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Molecular Mechanisms of RNA-Triggered Gene Silencing Machineries

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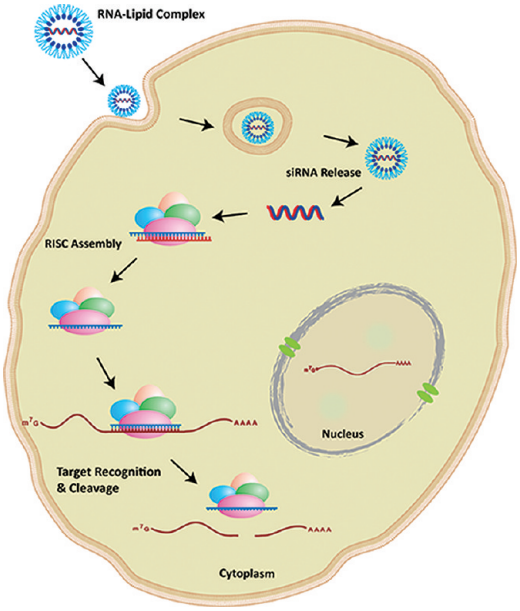
CONSPECTUS

Gene silencing by RNA triggers is an ancient, evolutionarily conserved, and widespread phenomenon. This process, known as RNA interference (RNAi), occurs when double-stranded RNA helices induce cleavage of their complementary mRNAs. Because these RNA molecules can be introduced exogenously as small interfering RNAs (siRNAs), RNAi has become an everyday experimental tool in laboratory research. In addition, the number of RNA-based therapeutics that are currently in clinical trials for a variety of human diseases demonstrate the therapeutic potential of RNAi.

In this Account, we focus on our current understanding of the structure and function of various classes of RNAi triggers and how this knowledge has contributed to our understanding of the biogenesis and catalytic functions of siRNA and microRNA in mammalian cells. Mechanistic studies to understand the structure and function of small RNAs that induce RNAi have illuminated broad functions of the ancient RNAi machinery in animals and plants. In addition, such studies have provided insight to identify endogenous physiological gene silencing RNA triggers that engage functional machineries similar to siRNAs. Several endogenous small RNA species have been identified: small noncoding RNAs (microRNAs), piwi-interacting RNAs (piRNAs), and endogenous siRNAs (endo-siRNAs). microRNAs are the most widespread class of small RNAs in mammalian cells. Despite their importance in biology and medicine, the molecular and cellular mechanisms of microRNA biogenesis and function are not fully understood. We provide an overview of the current understanding of how these molecules are synthesized within cells and how they act on gene targets. Interesting questions remain both for understanding the effects of modifications and editing on microRNAs and the interactions between microRNAs and other cellular RNAs such as long noncoding RNAs.



1. Introduction

Since the discovery in *Caenorhabditis elegans* (*C. elegans*) that double-stranded (ds) RNA could trigger a potent gene-specific silencing phenomenon, termed RNA interference (RNAi),[1] considerable effort has been made in many biological disciplines to address some of the fundamental questions surrounding RNAi. For example, is RNAi a general mechanism for gene regulation that is conserved across species? What are the physiological triggers of RNAi and how does it play a role in biological processes? Work designed to address such questions has led to the recognition that RNAi is a widespread natural phenomenon that is conserved across fungi, plants, and animals.

Long dsRNAs generate potent RNAi and silence target genes by inducing cleavage of their mRNA. However, in mammals, long dsRNA activates the innate immune response by inducing interferon pathways. Further mechanistic studies led to the discovery that mRNA cleavage induced by RNAi was guided by small ~21 nucleotide (nt) RNA fragments derived from long dsRNAs, which revealed that these small interfering RNAs (siRNAs) are the essential triggers for RNAi. Since these discoveries were made, great effort has

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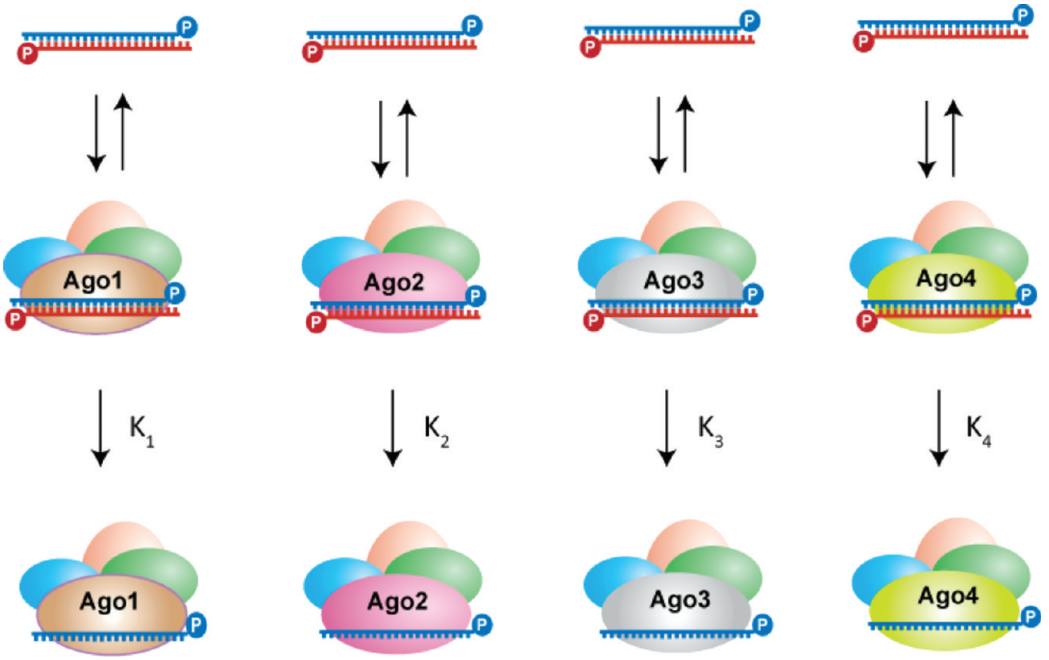

