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Review

MicroRNA-mediated gene silencing: are we close to a unifying model?

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Abstract

MicroRNAs (miRNAs) comprise a group of small non-coding RNA ~21 nucleotides in length. They act as post-transcriptional regulators of gene expression by forming base pairing interactions with target messenger RNA (mRNA). At least 1000 miRNAs are predicted to be expressed in humans and are encoded for in the genome of almost all organisms. Functional studies indicate that every cellular process studied thus far is regulated at some level by miRNAs. Given this expansive role, it is not surprising that disruption of this crucial pathway underlies the initiation of, or in the least, contributes to the development and progression of numerous human diseases and physiological disorders. This review will focus on the latest developments in uncovering the mechanism(s) of miRNA-mediated silencing with specific reference to the function of terminal effector proteins, how translation of target mRNA is inhibited and whether we are moving towards understanding this fundamental gene silencing paradigm.

Keywords: argonaute; microRNA; silencing; translation.

Introduction

The biogenesis pathway to produce active mature microRNAs (miRNAs) initiates with the processing of a larger primary molecule (pri-miRNA). Once transcribed from genomic DNA, the pri-miRNA forms a hairpin structure with imperfectly paired stems that are subsequently processed by the RNase III endonuclease Drosha and its interacting partner DiGeorge syndrome critical region gene 8 (DGCR8) (or Pasha in *Drosophila melanogaster*) into a 70-nucleotide (nt) hairpin structure termed pre-miRNA (1, 2). The formation of this processing complex is controlled by Drosha and DGCR8 co-regulating each other. DGCR8 is reported to stabilise Drosha *via* protein-protein interactions (3), while Drosha is capable of cleaving hairpin structures within *DGCR8* mRNA triggering degradation (4). This level of co-regulation enables tight control over miRNA biogenesis at this preliminary stage. Pre-miRNAs are transported to the cytoplasm in an exportin-5-dependent manner, where processing is completed by Dicer, another RNase III endonuclease in complex with TAR RNA binding protein (TRBP) that cleaves the pre-miRNA into ~20-nt RNA duplexes with 2-nt 3′ overhangs. One strand of the duplex is then selected as the mature miRNA, and the remaining strand (passenger strand or miRNA*) is degraded (Figure 1). However, in some instances, both strands function as mature miRNAs and both are loaded onto Argonaute (Ago) proteins to form part of an assembled ribonucleoprotein complex known as the miRNA-induced silencing complex (miRISC). Control of miRNA loading has been shown to be dependent on the MID domain of the Ago protein and its ability to interact with the nucleotide in the 5′ position of the miRNA Frank et al. (5) identified a nucleotide specificity loop within the phosphate binding pocket of the MID domain. The use of UMP, CMP, AMP and GMP to mimic the 5′ end of the miRNA demonstrated that the hydrogen bonding patterns presented by UMP and AMP, which are opposite to those produced by CMP and GMP, allow for interaction with the loop and phosphate binding pocket and account for the selection specificity of miRNA strands with a U or A nucleotide in the 5′ position.

Four Ago proteins (Ago 1–4) exist within the mammalian system. The function of this family of proteins has been demonstrated by their ability to associate with miRNA and repress translation when tethered to mRNA 3′UTRs (6–8). Ago2 differs from the other Ago proteins due to its ability to induce endonucleolytic cleavage *via* the P-element-induced wimpy testis domain (9). This ability enables Ago2 to be utilised in both the siRNA and miRNA pathways. This distinction is clearly seen in *D. melanogaster*, in which Ago1 and Ago2 activities are predominantly dedicated to miRNA or siRNA pathways, respectively (10). In plants, evidence of loading small RNAs onto specific Ago proteins has been reported and appears to be dependent on the 5′ nucleotide of the RNA sequence. Changing the 5′ nucleotide allows redirection of the small RNA to a different Ago partner (11, 12). As yet, evidence for a sorting signal to direct specific miRNAs to Ago partners has not been found. The nucleotide specificity loop identified in human Ago2, which biases loading of miRNAs with A or U nucleotides at the 5′ position, is well conserved between the other Ago proteins. Whether this loop or other regions of the Ago protein or other proteins present in RISC act to further sort the loading of different small RNAs onto specific Ago proteins requires further investigation (5).

To elicit silencing, the miRNA directs miRISC to the target mRNA through base pairing. Animal and mammalian

