RNA Interference, Mechanisms and Proteins Involved in

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RNA interference (RNAi) and microRNA (miRNA) pathways participate in posttranscriptional gene expression regulation. RNAi and miRNA biochemistry involve short (~22 nucleotide) regulatory RNAs: small interfering RNA (siRNA) and miRNA. Both siRNAs and miRNAs guide RNA–protein complexes to ''target'' complementary messenger RNAs (mRNAs) for cleavage or translational repression. Many proteins involved in these pathways have been elucidated. The biochemical mechanisms of miRNA/siRNA processing have been dissected in detail in plants and in animals using biochemical and genetic approaches. Here we review the current literature on RNAi and miRNA mechanisms with a special focus on the protein components.

Introduction

Much gene expression regulation occurs after mRNA transcription (1), and it is becoming increasingly clear that noncoding "regulatory" RNAs play central roles in posttranscriptional gene expression regulation. The breakthrough discovery of small regulatory RNAs and their multiple functions in gene regulation has heralded a fascinating new field in biology. Pioneering observations on the phenomenon of posttranscriptional gene silencing were reported in plants (2–5). However, the broader relevance of gene silencing mechanisms is confirmed in many other organisms, including the Nobel Prize winning discovery of RNA interference (RNAi) in worms (6). RNAi-related biological phenomena have now been elucidated in all known eukaryotic organisms. The similarities in RNAi mechanisms between plants and animals underscore the importance of this ancient gene regulatory mechanism. Moreover, in addition to mRNA "gene expression regulation," RNAi-related mechanisms were shown to have diverse functions including transposon silencing (7), chromosome remodeling (8), and DNA methylation (9). The molecular processes responsible for RNAi and its related pathways have been elucidated extensively over the last a few years. Here we review the literature about the mechanisms of RNAi and miRNA pathways with a focus on the proteins involved.

Complementary Research Strands Come Together

In 1990, Jorgensen (10) attempted to enhance flower color by overexpressing an extra copy of chalcone synthase (*chsA*) gene in transgenic petunia plants (3). Unexpectedly, among many transgenic petunia plants both the endogene and the transgene of chalcone synthase were silenced. The transgenic flowers showed multiple colors or generated white segments. Loss of cytosolic *chsA* mRNA was not associated with a reduction of the gene transcription (11), which indicates that the phenotype resulted from a post-gene transcriptional event. The phenomenon of silencing an endogenous plant gene, triggered by extra copies of a transgene with the same sequence, was termed "cosuppression" (10, 12).

Important advancements in the field that would eventually encompass RNAi and miRNAs came from the laboratory of Victor

Ambros. These experiments employed genetic screens to dissect the heterochronic pathway in *Caenorhabditis elegans*. Ambros et al. discovered that short noncoding RNAs (the product of the *lin-4* gene) regulated the translation of another gene product (*lin-14*) via partially complementary sequences in the 3′UTR of *lin-14* mRNA (13, 14). The significance of these data remained temporarily obscure—it was not until seven years later that a similar small regulatory RNA was discovered (15). Thereafter, it was recognized that these molecules were representatives of a large, biologically important class of RNA (the designation "microRNAs" was first described in separate papers in 2001) (16–18). Although miRNAs work through pathways somewhat different from siRNAs (see below), the paradigm of antisense recognition by small regulatory RNAs, coupled with posttranscriptional regulation of mRNAs, was thus established in animals.

In 1995, when worms were injected with antisense and sense RNAs to target a specific gene for down-regulation, Gao and Kemphues (19) found that both the control sense and the antisense RNAs induced similar gene silencing effects in worms. This phenomenon could not be explained fully at that time. In the seminal work of Fire et al. (6), dsRNA was finally demonstrated as a potent trigger in gene silencing by introducing tiny amounts of purified dsRNAs into *C. elegans*, and this phenomenon was termed "RNA interference".

