



## Review

## The art of microRNA: Various strategies leading to gene silencing via an ancient pathway

Guiliang Tang<sup>a,\*</sup>, Xiaoqing Tang<sup>b</sup>, Venugopal Mendu<sup>a</sup>, Xiaohu Tang<sup>a</sup>, Xiaoyun Jia<sup>a</sup>,  
Qi-Jun Chen<sup>a</sup>, Liheng He<sup>a</sup>

<sup>a</sup> Gene Suppression Laboratory, Department of Plant and Soil Sciences and KTRDC, University of Kentucky, Lexington, KY 40546, USA

<sup>b</sup> Department of Molecular and Cellular Biochemistry, University of Kentucky College of Medicine, 741 South Limestone, Lexington, KY 40536, USA

## ARTICLE INFO

## Article history:

Received 11 February 2008

Received in revised form 25 May 2008

Accepted 13 June 2008

Available online 20 June 2008

## Keywords:

miRNA

RISC

Target cleavage

Translational repression

## ABSTRACT

MicroRNAs (miRNAs), an endogenous type of small RNAs of ~22 nucleotides (nt), have long resided in the cells of plants and animals including humans, constituting an ancient pathway of gene regulation in eukaryotes. They have a simple structure in their mature form but carry enormous information that may regulate up to 90% of the human transcriptome. Furthermore, the multi-facets of a miRNA are tightly associated with diverse cellular proteins that make it broadly connected to various physiological and pathological processes. This review aims to examine miRNAs briefly from their biogenesis to their general functions with an emphasis on working mechanisms in regulation of their target mRNAs.

© 2008 Elsevier B.V. All rights reserved.

## 1. Introduction

MicroRNAs (miRNAs) are nature's gifts that biologists are just realizing play an important role in our cells. The last decade has witnessed a major paradigm shift regarding gene regulation by small RNAs with the discovery of RNA interference (RNAi) [1]. RNAi has now revolutionized modern biology theoretically, technologically, and practically, without which miRNAs would have remained obscure for a longer period. Biologists have fortunately accumulated enormous knowledge and approaches for the coming of the miRNA era since the elucidation of double-strand DNA helix in 1953 [2]. In just a few years, the lineage of a miRNA pathway has been extensively investigated in various species of both the plant and the animal kingdoms.

The coming of the miRNA era has brought a fortune to many medical scientists who intentionally or unintentionally found that this type of small RNA relates to research topics such as developmental timing, neuronal cell fate, apoptosis, proliferation, hematopoiesis, cancer development, neurological disorders, insulin secretion or exocytosis of the secretory pathway, metabolic disorders, B-cell development, adipocyte differentiation, cardiovascular disease, liver diseases, immune dysfunction, viral infection, and stem cell differentiation [3–6]. As a result, billions of dollars have been invested in human health research in association with miRNAs through the National Institute of Health, local governments and many private companies.

MiRNAs are also invaluable to plant scientists. Plant development has a tight relationship with miRNAs [7]. Many gene transcription factors that were previously revealed to control cell division, expansion, growth, differentiation, aging, organ identities, developmental phase transition, and plant hormone signaling turned out to be under the control of miRNAs [8,9]. Thus, miRNAs are indispensable to agricultural crops, because crop yield and quality in domestication [10] are directly or indirectly controlled by the miRNA-directed regulation of these transcription factor genes, some of which were implicated to be part of quantitative trait loci (QTL) in relation to crop production [10,11]. This review discusses the making of a miRNA, their intrinsic information, their modes of actions, and finally their wide variety of connections with cellular processes.

## 2. Silencing trigger: The biogenesis of a miRNA

## 2.1. The biogenesis of a canonical miRNA

Although the biogenesis process of a miRNA is a little different between plants and animals, the overall structures of their gene transcripts are similar. Most miRNA genes are located at intergenic regions [12], some are located inside introns of protein-coding genes [13,14]. MiRNA genes are transcribed into RNAs mostly by RNA polymerase II (pol-II) and in a few cases by RNA polymerase III (pol-III) [15,16]. The primary transcripts of a miRNA are usually termed primary miRNAs (pri-miRNAs). The processing of pri-miRNAs into miRNA precursors (pre-miRNAs) differs between plants and animals. In animals, pre-miRNAs are transported into cytoplasm before being processed into

\* Corresponding author.

E-mail address: [gtang2@uky.edu](mailto:gtang2@uky.edu) (G. Tang).

mature miRNAs by completely different sets of enzyme complexes. In contrast, plant pre-miRNAs remain in the nucleus and are processed by the same enzyme complex, resulting in mature miRNA duplexes inside the nucleus [17]. MiRNA duplexes are then transported into the cytoplasm to be assembled into RNA silencing effector complexes.

## 2.2. The biogenesis of a mirtron

Recently, it has been reported that the biogenesis of some pre-miRNAs in *Drosophila*, *Caenorhabditis elegans*, or even mammals can bypass the nucleus Drosha/Microprocessor by directly adopting spliced introns, and the mature miRNAs from these introns are thus termed “mirtrons” [18–20]. In the biogenesis of mirtrons, the primary miRNA transcripts are shared with the transcripts of specific protein-coding genes. The same promoter and terminator elements thus control the expression of both the coding genes and the mirtrons. After transcription, the spiced introns are debranched and folded back into “stem-loop” structures or “mirtronic pre-miRNAs (pre-mirtrons)” that are characteristic of the canonical pre-miRNA produced by Drosha/Microprocessor [18–20]. Pre-mirtrons are exported from nucleus into cytoplasm by sharing the same nuclear pre-miRNA transporter termed “Exportin-5” in *Drosophila* [19]. Mirtron pathway is an alternative route for miRNA biogenesis, but it is unclear whether mirtrons are widespread in evolution. The limited numbers of mirtrons in limited organisms suggest that mirtron pathway might be a prototype avenue for miRNA biogenesis before Drosha emergence in some organisms [18].

## 2.3. Proteins and their complexes for miRNA biogenesis

The protein components that process miRNA precursors to mature miRNAs are correspondingly different between plants and animals. In animals, the enzyme responsible for the production of pre-miRNA is the nuclear protein complex Microprocessor, composed of Drosha, a major catalytic enzyme of RNase-III family that cleaves pri-miRNA into pre-miRNA [21], and its partner “Pasha”, a dsRNA-binding protein that may stabilize the Drosha in the Microprocessor. Human Pasha was previously identified in the study of the human disease DiGeorge Syndrome (DGS)/Conotruncal Anomaly Face Syndrome (CAFS)/Velocardiofacial Syndrome (VCFS) and thus has a different name “DiGeorge Critical Region-8” (DGCR8) [22–24]. Pre-miRNAs are then transported from nucleus to cytoplasm by Exportin-5 [25–29]. Pre-miRNAs are further processed by Dicer enzyme complex into miRNA duplexes in cytoplasm. Similar to Drosha, human Dicer also has two alternative partners namely “transacting responsive (TAR) RNA element (from HIV-1)-binding protein” (TRBP) [30,31] and protein activator of interferon-induced protein kinase (PKR), PACT [32], to help process pre-miRNAs into mature miRNAs and deliver them to downstream proteins.

The nature of miRNA processing protein complexes and their components is reflected by the structures of their substrate pri-miRNA or pre-miRNA. Pri-miRNA and pre-miRNA are characterized by their shared double-stranded (ds) “core” region or “stem-loop” structure. Indeed, both Drosha and Dicer belong to double-stranded RNA (dsRNA) specific enzymes of RNase III family that cut the double-stranded “stem” region successively to produce the mature miRNA duplex.

Although Dicer and Drosha contain dsRNA-binding domain(s), these RNase III enzymes need additional dsRNA-binding protein partners to function. The interaction between RNase III enzymes (Dicer or Drosha) and their partners appears to be conserved from plants to animals. In *Drosophila*, Dicer 2 (DCR-2) and its partner R2D2 [33], a dsRNA-binding protein having 2 dsRNA-binding domains and associated with DCR-2, form complexes to function in the RNAi pathway [34]. On the other hand, *Drosophila* Drosha interacts with its partner dsRNA-binding protein Pasha [35] in the nucleus, and Dicer 1 (DCR-1) interacts with its partner R1D3 (also named *loqs*) [36–38], a dsRNA-binding protein having 3 dsRNA-binding domains and associated with DCR-1 in cytoplasm, forming complexes to function in the miRNA pathway.

