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Getting to the Root of miRNA-Mediated Gene Silencing

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MicroRNAs are ~22 nucleotide-long RNAs that silence gene expression posttranscriptionally by binding to the 3′ untranslated regions of target mRNAs. Although much is known about their biogenesis and biological functions, the mechanisms allowing miRNAs to silence gene expression in animal cells are still under debate. Here, we discuss current models for miRNA-mediated gene silencing and formulate a hypothesis to reconcile differences.

MicroRNAs (miRNAs) play important roles in a broad range of biological processes including development, cellular differentiation, proliferation, and apoptosis. Emerging evidence also implicates miRNAs in the pathogenesis of human diseases such as cancer and metabolic disorders. At least 100 miRNA genes have been identified in invertebrates, and 500–1000 in vertebrates and plants. Computational predictions of miRNA targets estimate that each miRNA regulates hundreds of different mRNAs, suggesting that a large proportion of the transcriptome is subject to miRNA regulation (reviewed by Bushati and Cohen, 2007).

To perform their regulatory functions miRNAs assemble together with Argonaute family proteins into miRNA-induced silencing complexes (miRISCs). Within these complexes, miRNAs guide Argonaute proteins to fully or partially complementary mRNA targets, which are then silenced posttranscriptionally (reviewed by Bushati and Cohen, 2007).

Despite remarkable progress in our understanding of miRNA biogenesis and function, the mechanisms used by miRNAs to regulate gene expression remain under debate. Indeed, published studies indicate that miRNAs repress protein expression in four distinct ways: (1) cotranslational protein degradation; (2) inhibition of translation elongation; (3) premature termination of translation (ribosome drop-off); and (4) inhibition of translation initiation (Figure 1). In addition, animal miRNAs can induce significant degradation of mRNA targets despite imperfect mRNA-miRNA base-pairing (Figure 1). MicroRNAs might also silence their targets by sequestering mRNAs in discrete cytoplasmic foci known as mRNA processing bodies or P bodies, which exclude the translation machinery. Here, we discuss evidence supporting these different mechanisms of repression by miRNAs and the discrepancies between them.

Post-initiation Mechanisms

Early studies in the worm *Caenorhabditis elegans* and recent studies in mammalian cell cultures present persuasive evidence that miRNAs repress protein synthesis after translation is initiated (Seggerson et al., 2002; Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006). Although these studies differ in the details, their conclusions stem from a common observation: in sucrose sedimentation gradients, miRNAs and their targets are associated with polysomes. These polysomes were shown to be actively translating mRNA targets because they were sensitive to a variety of conditions that inhibit translation. For example, they dissociate into monosomes or ribosomal subunits following brief incubation with translation inhibitors, such as hippuristanol, puromycin, or pactamycin (Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006).

The paradoxical observation that the targets of miRNAs appear to be actively translated while the corresponding protein product remains undetectable prompted the proposal that the nascent polypeptide chain might be degraded cotranslationally (Figure 1B; Nottrott et al., 2006). This proposal is, however, based on negative rather than direct positive evidence. For example, the identity of the putative protease remains unknown; and the proteasome was excluded as a possibility because proteasome inhibitors do not restore protein expression from silenced reporters (Nottrott et al., 2006).

To investigate how miRNAs silence their targets, Petersen et al. (2006) designed a synthetic miRNA reporter containing a 3′ untranslated region (UTR) with six identical sites partially complementary to a transfected siRNA (that is, an miRNA mimic). When this reporter was transiently expressed it associated with polysomes, although its expression was repressed by the siRNA. But if translation initiation was inhibited, then these ribosomes dissociated more rapidly than those associated with a control (unrepressed) mRNA. This led to the suggestion that miRNAs cause premature ribosome dissociation or ribosome drop-off (Figure 1A).

There is additional evidence that miRNAs mediate repression after translation initiation: silencing occurs even when reporter mRNA translation is initiated by a 5′ UTR containing an internal ribosome entry site (IRES). Because IRESs initiate translation of mRNAs independently of the mRNA cap structure, these results indicate that miRNAs repress translation at a step downstream of cap recognition (Petersen et al., 2006).