Although RNAi phenomena were described in different experimental contexts (labeled cosuppression in plants, RNAi in animals, and quelling in fungi), all these gene-silencing mechanisms have converged on a general paradigm. The common features of this paradigm share at least three characteristics: 1) Short/small regulatory RNAs (siRNAs) of 21–23 nucleotides (nts) are the key players in mediating specific RNA degradation, 2) the degradation of target RNA depends on specific sequence recognition of siRNAs, and 3) the machinery of the RNA-induced silencing complexes (RISCs) are similar in structure and function across most organisms.

The Mechanism of RNA Interference

Although RNAi and miRNAs were discovered in worms, major progress in revealing the RNAi pathway came from *in vitro* biochemical studies using a cell-free system from *Drosophila* syncytial blastoderm embryos, which was first carried out by Tuschl et al. (20). It was hypothesized that a population of 21–23 nt small RNAs could be generated in fly embryo lysates, which triggers the cleavage of radiolabeled target mRNA at 21–23 nt intervals (21). Experimentally, these short RNA fragments were indeed detected in extracts of *Drosophila* Schneider 2 (S2) cells that were transfected with dsRNA (22). Moreover, RNA molecules of similar size were also found to accumulate in transgene silenced plants (23). Based on their studies, Zamore et al. (21) proposed an early model of RNAi: Long dsRNAs are first cleaved into short dsRNA fragments of 21–23 nts. These short dsRNAs are then separated and integrated into a ribonucleoprotein complex, and the siRNA-ribonucleoprotein complex recognizes and binds the siRNA-specific complementary RNA that leads to destruction of the latter. This model laid a framework for the subsequent dissection of the RNAi pathway.

Generation of siRNAs and miRNAs

SiRNAs can be derived from several sources including long dsRNAs. dsRNAs can be produced many ways: They can be generated endogenously, for example, from transgene transcripts by RNA-directed RNA polymerase (RdRP) (24–27); by simultaneous transcription of sense and antisense DNA fragments from specific genomic loci (28); from viral replication intermediates (27, 29); or via introduction of genes that encode inverted repeats by genetic engineering (30, 31). Endogenously generated or exogenously introduced dsRNAs are then converted into 21–30 bp small RNAs by dicer (DCR), which is an RNase III family enzyme that initiates the RNAi (32).

MiRNA precursors, which are derived from endogenous genes, are processed differently from siRNAs. A single-stranded RNA can form a "hairpin" or "stem-loop" structure where the RNA folds back and base pairs with itself. The "stem" of this structure thus serves as dsRNA for additional processing. Some "stem-loop" RNAs are processed to become miRNAs. MiRNA genes are transcribed by RNA polymerase II (pol II), mostly or in a few cases by polymerase III (pol III) (33, 34), and the primary transcripts "pri-miRNAs" are processed by a microprocessor composed of a Drosha-like enzyme of RNase III family and a Pasha-like protein of dsRNA binding protein family (35–39). Or, in plants, pri-miRNAs are processed by the DCR family member DCL-1 (40). Like all RNase III enzymes, Drosha leaves two nt 3′ overhangs and 5′ monophosphate groups. Processed pre-miRNAs are transported to the cytoplasm via the export-receptor Exportin-5 (41–43).

MiRNAs not only are generated from miRNA genes located within intergenic regions, but also are derived from introns. A subset of intronic miRNA precursors from *Drosophila* can bypass the microprocessor and directly serve as DCR substrates (44, 45). These miRNAs are known as mirtrons. The spliced-intron lariats or mirtron precursors are debranched and refolded into stem-loop structures that have similar Drosha ends and are recognized by DCR1 in *Drosophila* (44, 45).