*C. elegans* has only one Dicer that functions in both RNAi and miRNA pathways. Worm Dicer interacts with its partner dsRNA-binding protein named RNAi deficient 4 (RDE-4) in the cytoplasm [39–40] functioning in siRNA pathway. However, RDE-4 does not seem to be required for the biogenesis of miRNAs since no apparent developmental defect was observed in *rde-4* mutant and no miRNA predicted target genes seemed upregulated in *the mutant* by microarray analysis [42]. The presence of dsRNA-binding protein as Dicer partner for miRNA biogenesis in worms needs further investigation. Nevertheless, it is clear that worm Drosha does require its partner Pasha in nucleus for the miRNA biogenesis [35]. Similarly, mammals also have only one Dicer. However, mammalian Dicer does have its dsRNA-binding protein partner TRBP/PACT for miRNA biogenesis [30–32,43], in collaboration with Drosha and Drosha partner DGCR8 [23,24].

Likewise in plants, HYPONASTIC LEAVES1 (HYL1), another dsRNA-binding protein, interacts with plant Dicer 1 (DCL1) [17,44–47]. DCL1, HYL-1, and other proteins, such as the zinc-finger-domain protein Serrate (SE) [48], colocalizing in discrete nuclear bodies termed nuclear dicing bodies (D-bodies) [17,45] that are similar to animal Microprocessors in miRNA biogenesis but different from the previously reported Cajal bodies in which the biogenesis of Argonaute 4 (AGO4)-associated siRNAs was implied [49,50]. Thus, dsRNA-binding proteins either stabilize Dicer proteins for small RNA production (e.g., miRNA biogenesis) or help the small RNAs to be delivered to downstream protein complexes, such as RNA-induced silencing complexes (RISCs) [34,36].

In addition to the role of Dicer and its dsRNA-binding partner in miRNA biogenesis, recent data suggests that AGO proteins may also be involved in miRNA biogenesis before the second step of miRNA maturation. Evidence came from the observation that AGO2 is recruited before the Dicer cleaves the pre-miRNA into mature miRNA, resulting in the production of AGO2-cleaved pre-miRNA (ac-pre-miRNA) [51]. The AGO2 cleavage site on the pre-miRNA is comparable to the cleavage position of the passenger strand of a siRNA duplex by the guide-associated RISC in RNAi [52–56]. Interestingly, Ac-pre-miRNA is still a good substrate for Dicer to produce mature miRNA that can be further assembled into miRNA-associated RISC. This observation further demonstrates that proteins of the miRNA pathway may interact with each other much earlier than appreciated and miRNA biogenesis involves both upstream (e.g., Dicer) and downstream (e.g., AGO) proteins.

## 2.4. Methylation of miRNAs in plants

Different from animal miRNAs, plant mature miRNAs are further methylated on the 2' hydroxyl group of the 3' terminal ribose by Hua Enhancer (HEN1), a plant miRNA or siRNA methyltransferase [57–59]. Methylation of miRNAs was not found in animals, whereas 2'-O-methylation of PIWI-associated siRNA (piRNA) by HEN1 homologs was similarly observed in *Drosophila* and mice [60–62]. The function of the methyl group at the 3' end of miRNAs in plants was proposed to stabilize miRNAs or prevent the miRNA serving primers for RNA-dependent RNA polymerase in cells [59]. Mutation of HEN1 led to oligouridylation generated by an unidentified enzyme at the 3' end of the miRNAs, which was thought to trigger the degradation of the uridylylated miRNAs in plants [63].

## 3. Effector complex: The making of a miRNA associated protein complex (miRNP)

### 3.1. Functional miRNA and miRNA\* are protected by miRNPs

A single-stranded miRNA may not be stable without forming a complex with cellular proteins. MiRNA duplexes (miRNA/miRNA\*) are produced by Dicer complexes and also need Dicer complexes to be assembled into effector complexes commonly termed miRNPs or sometimes miRNA associated RISCs (miRISCs) that contain at least a single-stranded miRNA and an AGO protein. Due to evolutionally

formed asymmetric structures (thermodynamic stability of the miRNA duplex structures) of pre-miRNAs, the majority of miRNA duplexes have a preference for the miRNA strand to be assembled into and protected by the miRNP, while the miRNA\* strand is excluded from miRNP and subsequently degraded [64,65].

Small RNA cloning and deep sequencing showed that both strands of many miRNA duplexes actually exist in specific type of cells and many of the miRNA\*s are assembled into miRNPs and target endogenous gene transcripts for regulation [66–68]. This phenomenon is likely due to the symmetric structures of some miRNA/miRNA\* duplexes or a switch of asymmetric arms of different specific miRNA family members in RISC assembly in different tissues. In some cases, the abundance of miRNA and miRNA\* populations in different tissues may not always follow the asymmetric structures of the miRNA/miRNA\* duplexes [67]. This suggests that other unidentified cellular proteins may function as catalytic factors for such unconventional strand selection. Alternatively, miRNA/miRNA\* duplex intermediates are somehow stabilized in cells/tissues waiting for asymmetric unwinding for miRNP assembly and are thus caught by small RNA deep sequencing. In summary, miRNA\* assembly into miRNPs may still be largely governed by thermodynamic rules [64,65] and the gene regulation directed by miRNA\* strands seemingly constitutes a more significant part of miRNA biology than previously appreciated [68].

### 3.2. The making of a miRNP is a sequential process

The making of a RISC is not an isolated process. Initially, RISC activity was biochemically purified from *Drosophila* S2 cells and subsequent protein sequencing of the active complex resulted in the discovery of a key RISC component AGO2 [69]. T7/His tagged recombinant AGO2 was found to be associated with ~ 22 nt luciferase siRNAs by Western and Northern blot analyses [69]. Later, biochemical results from various organisms indicated RISC assembly as a fairly instant process that could be understood only in the context of upstream and downstream biochemical processes [43,70–74].

As mentioned above, Dicer does not always need its partner, dsRNA-binding protein, to produce siRNAs and/or miRNAs [33,34,36–38]. However, the dsRNA-binding protein functions as an important sensor in determining the strand selection of small RNA duplexes in RISC assembly [74]. This strand selection and subsequent asymmetric assembly of RISCs, as determined by R2D2, was beautifully demonstrated in *Drosophila* RNAi pathways [74]. This work further implied that miRNA associated RISC assembly might also follow a similar rule [38,75]. It is now known that a RISC assembly needs the upstream Dicer proteins and their partners, and the downstream “core” proteins, the AGOs. Biochemical evidence indicates that such a RISC assembly process can be simplified into two steps: the RISC loading process that forms a RISC loading complex (RLC) and the RISC maturation process that forms a mature RISC [74,76]. RLC is a premature RISC, components of which have not been fully characterized.

### 3.3. “Core” RISCs and “holo” RISCs

UV-crosslinking, gel filtration, gel electrophoresis, and immunoprecipitation analyses have revealed the existence of both simple and complicated RISC complexes in cells [43,71,72,74,77]. The smallest or minimal RISC, sometimes named “core” RISC [76], may be constituted of only two components, an AGO protein and a small RNA [73,78]. In contrast, the largest RISC termed “holo” RISC is often composed of most RNAi or miRNA pathway proteins including Dicer, dsRNA-binding protein, RNA helicase, AGO, and proteins from other pathways, such as ribosomal proteins and specific disease pathway proteins [71]. With detailed dissection of mechanisms of miRNA-directed gene silencing, increasing evidence suggests that “holo” and “core” RISCs may play differential roles in miRNA-directed translational regulation and interactions between miRNA and many other pathways.

## 4. The gene regulator: a simple structure containing enormous information

### 4.1. “miRNA code” and its difference between plants and animals

The prediction that one-third of the human transcriptome is under the control of miRNAs, via a “seed” region to target the 3′ UTRs of transcripts [79,80], points to the fascinating information a miRNA carries. The puzzling information of a miRNA of ~21 nt is preferably referred to as a “miRNA code” in this article. The “miRNA code” embedded in the miRNA sequences is apparently different between plants and animals. In plants, a full or extensive sequence complementarity between a miRNA and the target mRNAs is necessary for miRNAs to regulate their target genes [9,81]. Thus, plant miRNAs encode very specific information to target limited numbers of their target genes.

In contrast, a miRNA code embedded in an animal miRNA is overwhelmingly determined by the “seed” region of 7 nucleotides between positions 2 and 8 from the 5′ end of the miRNA [79,80]. This feature greatly enlarges the intrinsic information of a miRNA in animals. Consequently, an animal miRNA is estimated to regulate about 200 target genes through translational repression mechanisms via the regulatory regions on the 3′ UTRs of the transcripts [79,80,82]. If the 5′ UTRs and coding regions are considered as targeting sites by miRNAs, up to about 90% of the human transcriptome will be regulated by the miRNAs [83]. To make it more complex, the same target gene transcripts can have more than one miRNA targeting sites that can be regulated by the same or different miRNAs [82].