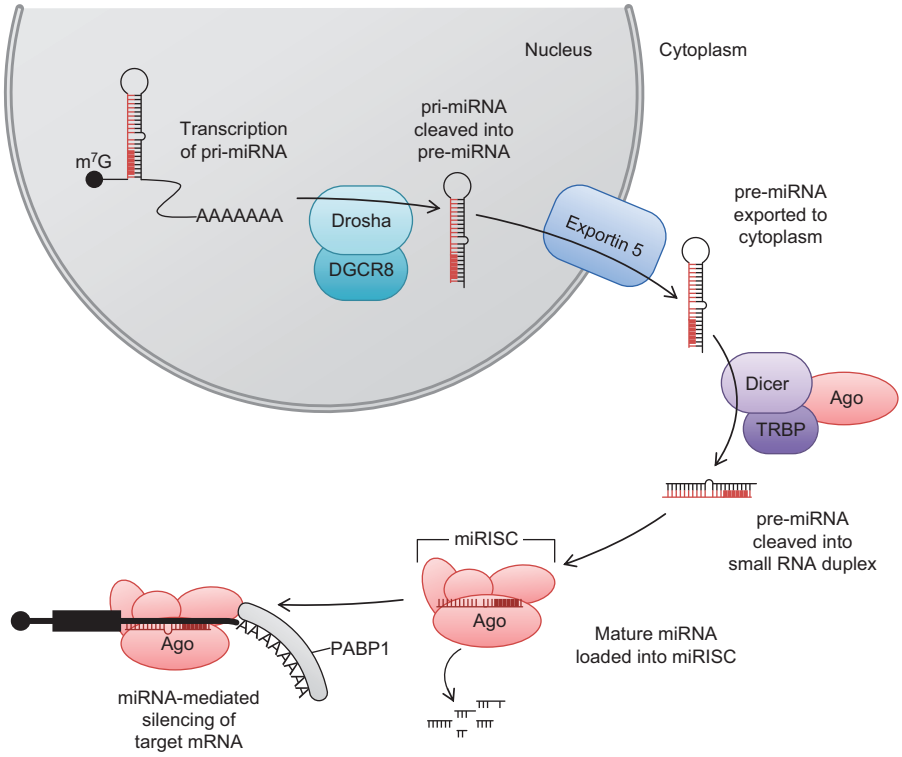

Figure 1 Schematic depicting the biogenesis of miRNA from transcription to silencing.
Transcribed pri-miRNA is processed in the nucleus into ~70-nt stem loops (pre-miRNA) by Drosha and its interacting partner DGCR8. The pre-miRNA is actively transported into the cytoplasm by exportin-5. Once in the cytoplasm, the pre-miRNAs are further processed by Dicer and TRBP into short RNA duplexes, from which the mature miRNA strand is incorporated into the RISC complex *via* recruitment by Ago proteins. The resulting complexes then target mRNA directed by the miRNA sequence and induce translational silencing.

models have shown this base pairing to be imperfect between the miRNA and mRNA sequence, with a key 5′ seed region (miRNA nt 2–7) that provides the most paring specificity (13–15). In addition to the seed region, two other regions of highly conserved miRNA sequence can also influence mRNA target binding, the 3′ region and centred sites. Base pairing of the 3′ region (nt 13–16) can enhance target recognition in conjunction with the seed region but can also be compensatory when mismatches occur within the seed sequence. However, this is predicted to affect <10% of binding sites (16). Complementary base pairing at positions nt 4–14 or 5–15 represent a proposed centred site, which though rare have been shown to effectively repress translation (17). Furthermore, some mRNA, such as E2F2 appears to be regulated directly by miRNAs, despite lacking a recognisable binding site, suggesting that the full myriad of differential RNA sequence factors that promote miRNA-mRNA interactions remains to be elucidated (18). Within plants, miRNAs commonly have complete complementarity with their target mRNA and induce endonucleolytic cleavage. However, evidence suggests that a system of non-perfect pairing similar to that seen in animals may also operate (19).

Despite intensive research in this field, precisely how miRNAs elicit translational silencing of their specific target mRNA remains unclear. Multiple models have emerged, which in this review we have broadly allocated into two groups: (i) those supporting miRNA-mediated silencing occurring after translation has begun and (ii) those supporting a block at translation initiation as the primary cause for silencing. Contrasting findings within the literature have created feverish debates as to which multiple mechanisms of miRNA-mediated silencing are valid, or indeed if continuous interlaboratory experimental variation is obscuring the picture of a single unifying mechanism, which may include several of the current distinct models that are simply executed in a specific temporal order. Within this review, we will discuss the evidence for the emerging models for pre- and post-initiation silencing, with a particular focus on the growing role of m^{7}G-cap recognition and the involvement of classical translation initiation factors. We will then explore the latest evidence for the contribution of both translational repression and RNA degradation to miRNA-mediated silencing, discussing the key question of whether these two mechanisms are mutually exclusive or temporally linked, the latter therefore implying that a single unified process or model may be present.

Overview of currently proposed models of translational repression

Post-initiation silencing

Within the post-initiation silencing field, two proposed models have dominated: (i) ribosome dissociation and