Inhibition of Translation Initiation

In contrast to these studies, Pillai et al. (2005) have shown that miRNAs and their targets are not associated with the

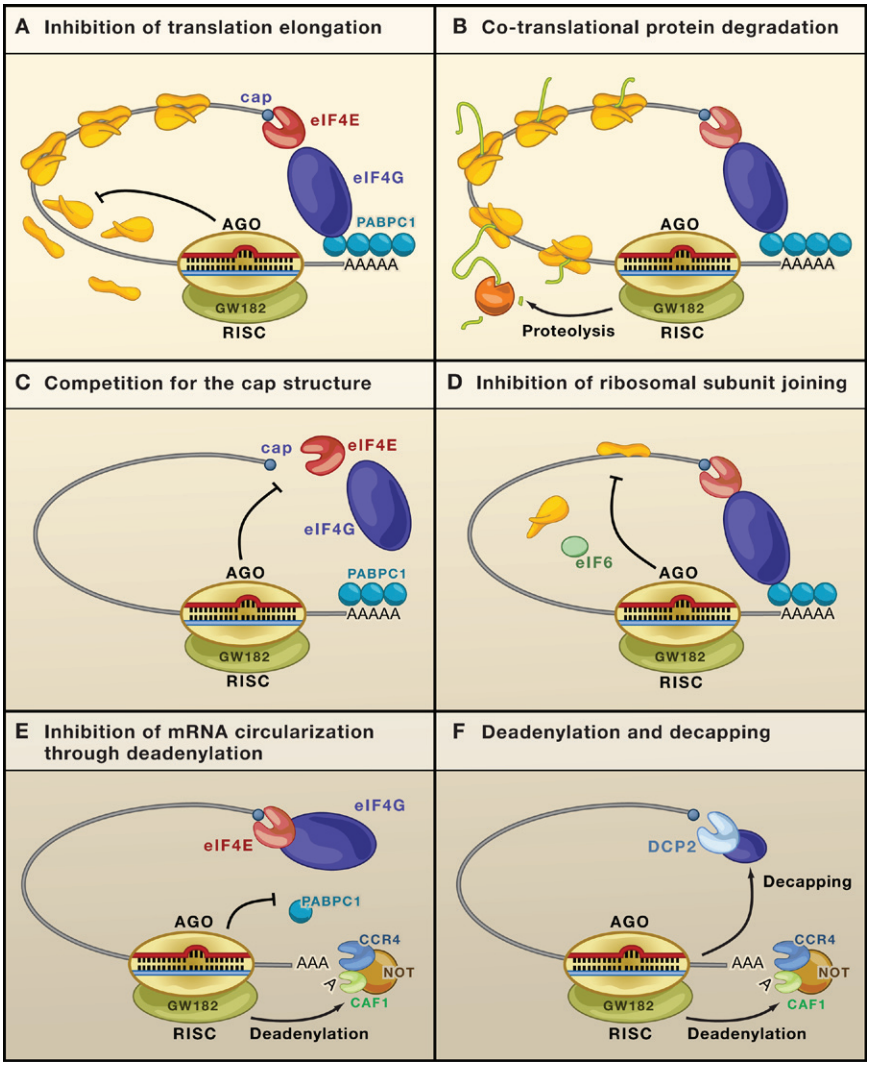

Figure 1. Mechanisms of miRNA-Mediated Gene Silencing

(A) Postinitiation mechanisms. MicroRNAs (miRNAs; red) repress translation of target mRNAs by blocking translation elongation or by promoting premature dissociation of ribosomes (ribosome drop-off).

(B) Cotranslational protein degradation. This model proposes that translation is not inhibited, but rather the nascent polypeptide chain is degraded cotranslationally. The putative protease is unknown.

(C–E) Initiation mechanisms. MicroRNAs interfere with a very early step of translation, prior to elongation. (C) Argonaute proteins compete with eIF4E for binding to the cap structure (cyan dot). (D) Argonaute proteins recruit eIF6, which prevents the large ribosomal subunit from joining the small subunit. (E) Argonaute proteins prevent the formation of the closed loop mRNA configuration by an ill-defined mechanism that includes deadenylation.