### 4.2. The complexity of a “miRNA code” in animals

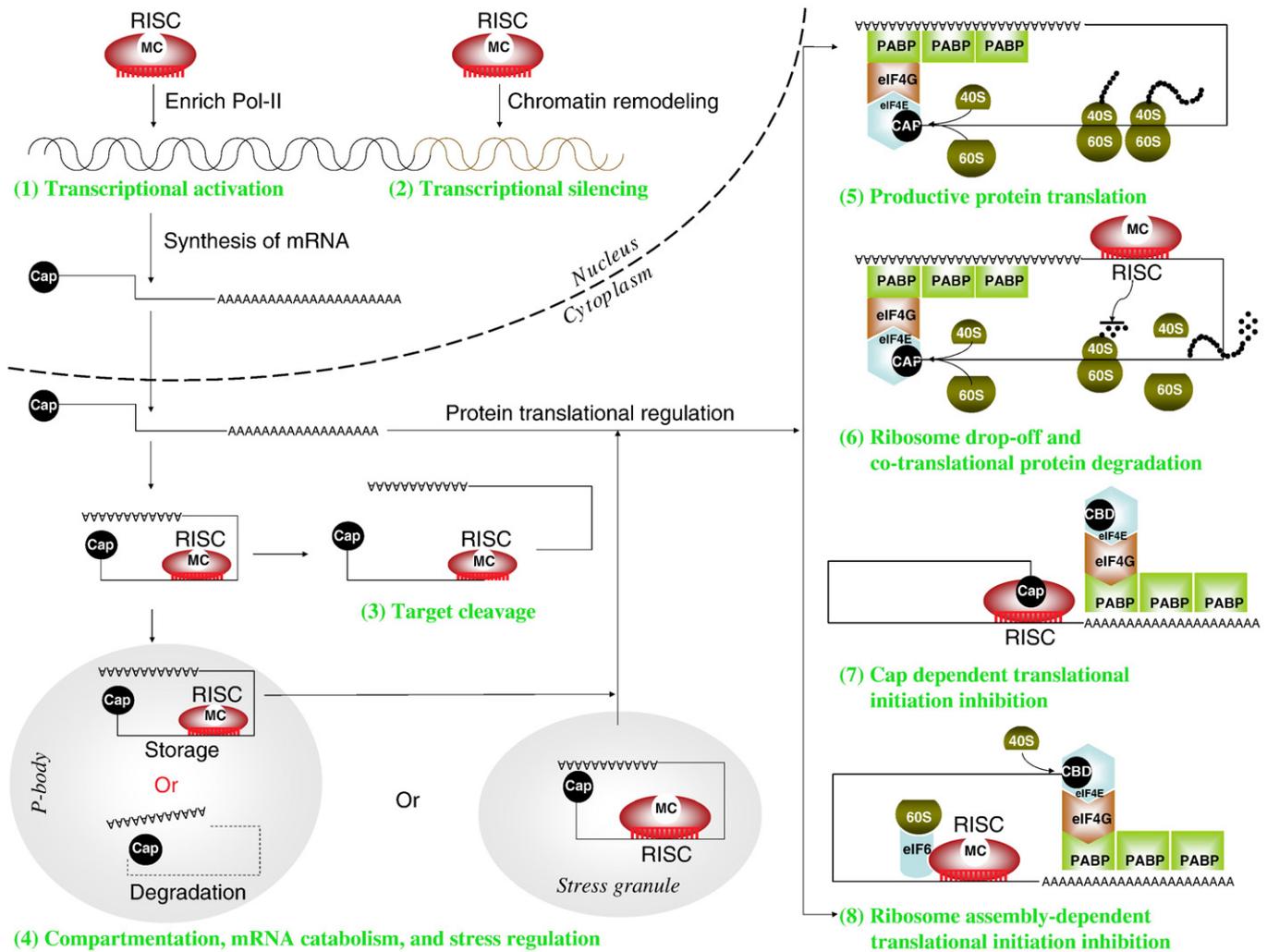
Not all the predicted miRNA targets are actually the *bona fide* targets; this suggests that information other than that from the “seed” region also contributes to the recognition between a miRNA and its targets. Additional determinant information could be found from regions other than the “seed” on a miRNA, from targets outside the miRNA binding regions, or from cellular protein factors that interact with miRNAs or miRNA targets. A well-studied example of this case is the miRNA let-7 mediated down regulation of its target gene *lin-41* in *C. elegans* [84].

In worms, let-7 molecules imperfectly bind to the 3′ UTR of *lin-41* at two regions termed let-7 complementary sites (LCS1 and LCS2) linked by 27 nt intervening sequence (abbreviated as LCS1-27-LCS2). Point mutation and LCS swap and recombination experiments demonstrated that residues outside of the “seed” region of the let-7 binding site and the context of the LCSs are critical for let-7 mediated suppression of *lin-41* [84]. First, a modification of the endogenous architecture “LCS1-27-LCS2” to “LCS1-27-LCS1” or “LCS2-27-LCS2” on the 3′ UTR of *lin-41* disrupted the regulatory role of let-7. Second, mutations of Non-Seed region, including the 5′ and 3′ base pairs, the bulged and looped residues, and 5′ overhangs, disrupted let-7 directed down regulation of *lin-41*. Finally, either shortening the length of the 27 nt spacer to 4 nt or 12 nt linker or changing the 27-nt specific sequence without altering the spacer length disrupted the down regulation of the reporter gene by let-7. Thus, the specific LCS1-27-LCS2 architecture including Non-Seed regions and the 27 nt spacer, is important for miRNA-directed target gene regulation at least in the case of *let-7::lin-41* interaction [84]. This case also seems true in humans [85].

## 5. Actions: All paths lead to a silencing destination

### 5.1. Two basic modes of action: miRNA-directed mRNA cleavage/instability and translational repression

The actions of miRNAs on their targets are most fascinating. Early studies have generalized two basic modes of actions of miRNAs on their targets: target cleavage and target translational repression.



**Fig. 1.** Various strategies of regulation of gene expression by miRNAs. MiRNA-directed regulations of gene expression are from nucleus to cytoplasm. MiRNA associated RISCs are the key players in these processes: (1) transcriptional activation, (2) transcriptional gene silencing, (3) target mRNA cleavage, (4) compartmentation, mRNA catabolism, and stress regulation, (5) productive protein translation without miRNA regulation, (6) ribosome drop-off and cotranslational protein degradation, (7) Cap-dependent translational initiation repression, and (8) ribosome assembly dependent translational initiation repression. RISC, RNA-induced silencing complex; MC, a motif at the AGO Middle domain (Mid); Pol-II, RNA polymerase II; Cap, the 5' end structure of a mRNA; PABP, poly(A) binding protein; eIF4G, eukaryotic translation initiation factor 4G; eIF4E, eukaryotic translation initiation factor 4E; 40S, ribosome small subunit; 60S, ribosome large subunit; CBD, Cap binding domain; eIF6, eukaryotic translation initiation factor 6.

There are several factors that determine whether a target mRNA will be cleaved or repressed for translation by a miRNA (for reviews see [76]). First, the cleavage of a miRNA target mRNA is executed by AGO proteins. AGO proteins have endonuclease or “slicer” activities conferred by the PIWI domain [56,86,87]. Some AGOs retain the slicer activities but others lose the activity during evolution [86]. Thus, only miRNAs assembled into AGOs that have slicer activities will have the potential to direct their target mRNAs for cleavage, while miRNAs associated with AGOs without slicer activities will likely function to suppress the target mRNA translation. Second, even if miRNAs are associated with AGOs with slicer activities, the cleavage of the target mRNAs will still depend on the complementary conditions between the miRNAs and their targets. Only full or extensive complementarity will result in a cleavage of the target mRNAs, while partial complementarity, especially no complementarity around the slicer cleavage site (a position between the nucleotides paired to base 10 and 11 of the miRNA from the 5' end), will likely mediate target mRNA translational repression [88].

Although translational repression was reported in a few cases [89–91] and recent data demonstrated it to be widespread and genetically separable from target cleavage [92], the majority of plant miRNAs were thought to mediate the cleavage of their target mRNAs. In

contrast, animal miRNAs act on, in most cases, target translational repression with so far only one case showing miRNA-directed target cleavage [93]. Yet, the action of miRNAs on their targets in animals is not that simple. Emerging evidence indicates that animal miRNA can induce the instability of the target mRNAs, which may be mediated by specific RNase (e.g., exonuclease *xrn-1* in *C. elegans* [94]) or by sequestering the target mRNAs into a cytoplasmic processing body (P-body) for temporary storage or degradation [95–97]. These indicate that animal miRNA-mediated target translational repression is mediated by complex mechanisms (Fig. 1).

