Leading Edge
Minireview
Cell

miRNAs Get an Early Start on Translational Silencing

Gunter Meister[1,*]
[1]Munich Center for integrated protein science (CiPSM), Laboratory of RNA Biology, Max-Planck-Institute of Biochemistry, Am Klopferspitz 18, 82152 Martinsried, Germany
*Correspondence: meister@biochem.mpg.de
DOI 10.1016/j.cell.2007.09.021

MicroRNAs (miRNAs) control gene expression by regulating mRNA stability and translation. Using cell-free in vitro systems, several labs have recently reported insights into the molecular mechanisms underlying miRNA-guided translational repression (Kiriakidou et al., 2007; Mathonnet et al., 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007). These new findings indicate that miRNAs inhibit translation at early steps of initiation.

MicroRNAs (miRNAs) constitute a class of small noncoding RNAs that have central roles in gene silencing and function as part of large gene regulatory networks. Although genes encoding miRNAs have been found in organisms as diverse as plants, animals, and viruses, the cellular functions of most of them are unknown. After transcription by RNA polymerases II or III, primary miRNA transcripts are processed into stem-loop-structured miRNA precursors that are further processed by the RNase III enzyme Dicer to double-stranded miRNA/miRNA* intermediates that are ~21 nucleotides long (Filipowicz et al., 2005; Zamore and Haley, 2005). Downstream unwinding and processing activities preferentially select one strand to become the mature miRNA, whereas the miRNA* strand is rapidly degraded. MicroRNAs specifically interact with members of the Argonaute (Ago) protein family and are incorporated into large ribonucleoprotein effector complexes termed miRNPs (Parker and Barford, 2006; Peters and Meister, 2007).

In animals, miRNAs hybridize to partially complementary binding sites typically located in the 3′ untranslated region (UTR) of target mRNAs and repress their expression. Efficient repression is either achieved by interfering with translation or by guiding processes for mRNA degradation that are initiated by deadenylation and decapping of the mRNA (Pillai et al., 2007). In plants and in rare cases also in animals, mRNAs contain highly complementary miRNA-binding sites and therefore miRNAs guide the sequence-specific cleavage of the mRNA in a process similar to RNA interference (RNAi) (Peters and Meister, 2007). Due to extensive efforts in recent years, the mechanistic details of sequence-specific RNA cleavage in RNAi are well understood. However, the molecular mechanisms underlying miRNA-guided translational repression and mRNA destabilization are only poorly characterized.

In recent work, four different groups have gained new insights into miRNA function by using cell-free in vitro systems (Mathonnet et al., 2007; Thermann and Hentze, 2007; Wakiyama et al., 2007; Wang et al., 2006). These findings mark an important turning point for the miRNA field, as these cell-free in vitro assays allow for a detailed biochemical dissection of the mechanisms underlying miRNA-guided gene silencing.

miRNAs Interfere with Translational Initiation

Although a steady stream of miRNA targets are being reported, the majority of mRNAs regulated by miRNAs remain unknown. Therefore, studies on the mechanism of miRNA-guided translational repression have been carried out using artificial luciferase constructs with imperfect miRNA-binding sites. Transfection of such reporters into cells demonstrated that efficient miRNA-guided translational inhibition requires a functional 7-methyl-guanine (m^7G) cap (Humphreys et al., 2005; Pillai et al., 2005). However, one study demonstrated that the presence of a poly(A) tail is required (Humphreys et al., 2005), whereas another paper reported that miRNA-guided translational repression is independent of a poly(A) tail (Pillai et al., 2005).

In order to investigate miRNA function in translation using biochemistry, Wang et al. (2006) utilized a rabbit reticulocyte lysate system and showed that miRNA-guided translational inhibition requires a functional m^7G-cap as well as a poly(A) tail (Wang et al., 2006). Now Wakiyama et al. (2007) have established a cell-free extract system derived from human embryonic kidney (HEK) 293 cells that overexpress known RNAi components, such as Ago2 and GW182. Human GW182, which is also known as TNRC6A, belongs to a family of tri-nucleotide repeat-containing proteins. TNRC6A and TNRC6B have been shown to be essential for miRNA-guided gene silencing in various organisms. Using a biotinylated capped and polyadenylated reporter mRNA containing six artificial binding sites in its 3′UTR for the miRNA *let-7*, they show that FLAG-tagged Ago2 and FLAG-tagged GW182 are specifically recruited to the reporter mRNA. In this cell-free system, efficient repression of translation requires an m^7G-cap