(F) MicroRNA-mediated mRNA decay. MicroRNAs trigger deadenylation and subsequent decapping of the mRNA target. Proteins required for this process are shown including components of the major deadenylase complex (CAF1, CCR4, and the NOT complex), the decapping enzyme DCP2, and several decapping activators (dark blue circles). (Note that mRNA decay could be an independent mechanism of silencing, or a consequence of translational repression, irrespective of whether repression occurs at the initiation or postinitiation levels of translation.) RISC is shown as a minimal complex including an Argonaute protein (yellow) and GW182 (green). The mRNA is represented in a closed loop configuration achieved through interactions between the cytoplasmic poly(A) binding protein (PABPC1; bound to the 3′ poly(A) tail) and eIF4G (bound to the cytoplasmic cap-binding protein eIF4E).

polysomal fraction in sucrose gradients but rather with the free mRNP pool in mammalian cells, indicating that translation inhibition occurs at the initiation step. Furthermore, in this and other studies, mRNAs translated through cap-independent mechanisms (that is, through an IRES) were refractory to repression by miRNAs, further supporting the notion that miRNAs inhibit cap-dependent translation initiation (Humphreys et al., 2005; Pillai et al., 2005; Mathonnet et al., 2007; Wakiyama et al., 2007).

In agreement with a role for miRNAs in blocking translation initiation, Kiriakidou et al. (2007) reported an unexpected observation: the central domain of Argonaute proteins exhibits sequence similarities to the cytoplasmic cap-binding protein eIF4E (eukaryotic translation initiation factor 4E), which is essential for cap-dependent translation initiation. eIF4E binds to the m^7Gppp-cap structure of mRNAs by stacking the methylated base of the cap between two tryptophans. At the equivalent position of the tryptophans in eIF4E, Argonaute proteins have phenylalanines that could mediate a similar interaction. Consistently, Kiriakidou et al. (2007) showed that human Argonaute 2 (AGO2) binds to m^7GTP on Sepharose beads, and that there is competition for this binding by a methylated cap analog (e.g., m^7GpppG) but not by unmethylated GpppG. The authors then showed that substituting one or both AGO2 phenylalanines with valine residues abrogated the silencing activity. These results support the idea that miRNAs inhibit translation at the cap-recognition step by displacing eIF4E from the cap structure (Figure 1C).

Evidence suggesting that miRNAs inhibit an early translation step (before elongation) was also reported by Chendrimada et al. (2007). Using human cells they showed that AGO2 associates with both eIF6 and large ribosomal subunits. By binding to the large ribosomal subunit, eIF6 prevents this subunit from prematurely joining with the small ribosomal subunit. Thus, if AGO2 recruits eIF6, then the large and small ribosomal subunits might not be able to associate, causing translation to be repressed (Figure 1D; Chendrimada et al., 2007).

Although both Kiriakidou et al. (2007) and Chendrimada et al. (2007) present results indicating that miRNAs repress translation prior to the elongation step, they propose mutually exclusive underlying mechanisms. Clearly, we need to investigate more fully how eIF6 and Argonaute's putative cap-binding domain contribute to mRNA silencing. In particular, without further structural information on eukaryotic Argonaute

proteins, another viable interpretation of Kiriakidou's results is that mutating the phenylalanine residues affects the protein activity through an unrelated mechanism. Similarly, eIF6 is required for 60S ribosomal subunit biogenesis and its depletion may have secondary effects that we do not yet fully appreciate.