## 5.2. Diverse mechanisms for miRNA-directed translational repression

Several protein-protein and protein-RNA interacting mechanisms have been proposed to explain miRNA associated RISC-directed target mRNA translational inhibition from the upstream to downstream steps of the protein translation machinery. These are roughly categorized into: (1) RISC-directed translational repression at the stages of translation initiation [98–101] or post-initiation [102–106]; (2) RISC-directed relocation or sequestration of miRNA target mRNAs to P-bodies [97,99,107] or stress granules (SGs) [108]; (3) RISC-guided mRNA decay through a rapid deadenylation [94,95,109,110]; and (4) RISC-directed

immediate and/or fast protein degradation following translation [106] also termed cotranslational protein degradation [111].

### 5.2.1. miRNA-directed translational repressions before and after translation initiation

Most early and a few recent studies demonstrated that miRNA-directed translational repression happened at post-translational initiation stages [102–104]. In these cases, the observations of translational repression were accompanied by the presence of polysomes. It was suggested that the translational repression might be due to ribosome “drop-off” during the elongation of translation [102]. More recent studies suggested that translational repression by miRNAs happened at the translational initiation stage [98,99], as the blockage of translation by miRNAs was shown in a m<sup>7</sup>G-cap-dependent manner. A recent study supported that translational repression occurred through the interaction between the mRNA cap and miRNA-associated RISCs. The binding of eukaryotic initiation factor 4E (eIF4E) to the m<sup>7</sup>G cap is essential for the initiation of the translation of most eukaryotic mRNAs [112]. Kiriakidou et al identified a cap-binding-like domain that is similar to the eIF4E in the middle domain (MC) of AGO2 and showed this MC domain binds to the m<sup>7</sup>G cap and may be required for translational repression by miRNA-associated RISCs [101]. The authors thus proposed that AGO represses the initiation of mRNA translation by binding to and precluding eIF4E from the m<sup>7</sup>G cap of the mRNA targets. This mechanism was further supported by direct evidence that increasing amount of purified eukaryotic translation initiation factor 4F (eIF4F) containing eIF4E suppressed the endogenous let-7 miRISC-directed translational repression in vitro [100].

Intriguingly, Thermann and Hentze showed evidence that *Drosophila* miR-2 associated RISC inhibits m<sup>7</sup>G cap-mediated translation initiation, accompanied by the formation of large RNPs, namely “pseudopolysomes” [113]. Though the biochemical components are unknown, the pseudopolysomes could be distinguished from the *bona fide* polysomes by applying the polysome disrupting agent puromycin. This demonstrated that the formation of the pseudopolysomes was independent of the formation of *bona fide* polysomes. That the pseudopolysomes inhibited the formation of 80S ribosome but could not be distinguished from polysomes in size, reminds us of the need for careful interpretation regarding the miRNA-mediated translational repression mechanism. The remaining questions are whether miR2-mediated translational repression is pseudopolysome dependent or not, and what are the components of pseudopolysome? In addition, it is not known if the formation of pseudopolysomes associated with miR2 is a general phenomenon. Similar methods applied to the analyses of other miRNAs, such as miR6 that shares 8 nucleotides with miR2 at the 5' end, will shed light on the nature and the formation of such pseudopolysomes.

Quite a few investigations demonstrated that translational repression by miRNAs at the translational initiation stage is in m<sup>7</sup>G cap-dependent manner as discussed above. Other experimental results indicated that eIF6, also termed p27BBP, was recruited by RISCs that contain TRBP-like protein, Dicer and AGO, leading to translational repression in both human and worm cells at the translation initiation step [114]. Protein translation requires productive assembly of 80S ribosome composed of the 40S and 60S subunits. In the cytoplasm, eIF6 is bound to free 60S but not to 80S. Release of eIF6 activates the 60S subunits, leading to productive assembly of 80S [115]. Loading 60S subunits with eIF6 caused a dose-dependent translational block and impairment of 80S formation. Interestingly, depletion of the protein eIF6 abrogates miRNA-mediated translational repression of the target mRNAs. Thus, translational repression by a RISC may be mediated by interaction between the 60S subunits and eIF6 that is recruited by the RISC, which is different from the mechanism of m<sup>7</sup>G cap-dependent translational repression.

### 5.2.2. miRNA-directed translational repressions via compartmentation

In addition to the above-mentioned mechanisms of protein-protein and protein-RNA interactions mediated translational repression,

cellular compartment is another factor that contributes to miRNA-directed protein translational repression. The AGO proteins and the repressed mRNAs are often enriched in a special cytosolic compartment, the processing bodies (P-bodies, also known as GW-bodies) [97,99,107]. P-bodies represent discrete cytoplasmic foci that are enriched in proteins/enzymes involved in mRNA decay and translational repression. These proteins include deadenylases, decapping enzymes (e.g., DCP1 and DCP2), and 5'→3' exonucleases (e.g. Xrn1) [116]. On the other hand, P-bodies are physically separated from ribosomes and lack protein translation machinery. In P-bodies, mRNAs can either undergo decapping and degradation [117] or be stored temporarily, thus leading to the proposition that miRNA-associated RISCs have dual functions: mediating mRNA degradation and storing the mRNA in P-bodies. Under certain conditions (e.g. stress) [118], miRNA target mRNAs can be released from the P-bodies and recruited by ribosomes, and thus resume the translation.

However, evidence also suggests that formation of a P-body is not required for miRNA-directed mRNA translational repression, but rather, a consequence of translation repression [119]. Chu and Rana showed that in some human cells miRNA function requires RCK/p54, a DEAD-box helicase that contains ATP-dependent RNA-unwinding activity and is known to be essential for translational repression. RCK/p54 interacts with AGO1 and AGO2 in vitro and in vivo, facilitates formation of P-bodies, and serves as a general repressor of translation [119]. Disrupting P-bodies did not affect interactions of RCK/p54 with AGO proteins and its function in miRNA-mediated translation repression. Depletion of RCK/p54 disrupted P-bodies and dispersed AGO2 throughout the cytoplasm but did not significantly affect RISC functions.

Theoretically, translational repression mediated by miRNAs does not affect the level of mRNAs. In reality, this is not always the case. Recent experimental evidence clearly suggests that miRNA-directed translational repression is also associated with a substantial degradation of target mRNAs in some cases [94,120–122]. In *Drosophila* Schneider 2 (S2) Cells, mRNAs targeted by miRNA-associated RISCs are first deadenylated, decapped, and subsequently degraded, which needs CCR4:NOT1 (a deadenylase complex), GW182, and the DCP1:DCP2 decapping complexes [94,95,109,123].

## 6. MiRNA-induced gene activation: A common or a rare phenomenon?

### 6.1. miRNA-directed translational activation via mRNA translocation

Most intriguingly, while miRNAs have been generally identified as negative regulators of expression of the target mRNAs, in most cases there is now accumulating evidence that in some circumstances miRNAs are found to enhance protein translation from their target mRNAs. In some cases (e.g. under stress conditions), miRNA associated RISCs simply help the repressed target mRNAs be released from the P-body and recruited by ribosomes resuming protein translation [118,124]. This derepression of the target mRNA translation by miRNA needs protein cofactors that are likely induced by stresses and are able to release translational repression by interacting with the 3' UTR of the target mRNA or by helping the target mRNA to re-associate with polysomes. The currently identified protein cofactors include AU-rich-element binding protein HuR [118,124] and apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G or A3G) [125].

### 6.2. miRNA-directed transcriptional activation

Moreover, synthetic siRNAs targeting the promoter regions of the human genes E-cadherin, cyclin kinase inhibitor p21<sup>(WAF1/CIP1)</sup> (p21), and vascular endothelial growth factor (VEGF) induced the expression of these genes through a chromatin remodeling related mechanism that requires the human AGO2 protein [126]. It seems

that siRNA-AGO2-programmed RISC can mark the gene promoter region and modify the structure, resulting in an activation of the gene transcription (Fig. 1). The mechanism, though not yet known, of siRNA-induced gene transcription by targeting the gene promoter seems conserved also in the case of miRNA-induced gene up-regulation. By scanning gene promoters for potential targeting regions by known miRNAs, miR-373 was identified to target promoters of E-cadherin and cold-shock domain-containing protein C2 (CSDC2), respectively [127]. Introduction of synthetic miR-373 or its precursor pre-miR-373 into PC-3 cells readily induced both gene expressions. The induction of E-cadherin expression by pre-miR-373 required Dicer, indicating that mature miR-373, rather than its precursor, plays a role in gene activation. MiR-373 targeting gene promoter was found to induce enrichment of RNA polymerase II (pol-II) at the promoters via an unidentified mechanism.