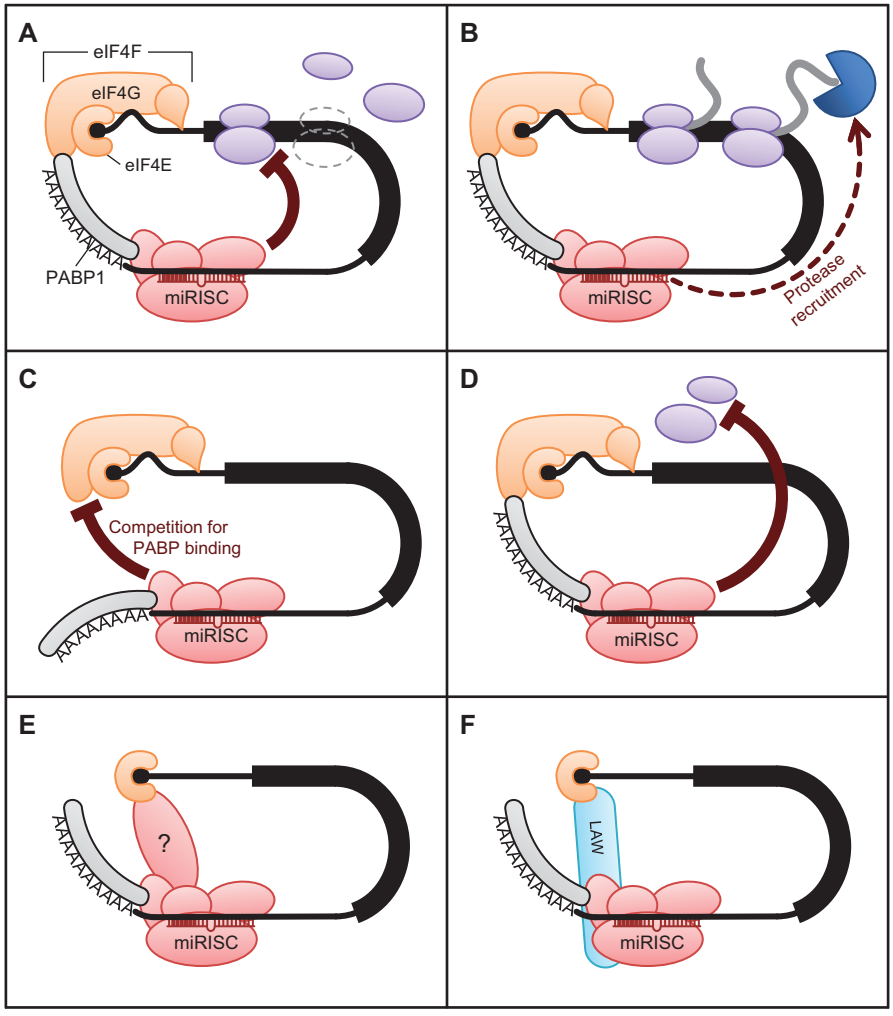

Figure 2 Current proposed models of miRNA-mediated silencing.
(A) Ribosome drop off. The interaction of RISC with the target mRNA causes rapid polysome dissociation repressing translation (25). (B) Co-translational degradation. Targeting by RISC causes the simultaneous degradation of the peptide as it is produced, likely by the recruitment of an as yet unidentified protease (24). (C) Formation of an inhibitory open loop complex. This model resides on evidence of the interaction between GW182 (TNRC6A-C) and PABP1, likely causing competition for eIF4G interaction with PABP1, preventing the assembly of closed-loop complex required for efficient translation (41, 42). (D) Inhibition of ribosome subunit assembly. The presence of RISC prevents the assembly of the ribosomal subunits preventing translation initiation, potentially by inhibition of eIF6 (37, 40). (E) Formation of an inhibitory closed loop. This model first hypothesised by Chan and Slack (43) proposes that RISC could compete for the CAP structure of targeted mRNA preventing the formation of the translation initiation complex. (F) Proposed model for the formation of an inhibitory closed-loop complex through LIMD1 and family member proteins Ajuba and WTIP (LAW). The LAW proteins have been identified as critical effectors of miRNA-mediated silencing and have the ability to interact with components of both RISC and the m^7G-cap complex (46), thus indicating that these proteins may function as a molecular scaffold between these complexes creating an inhibitory closed loop that prevents translation initiation.

(ii) co-translational degradation (Figure 2A,B). The evidence supporting these models has largely been derived from polysome sedimentation analysis, whereby mRNA and associated miRISC components are found to accumulate in polysome-occupied fractions. Early studies assessing targets of *lin-4* miRNA demonstrated the association of these targets with translating polysomes, which was accompanied by a reduction in protein levels during *Caenorhabditis elegans* larval development (20). Later studies in mammalian cell systems supported these findings and identified the presence of RISC components co-sedimenting with polysome fractions, all indicating a silencing mechanism occurring post-initiation (21–25).

A model of co-translational degradation was proposed to explain the above findings by Olsen and Ambrose (20) and Nottrott et al. (24). This model depicts the simultaneous degradation of a nascent peptide as it is produced (Figure 2B). However, to date, no candidate protease has been identified and no additional evidence supporting this model has been found. Furthermore, protection of polypeptides from proteolysis by targeting to the endoplasmic reticulum has been shown to have no effect on miRNA-mediated repression (26).

An alternative model proposed by Petersen et al. (25) implicates early ribosome dissociation or 'drop off'; this theory is supported by evidence of more rapid polysome dissociation from repressed mRNA than control non-repressed mRNA upon treatment of translation inhibitors (Figure 2A). However, as the mechanics of miRNA-mRNA target recognition are uncovered, it has become clear that the degree of translational repression may be dependent on the extent of miRNA site recognition, positioning and the number of sites within the mRNA (13–15, 17). This raises the possibility that co-sedimenting RISC may not always be repressing associated mRNA. Furthermore, Thermann et al. (27) identified the formation of pseudo-polysomes upon translational repression by *miR2* under conditions that prevent 60S ribosomal subunit joining and translation elongation. The pseudo-polysomes recapitulated the same sedimentation characteristics as polysomes and may provide some explanation for the presence of miRNA and RISC components in non-monosomal fractions.

Internal ribosome entry site – mediated translation

Further evidence in support of a post-initiation model has also been obtained from the manipulation of cap-independent translation driven by internal ribosome entry sites (IRES). Petersen et al. (25) demonstrated that translation driven by a hepatitis C virus (HCV) or cricket paralysis virus (CrPV) IRES could be silenced by miRNA. These findings were supported by Lytle et al. (28), where a comprehensive system of both plasmid-based reporter systems and *in vitro* transcribed RNA were used to show again that the presence of an HCV-IRES is not sufficient to overcome miRNA-mediated silencing. The limited number of classical translational factors required to initiate translation in these systems is highly suggestive of silencing occurring at a step independent of translation initiation. However, these findings are in stark contrast to those by Pillai et al. (26) and Humphreys et al. (29) who demonstrated *in vitro* transcribed HCV IRES-containing mRNAs to be resistant to miRNA-mediated silencing. There are several technical differences between the models used in these studies that may explain some of the conflicting findings currently within the literature (reviewed in Table 1). The method of transfection presents an initial problem. Lytle et al. (28) demonstrated differences in miRNA-mediated repression dependent on the use of cationic lipids or electroporation. Such differences may be the result of the increased transfection efficiency gained through use of cationic lipids, saturating the miRNA system and preventing repression being observed. Transfection of RNAs or plasmid DNAs could also affect observable miRNA repression, as transfected RNAs are likely to remain in the cytoplasm while transcription from plasmid DNA would generate the assembly of RNA-protein complexes that are exported into the cytoplasm; the latter may more closely recapitulate the functional biological complex with the reporter mRNA acquiring a correct and specific nuclear history. Finally, the use of a poorly functioning IRES, such as CrPV, shown to have only 2% of the translation efficiency of capped RNA, makes the determination of significant changes in cap-independent translation difficult (29). The use of such reporter systems, which themselves are not fully characterised, in multiple formats and cell types can only lead to more confusion rather than clarity at this stage. Further investigation of these complex systems is required to find a more conclusive answer.