miRNA-Mediated mRNA Decay

Initial studies reported that animal miRNAs repress translation without significantly affecting the abundance of target mRNAs. More recently, however, several reports showed that animal miRNAs do induce significant degradation of target mRNAs (Bagga et al., 2005; Wu and Belasco, 2005; Behm-Ansmant et al., 2006; Giraldez et al., 2006; Wu et al., 2006; Chendrimada et al., 2007; Eulalio et al., 2007a). In agreement with this, the levels of predicted and validated miRNA targets increase in cells in which the miRNA pathway is inhibited, for example, by depletion of Dicer or Argonaute proteins (Giraldez et al., 2006; Rehwinkel et al., 2006; Schmitter et al., 2006). Conversely, if specific miRNAs are ectopically expressed, then transcripts containing binding sites for those miRNAs become less abundant (Lim et al., 2005). Likewise, several examples show that expressing a given miRNA correlates with downregulation of transcripts containing complementary binding sites. For example, in zebrafish embryos at the onset of zygotic transcription, the dramatic increase of miR-430 expression correlates with the degradation of a large number of maternal mRNAs containing miR-430 binding sites in their 3′ UTRs (Giraldez et al., 2006).

In animal cells, miRNAs cause decay of mRNAs not through endonucleolytic cleavage by Argonaute proteins but rather by directing mRNAs to the general mRNA degradation machinery (except when the miRNA is fully complementary to the target). This observation is supported by studies in zebrafish embryos, *C. elegans*, *D. melanogaster*, and human cells showing that miRNAs accelerate deadenylation and decapping of their targets (Behm-Ansmant et al., 2006; Giraldez et al., 2006; Wu et al., 2006; Eulalio et al., 2007a).

Messenger RNA decay mediated by microRNAs requires Argonaute proteins, the P body component GW182, the CAF1-CCR4-NOT deadenylase complex, the decapping enzyme DCP2, and several decapping activators including DCP1, Ge-1, EDC3, and RCK/p54 (Figure 1F; Behm-Ansmant et al., 2006; Eulalio et al., 2007a). Current evidence indicates that GW182 is recruited to miRNA targets through direct interactions with the Argonaute proteins, contributing to translational repression (reviewed by Ding and Han, 2007; Eulalio et al., 2007b). GW182 also marks the transcript as a target for decay via deadenylation and decapping (reviewed by Ding and Han, 2007; Eulalio et al., 2007b).

Although compelling evidence shows that miRNAs trigger degradation of their targets, a critical question remains open: Is degradation an independent mechanism by which silencing is accomplished, or is it a consequence of a primary effect on translation? Some evidence suggests that miRNA-mediated mRNA decay can be uncoupled from translation. In human cells, an miRNA target whose translation was inhibited by inserting a strong stem loop structure in its 5′ UTR was nevertheless deadenylated in an miRNA-dependent manner (Wu et al., 2006). Likewise, in zebrafish embryos and human cell extracts, miRNA targets were deadenylated despite having a defective cap structure (Appp-cap) that impairs translation (Mishima et al., 2006; Wakiyama et al., 2007). Furthermore, in *D. melanogaster* cells and human cell extracts, miRNA-mediated mRNA decay could occur in the absence of active translation (Eulalio et al., 2007a; Wakiyama et al., 2007).

The extent of degradation is clearly specified by the mRNA target and not by the miRNA itself because the same miRNA can either repress translation or induce mRNA decay in a target-specific manner (Eulalio et al., 2007a). Alemán et al. (2007) found that whether miRNA mimics elicit decay or translational repression depends on the structure of the miRNA-mRNA duplexes (that is, the number, type, and position of mismatches). Grimson et al. (2007) found that the number of miRNA binding sites, the distance separating these sites, their position within the 3′ UTR, and the RNA context strongly influence the magnitude of the regulation, although the relative contributions of translational repression and decay in each case were not established. Thus, whether or not miRNAs elicit mRNA degradation strongly depends on specific features of the miRNA-binding site and its RNA context and so, most likely, on the specific complement of proteins associated with a given target.

Sequestration in P Bodies

Argonaute proteins, miRNAs, and miRNA targets colocalize to cytoplasmic foci known as P bodies. Additional components of P bodies include GW182, the CAF1-CCR4-NOT deadenylase complex, the decapping enzyme DCP2, decapping activators (e.g., DCP1, EDC3, Ge-1), and the RNA helicase RCK/p54, all of which have been implicated in miRNA function (reviewed by Eulalio et al., 2007b; Parker and Sheth, 2007).