### 6.3. miRNA-directed cell-cycle dependent translational activation

In addition to the above-mentioned translational up-regulation via miRNA-direct mRNA translocation to different compartmentations or transcriptional up-regulation via miRNA-directed enrichment of pol-II at the promoter region, miRNA-directed translational up-regulation has also been reported in cell-cycle-arrest dependent manner [128,129]. AU-rich elements (AREs) and miRNA targeting sites are often located at the 3' UTRs of the target mRNAs and play an important role in control of post-transcriptional gene regulation. Upon cell-cycle arrest, the AREs are readily transformed into a translation activation signal, recruiting AGO and fragile X mental retardation-related protein 1 (FXR1) associated miRNPs or RISCs. For example, Human miR369-3 associated miRNPs/RISCs interact with the AREs and activate protein translation.

Previous report showed that miR16, a human miRNA that contains an UAAUAAU sequence and binds to the ARE sequence, and ARE binding protein tristetraprolin (TTP) that interacts with AGO/eIF2C and forms complex with miR16, are required for ARE-mediated mRNA degradation [130]. A recent study suggested that miR16/ARE-mediated degradation of mRNAs can be inhibited by stimulating cells with different stresses, which was mediated by inhibiting mRNA deadenylation [131]. Thus, distinct miRNA-associated miRNPs/RISCs are able to switch post-transcriptional gene regulation to both directions: up- and down regulation of the target gene expressions.

Interestingly, a single Let-7 can control its target mRNA translation both ways during the cell cycle: up-regulation of the target mRNA translation upon cell-cycle arrest and down regulation the process in proliferating cells [132]. It was thus hypothesized that miRNP/RISC-directed translation regulation may oscillate between repression and activation during the cell cycle. This kind of novel dual regulation has not been observed for siRNA-direct post-transcriptional gene regulation in the cell cycle [132]. These findings suggest a more diverse role for miRNAs and siRNAs in the regulation of gene expression than previously appreciated.

## 7. Concluding remarks: the art of miRNA is about connections

If *lin-4* [133,134] was initially shaped in worms just as a tiny RNA orphan with no specific role of regulation in heterochronic pathway, there would be no sense of the art of miRNAs as we appreciate so much today. The discovery of RNAi made the long obscured miRNAs known to us with a sense of their essential roles in gene regulation via an ancient pathway. After being born with the help of “Drosha” and “Dicer”, and then coupled with “Slicer”, miRNAs execute their extensively regulatory roles in a wide variety of biological processes. To date, miRNAs have been implicated in wiring many biological processes, deregulation of which will result in various physiological and pathological consequences.

First, the expression of ~30% human coding gene is wired primarily with a few hundred miRNAs via the interaction between miRNAs and

their target mRNAs on the 3' UTR [79,80,82]. If the expression of these 30% human coding genes is considered to have a direct or indirect impact on expression of the remaining human genes, or if miRNA target regions are extended to the 5' UTR and the coding region [83], miRNAs are likely regulating the entire human transcriptome. Second, due to their widely regulatory roles, miRNAs have been wired with many biological, physiological, and pathological processes, showing their regulatory roles in development, cellular metabolism and development of various diseases. Finally, the connection of miRNAs to various kinds of biological processes is remarkably reflected by their multiple action mechanisms from simple cleavage of miRNA-targeted mRNAs to complicated translational repression of their target mRNAs. In particular, the regulatory roles of miRNAs in various biological processes in animals are probably mediated through the more and more complex and puzzling protein translational regulation. Shortly, we shall be able to fully appreciate the art of miRNAs in the ancient pathway of gene regulation.

## Acknowledgments

Particular thanks to Dr. Phil Zamore for the email discussion of specific issues during the preparation of this article and thanks also to several anonymous reviewers for their constructive comments and suggestions. G.T. is supported by the Kentucky Tobacco Research and Development Center (KTRDC), the USDA-NRI grants 2006-35301-17115 and 2006-35100-17433, the NSF MCB-0718029 (Subaward No. S-00000260), the NIH 5 R03 AI 068934-02, and an award from the Kentucky Science and Technology Corporation under Contract # KSTC-144-401-08-029.

## References

- [1] A. Fire, S. Xu, M.K. Montgomery, S.A. Kostas, S.E. Driver, C.C. Mello, Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*, *Nature* 391 (1998) 806–811.
- [2] J.D. Watson, F.H. Crick, Molecular structure of nucleic acids; a structure for deoxyribose nucleic acid, *Nature* 171 (1953) 737–738.
- [3] K. Jeyaseelan, W.B. Herath, A. Armugam, MicroRNAs as therapeutic targets in human diseases, *Expert Opin. Ther. Targets* 11 (2007) 1119–1129.
- [4] R.J. Perera, A. Ray, MicroRNAs in the search for understanding human diseases, *BioDrugs* 21 (2007) 97–104.
- [5] M.P. Perron, V. Boissonneault, L.A. Gobeil, D.L. Ouellet, P. Provost, Regulatory RNAs: future perspectives in diagnosis, prognosis, and individualized therapy, *Methods Mol. Biol.* 361 (2007) 311–326.
- [6] C. Zhang, MicroRNomics: a newly emerging approach for disease biology, *Physiol. Genomics* 33 (2008) 139–147.
- [7] M.R. Willmann, R.S. Poethig, Conservation and evolution of miRNA regulatory programs in plant development, *Curr. Opin. Plant. Biol.* 10 (2007) 503–511.
- [8] M.W. Jones-Rhoades, D.P. Bartel, B. Bartel, MicroRNAs and their regulatory roles in plants, *Annu. Rev. Plant. Biol.* 57 (2006) 19–53.
- [9] B. Bartel, D.P. Bartel, MicroRNAs: at the root of plant development? *Plant Physiol.* 132 (2003) 709–717.
- [10] J.F. Doebley, B.S. Gaut, B.D. Smith, The molecular genetics of crop domestication, *Cell* 127 (2006) 1309–1321.
- [11] H. Wang, T. Nussbaum-Wagler, B. Li, Q. Zhao, Y. Vigouroux, M. Faller, K. Bomblied, L. Lukens, J.F. Doebley, The origin of the naked grains of maize, *Nature* 436 (2005) 714–719.
- [12] A.C. Mallory, H. Vaucheret, MicroRNAs: something important between the genes, *Curr. Opin. Plant. Biol.* 7 (2004) 120–125.
- [13] S.Y. Ying, S.L. Lin, Intronic microRNAs, *Biochem. Biophys. Res. Commun.* 326 (2005) 515–520.
- [14] S.L. Lin, H. Kim, S.Y. Ying, Intron-mediated RNA interference and microRNA (miRNA), *Front Biosci.* 13 (2008) 2216–2230.
- [15] Y. Lee, M. Kim, J. Han, K.H. Yeom, S. Lee, S.H. Baek, V.N. Kim, MicroRNA genes are transcribed by RNA polymerase II, *EMBO J.* 23 (2004) 4051–4060.
- [16] G.M. Borchert, W. Lanier, B.L. Davidson, RNA polymerase III transcribes human microRNAs, *Nat. Struct. Mol. Biol.* 13 (2006) 1097–1101.
- [17] Y. Fang, D.L. Spector, Identification of nuclear dicing bodies containing proteins for microRNA biogenesis in living Arabidopsis plants, *Curr. Biol.* 17 (2007) 818–823.
- [18] J.G. Ruby, C.H. Jan, D.P. Bartel, Intronic microRNA precursors that bypass Drosha processing, *Nature* 448 (2007) 83–86.
- [19] K. Okamura, J.W. Hagen, H. Duan, D.M. Tyler, E.C. Lai, The mirtron pathway generates microRNA-class regulatory RNAs in *Drosophila*, *Cell* 130 (2007) 89–100.
- [20] E. Berezhikov, W.J. Chung, J. Willis, E. Cuppen, E.C. Lai, Mammalian mirtron genes, *Mol. Cell* 28 (2007) 328–336.