Pre- and post-initiation silencing determined by genomic context

In support of the existence of both pre- and post-initiation translational repression, Kong et al. (30) identified that the promoter type may be intrinsic to the type of silencing that occurs. Transcripts derived from a Simian vacuolating virus 40 promoter, which contained Let-7 sites in the 3′UTR, were found to be efficiently repressed (translation efficiency reduced by 88%) and upon sucrose gradient density analysis fractionated with subpolysomes. In contrast, when an identical mRNA was derived from a thymidine kinase promoter, it was also repressed to a similar extent (translation efficiency reduced by 97%), but these repressed mRNA were found to associate predominantly with polysomes. These data support the presence of two mutually exclusive mechanisms of silencing occurring at different points of translation, the determination of which is reliant on the transcriptional history and promoter type of the mRNA itself.

Pre-initiation silencing

Current models of miRNA-mediated silencing at the point of translation initiation have centred upon inhibition or disruption of the assembly of translational initiation complexes, although more recent studies highlight rapid mRNA decay as a potential mechanism preventing translation. Effective translation of mRNA requires the presence of a 5′ m^7G-cap structure and a 3′ poly(A) tail. The recruitment of translation initiation factors, such as eukaryotic translation initiation factors 4 G and E (eIF4G/eIF4E) and poly(A)-binding protein (PABP) to these regions, respectively, allows circularisation of the mRNA into a closed-loop structure (31–33). This structure enhances ribosome recruitment and mediation of translation termination, as well as affords protection against decapping and degradation (34–36). Therefore, disruption of any of these factors effecting circularisation and ribosomal recruitment are predicted to inhibit translation initiation, eliciting the effect of miRNA-mediated silencing. Three models describing this theory are (i) prevention of ribosome recruitment, (ii) disruption of circularisation causing an open loop structure and (iii) formation of an inhibitory closed-loop complex through competition with translation initiation factors for the mRNA m^7G-cap structure.

Disruption of ribosome recruitment was demonstrated by Wang et al. (37) whereby *CXCR4* artificial miRNA were able to inhibit translation by preventing the recruitment of the 60S subunit, likely mediated by eukaryotic translation initiation factor 6 (eIF6) in rabbit reticulocyte lysate. Studies in *D. melanogaster* Schneider cells (S2) showed that depletion of eIF6 had no effect on miRNA-mediated translational repression, or miRNA-dependent degradation of luciferase reporter

Table 1 Comparison of IRES mediated translation studies.

Study	IRES type and generation	Experimental design	Conclusions of study	Comment
Humphreys et al. (29)	Appp-CrPV-Luc Appp-EMCV-Luc *In vitro* transcribed Transfection by cationic lipid reagent	Comparison of capped and polyadenylated mRNA +/- CXCR4 sites with A-cap, non-polyadenylated CrPV or EMCV IRES-containing mRNA +/- CXCR4 sites.	miRNA unable to overcome translation initiating from uncapped CrPV or EMCV IRES-containing mRNA. Identifying initiation as the target of miRNA silencing.	No comparison of translation efficiencies of different reporter constructs to demonstrate functionality of CrPV IRES. EMCV also used but only in comparison to A-cap transcripts.
Pillai et al. (26)	EMCV-Luc HCV-Luc m^7G-Luc *In vitro* transcribed Transfection by cationic lipid reagent	Uncapped EMCV or HCV containing luciferase mRNA containing Let-7 sites was tested. Controls included inhibition of endogenous Let-7 and co-transfection of capped-luciferase mRNA containing Let-7 sites.	IRES-driven luciferase translation not overcome by Let-7-mediated silencing. Only capped-luciferase mRNA was susceptible to silencing.	No comparison of the difference in translation efficiency between capped and IRES-driven reporters. However, both reporter types were compared in the same assay.
Walters et al. (47)	HCV-Luc 5′M^7G-HCV-Luc HCV-Poly(A)-Luc 5′M^7G-HCV-Poly(A)-Luc c-myc-Luc 5′M^7G -c-myc-Luc *In vitro* transcribed Transfection by cationic lipid reagent	Comparison of IRES reporters alone and with combinations of canonical features 5′Cap tail, all containing miR30 sites.	IRES-mediated translation alone insensitive to miR 30 activity. The presence of both 5′Cap and 3′ poly(A) tail mediates susceptibility to miR30 silencing. The presence of 5′Cap alone is sufficient to mediate susceptibility.	IRES reporters containing miR30 sites were compared in the presence of transfected miR21 or miR30, and changes in translation were determined by normalising between these two conditions. No direct comparison was made between IRES-containing and empty vector Luc reporters to determine translation efficiency of the HCV IRES.
Petersen et al. (25)	Discistronic HCV-Luc or CrPV-Luc (+/- CXCR4 sites) DNA transfected by cationic lipid reagent	Hippuristanol used to inhibit Cap-dependent translation, changes in upstream and downstream luciferase levels compared +/- treatment.	Use of hippuristanol caused a 20-fold reduction in cap-dependent translation and only a 2-fold decrease in IRES-mediated translation. Addition of CXCR4 repressed both cap-dependent and IRES-driven luciferase translation.	Protein yield of IRES-driven luciferase was set to one, and treatments were compared to this not discistronic vector only; no indication of translation efficiency of the IRES was given.
Lytle et al. (28)	HCV-Luc m^7G-HCV-Luc Appp-HCV-Luc (all containing Let-7 sites in 5′ positions) DNA and *in vitro* transcribed RNA, transfected by cationic lipid reagent and electroporation	Demonstrated HCV-Luc with Let-7 sites insensitive to silencing. However, when sites were placed in the 5′UTR silencing occurred. Let-7-mediated silencing occurred independent of Let-7 site position with m^7G and A- capped luc reporters.	Concluded that miRNA-mediated silencing occurs at a step downstream of translation initiation as HCV IRES-mediated translation was overcome by the presence of Let-7 sites. However, it remains unclear if this is dependent on the position of the site, as when sites were positioned in the 3′UTR and reporters were transfected as DNA by cationic lipid, translation was not silenced. This raises the question of whether transcription from a DNA plasmid allows assembly of a nuclear RNA-protein complex before export to the cytoplasm, which affects the silencing outcome, and whether this recapitulates the *in vivo* mechanism more faithfully.	Highlighted the differing results that can occur depending on the type of transfection used, particularly with the use of DNA plasmids. Results expressed relative to mRNA level to provide translation efficiency. However, normalisation is between reporters with Let-7 sites in the sense orientation compared with those in the antisense (expressed as 1); no comparison was made of the difference in translation rates of G-capped, A-capped or HCV-driven translation.

