular signal. This is another example of mutually exclusive expression of an miRNA and its target because *Yan* is downregulated when *miR-7* expression is induced.

A similar negative-feedback loop operates during granulocytic differentiation (Fazi et al.

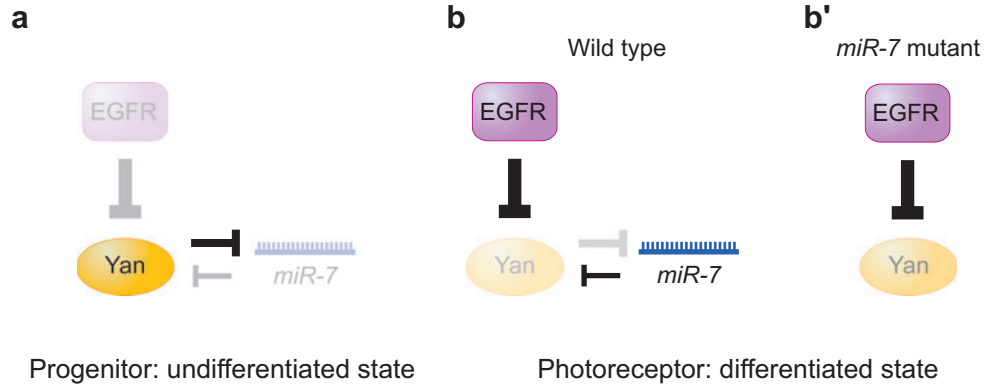

Figure 6

miR-7 reinforces photoreceptor differentiation. (*a*) Yan represses *mir-7* transcription in the progenitor. (*b*,*b*′) During photoreceptor differentiation, epidermal growth factor receptor (EGFR) signaling induces Yan degradation, which leads to the release of *miR-7* repression. (*b*) *miR-7* then reinforces Yan downregulation. (*b*′) In the absence of *mir-7*, Yan is still repressed by EGFR, and therefore photoreceptor differentiation occurs normally.

2005): In undifferentiated myeloid precursors, the transcription factor nuclear factor I-A (NFI-A) keeps levels of *miR-223* expression low. When ectopically expressed, *miR-223* enhances the differentiation of myeloid precursors into granulocytes. Upon stimulation with retinoid acid, which induces differentiation, CCAAT/enhancer binding protein α (C/EBPα) replaces NFI-A and induces high levels of *miR-223* transcription. *miR-223* in turn represses NFI-A translation via a binding site in its 3′UTR, thus ensuring its own expression. Depletion of *miR-223* followed by stimulation with retinoic acid leads to reduced expression of a granulocytic differentiation marker (Fazi et al. 2005).

In *C. elegans*, *miR-61* is directly transcriptionally activated in secondary vulval precursor cells by LIN-12/Notch (Yoo & Greenwald 2005). *miR-61* represses Vav-1, which would otherwise repress LIN-12, thereby inducing a positive-feedback loop, reinforcing LIN-12 activation and specification of secondary vulval cell fate. In contrast to *miR-7*, which ensures robustness of a decision made by EGFR signaling, *miR-61* may play a major role in cell-fate specification.

Thresholding

Flies lacking *miR-9a*, a conserved nervous system–specific miRNA, produce extra sense organs (Li et al. 2006). This defect is due to failure to repress the *miR-9a* target *senseless*. Indeed, the *miR-9a*-mutant phenotype is suppressed by the removal of one copy of the *senseless* gene, which limits the extent to which *senseless* can be overexpressed. An interesting aspect of this mutant is that loss of *senseless* regulation causes a sporadic defect, with up to 40% of animals affected. Why is that so? During selection of the sensory organ precursor (SOP) from cells of a proneural cluster, *senseless* expression is turned on by proneural proteins and feedbacks positively to reinforce proneural gene expression. The resulting high level of proneural activity induces a signal that the SOP uses to repress SOP fate in surrounding cells. Early in the process, *miR-9a* is broadly expressed in the neuroectoderm and limits proneural gene expression by keeping *senseless* expression low. During this phase, *miR-9a* sets a threshold that *senseless* expression has to overcome to induce SOP fate, thereby selecting only the cell with the highest transcriptional peak. In animals lacking *miR-9a*, *senseless* levels sporadically exceed

Sensory organ precursor (SOP): the cell that gives rise to the sensory organ

Proneural cluster: a group of cells that are competent to become neuronal precursors

the threshold, leading to ectopic SOP formation. It should be noted that the miRNA is not part of a feedback loop but limits the level at which a transcriptional feedback system can be activated. Whether other miRNAs have comparable roles in thresholding (Cohen et al. 2006) remains to be seen.