The final stages of miRNA maturation are deceptively complex. As indicated above, both long dsRNA and stem-loop RNA structures such as miRNA precursors are recognized and processed by the cytoplasmic RNase III enzyme DCR (32). Humans and *C. elegans* encode only one DCR, which can process both dsRNA and miRNA precursors; *Drosophila* has two DCR genes, and the *Arabidopsis* genome encodes four DCR homologs with each responsible for distinct pathways (46). DCR binds and cleaves dsRNA endonucleolytically and generates a 21–25 bp long dsRNA (47–50). The short dsRNA fragments are separated, and one strand is incorporated into an effector complex, called RNA-induced silencing complex (RISC) (22), where it binds directly to a member of the Argonaute protein family. Argonaute proteins—in plants as well as in animals—are at the heart of the function of RNAi and its related pathways.

The activity of Argonaute is affected dramatically by the sequence specificity of the loaded small regulatory RNA (i.e., the degree to which the small RNA is complementary to a "target" mRNA) (see below). In plants, miRNA-containing RISC guides predominately target cleavage because plant miRNAs are usually extensively complementary to their target genes (51), although bona fide translational repression has been described in plants (52). In contrast, mammalian miRNAs are usually not fully complementary, and miRNA-guided posttranscriptional regulation leads primarily to the repression of gene expression via translational regulation. To distinguish them from mRNA cleavage reactions mediated by RISC, effector complexes that contain mammalian miRNAs are often referred to as miRNPs (53, 54).

The RISC Components and Function

The action of RNA interference is carried out by RISC. RISC is a ribonucleoprotein particle (RNP) with a "core" structure composed of a single-stranded siRNA or miRNA and an Argonaute protein (AGO). Depending on both the isoform of the AGO and the degree of complementarity between the siRNA/miRNA and the target sequence, interaction of RISC with mRNAs can result in multiple silencing events including endonucleolytic cleavage, translational repression, sequestration to P-body compartment, and mRNA degradation (**Fig. 1**).

Although AGO and small regulatory RNAs are the *sine qua non*, other proteins are also important to regulate the function of RISC. The complete protein repertoire of RISCs is not elucidated fully. The reported sizes of RISC vary from 160 kDa (minimal RISC) to 80 S (holo-RISC). The "holo" RISCs were found to be associated with many proteins in addition to the AGO protein and the small RNA. Both *in vitro* and *in vivo* studies showed that an AGO protein and a guide strand siRNA alone can form a functional "core" RISC to cleave the target mRNA. For example, either recombinant human AGO2, or biochemical-purified RISC from human or *Drosophila* cell lysate, are active complexes capable of substrate binding and cleavage (22, 55–61). Similarly, in *Arabidopsis*, the immunoaffinity-purified AGO1 proteins can interact with single-stranded siRNAs to form active RISCs (62). Both recombinant and endogenous RISC-directed target cleavages occur in a divalent cation (Mg^{2+} or Mn^{2+})-dependent manner. Other examples, however, indicate that activity of RISCs needs additional protein components or cofactors for its function. For