EMCV, encephalomyocarditis virus; HCV, hepatitis C virus; CrPV, cricket paralysis virus.

constructs fused with the 3′UTRs of *nerfin-1* or *CG11206* (38). In addition, depletion of eIF6 in *C. elegans* also had no discernible effect on *Let-7* activity (39). These contrasting findings may be due to the differences in the model systems used, particularly differing effects on poly(A) processing by the use of nuclease-treated reticulocyte lysate. However, the suggestion of ribosomal disassembly being important in miRNA silencing was supported by the work of Chendrimada et al. (40) in which they confirmed the co-immunoprecipitation of Ago2 with both eIF6 and large ribosomal subunits, thus potentially preventing ribosome assembly and suppressing translation (Figure 2C). These data support a mechanism of silencing that occurs at the start of translation, although a full understanding of the impact of these interactions with eIF6 directly on miRNA-mediated silencing is yet to be fully determined.

The requirement of a closed-loop mRNA structure to enable efficient translation has been the focus for two opposing models of translation repression at initiation. The first of these models suggests that competition between the key RISC silencing effector GW182 (trinucleotide repeat-containing gene 6A protein TNRC6A-C in humans) with translation factor eIF4G for PABP1 interaction prevents the creation of a closed-loop mRNA structure (41). Mutation of the PAM2 motif in TNRC6A-C abolishes the interaction with PABP1, consequentially impairing silencing activity (42). This open-loop formation would likely inhibit ribosome recruitment and translation initiation (Figure 2D). This repression of translation could be coupled either sequentially or simultaneously with the promotion of decapping and deadenylation, causing destabilisation and degradation of the mRNA (41). It is yet to be determined, but important to know, the degree to which translation is suppressed by the inhibition of the eIF4G and PABP1 interaction by GW182 alone, conceding that destabilisation and degradation of the mRNA may be the ultimate effector function of GW182 within the miRNA silencing pathway.

The second emerging model proposes that components of miRISC interact with the mRNA m^{7}G-cap structure either directly or *via* competition for translation initiation factors, such as eIF4E. This bridging between the 3′ bound RISC complex and the 5′-cap complex would create an alternative inhibitory closed loop preventing the assembly of the correct translation factors and inhibiting translation initiation, and was first proposed by Chan and Slack (43) (Figure 2E).

Kiriakidou et al. (44) reported the interaction of human Ago2 with m^{7}GTP-bound Sepharose beads, which could be competed out by an m^{7}GpppG analogue. This interaction was determined to be mediated by the presence of two phenylalanines in the central mid-domain of Ago2, as substitution of one or both phenylalanines disrupted Ago2 silencing ability. Kiriakidou et al. made the observation that the phenylalanines occur in a sequence similar to that of the m^{7}G-cap binding region of eIF4E, proposing that the phenylalanines may mimic the interaction that occurs between the methylated base of the cap structure and the two tryptophans in this region. However, Eulalio et al. (38) later determined that the mutation of these residues disrupted the interaction between Ago proteins and GW182, an interaction shown to be critical for mediating silencing. Therefore, it remains to be determined whether this observed interaction with the m^{7}G-cap structure is mediated *via* another region of Ago2 or indirectly by an unidentified intermediary protein. Subsequent studies modelling the mid-domain indicate the presence of a site able to bind nucleotides, such as m^{7}G-cap, and that allosteric control of this site dependent on miRNA binding provides specificity to such interactions (45).

Recent studies published by our group (46) also support the model of an inhibitory closed loop preventing translation initiation. We discovered the presence of a group of three novel proteins, LIM domain-containing protein 1 (LIMD1), Ajuba and Wilms' tumour 1 interacting protein (WTIP), that have a functional role in miRNA-mediated silencing yet are not required for the function of siRNA. Characterisation of their interaction partners revealed that all three proteins are able to interact with components of the RISC complex as well as translation initiation factors and m^{7}GTP Sepharose (possibly *via* eIF4E). The presence of LIMD1 was also found to substantially increase the amount of human Ago2 that associates with eIF4E, thus providing compelling evidence for a link between these factors and supporting a closed-loop complex hypothesis (46). These data support the existing models proposing that miRNA silencing is mediated through a block on translation initiation, indicating LIMD1 along with Ajuba and WTIP facilitate a link between the bound miRISC complex and the m^{7}G-cap structure, likely disrupting the assembly of the translation initiation complex and repressing translation initiation (Figure 2F). However, we have not been able to rule out the involvement of rapid mRNA degradation in the observed silencing effect. These observations fit well with previous findings demonstrating the importance of m^{7}G-cap recognition in mRNA processing and regulation, such as the work by Walters et al. (47) who demonstrated the presence of a cap structure to be sufficient to allow miRNA-mediated silencing to occur.