An attempt to investigate *miR-9a* and other miRNA functions has been made by the injection of complementary 2′-*O*-methyl oligoribonucleotides into fly embryos (Leaman et al. 2005). However, where comparisons have been possible the results obtained by antisense-mediated depletion have differed significantly from those observed in the corresponding miRNA-null mutants. Depletion of *miR-1*, *miR-9a*, *miR-279*, *miR-6*, *miR-286*, and several miRNAs of the *miR-310* family was reported to cause a variety of severe embryonic defects. Comparable defects were not seen in embryos mutant for these miRNAs (Li et al. 2006, Sokol & Ambros 2005; N. Bushati & S.M. Cohen, unpublished data). It is unclear how to explain this discrepancy.

TARGET TYPES: SWITCHING OFF VERSUS TUNING TARGET EXPRESSION LEVELS

A few years ago, Bartel & Chen (2004) proposed the terms switch target and tuning target to describe two classes of relationships between miRNAs and targets (**Figure 7**). These terms were coined to describe what the miRNA does to its target and should not be confused with what regulation of the target does to the cell or organism.

A switch target is one for which the miRNA reduces target expression to a level below which it has any meaningful activity in the cell, effectively switching it off. Most of the targets of *miR-1* or *miR-124*, which are normally expressed at low levels in the miRNA-expressing cells, would likely qualify as switch targets because the job of the miRNA appears to be to reduce their potential leaky expression to inconsequential levels. In these and other examples discussed above, such as that of *miR-196*, the miRNAs do not appear to act as developmental switches. However, important miRNA targets, including those of the heterochronic miRNAs *lin-4* and *let-7*, may act as developmental switches (Lee et al. 1993, Wightman et al. 1993). Although these are also likely to be switch targets, it remains to be demonstrated that the residual expression after miRNA-mediated repression is without function.

The notion of a tuning target reflects the role of the miRNA in setting a defined level of target expression while being coexpressed with the miRNA. Tuning targets can make use of miRNAs to smooth out fluctuations in their expression or, by altering miRNA level, to ensure that target levels are suitable for the prevailing conditions. The critical distinction between the switch and tuning modes lies in whether the residual level of target expression is required, i.e., has a specific function, in that cell or if the job of the miRNA is merely to reduce target expression to an inconsequential level. One could make this distinction genetically, for example, by selectively eliminating the target gene in the miRNA cells, using a conditional knockout approach. To date no such analysis has been presented, but we suggest this as a rigorous standard.

miRNAs IN HUMAN DISEASE

One of the human diseases in which miRNAs have been implicated is the neuropsychiatric disorder Tourette's syndrome (TS) (Abelson et al. 2005). The 3′UTR of *SLITRK1* contains a *miR-189*-binding site, which is mutated in some TS patients. This polymorphism replaces a GU base pair with AU pairing, leading to stronger regulation by the miRNA. In situ hybridization of *SLITRK1* mRNA and *miR-189* revealed coexpression in neuroanatomical circuits most commonly implicated in TS. Obviously this mutation is only one out of many rare mutations leading to this complex disease, but it demonstrates how an miRNA can be involved in the establishment of a disease phenotype.