- [21] Y. Lee, C. Ahn, J. Han, H. Choi, J. Kim, J. Yim, J. Lee, P. Provost, O. Radmark, S. Kim, V.N. Kim, The nuclear RNase III Drosha initiates microRNA processing, *Nature* 425 (2003) 415–419.
- [22] A. Shiohama, T. Sasaki, S. Noda, S. Minoshima, N. Shimizu, Molecular cloning and expression analysis of a novel gene DGCR8 located in the DiGeorge syndrome chromosomal region, *Biochem. Biophys. Res. Commun.* 304 (2003) 184–190.
- [23] J. Han, Y. Lee, K.H. Yeom, Y.K. Kim, H. Jin, V.N. Kim, The Drosha-DGCR8 complex in primary microRNA processing, *Genes Dev.* 18 (2004) 3016–3027.
- [24] R.I. Gregory, K.P. Yan, G. Amuthan, T. Chendrimada, B. Doratotaj, N. Cooch, R. Shiekhattar, The Microprocessor complex mediates the genesis of microRNAs, *Nature* 432 (2004) 235–240.
- [25] Y. Zeng, B.R. Cullen, Structural requirements for pre-microRNA binding and nuclear export by Exportin 5, *Nucleic Acids Res.* 32 (2004) 4776–4785.
- [26] R. Yi, Y. Qin, I.G. Macara, B.R. Cullen, Exportin-5 mediates the nuclear export of pre-miRNAs and short hairpin RNAs, *Genes Dev.* 17 (2003) 3011–3016.
- [27] R. Yi, B.P. Doehle, Y. Qin, I.G. Macara, B.R. Cullen, Overexpression of exportin 5 enhances RNA interference mediated by short hairpin RNAs and microRNAs, *RNA* 11 (2005) 220–226.
- [28] V.N. Kim, MicroRNA precursors in motion: exportin-5 mediates their nuclear export, *Trends Cell Biol.* 14 (2004) 156–159.
- [29] M.T. Bohnsack, K. Czaplinski, D. Gorlich, Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs, *RNA* 10 (2004) 185–191.
- [30] A.D. Haase, L. Jaskiewicz, H. Zhang, S. Laine, R. Sack, A. Gatignol, W. Filipowicz, TRBP, a regulator of cellular PKR and HIV-1 virus expression, interacts with Dicer and functions in RNA silencing, *EMBO Rep.* 6 (2005) 961–967.
- [31] T.P. Chendrimada, R.I. Gregory, E. Kumaraswamy, J. Norman, N. Cooch, K. Nishikura, R. Shiekhattar, TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing, *Nature* 436 (2005) 740–744.
- [32] Y. Lee, I. Hur, S.Y. Park, Y.K. Kim, M.R. Suh, V.N. Kim, The role of PACT in the RNA silencing pathway, *EMBO J.* 25 (2006) 522–532.
- [33] Q. Liu, T.A. Rand, S. Kalidas, F. Du, H.E. Kim, D.P. Smith, X. Wang, R2D2, a bridge between the initiation and effector steps of the Drosophila RNAi pathway, *Science* 301 (2003) 1921–1925.
- [34] X. Liu, F. Jiang, S. Kalidas, D. Smith, Q. Liu, Dicer-2 and R2D2 coordinately bind siRNA to promote assembly of the siRISC complexes, *RNA* 12 (2006) 1514–1520.
- [35] A.M. Denli, B.B. Tops, R.H. Plasterk, R.F. Ketting, G.J. Hannon, Processing of primary microRNAs by the Microprocessor complex, *Nature* 432 (2004) 231–235.
- [36] X. Liu, J.K. Park, F. Jiang, Y. Liu, D. McKearin, Q. Liu, Dicer-1, but not Loquacious, is critical for assembly of miRNA-induced silencing complexes, *RNA* 13 (2007) 2324–2329.
- [37] K. Saito, A. Ishizuka, H. Siomi, M.C. Siomi, Processing of pre-microRNAs by the Dicer-1-Loquacious complex in Drosophila cells, *PLoS Biol.* 3 (2005) 2e235.
- [38] K. Forstemann, Y. Tomari, T. Du, V.V. Vagin, A.M. Denli, D.P. Bratu, C. Klattenhoff, W.E. Theurkauf, P.D. Zamore, Normal microRNA maturation and germ-line stem cell maintenance requires Loquacious, a double-stranded RNA-binding domain protein, *PLoS Biol.* 3 (2005) e236.
- [39] G.S. Parker, D.M. Eckert, B.L. Bass, RDE-4 preferentially binds long dsRNA and its dimerization is necessary for cleavage of dsRNA to siRNA, *RNA* 12 (2006) 807–818.
- [40] H. Tabara, E. Yigit, H. Siomi, C.C. Mello, The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DEXH-box helicase to direct RNAi in *C. elegans*, *Cell* 109 (2002) 861–871.
- [41] S. Parrish, A. Fire, Distinct roles for RDE-1 and RDE-4 during RNA interference in *Caenorhabditis elegans*, *RNA* 7 (2001) 1397–1402.
- [42] N.C. Welker, J.W. Habig, B.L. Bass, Genes misregulated in *C. elegans* deficient in Dicer, RDE-4, or RDE-1 are enriched for innate immunity genes, *RNA* 13 (2007) 1090–1102.
- [43] R.I. Gregory, T.P. Chendrimada, N. Cooch, R. Shiekhattar, Human RISC couples microRNA biogenesis and posttranscriptional gene silencing, *Cell* 123 (2005) 631–640.
- [44] F. Vazquez, V. Gasciolli, P. Crete, H. Vaucheret, The nuclear dsRNA binding protein HYL1 is required for microRNA accumulation and plant development, but not posttranscriptional transgene silencing, *Curr. Biol.* 14 (2004) 346–351.
- [45] L. Song, M.H. Han, J. Lesicka, N. Fedoroff, Arabidopsis primary microRNA processing proteins HYL1 and DCL1 define a nuclear body distinct from the Cajal body, *Proc. Natl. Acad. Sci. U. S. A.* 104 (2007) 5437–5442.
- [46] Y. Kurihara, Y. Takashi, Y. Watanabe, The interaction between DCL1 and HYL1 is important for efficient and precise processing of pri-miRNA in plant microRNA biogenesis, *RNA* 12 (2006) 206–212.
- [47] M.H. Han, S. Goud, L. Song, N. Fedoroff, The Arabidopsis double-stranded RNA-binding protein HYL1 plays a role in microRNA-mediated gene regulation, *Proc. Natl. Acad. Sci. U. S. A.* 101 (2004) 1093–1098.
- [48] L. Yang, Z. Liu, F. Lu, A. Dong, H. Huang, SERRATE is a novel nuclear regulator in primary microRNA processing in Arabidopsis, *Plant J.* 47 (2006) 841–850.
- [49] C.F. Li, O. Pontes, M. El-Shami, I.R. Henderson, Y.V. Bernatavichute, S.W. Chan, T. Lagrange, C.S. Pikaard, S.E. Jacobsen, An ARGONAUTE4-containing nuclear processing center colocalized with Cajal bodies in *Arabidopsis thaliana*, *Cell* 126 (2006) 93–106.
- [50] O. Pontes, C.F. Li, P.C. Nunes, J. Haag, T. Ream, A. Vitins, S.E. Jacobsen, C.S. Pikaard, The Arabidopsis chromatin-modifying nuclear siRNA pathway involves a nucleolar RNA processing center, *Cell* 126 (2006) 79–92.