The detection of Argonaute proteins, miRNAs, and miRNA targets in P bodies led to a model in which miRNA targets get sequestered in P bodies, where they are shielded from the translation machinery and may undergo decay (Eulalio et al., 2007b; Parker and Sheth, 2007). It has been debated whether the localization to P bodies is a cause or a consequence of silencing. However, recent studies demonstrate that the miRNA pathway remains unaffected in cells lacking detectable microscopic P bodies (Chu and Rana, 2006; Eulalio et al., 2007c). Thus, although P body components play crucial roles in miRNA-mediated silencing, aggregation into microscopic P bodies is not required for miRNA function. These results imply that silencing is initiated in the soluble cytoplasmic fraction, and that the localization of the silencing machinery in P bodies is a consequence rather than a cause of silencing (Humphreys et al., 2005; Pillai et al., 2005; Chu and Rana, 2006; Eulalio et al., 2007c).

Insights from In Vitro Studies

The inhibition of translation initiation by miRNAs has been recapitulated in vitro, in cell-free extracts of diverse origin. These include rabbit reticulocyte lysates (Wang et al., 2006), *D. melanogaster* embryo extracts (Thermann and Hentze, 2007), and extracts from two

different mammalian cell lines, mouse Krebs-2 ascites, and human HEK293F cells (Mathonnet et al., 2007; Wakiyama et al., 2007). In these extracts, miRNAs silenced translation of m^7Gppp-capped mRNAs but not of transcripts carrying an artificial Appp-cap structure (irrespective of the poly(A) tail). In mouse and human cell extracts, transcripts initiating translation in an IRES-dependent manner (thus cap-independent) were refractory to miRNA regulation (Mathonnet et al., 2007; Wakiyama et al., 2007).

Few studies have investigated the poly(A) tail's role in silencing; but when this issue was addressed explicitly, nonpolyadenylated mRNAs were either silenced in vivo (Pillai et al., 2005; Wu et al., 2006) or were partially or fully refractory to silencing in vivo and in vitro, respectively, regardless of the cap structure (Humphreys et al., 2005; Wang et al., 2006; Wakiyama et al., 2007).

Wakiyama et al. (2007) observed a strict requirement for both the cap structure and the poly(A) tail for silencing. Moreover, their extracts recapitulated miRNA-mediated deadenylation as previously observed in zebrafish embryos and in human and *D. melanogaster* cells (Behm-Ansmant et al., 2006; Giraldez et al., 2006; Wu et al., 2006). Based on these observations, Wakiyama et al. (2007) proposed that miRNAs trigger deadenylation; consequently in this model, translation is repressed because the cap structure and the poly(A) tail cannot synergize (Figure 1E). It is well established that if the cytoplasmic poly(A)-binding protein (PABPC1) is bound to an mRNA poly(A) tail, it can interact with translation initiation factor 4G (eIF4G); eIF4G is bound to the cap structure through interactions with eIF4E. This interaction induces mRNA to form a closed loop (Figure 1B), which greatly enhances translation (see Wakiyama et al., 2007 and references therein). Silenced mRNAs will not form a closed loop because they are deadenylated.

Notably, there is also debate about whether deadenylation is the cause or the consequence of silencing. Wakiyama et al. (2007) found that all mRNAs containing miRNA-binding sites were deadenylated in an miRNA-dependent manner, including those refractory to silencing because of the presence of an Appp-cap or an IRES in their 5′ UTR; this finding is in agreement with results reported in zebrafish embryos (Mishima et al., 2006). Deadenylation also occurred in the presence of cycloheximide, suggesting that it does not require ongoing translation. These results were interpreted as evidence that deadenylation causes the translational repression (Wakiyama et al., 2007).

Other studies suggest that beyond deadenylation, additional mechanisms cause translational repression. For example, depleting an essential component of the deadenylase complex (that is, NOT1) in *D. melanogaster* cells prevented miRNA-mediated mRNA decay; however, protein expression was not fully restored indicating that the reporters remained silenced at the translational level (Behm-Ansmant et al., 2006). Similarly, Wu et al. (2006) showed that, in human cells, a reporter mRNA in which the poly(A) tail was replaced by a histone-stem loop structure was nevertheless repressed by an miRNA, demonstrating that translational repression is not caused by deadenylation.