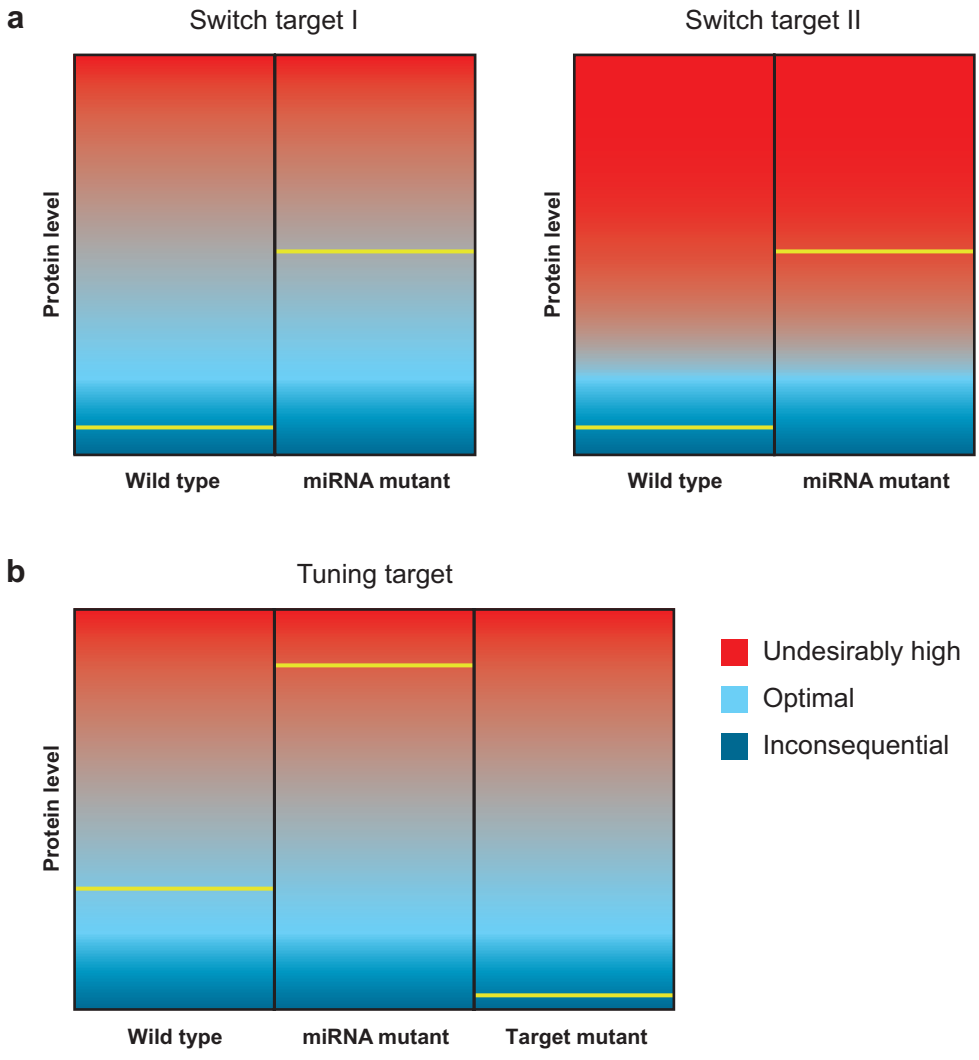

Figure 7

Switch and tuning targets revisited. Red, light blue, and dark blue indicate areas in which protein levels are undesirably high, optimal, and inconsequential, respectively. (*a*) Switch targets are downregulated to inconsequential levels. In the absence of the miRNA, these targets are expressed at levels that may be harmless (switch target I) or at detrimental levels (switch target II). The consequences can be subtle or severe, depending on the type of target gene and its relative level of misexpression. (*b*) miRNAs adjust the expression of tuning targets to optimal levels. In the absence of the miRNA, the tuning target is expressed at a detrimental level. In contrast to switch targets, tuning targets have a function in the miRNA-expressing cell, so reducing them further has a negative effect.

Ninety percent of patients suffering from DiGeorge syndrome lack one copy of the chromosomal region 22q11 (Lindsay 2001). Interestingly, the Drosha partner DGCR8 maps to this region (Denli et al. 2004, Gregory et al. 2004, Han et al. 2004, Landthaler et al. 2004, Shiohama et al. 2003), but it remains to be determined if reduced miRNA levels are an underlying cause of DiGeorge syndrome.

Expansion of the polyglutamine repeats in Ataxin-3 has been linked to neurodegeneration. Depletion of *dicer* in human cells led to a significant enhancement of Ataxin-3-induced toxicity (Bilen et al. 2006). Which miRNAs are involved in this human neurodegenerative disorder remains to be determined.

miRNAs as Oncogenes and Tumor Suppressors

miRNA expression levels are altered in primary human tumors (Calin et al. 2004, Lu et al. 2005). One study reported that global miRNA expression was lower in cancer tissues than in normal tissues (Lu et al. 2005), but another did not find this trend (Volinia et al. 2006). Because many miRNAs are expressed in differentiated cell types, global miRNA levels may reflect the differentiation state of the tissue and perhaps may be attributable to a failure of Drosha processing (Thomson et al. 2006). Intriguingly, significantly differing miRNA profiles can be assigned to various types of tumors, suggesting that miRNA profiling has diagnostic and perhaps prognostic potential (Calin & Croce 2006, Lu et al. 2005). However, for most miRNAs it is unknown whether they actually play an active role in tumor formation.

Loss of miRNAs in cancer tissue may suggest a role of miRNAs as tumor suppressors. The *miR-15a* and *miR-16-1* genes are located in chromosome region 13q14, which is deleted in most cases of chronic lymphocytic leukemia (Calin et al. 2002). These miRNAs target *B cell lymphoma 2* (*Bcl2*), an antiapoptotic gene, suggesting that loss of *miR-15a* and *miR-16-1* in B cells may lead to the inhibition of apoptosis, giving rise to malignancies (Cimmino et al. 2005). However, causality has not been established, and the correlation is not universal. The same miRNAs are overexpressed in a type of pancreatic tumor (Volinia et al. 2006).