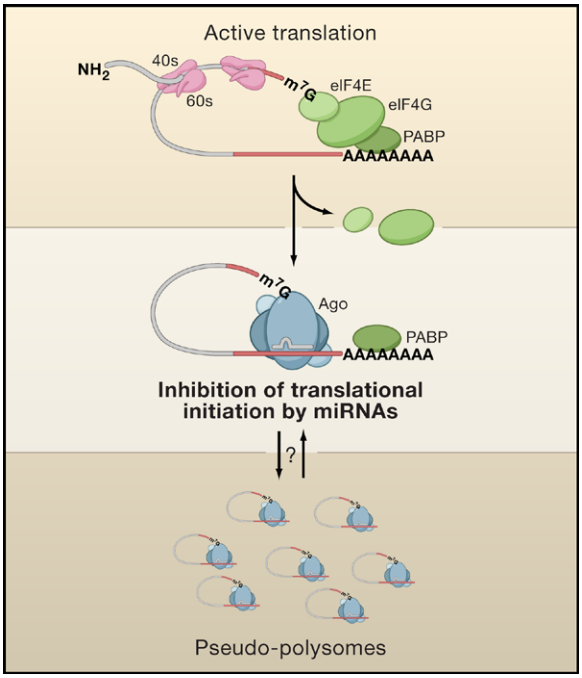

Figure 1. miRNA-Guided Repression of Translational Initiation
In the absence of miRNAs, the translation initiation factor eIF4E recognizes and binds to the 7-methyl-guanine (m^7G) cap of the mRNA. eIF4G binds to both eIF4E and the poly(A)-binding protein (PABP) and therefore allows for the establishment of a closed loop, which is required for efficient translation initiation. Upon miRNP binding to the 3′ untranslated regions (UTRs) of the mRNA, Ago proteins compete with eIF4E for cap binding. The interaction of Ago with the cap releases eIF4E/G and inhibits initiation. Repressed mRNA-protein assemblies (mRNPs) aggregate into larger structures termed pseudo-polysomes that might be disaggregated to allow for re-entry into active translation. AAA, poly(A) tail; NH_2, amino terminus of the nascent polypeptide chain.

as well as a poly(A) tail. Interestingly, it was found in the HEK293 cell lysates that the mRNA containing *let-7*-binding sites in its 3′UTR was efficiently deadenylated and that this was dependent on *let-7*. Deadenylation was independent of translation, as addition of cycloheximide, which blocks translation elongation, had no effect on deadenylation. However, translation efficiency was strongly correlated to deadenylation of the reporter mRNA (Wakiyama et al., 2007). Notably, Wakiyama et al. coexpressed GW182, a protein known to recruit deadenylation enzymes to mRNAs (Behm-Ansmant et al., 2006). miRNA function might therefore be biased towards deadenylation in this system, and it would be interesting to see if similar observations could be made in a wild-type HEK293 lysate.

The recent study by Sonenberg and coworkers utilized unmodified mammalian cell extracts (Mathonnet et al., 2007). Mathonnet et al. (2007) used extracts from mouse Krebs-2 ascites cells and luciferase constructs carrying six artificial *let-7*-binding sites to show that translation of the reporter is inhibited in a concentration-dependent manner reflecting the levels of endogenous *let-7*. By incubating radiolabeled mRNAs in cell lysates, the authors demonstrate that mRNA levels of the *let-7* reporter and a control reporter behave similarly, at least in the first 40 min of the reaction. Therefore, differences in activity are due to effects on translation. Interestingly, at later time points mRNA levels decrease and this effect is dependent on *let-7*, suggesting that inhibition of translation is an early event in miRNA-guided gene silencing and is at least to some extent followed by mRNA degradation. Sonenberg and coworkers then took advantage of their cell-free system to analyze the step of translation that is inhibited by *let-7*. Using glycerol gradients, they found that formation of the 80S ribosome complex is significantly reduced when the *let-7* reporter was used, demonstrating that *let-7* inhibits ribosome recruitment to the mRNA. Moreover, addition of recombinant eIF4F (the cap-binding complex) interfered with *let-7*-guided inhibition, indicating that miRNAs inhibit translational initiation by targeting the mRNA cap structure.