Together, these studies provide convincing evidence to support a model whereby inhibition of translation at the point of initiation *via* recognition of m^{7}G-cap, and prevention of the assembly of the translation initiation complex is important for mediating miRNA silencing. Whether this effect is universal to all mRNAs, or to all RISC complexes dependent on their constitutive components, remains to be elucidated.

However, despite strong evidence to support the role of m^{7}G-cap in silencing (which will be discussed in more detail in the next section), several studies challenge the view that simply blocking translation induces silencing. Several studies now report that miRNAs reduce target mRNA levels, making rapid mRNA degradation an important feature of the silencing effect. Wu et al. (48) report the ability of *Let-7* and *miR0125b* to enhance poly(A) removal, a step that leads to decapping and rapid degradation of targeted mRNA. Although foreseeably both a reduction in translation and increased deadenylation caused by miRNA could occur as a single event, the authors indicate they do not believe this is the case. They demonstrate that the poly(A) tail is not required for repression of translation; therefore, it is likely to be a mechanism independent of poly(A) shortening.

In agreement with the role of rapid deadenylation and degradation, more recent studies have utilised tethering studies to determine that all Ago proteins and TNRC6C can trigger mRNA decay. These findings highlight the intrinsic role of mRNA decay in promoting irreversible silencing and the use of miRISC to recruit the factors required (49). Furthermore, the widespread role of deadenylation was demonstrated by Izaurralde et al. (50) who confirmed that a poly(A) tail and thus mRNA circularisation were not required for this process.

These studies provide strong support for deadenylation and mRNA decay being the major terminal effect of miRNA silencing. However, as alluded to by Wu et al. (48), it is still not clear whether this process represents a separate mechanism that prevents or suppresses translation by rapid decay or whether it is linked to a block at translation initiation. This paradigm will be discussed further in subsequent sections.

Recognition of m^{7}G-cap

The data accumulating for a pre-initiation model contribute to a growing body of evidence that supports a model whereby the recognition of, and interaction with the m^{7}G-cap structure of mRNA may be a critical feature of miRNA-mediated silencing.

Several studies have highlighted the importance of the m^{7}G-cap structure when observing silencing or artificial mRNA. Humphreys et al. (29) demonstrated that the lack of a functional m^{7}G-cap structure reduces the efficiency of miRNA-mediated silencing (~2-fold repression) when compared with similar mRNA possessing an m^{7}G-cap (~5-fold repression) in cell culture studies. These conclusions have also been fully supported by cell-free systems (27, 51). In addition, the use of mRNA containing a viral IRES to create cap-independent translation has been found to be refractory to miRNA silencing, consolidating the importance of the m^{7}G-cap structure (26, 29). However, as previously highlighted, a degree of controversy exists in the literature over the interpretation of such results between contrasting studies; these are reviewed in Table 1. Recent findings by Walters et al. (47) may allude to a more definitive explanation. Walters et al. demonstrate that translation initiating from an HCV-IRES can be silenced by miRNA, but the presence of the m^{7}G-cap structure and polyadenylation factors are required to mediate this silencing. These data again denote the presence of the m^{7}G-cap structure and other features, such as the poly(A) tail to be critical in determining miRNAs' ability to silence. Further support for m^{7}G -cap recognition was discerned through the alleviation of silencing by *Let-7* upon the introduction of purified eIF4F complex to Krebs extract, effectively competing out miRISC targeting of the m^{7}G-cap structure (52). However, data concerning the requirement for the poly(A) tail is more varied. Repression of reporter transcripts has been observed to be stronger in the presence of a poly(A) tail (29), although this was not confirmed by others (26) and the possibility of *in vivo* polyadenylation of the transcripts was not ruled out. The requirement of a poly(A) tail was also deemed non-essential by Wu et al. (48) who demonstrated translation repression could still occur when the poly(A) tail of a target mRNA was replaced with a 3′ histone stem-loop.

To summarise, it would seem the formation of active translation initiation complexes at m^{7}G-cap structures appear an attractive target for miRNA-directed silencing, using RISC directly or associated proteins to block translation initiation. These data strongly support a direct role for cap recognition being required for the silencing process. However, it remains to be elucidated whether a mechanism, such as a blocking translation complex assembly would alone be sufficient to mediate silencing or whether this may represent an initial step that acts in concert with other observed mechanisms, such as recruitment of decapping and degradation factors and transport to processing bodies in a temporal fashion to induce a more complete and irreversible silencing effect.

Degradation and translational repression, independent or related mechanisms?

Evidence exists to support both targeting of translation at the point of initiation and repression of translation through the rapid decay of targeted mRNA. It is not yet clear whether these mechanisms are exclusive or dependent on each other, and if so whether they occur simultaneously or in a sequential manner. Clearly, our knowledge from canonical m^{7}G-cap-dependent deadenylation data support a variation on this theme, whereby m^{7}G-cap-dependent translation is inhibited by the initial shortening of the poly(A) tail, followed by either decapping and 5′-3′ degradation or 3′-5′ exonuclease decay (53, 54). Poly(A)-specific ribonuclease (PARN) is the best characterised mammalian deadenylase. During mRNA decay, PARN has been shown to interact with the 5′ m^{7}G-cap (55, 56). This interaction appears to increase the rate of poly(A) shortening. Studies carried out under serum-deprived conditions indicate that PARN may exert competition with eIF4E for the 5′ m^{7}G-cap, thus preventing translation and mediating deadenylation. This competition appears to be regulated by post-translational modification of both proteins under specific conditions (57). These findings again point to the close proximity between the 5′ and 3′ termini of the mRNA for efficient processing and indicate that the competitive interplay for these 5′-3′ interactions may be critical in regulating miRNA silencing and the fate of the silenced mRNA.