- [51] S. Diederichs, D.A. Haber, Dual role for Argonautes in MicroRNA processing and posttranscriptional regulation of MicroRNA expression, *Cell* 131 (2007) 1097–1108.
- [52] C. Matranga, Y. Tomari, C. Shin, D.P. Bartel, P.D. Zamore, Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes, *Cell* 123 (2005) 607–620.
- [53] T.A. Rand, S. Petersen, F. Du, X. Wang, Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation, *Cell* 123 (2005) 621–629.
- [54] P.J. Leuschner, S.L. Ameres, S. Kueng, J. Martinez, Cleavage of the siRNA passenger strand during RISC assembly in human cells, *EMBO Rep.* 7 (2006) 314–320.
- [55] M. Hossbach, J. Gruber, M. Osborn, K. Weber, T. Tuschl, Gene silencing with siRNA duplexes composed of target-mRNA-complementary and partially palindromic or partially complementary single-stranded siRNAs, *RNA Biol.* 3 (2006) 82–89.
- [56] K. Miyoshi, H. Tsukumo, T. Nagami, H. Siomi, M.C. Siomi, Slicer function of Drosophila Argonautes and its involvement in RISC formation, *Genes Dev.* 19 (2005) 2837–2848.
- [57] K.L. Tkaczuk, A. Obarska, J.M. Bujnicki, Molecular phylogenetics and comparative modeling of HEN1, a methyltransferase involved in plant microRNA biogenesis, *BMC Evol. Biol.* 6 (2006) 6.
- [58] Z. Yang, Y.W. Ebright, B. Yu, X. Chen, HEN1 recognizes 21–24 nt small RNA duplexes and deposits a methyl group onto the 2' OH of the 3' terminal nucleotide, *Nucleic Acids Res.* 34 (2006) 667–675.
- [59] B. Yu, Z. Yang, J. Li, S. Minakhina, M. Yang, R.W. Padgett, R. Steward, X. Chen, Methylation as a crucial step in plant microRNA biogenesis, *Science* 307 (2005) 932–935.
- [60] K. Saito, Y. Sakaguchi, T. Suzuki, H. Siomi, M.C. Siomi, Pimet, the Drosophila homolog of HEN1, mediates 2'-O-methylation of Piwi-interacting RNAs at their 3' ends, *Genes Dev.* 21 (2007) 1603–1608.
- [61] M.D. Horwich, C. Li, C. Matranga, V. Vagin, G. Farley, P. Wang, P.D. Zamore, The Drosophila RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC, *Curr. Biol.* 17 (2007) 1265–1272.
- [62] Y. Kirino, Z. Mourelatos, The mouse homolog of HEN1 is a potential methylase for Piwi-interacting RNAs, *RNA* 13 (2007) 1397–1401.
- [63] J. Li, Z. Yang, B. Yu, J. Liu, X. Chen, Methylation protects miRNAs and siRNAs from a 3'-end uridylation activity in Arabidopsis, *Curr. Biol.* 15 (2005) 1501–1507.
- [64] D.S. Schwarz, G. Hutvagner, T. Du, Z. Xu, N. Aronin, P.D. Zamore, Asymmetry in the assembly of the RNAi enzyme complex, *Cell* 115 (2003) 199–208.
- [65] A. Khvorova, A. Reynolds, S.D. Jayasena, Functional siRNAs and miRNAs exhibit strand bias, *Cell* 115 (2003) 209–216.
- [66] S. Ro, C. Park, K.M. Sanders, J.R. McCarrey, W. Yan, Cloning and expression profiling of testis-expressed microRNAs, *Dev. Biol.* 311 (2007) 592–602.
- [67] S. Ro, C. Park, D. Young, K.M. Sanders, W. Yan, Tissue-dependent paired expression of miRNAs, *Nucleic Acids Res.* 35 (2007) 5944–5953.
- [68] K. Okamura, M.D. Phillips, D.M. Tyler, H. Duan, Y.T. Chou, E.C. Lai, The regulatory activity of microRNA\* species has substantial influence on microRNA and 3' UTR evolution, *Nat. Struct. Mol. Biol.* 15 (2008) 354–363.
- [69] S.M. Hammond, S. Boettcher, A.A. Caudy, R. Kobayashi, G.J. Hannon, Argonaute2, a link between genetic and biochemical analyses of RNAi, *Science* 293 (2001) 1146–1150.
- [70] E. Maniatakis, Z. Mourelatos, A human, ATP-independent, RISC assembly machine fueled by pre-miRNA, *Genes Dev.* 19 (2005) 2979–2990.
- [71] J.W. Pham, J.L. Pellino, Y.S. Lee, R.W. Arthurs, E.J. Sontheimer, A Dicer-2-dependent 80s complex cleaves targeted mRNAs during RNAi in Drosophila, *Cell* 117 (2004) 83–94.
- [72] K. Kim, Y.S. Lee, R.W. Carthew, Conversion of pre-RISC to holo-RISC by Ago2 during assembly of RNAi complexes, *RNA* 13 (2007) 22–29.
- [73] I.J. MacRae, E. Ma, M. Zhou, C.V. Robinson, J.A. Doudna, In vitro reconstitution of the human RISC-loading complex, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 512–517.
- [74] Y. Tomari, C. Matranga, B. Haley, N. Martinez, P.D. Zamore, A protein sensor for siRNA asymmetry, *Science* 306 (2004) 1377–1380.
- [75] F. Jiang, X. Ye, X. Liu, L. Fincher, D. McKearin, Q. Liu, Dicer-1 and R3D1-L catalyze microRNA maturation in Drosophila, *Genes Dev.* 19 (2005) 1674–1679.
- [76] G. Tang, siRNA and miRNA: an insight into RISCs, *Trends Biochem. Sci.* 30 (2005) 106–114.
- [77] J.W. Pham, E.J. Sontheimer, Molecular requirements for RNA-induced silencing complex assembly in the Drosophila RNA interference pathway, *J. Biol. Chem.* 280 (2005) 39278–39283.
- [78] F.V. Rivas, N.H. Tolia, J.J. Song, J.P. Aragon, J. Liu, G.J. Hannon, L. Joshua-Tor, Purified Argonaute2 and an siRNA form recombinant human RISC, *Nat. Struct. Mol. Biol.* 12 (2005) 340–349.
- [79] B.P. Lewis, C.B. Burge, D.P. Bartel, Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets, *Cell* 120 (2005) 15–20.
- [80] X. Xie, J. Lu, E.J. Kulbokas, T.R. Golub, V. Mootha, K. Lindblad-Toh, E.S. Lander, M. Kellis, Systematic discovery of regulatory motifs in human promoters and 3' UTRs by comparison of several mammals, *Nature* 434 (2005) 338–345.
- [81] D.P. Bartel, MicroRNAs: genomics, biogenesis, mechanism, and function, *Cell* 116 (2004) 281–297.
- [82] A. Krek, D. Grun, M.N. Poy, R. Wolf, L. Rosenberg, E.J. Epstein, P. MacMenamin, I. da Piedade, K.C. Gunsalus, M. Stoffel, N. Rajewsky, Combinatorial microRNA target predictions, *Nat. Genet.* 37 (2005) 495–500.
- [83] K.C. Miranda, T. Huynh, Y. Tay, Y.S. Ang, W.L. Tam, A.M. Thomson, B. Lim, I. Rigoutsos, A pattern-based method for the identification of MicroRNA binding sites and their corresponding heteroduplexes, *Cell* 126 (2006) 1203–1217.
- [84] M.C. Vella, K. Reinert, F.J. Slack, Architecture of a validated microRNA::target interaction, *Chem. Biol.* 11 (2004) 1619–1623.