Argonaute Proteins Interact with the m^7G-cap of mRNAs

The data obtained from the in vitro assays by Mathonnet et al. (2007) and Wakiyama et al. (2007) raised the question of how miRNPs that are bound to the 3′UTR of a target mRNA interfere with translational initiation. By analyzing the amino acid sequence of human Ago proteins in detail, Mourelatos and coworkers recently reported in *Cell* significant progress toward answering this question (Kiriakidou et al., 2007). Ago proteins contain a highly conserved motif that shows similarity to the m^7G-cap-binding motif of eIF4E. Two amino acids with aromatic side chains that specifically bind the m^7G-cap are a characteristic feature of this motif. Indeed, tagged human Ago2 isolated from HEK293 cells efficiently interacted with m^7G-sepharose, whereas an Ago2 variant in which the two critical phenylalanines have been mutated did not. Interestingly, the Ago2 mutant retained the ability to cleave target mRNAs, indicating that the mutations did not alter Ago2 folding. Ago proteins inhibit translation when artificially tethered to the 3′UTR of mRNAs (Pillai et al., 2004). However, the Ago2 variant with mutations in the cap-binding motif no longer inhibited translation, indicating that the interaction of Ago proteins and the mRNA cap structure are required for translational repression. Based on these observations a model for miRNA-guided interference of translational initiation can be proposed (Figure 1). In this model Ago proteins compete with eIF4E for cap binding. Once an Ago protein is bound, the cap is no longer accessible for eIF4E and translational initiation is repressed.

miRNAs Induce Formation of Pseudo-Polysomes

Further mechanistic insights into miRNA-guided translational repression are provided by Thermann and Hentze (2007). They introduced six *miR-2*-binding sites of the *reaper* mRNA into the 3′UTR of a fire-

fly luciferase reporter and incubated it with *Drosophila* embryo lysates. Similar to the in vitro systems described above, translation of the *miR-2* reporter was inhibited by endogenous *miR-2*, and this inhibition required an intact cap structure of the mRNA. 80S complex formation on the reporter mRNA containing the *reaper miR-2*-binding sites was inhibited by *miR-2* as observed in mouse cell lysates. Interestingly, Thermann and Hentze realized that the *miR-2* reporter construct shifts toward heavier fractions in sucrose gradients when repressed by *miR-2*. Moreover, this shift occurred in the presence of cycloheximide, which blocks translational elongation and therefore the establishment of polysomes. Due to their comigration with polysomes in density gradients the authors referred to these structures as "pseudo-polysomes." A closer investigation revealed that *miR-2* is found in pseudo-polysomes and that these structures constitute large EDTA-sensitive mRNA-protein (mRNP) assemblies. What are pseudo-polysomes and how do they contribute to miRNA-guided gene silencing? Pseudo-polysomes appear to be large protein-mRNA aggregates, and therefore it is tempting to speculate that such assemblies might resemble cytoplasmic processing bodies (P bodies). P bodies are highly dynamic structures that can adopt different dimensions and have been implicated in miRNA function (Eulalio et al., 2007; Franks and Lykke-Andersen, 2007). EDTA sensitivity might indicate that pseudo-polysomes are reversible structures, which has been shown for P bodies as well, and suggests that repressed mRNPs that aggregate into pseudo-polysomes could disaggregate and return to active translation. This would explain recent findings that mRNAs targeted by miRNAs localize to P bodies and that miRNA-guided translational repression is reversible (Bhattacharyya et al., 2006). Notably, pseudo-polysomes have only been observed in the system that uses *Drosophila* embryo lysates, but not in extracts of mouse Krebs-2 ascites cells. It is therefore unclear whether this mechanism of miRNA-guided pseudo-polysome formation is a general phenomenon.

One surprising aspect of the work by Thermann and Hentze (2007) is that an intact m^7G-cap structure is not essential for the formation of pseudo-polysomes, as pseudo-polysomes also form on mRNAs containing artificial, nonfunctional cap structures (A-cap, ApppN) that do not support translational initiation. However, *miR-2*-guided repression of 80S complex assembly on the luciferase reporter mRNA strictly requires an m^7G-cap. These findings suggest that the formation of pseudo-polysomes and presumably also P bodies does not require active translational repression by miRNAs. miRNA binding to the 3′UTR and inhibition of translation initiation seem to be enough to form large pseudo-polysomes, even when the inhibition is independent of the miRNA pathway.

Unresolved Aspects of miRNA-Guided Translational Repression

The cell-free translation systems that have been recently reported clearly put forward a model in which miRNAs interfere with translational initiation. However, initial studies in *C. elegans* and more recent studies in mammals have shown that miRNAs cosediment with polyribosomes, which has led to the suggestion that miRNAs are involved in regulating translational elongation (Maroney et al., 2006; Nottrott et al., 2006; Olsen and Ambros, 1999; Petersen et al., 2006; Seggerson et al., 2002). In some of these studies, cosedimentation with polysomes was abolished after puromycin treatment, which disassembles polysomes. It has also been shown that nascent polypeptides are rapidly degraded when mRNAs are under miRNA regulation (Nottrott et al., 2006). Moreover, it has also been observed that ribosomes drop off mRNAs when miRNAs bind to the 3′UTR, which has led to a ribosome drop-off model of miRNA function (Petersen et al., 2006).