TABLE 1. Small RNAs Involved in Gene Silencing

class	size (nt)	function	origin	species
microRNAs	21−25	translation repression and mRNA destabilization	miR-coding genes, introns (mirtrons)	C. elegans, D. melanogaster, A. thaliana, O. sativa, mammals
endo-siRNAs	21−25	transposon silencing and mRNA degradation	transposons, pseudogenes	D. melanogaster, C. elegans, mammals
siRNAs	21−25	mRNA degradation and transposon silencing	intergenic regions, exons and introns	C. elegans, D. melanogaster, S. pombe, A. thaliana, O. sativa
piRNAs	24−31[a]	transposon silencing, spermatogenesis	transposons and other repeat sequences	C. elegans, D. melanogaster, Danio rerio, mammals

[a] piRNAs in C. elegans are ~21 nt in length.

been directed at identifying endogenous physiological triggers that have similar properties to siRNAs. Several endogenous small RNA species have been identified, including small noncoding RNAs (microRNAs), piwi-interacting RNAs (piRNAs), and endogenous siRNAs (endo-siRNAs).

MicroRNAs (miRNAs) are single-stranded RNAs ~21 nt in length that are involved in almost every area of biology, including developmental processes, disease pathogenesis, and host−pathogen interactions.[2−4] The biogenesis of mature miRNAs relies mainly on digestion of the precursor RNA hairpin structure by two members of the RNase III family, Drosha and Dicer, while other miRNAs can be generated through splicing of miR-coding introns. MicroRNAs are loaded into a functional ribonucleoprotein assembly called the RNA-induced silencing complex (RISC), which serves as the catalytic engine for miRNA-mediated post-transcriptional regulation. Although some studies have suggested a potential role for miRNAs in translational activation, the more common mechanism of miRNA-mediated gene regulation involves repression. In general, miRNAs bind imperfectly to the 3′ UTR of target mRNA and block their expression by directly inhibiting the translational steps and/or by enhancing mRNA destabilization.[5−7]

Piwi-interacting RNAs, piRNAs, are germ cell-specific and larger than miRNAs, spanning ~24−29 nts in length. piRNAs were discovered in *Drosophila melanogaster* development studies, and most of these RNAs matched to intergenic repetitive element sequences including retrotransposons. Distinct from miRNAs, piRNAs directly interact with Piwi proteins and have been shown to regulate transposon activities in *Drosophila*.[8] piRNAs associate with Piwi proteins, and their biogenesis does not involve Drosha or Dicer activities. Although not tested, it is possible that Piwi proteins provide nuclease function to generate piRNAs. piRNAs are

encoded in clusters throughout the genome. Since Piwi proteins exhibit RNA cleavage activities, a unique amplification loop has been proposed for piRNA biogenesis, in which each piRNA-mediated cleavage creates the 5′ end of a new piRNA.[9]

A third class of small RNAs, the endo-siRNAs, was originally discovered in *Drosophila*[10,11] where they were shown to be expressed in both gonadal and somatic tissues, and bind mainly to the Ago2 protein.[11] Endo-siRNAs can be generated from such distinct loci as transposon elements, natural antisense transcripts (NAT), and pseudogenes, as well as from other long hairpin mRNAs.[10,11] However, the biogenesis of these small RNAs remains unclear and, at least in Drosophila, requires the involvement of the protein Loquacious (LOQS).[11] Classes of small RNAs in various species and their origin and function are summarized in Table 1. In mammals, there are four Ago proteins that possibly assemble small RNAs into RISC with different rate constants (Scheme 1).

Although piRNAs and endo-siRNAs exhibit an interesting ability to regulate certain genomic loci elements, miRNAs are the most abundant species of small RNAs in mammalian cells. Despite their importance in biology and medicine, the molecular and cellular mechanisms of miRNA biogenesis and function are not fully understood. In this Account, we focus on our current understanding of the structure and function of RNAi triggers and how this knowledge contributes to our understanding of miRNA function in mammalian cells.

2. The RNAi Triggers

A variety of RNA molecules are able to induce RNAi, including hairpin RNAs, long double-stranded RNAs, RNA viruses, transposon elements, and exogenously introduced siRNAs.[12] Hairpin RNAs and long dsRNAs induce RNAi after processing by the enzyme Dicer, an RNase III family endoribonuclease (Figure 1). The products of Dicer activity are small RNAs with a 2 nt overhang at the 3′ end of each strand, and a monophosphate at the 5′ end. Dicer binds to both linear dsRNAs and hairpin RNAs; thus, these molecules could be expressed by DNA vectors in target cells to induce efficient gene silencing. After cleavage by Dicer, the resulting ~21 nt RNAs are loaded into an RNA–protein complex called the RNA-induced silencing complex (RISC). Alternatively, exogenous siRNAs of the same length can be directly introduced into cells and loaded into RISCs without Dicer processing;[12] this has become the standard experimental method to induce transient gene silencing in mammalian cells. Depending on the original source of the small RNAs, RISCs are termed miRISCs or siRISCs. Once loaded into RISCs,