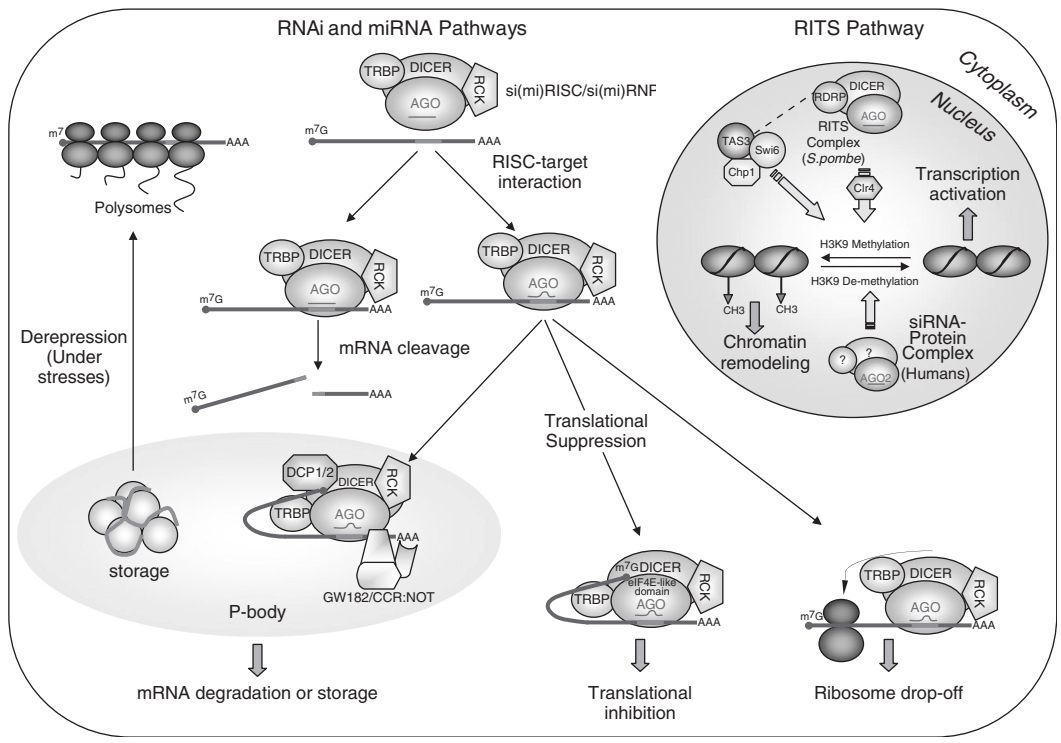

Figure 1 Mechanisms of small RNA–protein complex mediated gene suppression. In cytoplasm, siRNA and miRNAs are sorted and integrated into different types of RISC/RNP complexes. RISCs/RNPs that contain AGO proteins with or without RNase H activity (slicer) and the loaded siRNAs or miRNAs, execute gene suppression function via different mechanisms: 1) target mRNA cleavage by the slicer; 2) prevention of translation initiation by excluding the translation initiation factor eIF4E from binding the mRNA cap (m^7G) via an eIF4E-like domain (MC) located on the AGO proteins; 3) ribosome dropoff from the target mRNA, which is induced by the RISCs/RNPs; 4) sequestration of target mRNAs to P-bodies for temporary storage or degradation. Under certain conditions (e.g., stresses), miRISCs help release the target mRNAs from P-bodies to resume the target mRNA translation. In the nucleus, siRNA-loaded RITS complex directs Clr4 to methylate of the ninth lysine of histone H3. RITS complex recruits the cellular proteins such as Chp1, Swi6, and TAS3, which leads to the chromatin remodeling and shutoff of gene expression in *S. pombe*. In humans, synthetic siRNAs that target promoter regions can interact with AGO2 protein and demethylate the H3K9, which leads to the activation of the target genes.

example, the RISC-directed multiple turnover target cleavage needs ATP, although a component that binds and hydrolyses ATP has not yet been identified (58, 60, 61, 63). Several key components such as DCR, helicases, the GEMIN3 (a DEAD box helicase) (53) and its interacting protein GEMIN4, and dsRNA endonucleases have been identified in different organisms for their roles in RISC function.

RLC and siRNA Loading onto RISC

After cleaving dsRNA to produce siRNAs, DCR may remain in association with siRNAs through interactions with its partner proteins, which change under different circumstance and different species (64, 65). These partner proteins include RDE-4 in *C. elegans* (66), the R2D2 in flies (67), and the TRBP in humans (68). DCR interacts with these small RNA binding proteins and helps sorting small RNA duplex to form the RISC-loading complex (RLC). As its name suggests, RLC is responsible partly for loading the siRNA or miRNA into RISC. The transition from double-stranded to single-stranded RNAs during RISC assembly is achieved via RNA-protein and protein-protein interactions. The RLC is partially characterized in humans (69), *Drosophila* (see below) and *C. elegans*, but the exact composition is unknown.