miRNAs with oncogenic potential are expressed from the *miR17-92* locus 13q31, which is amplified in some tumors, e.g., tenfold in B cell lymphoma samples (He et al. 2005). Overexpression of this cluster in a mouse model of human B cell lymphoma accelerated *c*-Myc-induced tumorigenesis, and the apoptosis normally seen in *c*-Myc-induced tumors was suppressed (He et al. 2005). *c*-Myc can directly transcriptionally activate the *miR17-92* cluster and the proapoptotic E2F1, which in turn is targeted by two miRNAs of the cluster (O'Donnell et al. 2005). These miRNAs would therefore support a shift from apoptosis toward proliferation by the downregulation of E2F1. Moreover, *c*-Myc-induced activation of the *miR17-92* cluster leads to enhanced tumor angiogenesis in mouse colonocytes, probably via direct, miRNA-mediated downregulation of antiangiogenic proteins (Dews et al. 2006).

Primary human fibroblasts expressing the clustered miRNAs *miR-372* and *miR-373* were able to overcome oncogenic Ras-mediated arrest and, therefore, induced tumorigenesis (Voorhoeve et al. 2006). In part, this effect is mediated by targeting of the tumor suppressor LATS2. *miR-372* and *miR-373* are expressed specifically in testicular germ cell tumors (Voorhoeve et al. 2006).

Interestingly, both miRNA clusters are highly expressed in embryonic stem cells (Suh et al. 2004, Thomson et al. 2004), which suggests that they contribute to tumorigenesis by exerting their normal function at the wrong time and place in the organism. Another miRNA with oncogenic potential is *miR-155*, which is overexpressed in several kinds of B cell lymphomas and can induce preleukemic pre–B cell proliferation in mice when it is overexpressed specifically in B cells (Costinean et al. 2006). For a more in-depth review of miRNAs in cancer, see Calin (2006).

miRNAs and Viruses

Viruses use miRNAs in their effort to control their host cell; reciprocally, host cells use miRNAs to target essential viral functions.

miRNAs have been found in nuclear DNA viruses like the herpesvirus, but to date none have been found in RNA viruses (Cullen 2006; Pfeffer et al. 2004, 2005). Most RNA viruses are restricted to the cytoplasm and are therefore not expected to encode miRNAs because miRNA transcripts need to be processed in the nucleus by Drosha.

The SV40-encoded miRNA *miR-S1* helps keep the infected cell hidden from the immune system. It is expressed late in the viral replication cycle, when it acts to degrade early viral mRNAs encoding T antigen, limiting exposure of the infected cell to cytotoxic T lymphocytes (Sullivan et al. 2005). *miR-LAT* of herpes simplex virus-1 inhibits apoptosis of latently infected neurons by targeting the proapoptotic transforming growth factor-β (TGF-β) and one of its mediators, SMAD3 (Gupta et al. 2006). The miRNA thereby keeps its host cell alive.

Endogenous, cellular miRNAs that target viral RNAs have been reported as well. In one scenario, the cell uses the miRNA to impede viral replication. *miR-32* restricts the replication of the retrovirus PFC-1 in cell culture (Lecellier et al. 2005). In another scenario, the virus takes advantage of an endogenous cellular miRNA. Replication of hepatitis C viral RNA is facilitated by binding of the liver-specific miRNA *miR-122* to the 5′ noncoding region of the viral genome (Jopling et al. 2005).

CONCLUSIONS

miRNAs have captured the attention of the biological sciences community in part because of their novelty and in part because of the excitement of exploring the regulatory potential of a new type of molecule. This review aims to illustrate that the analysis of miRNA functions in vivo has begun to shed light on the types of biological processes that miRNAs regulate. As well, we have begun to understand the variety of ways miRNAs can act in the context of other regulatory mechanisms. It appears that their biological roles in development and disease as well as their modes of action will be diverse.

SUMMARY POINTS

1. A developmental switch is a change in the program of a cell in response to the input of new information. This can be accomplished by a change in transcription or an miRNA-mediated change in posttranscriptional gene expression.
2. Resistance to perturbation is a common property of developmental systems. miRNA-mediated posttranscriptional gene regulation is thought to contribute to robustness, in part through noise reduction.
3. miRNA targets are often expressed at very low levels, possibly at that of noise, in miRNA-expressing cells. In such cases, the job of the miRNA is to keep low-level expression to inconsequential levels.
4. Some miRNAs act as components of regulatory feedback loops. In some cases they are key components. In other cases, they are modulators that ensure robustness or set a threshold for switch activation.
5. Some miRNAs switch off target gene expression, reducing them to inconsequential levels. Failure to regulate targets in this way may have severe consequences or subtle effects, depending on the nature of the targets.

6. Some miRNAs may maintain the level of target gene expression within an optimal window, neither too high nor too low. Here the critical features are that the target has a specific function in the cell at the level set by the miRNA and that expression above or below the set level may be detrimental.

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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