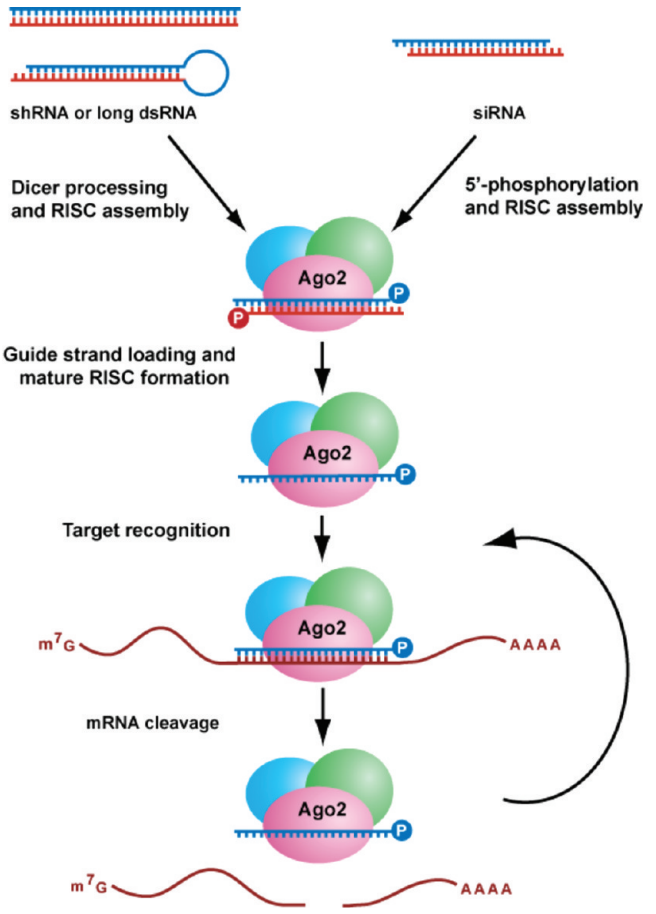

FIGURE 1. Steps in RISC function. Double-stranded (ds) or short hairpin (sh) RNAs are first bound and cleaved by Dicer into small interfering RNAs (siRNAs; ~21 nt) with 2 nt overhangs and 5′ phosphates. These siRNAs are then loaded into protein complexes termed RNA-induced silencing complex (RISCs). Ago2, a component of RISCs, binds the double-stranded siRNAs and cleaves the passenger strand, which induces its dissociation from the RISC complex and degradation. The remaining guide strand then leads the activated RISCs to find target mRNAs that contain perfectly matched complementary sequences to the guide strand. Binding of RISCs to the target mRNAs induces conformational changes and results in cleavage of the mRNA by Ago2. Cleaved mRNAs are then subject to mRNA decay or degradation, thus silencing the target gene expression.

the two strands of the RNA duplex have distinct fates. The sense (passenger) strand that has the same sequence as the target mRNA will be cleaved and degraded. In contrast, the antisense (guide) strand that has the complementary sequence to the target mRNA will remain in the RISC and direct recognition and cleavage of the target mRNA (Figure 1). Target gene expression is silenced by cleaving the mRNA 10–11 nt upstream of the 5′ end of the guide strand. This is mediated through the activity of Ago2, which is one of the main components of RISCs and contains an enzymatically competent RNase H-like domain. Ago2 lies at the heart of RNAi pathways and is the catalytic center of RISC function.

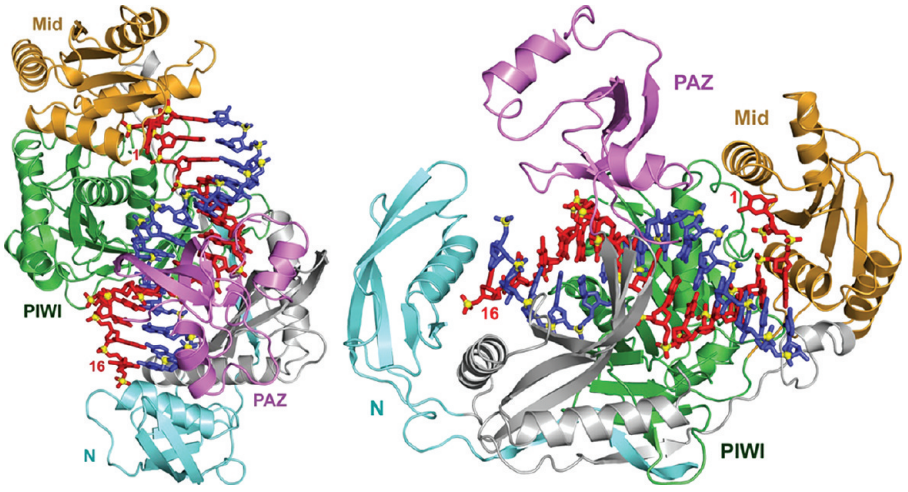

FIGURE 2. Crystal Structure of *T. thermophilus* Ago (Asn478) bound with 21 nucleotide guide DNA and 19 nucleotide target RNA. Two views of the 2.8 A crystal structure of the ternary complex. The structure was generated using mutant Ago of *T. thermophilus*, which is unable to cleave the target RNA, thus facilitating detailed examination of the cleavage site at position 10−11. The guide strand DNA (red) is traced for nucleotides 1−16, which are perfectly matched with its target mRNA (blue). Target RNA is traced for nucleotides 2′−16′. Only the 5′ end of the guide strand is anchored in this ternary complex. The two strands retain the conformation to one turn of A-form helix (12 nt) upon binding,[13,14] and the cleavage site of nt 10−11 stack on each other in a catalytically competent conformation. The N-domain of Ago seems to block the interaction between the guide strand and target mRNA beyond position 16, thus the 3′ end could be released from the PAZ domain. Adapted from ref 17.

After the target mRNA is cleaved, the RISCs are recycled and proceed through several rounds of cleavage events.