SiRNA-directed RISC assembly in *Drosophila* is one of the most experimentally dissected and understood systems *in vitro*. One of *Drosophila's* two DCR homologs (DCRs), DCR2, is involved in siRNA production and subsequently siRISC assembly, whereas DCR1 is involved in miRNA biogenesis (70), and its dsRNA binding partner is Loquacious (71, 72). Recent data indicate that the two pathways in *Drosophila* are interchangeable. A subset of miRNA duplexes can be sorted into the siRNA pathway after their biogenesis (64, 65). The sorting of miRNAs into RNAi pathway requires the disassociation of miRNA duplex from DCR1 complex and a recruitment of the duplex to DCR2 complex. DCR2 is tightly associated with its partner, R2D2, to form a stable heterodimer (DCR2-R2D2), without which both are unstable in cells (67). The formation of RLC starts from the interaction between the siRNA duplex and the DCR2-R2D2 heterodimer (73). R2D2 is a sensor protein that tends to bind the thermodynamically more stable siRNA duplex end and places the siRNA duplex onto DCR2-R2D2 in the appropriate orientation (73, 74). This directional positioning of the siRNAs on RLC during RISC assembly referred to as "asymmetric assembly of RISCs" or "asymmetric assembly of RLC" is a key function of RLC. The asymmetric complex assembly is defined mainly by R2D2 and the structures of the small RNA duplexes (73).

Whether siRNA/miRNA are loaded as double stranded, or are first unwound with only single-stranded guide siRNA being loaded into RISC, is still not fully understood. Initially, it was thought that a nonprocessive helicase that separated the two strands of the duplex was involved in RLC (74). In later studies, it has been suggested that siRNAs are loaded initially into RISC as duplexes, and AGO2 cleaves the passenger strand of the siRNA, which facilitates its displacement and leaves the siRNA guide strand bound stably to AGO2 (75–78). A recent report by Robb and Rana (79) demonstrated that human RNA helicase A (RHA) is an siRNA-loading factor. RHA interacts in human cells with siRNA, AGO2, TRBP, and DCR, and functions in the RNAi pathway. In RHA-depleted cells, RNAi was reduced as a consequence of decreased intracellular concentration of active RISC assembled with the guide-strand RNA and AGO2. It is envisaged that other components of RISC are waiting to be identified.

RISC-Mediated Target mRNA Interaction

The rules for the interactions between RISC and target mRNAs probably differ somewhat between species and perhaps between tissues in a given species. Two recent papers from the Zamore laboratory (64, 65) provided their important findings about RISC-target interaction in *Drosophila* S2 cells or embryo lysate. Despite distinct pathways of siRNAs and miRNAs biogenesis, the authors showed that siRNAs and miRNAs are sorted by a common process that involves two types of functionally distinct Argonaute protein complexes: AGO1 and AGO2. The sorting of small RNAs between AGO1 and AGO2 is an active process that depends on the structure of the double-stranded siRNA and the double-stranded assembly intermediates. For example, DCR2-R2D2 acts as a gatekeeper for the assembly of AGO2-RISC, which promotes the incorporation of siRNAs and disfavors the integration of miRNAs. An independent mechanism (yet to be identified) acts in parallel to favor assembly of miRNA/miRNA* duplexes into AGO1-RISC and to exclude siRNAs from incorporation into AGO1. Partitioned small RNAs may be loaded onto either AGO1 or AGO2, and this association then determines how the small RNA functions to repress gene expression either via transcript cleavage or via translation inhibition.

Both siRNA- and miRNA-guided RISCs can lead either to the destruction of the target mRNAs or to the suppression of the target mRNA translation. At least three conditions are essential for target mRNA to be cleaved site-specifically by RISC: 1) the sequence complementarity between the guide RNA and the mRNA target must be complete via the nucleotides numbered 2-11 complementary to the 5′ "seed" region of the siRNA, 2) RISC must comprise an AGO protein (e.g., AGO2 in humans) with RNase H-like endonuclease "slicer" activity for forming a cleaving RISC, and 3) a scissile phosphodiester bond must exist on the mRNA target accessible to the endonuclease active motif located in the PIWI-domain of the AGO protein.