How could such discrepancies related to the function of miRNAs be explained? Although a role for miRNAs in initiation of translation is now well documented, it cannot be excluded that miRNAs also function during other steps of translation. It is reasonable to propose that miRNAs have distinct functions on different mRNAs or during different cellular or developmental stages. Notably, most studies on miRNA function have been performed with reporters containing artificial or isolated miRNA-binding sites. Such reporters are very helpful in elucidating the basic mechanistic steps of this process; however, these functions may differ in the context of large native 3′UTRs where location of the binding sites as well as the interplay with other regulatory proteins is important. In some studies, however, it is also possible that the comigration of miRNAs or protein components of the miRNA pathway with polysomes has been misinterpreted as polysome association. In light of the recent study by Thermann and Hentze (2007), such structures might be large miRNP aggregates or pseudo-polysomes.

Although still controversial, it is becoming increasingly apparent that at least one function of miRNAs is the inhibition of translational initiation. The reported data from the cell-free translation systems raise many new questions. How are pseudo-polysomes formed and how is this process regulated? How do such structures disaggregate to allow for re-entry into active translation? What are the protein components of pseudo-polysomes and how are they related to P bodies? The use of these new biochemical systems in conjunction with proteomic approaches and other cell biological studies will help to further elucidate miRNA function.

ACKNOWLEDGMENTS

I thank A. Frohn, C. Ender, L. Weinmann, and M. Kiebler for helpful discussions. Our research is supported by the Max-Planck-Society, the Deutsche Forschungsgemeinschaft, the Deutsche Krebshilfe, and the European Union.

REFERENCES

Behm-Ansmant, I., Rehwinkel, J., Doerks, T., Stark, A., Bork, P., and Izaurralde, E. (2006). Genes Dev. *20*, 1885–1898.

Bhattacharyya, S.N., Habermacher, R., Martine, U., Closs, E.I., and Filipowicz, W. (2006). Cell *125*, 1111–1124.

Eulalio, A., Behm-Ansmant, I., and Izaurralde, E. (2007). Nat. Rev. Mol. Cell Biol. *8*, 9–22.

Filipowicz, W., Jaskiewicz, L., Kolb, F.A., and Pillai, R.S. (2005). Curr. Opin. Struct. Biol. *15*, 331–341.

Franks, T.M., and Lykke-Andersen, J. (2007). Genes Dev. *21*, 719–735.

Humphreys, D.T., Westman, B.J., Martin, D.I., and Preiss, T. (2005). Proc. Natl. Acad. Sci. USA *102*, 16961–16966.

Kiriakidou, M., Tan, G.S., Lamprinaki, S., De Planell-Saguer, M., Nelson, P.T., and Mourelatos, Z. (2007). Cell *129*, 1141–1151.

Maroney, P.A., Yu, Y., Fisher, J., and Nilsen, T.W. (2006). Nat. Struct. Mol. Biol. *13*, 1102–1107.

Mathonnet, G., Fabian, M.R., Svitkin, Y.V., Parsyan, A., Huck, L., Murata, T., Biffo, S., Merrick, W.C., Darzynkiewicz, E., Pillai, R.S., et al. (2007). Science published online July 26, 2007. 10.1126/science.1146067.

Nottrott, S., Simard, M.J., and Richter, J.D. (2006). Nat. Struct. Mol. Biol. *13*, 1108–1114.

Olsen, P.H., and Ambros, V. (1999). Dev. Biol. *216*, 671–680.

Parker, J.S., and Barford, D. (2006). Trends Biochem. Sci. *31*, 622–630.

Peters, L., and Meister, G. (2007). Mol. Cell *26*, 611–623.

Petersen, C.P., Bordeleau, M.E., Pelletier, J., and Sharp, P.A. (2006). Mol. Cell *21*, 533–542.

Pillai, R.S., Artus, C.G., and Filipowicz, W. (2004). RNA *10*, 1518–1525.

Pillai, R.S., Bhattacharyya, S.N., Artus, C.G., Zoller, T., Cougot, N., Basyuk, E., Bertrand, E., and Filipowicz, W. (2005). Science *309*, 1573–1576.

Pillai, R.S., Bhattacharyya, S.N., and Filipowicz, W. (2007). Trends Cell Biol. *17*, 118–126.

Seggerson, K., Tang, L., and Moss, E.G. (2002). Dev. Biol. *243*, 215–225.

Thermann, R., and Hentze, M.W. (2007). Nature *447*, 875–878.

Wakiyama, M., Takimoto, K., Ohara, O., and Yokoyama, S. (2007). Genes Dev. *21*, 1857–1862.

Wang, B., Love, T.M., Call, M.E., Doench, J.G., and Novina, C.D. (2006). Mol. Cell *22*, 553–560.

Zamore, P.D., and Haley, B. (2005). Science *309*, 1519–1524.