Not all siRNAs are loaded into RISC with the same efficiency. Several studies have uncovered some key siRNA features that considerably affect their RISC loading efficiency, and thus also affect the downstream potency of RNA interference. One important feature is the RNA structure. The ideal RNAi triggers adopt an A-form helix, which is different from the typical B-form helix of DNA molecules. This helical geometry leads to a more tightly packaged RNAi molecule with a narrower and deeper major groove, making it more stable than the B-form helix. These observations are supported by the results of experiments with mutant siRNAs that contain internal bulge structures[13,14] or residues with chemical modifications on functional groups.[15] The bulge structures may distort the A-form helix by widening the major groove and increasing the accessibility of its functional groups.[16] Consistent with this, introducing bulge structures into the guide strand was found to completely abolish the RNAi activity of mutant siRNAs. These results, together with those using chemical modification of siRNA, have established the essential role of α-helical geometry in siRNA-mediated gene silencing.[12] Recent crystal structures of Ago bound to a guide strand and its target RNA further highlighted the significance of the A-form helix in RISC catalysis (Figure 2).[17] High-resolution crystal structures have been reported of *T. thermophilus* Ago catalytic mutant proteins bound to 5′-phosphorylated 21 nt guide DNA and complementary target RNAs of 12, 15, and 19 nt in length.[17] These structural and biochemical studies provide insight into the guide-strand-mediated recognition and cleavage of target RNA by Ago, as well as the importance of divalent metal ions in catalysis.[17] Ternary structures have determined that both ends of the guide strand are anchored forming one helical turn of the A-form helix with the 12 nt target RNA spanning the seed region and cleavage site. Analysis of base stacking between RNA and protein showed interesting interactions: the base at position 16 of the guide strand stacked on the aromatic ring of Tyr43 while the base at 16′ of the target strand stacked over the Pro44 ring. Base-pair stacking is disrupted for bases 17, 18, and 19, leading to separation of guide and target strands (Figure 2). These interactions demonstrate an unexpected role of the N domain in blocking the propagation of the guide strand-target RNA duplexes beyond position 16 in the 19 nt target ternary complexes (Figure 2).

A second RNAi feature that influences efficient RISC loading and RNAi is the requirement for 5′ phosphorylation of the guide strand. siRNAs generated from long dsRNAs by Dicer all contain 5′ monophosphates, while exogenously introduced siRNAs often have 5′ hydroxyl groups. This suggests that loading of siRNAs into functional RISCs may require 5′ phosphorylation of siRNAs. Indeed, this is supported by the observation that RNAi activity can be abolished by chemical modification of the 5′ end of siRNAs with amino groups and 3-carbon linkers to block phosphorylation.[13] This modification could also block the binding of

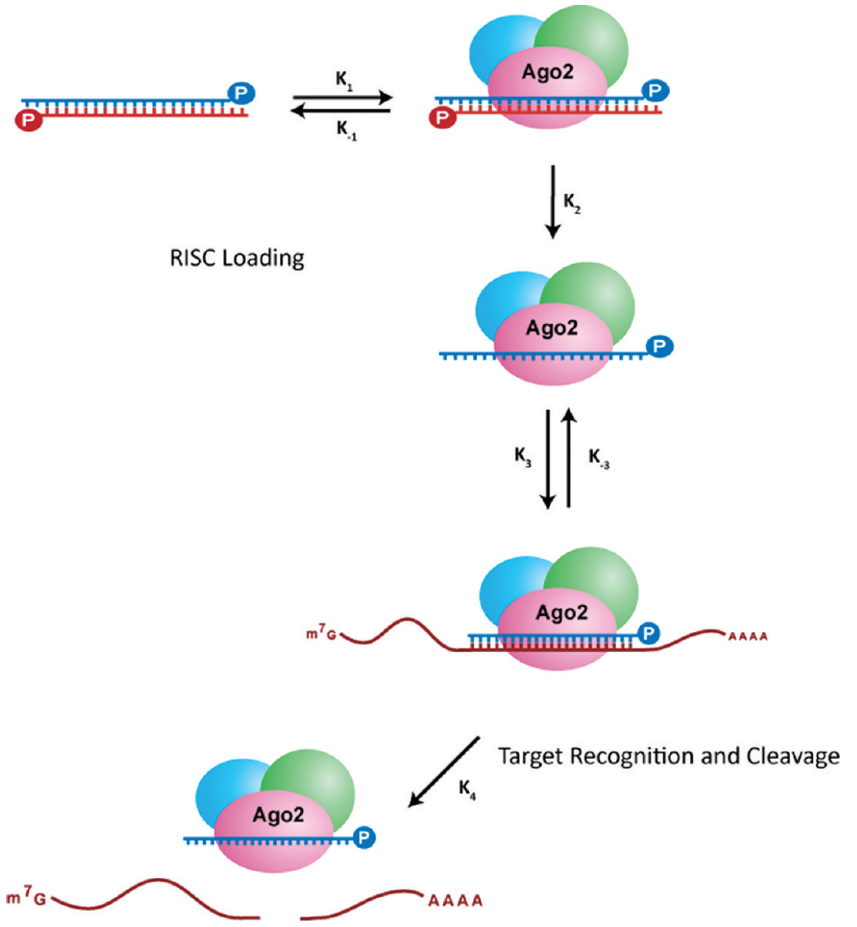

FIGURE 3. Kinetics of RISC assembly and function. The assembly and function of RISCs can be divided into at least two catalytic steps; for simplicity, only two checkpoints are considered here. The first checkpoint is RISC loading. siRNA binding by RISCs is denoted as K_1, and assembly of functional activated RISCs is denoted as K_2. K_2 can be affected by the thermodynamics of siRNAs. The second checkpoint involves target recognition and cleavage. After guide strands of siRNAs are loaded into RISCs, the protein complex is activated and led by the guide strand to target mRNAs. Target mRNAs are bound by functional RISCs, change their conformation to A-form helices and are finally cleaved by Ago2 at nt position 10−11 from the 5′ end of the guide strand. The target mRNA recognition by RISCs is denoted as K_3, and mRNA cleavage is denoted as K_4. K_3 could be affected by several factors such as the secondary structure of target mRNAs.

cellular factors that recognize the 5′ hydroxyl group. Interestingly, unlike the guide strand, modifications of the passenger strand, such as chemical modification and introduction of bulge structures, are well tolerated. Most passenger strand modifications will not negatively affect RNAi activity as long as the A-form helix structure of the siRNA duplex is maintained.[12] This includes capping the 5′ hydroxyl of the passenger strand to facilitate loading of the guide strand into functional RISCs.