In general, target recognition, binding, and cleavage by RISC are determined mainly by the base pairing between the 5′ portion (~10 nt) of a siRNA or miRNA and their target mRNA. When recognizing complementary mRNA, activated RISC forms an effector complex with the target mRNA (80, 81). After cleavage, the target mRNA is degraded. Activated RISC, as a multiturnover enzyme, is recycled to cleave additional mRNA targets (56).

Only a few AGO proteins, such as hAGO2 in humans (61, 82, 83); dAGO1 and dAGO2 in *Drosophila* (84); RDE1, PPW-1,

ALG1, and ALG2 in *C. elegans* (66); and *Arabidopsis* AGO1 and AGO4 (62, 85) have RNase H-like motifs at the PIWI domains that have been identified to have or potentially to have endonuclease ("slicer") activity. The cleaving activity of the AGO "slicers" is divalent cation- (e.g., Mg^{2+}) dependent (61, 86). It catalyzes the hydrolysis of the phosphodiester linkage of mRNA target at specific site and yields 3′ hydroxyl and 5′ phosphate termini on the cleavage products (59, 80). This specific cleavage site is predetermined on RISCs and is located precisely at the phosphodiester bond in the mRNA target between the nucleotides paired to base 10 and 11 of the guide RNA from the 5′ end (57, 61, 80, 87, 88).

Crystal structure studies and biochemical analysis revealed that the phosphate group at the 5′ end of the guide RNA has an impact on the cleavage site fidelity by a RISC "slicer" active site (89–93). The guide RNA normally positions on the AGO protein in a RISC effector with the 5′ end anchored into the phosphate-binding basic pocket in Mid-/Piwi-domain, the 3′ end bound with the PAZ-domain, and the middle sequence channeled with the "slicer" active site between the Mid-/Piwi-domain and the PAZ-domain. To make the cleavage event occur, the scissile phosphodiester bond at the target must be positioned conducively by the conformational changes induced by the interaction among the AGO protein, the guide small RNA, and the target mRNA.

The structure of target mRNAs also has a role on RNAi efficiency. Although the base pairing between a siRNA/miRNA and target site is important, *in vitro* approaches and bioinformatic prediction demonstrated that the sequence context that surrounds miRNA-binding sites influence the sensitivity to repression by a siRNA/miRNA (93–96). Sequence context could influence miRNA efficacy by mediating the binding of hypothetical cofactor proteins or by affecting the secondary structure of a target site and its accessibility to binding by the siRNA/miRNA. Multiple additional steps in the RNAi pathway including RISC assembly, siRNA strand selection, and target site accessibility might be affected by the target sequence composition. In a recent work by Ameres et al. (97), the authors demonstrate that RISC scans for its target sequences by transiently and nonspecifically binding single-stranded RNA, and it promotes siRNA-target RNA annealing when siRNA meets its target sequence. Their findings also show that cleavage competence of RISC is influenced by the secondary structure of the RNA target and that the 5′ portion of the siRNA determines the stable association of RISC with its target. This study provides insight into the cleavage event in RNAi and could improve the design of potent siRNAs.

RISC-Mediated Translation Inhibition

As stated, translation inhibition mediated by miRNPs/miRISC depends largely on the sequence complementarity of loaded miRNA to target mRNA and the type of AGO protein that is recruited in RISC. Partially complementary siRNAs/miRNAs loaded onto AGO protein that lacks "slicer" activity, is thought to produce translational inhibition. The following are at least four possible mechanisms to explain miRISC-mediated target mRNA translational inhibition: 1) RISC represses protein translation at the stage of translation initiation and postinitiation, 2) small RNA-guided RISC assists relocation and sequestration of the target mRNAs to cytoplasmic processing bodies (P-bodies, stress granules, etc.), 3) RISC guides mRNA decay triggered by rapid deadenylation, and 4) miRISC may cause immediate and/or fast protein degradation after translation (98).