- [85] A. Grimson, K.K. Farh, W.K. Johnston, P. Garrett-Engele, L.P. Lim, D.P. Bartel, MicroRNA targeting specificity in mammals: determinants beyond seed pairing, *Mol. Cell* 27 (2007) 91–105.
- [86] J. Liu, M.A. Carmell, F.V. Rivas, C.G. Marsden, J.M. Thomson, J.J. Song, S.M. Hammond, L. Joshua-Tor, G.J. Hannon, Argonaute2 is the catalytic engine of mammalian RNAi, *Science* 305 (2004) 1437–1441.
- [87] N. Baumberger, D.C. Baulcombe, Arabidopsis ARGONAUTE1 is an RNA Slicer that selectively recruits microRNAs and short interfering RNAs, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 11928–11933.
- [88] J.G. Doench, C.P. Petersen, P.A. Sharp, siRNAs can function as miRNAs, *Genes Dev.* 17 (2003) 438–442.
- [89] X. Chen, A microRNA as a translational repressor of APETALA2 in Arabidopsis flower development, *Science* 303 (2004) 2022–2025.
- [90] M.J. Aukerman, H. Sakai, Regulation of flowering time and floral organ identity by a MicroRNA and its APETALA2-like target genes, *Plant Cell* 15 (2003) 2730–2741.
- [91] M. Gandikota, R.P. Birkenbihl, S. Hohmann, G.H. Cardon, H. Saedler, P. Huijser, The miRNA156/157 recognition element in the 3' UTR of the Arabidopsis SBP box gene SPL3 prevents early flowering by translational inhibition in seedlings, *Plant J.* 49 (2007) 683–693.
- [92] P. Brodersen, L. Sakvarelidze-Achard, M. Bruun-Rasmussen, P. Dunoyer, Y.Y. Yamamoto, L. Sieburth, O. Voinnet, Widespread translational inhibition by plant miRNAs and siRNAs, *Science* 320 (2008) 1185–1190.
- [93] S. Yekta, I.H. Shih, D.P. Bartel, MicroRNA-directed cleavage of HOXB8 mRNA, *Science* 304 (2004) 594–596.
- [94] S. Bagga, J. Bracht, S. Hunter, K. Massirer, J. Holtz, R. Eachus, A.E. Pasquinelli, Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation, *Cell* 122 (2005) 553–563.
- [95] J. Rehwinkel, I. Behm-Ansmant, D. Gatfield, E. Izaurralde, A crucial role for GW182 and the DCP1:DCP2 decapping complex in miRNA-mediated gene silencing, *RNA* 11 (2005) 1640–1647.
- [96] J.J. Rossi, RNAi and the P-body connection, *Nat Cell Biol* 7 (2005) 643–644.
- [97] J. Liu, F.V. Rivas, J. Wohlschlegel, J.R. Yates III, R. Parker, G.J. Hannon, A role for the P-body component GW182 in microRNA function, *Nat. Cell Biol.* 7 (2005) 1261–1266.
- [98] D.T. Humphreys, B.J. Westman, D.I. Martin, T. Preiss, MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly (A) tail function, *Proc. Natl. Acad. Sci. U. S. A.* 102 (2005) 16961–16966.
- [99] R.S. Pillai, S.N. Bhattacharyya, C.G. Artus, T. Zoller, N. Cougot, E. Basyuk, E. Bertrand, W. Filipowicz, Inhibition of translational initiation by Let-7 MicroRNA in human cells, *Science (New York, N.Y.)* 309 (2005) 1573–1576.
- [100] G. Mathonnet, M.R. Fabian, Y.V. Svitkin, A. Parsyan, L. Huck, T. Murata, S. Biffo, W.C. Merrick, E. Darzynkiewicz, R.S. Pillai, W. Filipowicz, T.F. Duchaine, N. Sonenberg, MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F, *Science* 317 (2007) 1764–1767.
- [101] M. Kiriakidou, G.S. Tan, S. Lamprinaki, M. De Planell-Saguer, P.T. Nelson, Z. Mourelatos, An mRNA m(7)G Cap binding-like motif within human Ago2 represses translation, *Cell* 129 (2007) 1141–1151.
- [102] C.P. Petersen, M.E. Bordeleau, J. Pelletier, P.A. Sharp, Short RNAs repress translation after initiation in mammalian cells, *Mol. Cell* 21 (2006) 533–542.
- [103] K. Slegger, L. Tang, E.G. Moss, Two genetic circuits repress the *Caenorhabditis elegans* heterochronic gene lin-28 after translation initiation, *Dev. Biol.* 243 (2002) 215–225.
- [104] P.H. Olsen, V. Ambros, The lin-4 regulatory RNA controls developmental timing in *Caenorhabditis elegans* by blocking LIN-14 protein synthesis after the initiation of translation, *Dev. Biol.* 216 (1999) 671–680.
- [105] P.A. Maroney, Y. Yu, J. Fisher, T.W. Nilsen, Evidence that microRNAs are associated with translating messenger RNAs in human cells, *Nat. Struct. Mol. Biol.* 13 (2006) 1102–1107.
- [106] S. Nottrott, M.J. Simard, J.D. Richter, Human let-7a miRNA blocks protein production on actively translating polyribosomes, *Nat. Struct. Mol. Biol.* 13 (2006) 1108–1114.
- [107] G.L. Sen, H.M. Blau, Argonaute 2/RISC resides in sites of mammalian mRNA decay known as cytoplasmic bodies, *Nat. Cell Biol.* 7 (2005) 633–636.
- [108] A.K. Leung, J.M. Calabrese, P.A. Sharp, Quantitative analysis of Argonaute protein reveals microRNA-dependent localization to stress granules, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 18125–18130.
- [109] I. Behm-Ansmant, J. Rehwinkel, T. Doerks, A. Stark, P. Bork, E. Izaurralde, mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes, *Genes Dev.* 20 (2006) 1885–1898.
- [110] M. Wakiyama, K. Takimoto, O. Ohara, S. Yokoyama, Let-7 microRNA-mediated mRNA deadenylation and translational repression in a mammalian cell-free system, *Genes Dev.* 21 (2007) 1857–1862.
- [111] A. Eulalio, E. Huntzinger, E. Izaurralde, Getting to the root of miRNA-mediated gene silencing, *Cell* 132 (2008) 9–14.
- [112] J.D. Richter, N. Sonenberg, Regulation of cap-dependent translation by eIF4E inhibitory proteins, *Nature* 433 (2005) 477–480.
- [113] R. Thermann, M.W. Hentze, Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation, *Nature* 447 (2007) 875–878.
- [114] T.P. Chendrimada, K.J. Finn, X. Ji, D. Baillat, R.I. Gregory, S.A. Liebhaber, A.E. Pasquinelli, R. Shiekhattar, MicroRNA silencing through RISC recruitment of eIF6, *Nature* 447 (2007) 823–828.
- [115] M. Ceci, C. Gavrighi, C. Gorriani, L.A. Sala, N. Offenhauser, P.C. Marchisio, S. Biffo, Release of eIF6 (p27BBP) from the 60S subunit allows 80S ribosome assembly, *Nature* 426 (2003) 579–584.
- [116] C. Fillman, J. Lykke-Andersen, RNA decapping inside and outside of processing bodies, *Curr. Opin. Cell Biol.* 17 (2005) 326–331.
- [117] U. Sheth, R. Parker, Decapping and decay of messenger RNA occur in cytoplasmic processing bodies, *Science (New York, N.Y.)* 300 (2003) 805–808.
- [118] S.N. Bhattacharyya, R. Habermacher, U. Martine, E.I. Closs, W. Filipowicz, Stress-induced reversal of microRNA repression and mRNA P-body localization in human cells, *Cold Spring Harbor Symp. Quant. Biology* 71 (2006) 513–521.
- [119] C.Y. Chu, T.M. Rana, Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54, *PLoS Biol.* 4 (2006) e210.
- [120] L.P. Lim, N.C. Lau, P. Garrett-Engele, A. Grimson, J.M. Schelter, J. Castle, D.P. Bartel, P.S. Linsley, J.M. Johnson, Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs, *Nature* 433 (2005) 769–773.
- [121] J. Krutzfeldt, N. Rajewsky, R. Braich, K.G. Rajeev, T. Tuschl, M. Manoharan, M. Stoffel, Silencing of microRNAs in vivo with 'antagomirs', *Nature* 438 (2005) 685–689.
- [122] A.J. Giraldez, Y. Mishima, J. Rihel, R.J. Grocock, S. Van Dongen, K. Inoue, A.J. Enright, A.F. Schier, Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs, *Science (New York, N.Y.)* 312 (2006) 75–79.
- [123] L. Wu, J. Fan, J.G. Belasco, MicroRNAs direct rapid deadenylation of mRNA, *Proceedings of the Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 4034–4039.
- [124] S.N. Bhattacharyya, R. Habermacher, U. Martine, E.I. Closs, W. Filipowicz, Relief of microRNA-mediated translational repression in human cells subjected to stress, *Cell* 125 (2006) 1111–1124.
- [125] J. Huang, Z. Liang, B. Yang, H. Tian, J. Ma, H. Zhang, Derepression of microRNA-mediated protein translation inhibition by apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G) and its family members, *J. Biol. Chem.* 282 (2007) 33632–33640.
- [126] L.C. Li, S.T. Okino, H. Zhao, D. Pookot, R.F. Place, S. Urakami, H. Enokida, R. Dahiya, Small dsRNAs induce transcriptional activation in human cells, *Proc. Natl. Acad. Sci. U. S. A.* 103 (2006) 17337–17342.
- [127] R.F. Place, L.C. Li, D. Pookot, E.J. Noonan, R. Dahiya, MicroRNA-373 induces expression of genes with complementary promoter sequences, *Proc. Natl. Acad. Sci. U. S. A.* 105 (2008) 1608–1613.
- [128] S. Vasudevan, Y. Tong, J.A. Steitz, Switching from repression to activation: microRNAs can up-regulate translation, *Science* 318 (2007) 1931–1934.
- [129] S. Vasudevan, J.A. Steitz, AU-rich-element-mediated upregulation of translation by FXR1 and Argonaute 2, *Cell* 128 (2007) 1105–1118.
- [130] Q. Jing, S. Huang, S. Guth, T. Zarubin, A. Motoyama, J. Chen, F. Di Padova, S.C. Lin, H. Gram, J. Han, Involvement of microRNA in AU-rich element-mediated mRNA instability, *Cell* 120 (2005) 623–634.
- [131] J. Mols, A.V. Berg, M. Otsuka, M. Zheng, J. Chen, J. Han, TNF-alpha stimulation inhibits siRNA-mediated RNA interference through a mechanism involving poly-(A) tail stabilization, *Biochim. Biophys. Acta* (2008).
- [132] S. Vasudevan, Y. Tong, J.A. Steitz, Cell-cycle control of microRNA-mediated translation regulation, *Cell Cycle* 7 (2008).
- [133] R.C. Lee, R.L. Feinbaum, V. Ambros, The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14, *Cell* 75 (1993) 843–854.
- [134] B. Wightman, I. Ha, G. Ruvkun, Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in *C. elegans*, *Cell* 75 (1993) 855–862.