3. Kinetics of the Catalytic Engine Assembly

The assembly of RISCs requires a series of kinetic processes and can be divided into at least two catalytic steps: (1) RISC loading and (2) target recognition, cleavage, and release. These two events each contain several further steps, such as dsRNA binding, target recognition, cleavage, product release, and RISC recycling (Figure 3). For simplicity, only two checkpoints steps are considered here. The overall catalytic efficiency of RISC assembly can be represented by K_{cat}, which is the turnover number or the number of reactions that occur at the catalytic site per unit of time. The K_{cat} for RISC loading is designated as K_2, while that of the second catalytic step, target recognition and cleavage, is designated as K_4. Therefore, RISCs with high catalytic potentials would have high K_2 and K_4.

Several parameters may affect the rates of K_2 and K_4 and thus result in the assembly of RISCs with different performance characteristics. For example, the thermodynamics of double-stranded siRNAs could determine which strand gets loaded into the RISCs. siRNA duplexes with unstable 5′ ends in the guide strands will enable efficient incorporation of the guide strand into the functional RISCs (activated RISCs).

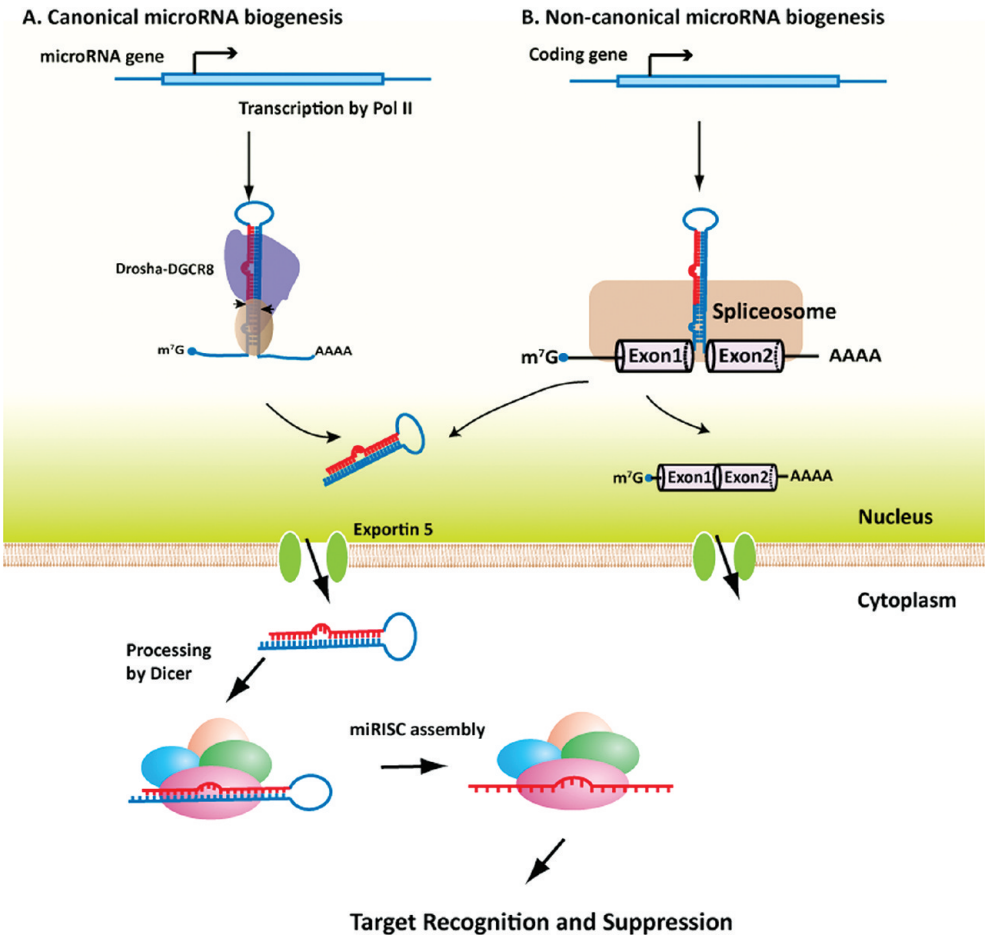

FIGURE 4. Canonical and noncanonical microRNA biogenesis pathways. Depending on the origin of miRNAs, two pathways have been proposed for miRNA biogenesis in vivo. (a) Canonical miRNA biogenesis. In this pathway, miRNA-encoding genes are first transcribed, usually through the Pol II promoter, into primary-miRNA-containing mRNAs. Hairpin structures within these mRNAs are then detected and bound by the Drosha–DGCR8 protein complex. Drosha cleaves the hairpin and generates ~70 nt long miRNA precursors, called pre-microRNAs. Pre-miRNAs are then transported from the nucleus into the cytoplasm through exportin 5, and are further processed by the Dicer complex. Processing by Dicer generates ~21 nt mature miRNAs which are then loaded by Ago2 to form functional RISCs and carry out downstream functions. (b) Noncanonical microRNA biogenesis. In this pathway, miRNAs are usually encoded in the intron regions of protein-coding genes, called mirtrons. After transcription, primary mRNAs are bound and processed by spliceosome protein complexes, which give rise to mature protein coding mRNAs and ~70 nt pre-miRNAs after debranching. Pre-miRNAs generated in this way then join the ones from the canonical pathway for transportation and Dicer processing.