Small RNA-guided RISC can inhibit translation at the initiation step of protein translation (99, 100) or at postinitiation stages (101–103). Pillai et al. (100) showed that in mammalian cells, let-7 miRNP inhibits translation at the initiation step. The authors employed two independent approaches to investigate the inhibition process. One involved tethering the human AGO protein to the 3′-UTR of a reporter gene to mimic the miRNA-mediated translational repression in HeLa cells. The second approach was to assay the endogenous let-7 miRNPs for their inhibition of protein translation of a reporter mRNA that contains the let-7 targeting sites. The results demonstrated that miRNA guided/associated miRNP/miRISC blocked the protein translation initiation in an m^7G-cap-dependent manner, which suggests that miRNPs interfere with recognition of the cap.

The 5′ end of eukaryotic mRNAs is modified by the addition of a 7-methyl guanosine cap. Eukaryotic initiation factor 4E (eIF4E) binds the m^7G cap directly, and this interaction is essential for the initiation of translation of most eukaryotic mRNAs. Regulation of translation initiation is the most common target of translational control, and preventing binding of eIF4E to the m7G cap is a commonly employed cellular strategy to inhibit translation (104). A recent study by Kiriakidou et al. (105) indicates that the translation inhibition mediated by miRNPs occurred at the initial translation stage. Kiriakidou et al. (105) identified a cap-binding-like domain (MC) in the middle domain of AGO2. The MC domain demonstrates m^7G cap binding activity and is required for translational repression but not for assembly with miRNA or endonucleolytic activity. In addition to their finding, the authors propose that AGO represses the initiation of mRNA translation by binding to the m^7G cap of mRNA targets, which is likely to preclude the recruitment of eIF4E. Intriguingly, Thermann and Hentze (106) have shown evidence that *Drosophila* miR-2 function is mediated by inhibiting m7GpppG cap-mediated translation initiation in association with the formation of large RNPs they call "pseudopolysomes."

RISC may also mediate dropoff of translating ribosomes by causing ribosomes to exit prematurely from their associated mRNAs (103). Alternatively, protein translation might not be affected, but the nascent protein is degraded rapidly by the ability of miRNPs to recruit proteolytic enzymes that would degrade nascent polypeptides that emerge from the actively translating ribosomes. The hypothesis that miRISC/miRNPs repress protein translation posttranslationally is also supported by the fact that many miRNAs are associated with actively translating, endogenous mRNAs in polysomes (107). A recent study also found that in human and worm cells alike, depletion of the protein eIF6 (an "anti-association" ribosome inhibitory protein known

to prevent productive assembly of the 80 S ribosome) abrogates miRNA-mediated regulation of target protein and mRNA levels (108).

The AGO proteins and the repressed mRNAs are enriched in the cytoplasmic processing bodies (P-bodies, also known as GW-bodies) (100, 109, 110). P-bodies represent discrete cytoplasmic foci, which are enriched in proteins involved in mRNA decay and translational repression. These proteins include deadenylases, decaping enzymes such as DCP1 and DCP2, and $5' \rightarrow 3'$ exonucleases (111). On the other hand, P-bodies lack ribosomes and protein translation machinery. In P-bodies, mRNAs can undergo decapping and degradation (112), or they can be stored temporarily. As such, under certain conditions (e.g., stress) (113), the target mRNAs sequestered to and stored in P-bodies can be released and re-recruited by the ribosome to resume the translation.

Chu and Rana (114) showed that in some human cells, miRNA function requires RCK/p54, which is a DEAD-box helicase 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 is a general repressor of translation. Disrupting P-bodies did not affect RCK/p54 interactions with AGO proteins or its function in miRNA-mediated translation repression. Depletion of RCK/p54 disrupted P-bodies and dispersed AGO2 throughout the cytoplasm but did not affect RISC functions significantly. These studies also suggest that translation suppression by miRISC does not require P-body structural integrity and suggests that location of miRISC to P-bodies is the consequence of translation repression.