Clear and confirmatory evidence is yet to be obtained that will determine whether deadenylation and subsequent mRNA degradation is the predominant mechanism of miRNA-mediated silencing and whether this process is temporally linked to translation repression. Iwasaki et al. (58) used *Drosophila* embryo extract to demonstrate that both Ago1 and Ago2 mediate miRNA silencing but *via* different mechanisms. Ago1-mediated silencing utilised both repression of translation and deadenylation and decay promoted by Ago1 interaction with GW182. In contrast, Ago2 was unable to interact with GW182, but can, however, interact with eIF4E, creating direct competition with eIF4G for eIF4E and

repressing cap-dependent translation initiation. This mechanism is not directly conserved in mammals as Ago2 is not seen to directly interact with eIF4E (59). However, a similar mechanism could be envisioned whereby intermediary proteins could bridge the interaction of RISC with eIF4E (43). LIMD1, Ajuba or WTIP have already been shown to interact with both RISC and eIF4E, with the overexpression of LIMD1 increasing the association of Ago2 with eIF4E (46). Of note is the fact that LIMD1 also binds DCP2 and is likely linked to TNRC6A-C *via* interactions with RISC, indicating the engagement of the associated decapping and deadenylation factors. Such interactions would support the role of LIMD1, Ajuba and WTIP in miRNA-mediated mRNA degradation with a possible initial very transient inhibition of translation initiation. However, no evidence to support the involvement of LIMD1 and family members in the degradation pathway has yet been obtained.

GW182 is recognised as the major factor in deadenylation of target mRNA, through recruitment of the evolutionarily conserved CCR4-Not1 complex (59). Depletion of the CCR4-NOT components abolishes the degradation activity associated with GW182 interaction (50, 60, 61). In addition, several decapping (DCP1, DCP2) and enhancing (Ge-1, EDC3, Pat, Me31B) factors also critical for mRNA destabilisation have been found to associate with silenced mRNA and the miRISC complex (60, 62).

As previously discussed, Chen et al. (49) demonstrated the clear involvement of all four Ago proteins and TNRC6C in inducing deadenylation when tethered to target mRNA. The ability of miRNA and thus miRISC to promote this process has also been established (48).

Further, in support of mRNA decay as the terminal underlying mechanism of silencing in the mammalian system, Guo et al. (63) demonstrated that mRNA destabilisation alone could attribute to a ≥84% decrease in protein production from mRNAs targeted by miR155 or miR1. However, this study was unable to rule out the involvement of translational repression acting in concert with mRNA destabilisation or as an initial trigger for silencing. In addition, inhibition of the deadenylation process cannot completely alleviate silencing (59), suggesting translational repression may be an essential feature of the silencing process and possibly preceding mRNA degradation. This is strongly supported by the observation of Zekri et al. (41) that the interaction between GW182 and PABP1, to prevent translation initiation, needs to occur before deadenylation can commence. In addition, other studies suggest that the fate of silenced mRNA is context dependent on the miRNA-mRNA combination. *Dicer* mRNA targeted by *Let-7* is maintained at a constant level despite translation being silenced, potentially allowing the release of the mRNA to re-enter the translation cycle (64). It is unclear whether such examples of silencing being predominantly regulated by translational repression are rare occurrences; thus, broader investigations are needed to determine whether silenced mRNA undergo the same processes or whether the fate of the silenced mRNA is determined by additional as yet unknown factors that potentially relate to its transcriptional history.

Expert opinion

Considerable advances have been made to our understanding of the complex processes that promote miRNA-mediated silencing. The discovery of novel components and extensive studies characterising the functional impact of key interactions have greatly progressed our understanding of the importance of m^7G-cap recognition and subsequent rapid degradation of silenced mRNA. However, it seems there remains sufficient evidence supporting multiple alternative models that prevents a conclusive single model of miRNA-mediated silencing emerging from this growing list. Currently, it is unclear how the transcriptional history of an mRNA influences latter silencing (30), such that reporter systems and *in vitro* transcribed mRNA lacking such transcriptional histories may not fully mimic the *in vivo* silencing mechanism (28). Although it is conceivable that multiple models of silencing exist, it is also possible to speculate that such subtle differences in methodology as outlined have masked support for a single mechanism of silencing and that what we are really discovering/dissecting/analysing and assaying in these experiments are multiple parts of a single regulatory pathway that may be temporally linked. If this hypothesis were to be the case, then the following may form a plausible model as depicted in Figure 3.