During this process, the passenger strand will be cleaved by Ago2 and subjected to further destruction. Removal of the passenger strand facilitates RISC formation.[18] Therefore, siRNAs with unstable 5′ ends in the guide strands will likely have a high K_2, which indicates that it will be more efficiently incorporated into the functional RISCs. As mentioned previously, modification of siRNAs would affect their loading efficiency into RISCs, which is another factor that could affect K_2. However, K_2 is not the only factor to consider for achieving efficient downstream target gene silencing. In reality, not all of the activated RISCs would have the same target mRNA recognition and cleavage, and thus the second K_{cat}, K_4, is postulated to be crucial as well. At least two parameters could control K_4. One is the accessibility of target mRNAs. The local environment of a target mRNA could indeed have a profound impact on the silencing efficiency of the same RISCs. Recent studies have shown that mRNA regions with strong secondary structures, such as hairpin and stem loops, are resistant to targeting by RISCs.[19−21] In this case then, high K_4 represents high accessibility of mRNAs for activated RISCs. Another factor that could affect K_4 is the structural flexibility of the RISC complex. Various studies have shown that RISCs formed in vivo (holo-RISCs) by delivery of exogenous siRNAs into the cell have lower K_{cat} (K_4) than RISCs formed in cell lysates (minimal RISCs), or recombinant RISCs.[19] This could be due to binding of additional cellular factors to the RISCs, thus restricting the structural flexibility of the assembled protein complex. It should be noted that

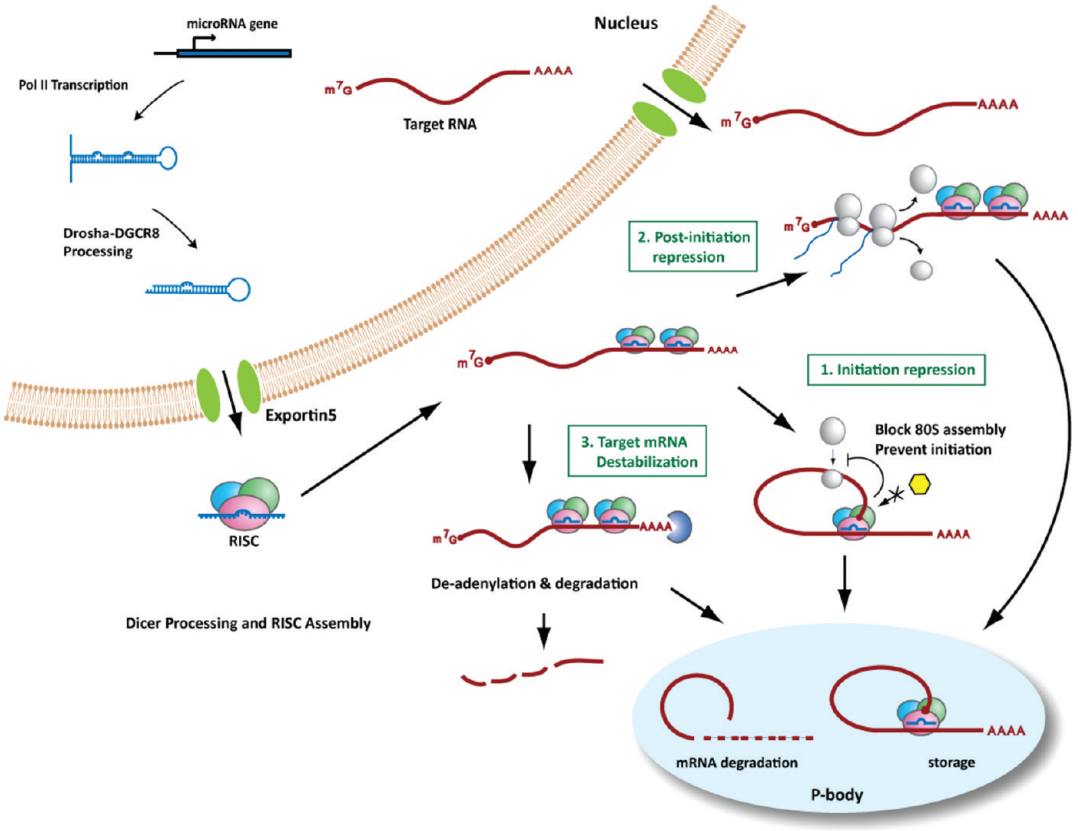

FIGURE 5. microRNA function. After loading with Ago proteins to form functional RISCs, miRNA-guided RISCs bind to the target mRNAs and inhibit target gene expression. Currently, there are at least three mechanisms that have been linked to miRNA-mediated gene silencing. (1) Repression of translation initiation. In this case, miRISCs inhibit initiation of translation by affecting the eIF4F-cap recognition, 40S small ribosomal subunit recruitment, and/or by inhibiting incorporation of the 60S subunit and formation of the 80S ribosomal complex. Some of the target mRNAs bound by miRISC is transported into P-bodies for storage and may re-enter the translation phase when induced. (2) Postinitiation translational repression. miRISCs could interfere after translation has been initiated by inhibiting elongation of ribosomes, causing ribosome drop-off from mRNAs, and/or by facilitating degradation of newly synthesized nascent peptides. (3) Destabilization of target mRNAs. miRISCs could cause destabilization of target mRNAs by directly interacting with CCR4-containing deadenylation complexes and facilitating the deadenylation of poly A tails of target mRNAs. Following deadenylation, the 5′ end-capping structures of target mRNAs are also removed by the DCP1−DCP2 complex.