Downregulation of gene expression by miRNAs is also associated with a substantial degradation of some target mRNAs (115–118). Some mRNAs that are destined to undergo miRNP-mediated decay are first deadenylated (115, 119, 120). The CCR4:NOT1 was identified as deadenylase complex in *Drosophila* S2 cells (119). In addition, the GW182 protein was identified as a key factor required for mRNA deadenylation. mRNA degradation by miRNAs requires GW182, the CCR4:NOT1 deadenylase, and the DCP1:DCP2 decapping complexes. The following is a model proposed for this posttranscriptional gene suppression: AGO1-containing RISCs bind to mRNA targets by means of base-pairing interactions with miRNAs; AGO1 may then recruit GW182, which in turn marks the transcripts as targets for decay via a deadenylation and decapping mechanism (115, 119, 121)

Although miRNAs generally have been identified as negative regulators of expression of the target mRNAs in most cases, accumulating evidence now suggests 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 the ribosome to resume protein translation as discussed above (113, 122). This derepression of the target mRNA translation by miRNA needs protein cofactors that are likely induced by stresses and can 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 (113 122) and apolipoprotein B mRNA-editing enzyme catalytic polypeptide-like 3G (APOBEC3G or A3G) (123). Moreover, synthetic siRNAs that target the promoter regions of the human genes E-cadherin, cyclin kinase inhibitor p21 ($^{WAF1/CIP1}$) (p21), and vascular endothelial growth factor induced the expression of these genes through chromatin remodeling related mechanism that requires the human AGO2 protein (124). These findings suggest a more diverse role for miRNAs and siRNAs in the regulation of gene expression than previously appreciated. However, the degree to which these mechanisms are biologically widespread remains to be observed.

RITS Complex-Directed Chromatin Remodeling

Different from cytoplasmic RNA interference pathways is the RNA silencing pathway, which is named RNA induced transcriptional silencing (RITS) and occurs in the nucleus. RITS plays a general role in the construction of centromeres (125, 126). Extensive studies, most notably in the fission yeast *Schizosaccharomyces pombe*, have established RNAi-dependent chromatin remodeling. H3K9 methylation is a central process for RNAi-dependent formation of heterochromatin, by which genes are silenced in regions that contain repetitious DNA sequences (127–130).

Three "RNAi" genes are required for this process: RNA-dependent RNA polymerase (RDP1), DCR1, and AGO1. RDP1 is thought to amplify or to produce the initial dsRNA trigger for centromeric silencing in *S. pombe*. DCR1 is presumed to generate siRNAs from the trigger dsRNA. AGO1 is a component of the RITS complex, which is the effector complex of siRNA-directed transcriptional silencing. In this pathway, siRNA homologous to the repeated sequences are generated by DCR and associate with an Argonaute via mechanisms not defined fully. These siRNA/RISCs direct histone-modifying components, such as Clr4, a H3K9-specific histone lysine methyltransferase (HKMT), to homologous loci. Clr4 then methylated lysine 9 of histone H3 (H3K9). Methylated H3K9 recruits effectors like Swi6, Chp1, and other components (e.g., TAS3), which lead eventually to the formation of condensed heterochromatin structures (8, 129, 131–133). Interestingly, synthetic siRNAs that target the promoter regions of several human genes activated the gene expression that needs the human AGO2 and is associated with a loss of lysine-9 methylation on histone 3 at siRNA-target sites (124). It seems that methylation and demethylation of H3K9 to switch off and on genes are via related small RNA pathways in *S. pombe* and humans.

Summary and Conclusions

RNAi and miRNA pathways involve ancient evolutionary mechanisms for gene expression regulation. These pathways overlap in many aspects with regard to the processing of small regulatory RNAs and to the targeting of mRNAs for post-transcriptional gene expression regulation. Organisms have

evolved different ways of using these mechanisms, and researchers are only beginning to understand the biologic importance of these pathways. If the prior scientific literature is any indication, then many more surprises will develop as we seek to uncover the roles played by small regulatory RNAs and their protein partners.

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