A striking finding has been reported by Mathonnet et al. (2007): adding purified initiation complex eIF4F (which includes the cytoplasmic cap-binding protein eIF4E, the scaffolding protein eIF4G, and the RNA helicase eIF4A) counteracted silencing. This fits nicely with the idea that the Argonaute proteins compete with eIF4E for binding to the cap structure as proposed by Kiriakidou et al. (2007). The displacement of eIF4E would prevent the circularization of the mRNA and consequently enhance deadenylation. In summary, although the in vitro studies differ in the details, they all point to a role for miRNAs in interfering with early steps of translation initiation.

Why So Many Mechanisms?

It is hard to reconcile the different reported modes of miRNA regulation of gene expression. Perhaps these different modes reflect different interpretations and experimental approaches. Another possibility is that miRNAs do indeed silence gene expression via multiple mechanisms. Finally, miRNAs might silence gene expression by a common and unique mechanism; and the multiple modes of action represent secondary effects of this primary event.

Are We Measuring What Matters in Silencing?

In principle, it should be easy to discriminate the post-initiation mode from the initiation mode of miRNA-mediated repression by analyzing the position of the silenced mRNA reporter in sucrose sedimentation gradients. In the first case, the reporter is expected to cofractionate with polysomes, whereas in the second case it should be present in the free RNP fraction (that is, at the top of the gradient). Unfortunately, these experiments do not give clear-cut results and their interpretation has proven problematic.

First, except for EDTA treatments, most conditions that disrupt polysomes (e.g., puromycin, hippuristanol, or pactamycin treatments) do not quantitatively shift mRNAs from the polysome fraction to the free mRNP fraction but rather from heavy to lighter fractions of the gradient. This shift could be, in some cases, within experimental variations of the measurements. Second, inhibitory effects of miRNAs are in the 2- to 5-fold range. Hence, 50%–20% of the reporter mRNA is not silenced. This heterogeneity of the reporter mRNA population likely obscures the results. Third, a fraction of repressed mRNA may be partially degraded (most studies cannot rule out 10%–15% degradation of the reporter); depending on the degree of regulation, this may represent a significant fraction of the silenced mRNA.

Given the difficulties mentioned above, it is not surprising that sedimentation profiles do not provide incontrovertible evidence for or against a specific mechanism. Aware of the limitations of this approach, many studies have validated their conclusions using an independent strategy. The use of IRES represents such an independent approach. The results obtained with IRES-containing reporters have been consistent with the analysis of polysome profiles within a given study but have been surprisingly inconsistent between different studies, suggesting an experimental bias.

An Experimental Approach Bias?

Most evidence that translation initiation is inhibited comes from studies of mRNAs synthesized in vitro and incubated in cell-free extracts or transfected into cultured cells. In vivo,

miRNA targets are not "naked" mRNAs but exist as ribonucleoprotein particles or mRNPs. It is generally accepted that RNA-binding proteins are deposited on mRNAs cotranscriptionally or during processing. Therefore, the full complement of proteins associating with mRNAs transcribed in vivo is likely to be different from that bound to the same mRNA in an in vitro system or following mRNA transfection. This may explain some discrepancies observed, for example, between studies by Humphreys et al. (2005) on the one hand and Petersen et al. (2006) on the other. The implication is that RNA-binding proteins strongly influence the final outcome of miRNA regulation.

Another potential source of discrepancy is the nature of the reporter. Some studies used artificial reporters containing multiple identical miRNA-binding sites inserted in a heterologous 3′ UTR. These artificial reporters require up to six binding sites for efficient silencing, whereas natural 3′ UTRs seldom have six identical, regularly spaced binding sites for the same miRNA. Thus, it is possible that the artificial reporters do not fully recapitulate miRNA regulation. Yet, when such reporters were used, different mechanisms could be observed; so these reporters alone do not explain all of the discrepancies (Humphreys et al., 2005; Petersen et al., 2006; Wakiyama et al., 2007).