RISCs with high K_4 might not be advantageous since the high structural flexibility could increase the risk of nonspecific mRNA destruction in cells.

There are two specific steps of RISC assembly that are rate-limiting (Figure 3). The first is the binding and loading of siRNAs into RISCs, and the second is the target recognition process. Two mechanisms have been envisioned by which activated RISCs could recognize target mRNAs. One is a mechanism similar to that used by ribosomes to locate the translation initiation site by scanning across the target mRNA and stopping at the first suitable site. Alternatively, RISCs could recognize the target mRNAs by a diffusion-controlled "hit-and-run" mechanism. To test the scanning model, 2′-*O*-methyl oligonucleotides were used to create blocks near the target site on the mRNAs. If the model is correct, RISCs will be arrested at these blocks due to high-affinity binding of the oligos on the mRNAs, which will prevent RISCs from further scanning. However, the 2′-*O*-methyl oligonucleotides were found to enhance cleavage of target mRNAs by RISC due to the removal of nearby secondary structures and increased accessibility of the targets.[19] Thus, target recognition of RISCs follows the diffusion-controlled model, where antisense strand-guided RISCs are continuously binding to different target mRNAs. Once a perfectly matched mRNA is bound, the complementary strands form an A-form helix and induce conformation changes in the RISCs, resulting in target mRNA cleavage. Interestingly, RISC is about 3-fold more active in the absence of translation and blocking scanning from both the 5′ and 3′ ends of an mRNA does not interfere with RISC function.[22]

4. The Origin of Natural Triggers

Currently, at least two pathways have been identified for miRNA biogenesis (Figure 4). The canonical miRNA biogenesis

pathway starts with the transcription of independent miRNA-encoding transcripts. These primary miRNA transcripts (pri-miRNAs) fold into hairpin structures and are processed in the nucleus by Drosha and its associated protein complex. Drosha is a member of the RNase III family of enzymes and, together with its cofactor DGCR8, cuts the pri-miRNA hairpins to generate ~70 nt miRNA precursors (pre-miRNAs). By contrast, the noncanonical miRNA biogenesis pathway is Drosha-independent. Instead, miRNAs generated through this pathway are usually encoded in the intron regions of protein-coding genes which are often referred to as mirtrons. Mirtron-containing primary transcripts are processed by spliceosomes to generate pre-miRNAs. Pre-miRNAs from both canonical and noncanonical biogenesis pathways are then exported into the cytoplasm by the exportin 5 complex and are further processed by Dicer to generate mature miRNAs. Finally, the miRNAs are loaded into Ago-containing RISC complexes (miRISCs) to carry out their downstream functions.

The regulation of miRNA biogenesis mainly relies on transcriptional regulation of miRNA-encoding genes. However, recent progress provides evidence that other steps in miRNA biogenesis are also tightly regulated.[3,6] In the canonical pathway, Drosha and DGCR8 can cross-regulate each other's expression. Binding of DGCR8 to Drosha's middle domain has a stabilizing effect, but excessive amounts of DGCR8 significantly compromise the processing activity of Drosha in vitro. It is likely that maintaining the correct ratio of Drosha to DGCR8 is crucial for optimal processing activity of the complex and for miRNA biogenesis. In addition to Drosha and DGCR8, Dicer is also regulated by its binding partner TRBP, as a decrease in TRBP levels results in destabilization of Dicer and defects in pre-miRNA processing. This is particularly important in certain diseases such as human carcinomas, where TRBP expression is diminished and causes impaired Dicer function. Since many miRNAs act as potent tumor suppressors, impaired miRNA biogenesis could contribute to the progression of these carcinomas. Collectively, these findings point to a sophisticated network that tightly regulates miRNA biogenesis.

5. MicroRNA-Mediated Post-Transcriptional Gene Regulation

Once formed, pre-miRNAs are exported into the cytoplasm where they are further processed by Dicer and loaded into functional miRISCs (Figure 5). The unique features of miRNAs results in miRISCs having different functions than siRISCs. While siRISCs induce gene silencing by cutting target mRNAs with perfectly complementary sequences to the guide strand, miRNAs induce gene silencing without cleaving the target mRNA, although cleavage activity is retained when a perfectly matched target is present. The seed region of miRNAs, 2−7 nt at the 5′ end of mature miRNAs, plays a key role in determining which target mRNAs are regulated by a given miRNA.[23] The first translation repression mechanism by an miRNA was shown when miRNA Lin-4 in *C. elegans* inhibited Lin-14 expression without causing a reduction in Lin-14 mRNA levels.[24−26] Based on recent developments in understanding miRNA biology and mechanisms, at least three main models can be proposed by which miRNAs could modulate gene expression post-transcriptionally: (1) inhibition of translation initiation, (2) postinitiation inhibition of translation, and (3) mRNA destabilization (Figure 5).