New 'LAW' of miRNA-mediated silencing

Data generated by Kong et al. (30) indicate that the promoter type plays a role in determining the observed mechanism of silencing as being type I translation initiation, or type II post-initiation. We would speculate that the transcriptional history of the mRNA in part dictated by the promoter type controls the initial silencing mechanism used likely *via* the selective recruitment to the mRNA of ancillary proteins that interact with the core miRISC creating different type I or type II subtypes. Subsequently, the fate of the mRNA would be determined by the miRISC subtype; those dictating type II or post-initiation silencing would seem likely to immediately recruit decapping and deadenylase factors through GW182, triggering rapid destabilisation and decay of the mRNA preventing translation (41, 42, 59, 60, 63). Subtypes specifying type I or silencing at initiation may include, among other proteins, the previously identified factors LIMD1, Ajuba and WTIP (LAW). These factors enable a block on translation initiation, in the case of LIMD1 by facilitating an interaction between the core miRISC and the eIF4E/m^7G-cap complex (46). Following a block on translation initiation, the silenced mRNA could be recruited to P-bodies where they are subjected to mRNA decay *via* GW182 recruitment of CCR4-NOT1 or returned to translation by release of the translation initiation block by an as yet unidentified factor (62, 64–66). Although this overarching/unifying model of silencing is speculative, it starts to become possible to see how different mechanisms may interact and be captured experimentally if they occur at temporal points in a larger chain of events, particularly if movement through such events is dependent on the cell cycle or other homeostasis factors. This notion could

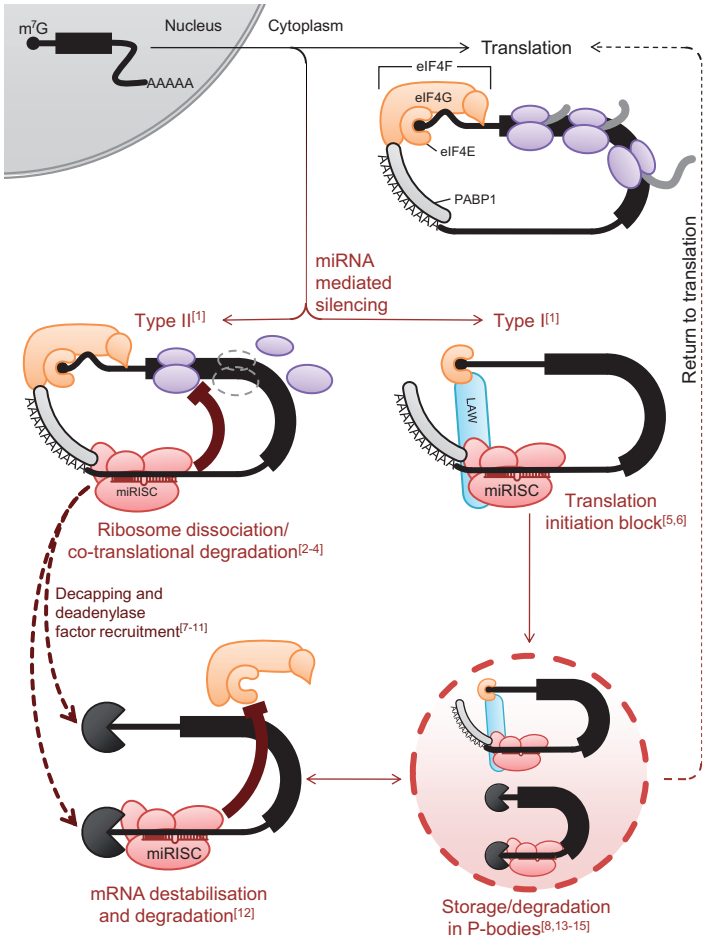

Figure 3 Proposed unifying model of miRNA-mediated silencing.
Transcriptional history dictates the subtype of miRISC associated with the target mRNA (30)[1]. Subsets of miRISC predisposing to a type II mechanism of silencing (20, 24, 25)[2–4] promote the recruitment of decapping and degradation factors to destabilise and degrade the mRNA (41, 42, 59, 60, 62, 63)[7–12]. Destabilised mRNA may or may not be stored in P-bodies to allow complete degradation to take place. Subtypes specifying a type I mechanism of silencing may include proteins, such as the newly identified LIMD1, Ajuba and WTIP, among others, to create a block on translation initiation (43, 46)[5,6]. Following a block on translation initiation, the silenced mRNA would likely be recruited to P-bodies and subjected to destabilisation and degradation, or stored until released back into the translating mRNA pool (62, 64–66)[8,13–15].

explain some contradictory findings, such as why inhibition of the deadenylation process alone has not been seen to fully repress silencing, while others have observed this process as a predominant feature (59, 63).

Outlook

Over the next decade, the continuing search for new components of the system and advances in our understanding of how these interactions form, and subsequently modulate each other to achieve functional miRNA-mediated silencing, will foreseeably begin to unravel the multiple- vs. single-model argument promoting mechanism(s) of silencing that account for the considerable variables and constraints of the system. The advancement of model systems and illumination of caveats within our current studies will hopefully begin to rectify the contradictions that currently exist and, together with the accompanying conceptual advances, will eventually explain how this highly refined and elegant system operates and begin to demonstrate how it interacts with other regulatory processes to control gene expression and an organism's homeostasis.

Highlights

- There is sufficient evidence supporting miRNA-mediated silencing acting at both translation elongation and initiation. To date, the most strongly supported set of models are those depicting silencing occurring at translation initiation.
- Mounting evidence supports the recognition of the m^7G-cap structure and/or translation machinery as a critical feature of miRNA silencing.

- Rapid degradation of targeted mRNA is seen to be the predominant consequence of silencing, although it is yet to be determined if this process alone is sufficient to prevent translation or is a secondary temporal or simultaneous event in the silencing process.
- We suggested that a single model of miRNA-mediated silencing may exist that links together several of the major models. Although speculative, future work should focus on not only understanding the complex nature of RISC interactions but also on the temporal element of this pathway.

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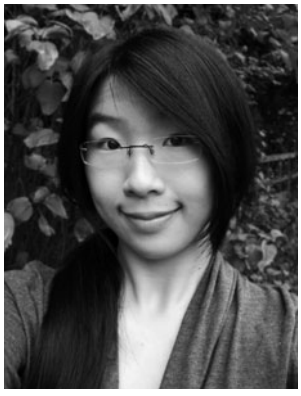

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