Additional experimental differences reside in the use of in vivo transcribed and processed miRNAs versus transfected siRNAs. Transfected siRNAs are believed to act as miRNAs, provided that the reporter contains partially complementary binding sites. In human cells, however, it is not clear how the biogenesis pathway or the structure of the double-stranded miRNA or siRNA intermediate influences which Argonaute protein they associate with. However, studies in *D. melanogaster* demonstrated that it is the structure of the small RNA duplex rather than the biogenesis pathway that specifies which Argonaute-containing complex is loaded with the miRNA or siRNA (Tomari et al., 2007). If this were the case also in human cells, endogenous miRNAs and transfected siRNAs could end up in different Argonaute complexes.

There are four human Argonaute paralogs; all are presumed to repress translation through a similar mechanism (although this was never investigated in great detail). Moreover, most studies did not establish which human Argonaute paralog was responsible for the silencing activity. This raises the following question: Do different Argonaute proteins silence partially complementary targets through similar or different molecular mechanisms? The corollary is whether discrepancies between different studies reflect the action of different Argonaute proteins or Argonaute complexes with distinct protein compositions. Indeed, biochemical purification of Argonaute-containing mRNA-protein complexes reveals partially nonoverlapping sets of mRNAs in association with individual Argonaute proteins, suggesting some degree of specificity in target selection (Beitzinger et al., 2007).

Multiple Mechanisms or Multiple Outputs?

Studies comparing natural full-length 3′ UTRs suggest that the final outcome of miRNA regulation depends on the features and sequence of the target's 3′ UTR (Behm-Ansmant et al., 2006; Eulalio et al., 2007a; Grimson et al., 2007). In these studies, the contribution of translational repression or mRNA degradation to gene silencing differed for each miRNA target pair.

Stress conditions provide additional evidence that the context of the 3′ UTR is important, as binding of a protein known as HuR to the 3′ UTR of cationic amino acid transporter 1 (CAT-1) mRNA relieves miR-122-mediated silencing. Although it remains to be determined whether HuR directly displaces miR-122 or interferes indirectly with its function in *cis*, it seems likely that other RNA-binding proteins might also counteract, modulate, or influence the extent and mode of miRNA regulation in a target-specific manner. This is supported by the increasing number of examples in which RNA-binding proteins modulate miRNA function (reviewed by Leung and Sharp, 2007).

Nevertheless, it is still possible that miRNAs silence gene expression through a common and unique mechanism, and that the multiple modes of action reflect secondary consequences of this primary event rather than independent mechanisms. These secondary effects will vary in a cell- or target-dependent manner. For instance, translational repression may represent a primary event that in a target- or cell-specific manner may or may not lead to mRNA decay. Indeed, proteins bound to the target mRNA may influence degradation. Cell-specific effects are also possible: for example, zebrafish *nanos1* mRNA is deadenylated and degraded by miR-430 in somatic cells yet is refractory to miR-430 regulation in the germline (Mishima et al., 2006).

Likewise, one could envisage cotranslational protein degradation as a consequence of a primary event that occurred during translation elongation; the silenced ribosome signals aberrant translation such that the nascent polypeptide chain is degraded. Examples showing nascent polypeptides being cotranslationally degraded have been described (Nottrott et al., 2006 and references therein).

Conclusion

A growing body of evidence indicates that miRNAs play important roles in the pathogenesis of human diseases such as cancer, neurodegenerative and metabolic disorders, and viral infection. A detailed mechanistic understanding of miRNA-mediated gene regulation is therefore of critical importance to evaluate the potential of miRNAs as therapeutic targets. Although we have begun to understand miRNA biogenesis and have identified key effectors of miRNA function, the mechanisms of action of miRNAs in animal cells are still being hotly debated. We consider it unlikely, though, that the diverse mechanisms observed result from different technical approaches. Rather, we favor a view whereby miRNA repression is manifested in distinct ways depending upon the specific features and composition of the mRNP target. Currently, we cannot determine whether these different modes of repression represent multiple independent silencing mechanisms or multiple outputs from a unique primary inhibitory event.

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