Inhibition of Translation Initiation. MicroRNA-mediated translation repression was observed in HeLa cells in which reporter expression was regulated by let-7 miRNA,[27] and no decrease of reporter mRNAs was detected. In addition, reporter mRNAs containing let-7 target sites shifted to a lighter fraction of polysomal gradients, suggesting that repression could be modulating translation initiation.[27] There are some observations suggesting that this inhibition of translation initiation could be cap-dependent as mRNAs with nontraditional cap structures (ApppG) were less repressed by Cxcr4 miRNA mimics in HeLa cells.[3] This was further supported by in vitro experiments using cell-free extracts.[3] miR-2-mediated repression was shown to be linked with inhibition of 40S ribosomal subunit recruitment and formation of 80S initiation complexes in fly embryo extracts. Additional evidence came from experiments where target mRNA with modified 5′ caps exhibited increased repression by miRNAs. Similarly, supplementing the protein extracts with eIF4F complexes, which directly recognize cap structures of mRNAs, also increased let-7-mediated translational repression of reporter mRNAs. Finally, there is additional evidence demonstrating that joining of 60S ribosomal subunits could also be inhibited by miRNAs.[6] Together, these results show that miRNA-mediated repression of target mRNA is cap-dependent and results from multiple inhibitory effects on translation initiation.

Postinitiation Inhibition. Several studies provide evidence that inhibition of target gene expression by miRNAs can occur at postinitiation steps.[6] Despite the observation that certain miRNAs and Ago proteins can be detected in polysomal fractions, IRES-containing target mRNAs have been reported to be repressed by miRNAs as well.[28,29] Some IRES-bearing mRNAs even showed cap-independent translation while still being efficiently repressed by

miRNAs.[28] One model proposed for postinitiation inhibition is ribosomal runoff, in which ribosomes fall off the mRNA prematurely. Although no direct evidence exists, this model is supported by observations in vitro that inhibition of translation initiation causes a more rapid loss of ribosomes on mRNAs targeted by miRNAs.[28] Premature termination is the simplest explanation for such observations.

mRNA Destabilization and Decay. mRNA degradation by miRNA was reported in *C. elegans* where partial base pairing of let-7 miRNA resulted in degradation of its lin-41 target mRNA.[5] This report raised the possibility that mRNAs containing partial miRNA complementary sites can be targeted for degradation in vivo. Destabilization of target mRNA by miRISCs in mammalian cells has recently been proposed as the main mechanism of miRNA gene regulation in mammalian cells.[7] This destabilization is likely due to deadenylation of target mRNAs.

How do miRNAs cause deadenylation of target mRNAs? Recent studies have revealed the molecular mechanism of miRNA-mediated mRNA deadenylation, in which one key protein, GW182, is centrally involved.[3,6] GW182 directly interacts with all members of the Ago protein family and is localized within P-bodies in the cytoplasm of mammalian cells.[6] Another P-body protein, RCK/p54, a DEAD box helicase, has been shown to interact with the argonaute proteins, Ago1 and Ago2, and modulate miRNA function.[30] RCK/p54 facilitates formation of P-bodies and is a general repressor of translation, suggesting that miRNAs are transferred to P-bodies for further decay or storage.[30] GW182 binds Ago proteins through GW repeats, and tethering of GW182 to the target mRNA promotes mRNA deadenylation[31,32] through GW182-dependent recruitment of the CCR4-containing deadenylation complex.[31] In addition, GW182 also interacts with poly(A) binding proteins (PABP) through its C terminal domain.[33] PABP has previously been reported to be involved in translation initiation by interacting with eIF4G; thus, interactions between GW182 and PABP may interfere with this process and have multiple effects on target gene expression. It is worth noting that mRNA decapping complexes such as DCP1-DCP2 may also be involved in miRNA-mediated gene silencing, as knockdown of DCP-1 and DCP-2 stabilizes deadenylated mRNAs and thus compromises miRNA-mediated inhibition of expression.[6,31]

Therapeutic Applications. Catalytic silencing of specific genes by RNA provided the rationale for RNAi-based therapeutic agents because siRNAs could be designed to treat diseases by lowering concentrations of disease-causing gene products. Similarly, disease-related miRNA dysregulation can be treated either by expressing miRNA mimics to enhance miRNA levels or by inhibiting high levels of disease-related miRNAs in cells. Development of such RNA-based therapies requires chemically stabilized RNA and vehicles for targeted delivery in vivo. Recent advances in understanding the rules for chemically modifying siRNA and miRNA sequences without compromising their gene-silencing efficiency have allowed the design and synthesis of therapeutically effective RNA molecules that can silence target genes in vivo.[34] The second remaining challenge to deliver RNA-based drugs to diseased organs is being addressed by rapid developments in bioengineering and nanotechnologies to design RNA cargo vehicles that can efficiently deliver and release RNA compounds at their target sites.[34] Based on this rapid progress in understanding RNA structure and function in gene silencing and their applications in disease models, it is likely that RNA-based therapeutics will become a reality in the very near future. It is remarkable to witness that, in the short period of time since the discovery of RNAi, a myriad of biotechnology and drug discovery companies using RNAi have been formed, and a number of RNA therapeutics are being tested in clinical trials.

6. Future Perspectives

Given the fundamental roles of miRNAs in regulating a variety of processes, our current understanding of the biogenesis, regulation, and function of miRNAs will no doubt expand considerably in the coming years. One area of particular interest is miRNA editing and modification. Several emerging lines of evidence suggest that modifications on miRNA termini could have a broad impact on their stability, downstream processing, and protein recruitment. In addition, variations have been observed in mature miRNA sequences from the same pre-miRNA, and addition of nucleotides to the miRNA 5′ end could have dramatic effects on its function since the 5′-end seed region determines the target mRNA population. Another potentially interesting area is the emerging role of long noncoding RNAs (lncRNAs) and possible crosstalk between lncRNAs and miRNAs. The lncRNAs could be the natural sponges for miRNAs, and the available miRISCs may be regulated by expression and binding of their corresponding lncRNAs. Additionally, different Ago proteins may regulate each other's function by competing for the available miRNAs (Scheme 1).

Studying these small noncoding RNAs and their potential relationship with protein-coding genes or lncRNAs should

shed light on the complexity of gene regulation and lead to the development of new technologies and therapeutics.

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FOOTNOTES

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