CHAPTER 1

microRNA BIOGENESIS AND FUNCTION
An Overview

René F. Ketting*

Abstract: During the last decade of the 20th century a totally novel way of gene regulation was revealed. Findings that at first glance appeared freak features of plants or *C. elegans* turned out to be mechanistically related and deeply conserved throughout evolution. This important insight was primed by the landmark discovery of RNA interference, or RNAi, in 1998. This work started an entire novel field of research, now usually referred to as RNA silencing. The common denominator of the phenomena grouped in this field are small RNA molecules, often derived from double stranded RNA precursors, that in association with proteins of the so-called Argonaute family, are capable of directing a variety of effector complexes to cognate RNA and/or DNA molecules. One of these processes is now widely known as microRNA-mediated gene silencing and I will provide a partially historical framework of the many steps that have led to our current understanding of microRNA biogenesis and function. This chapter is meant to provide a general overview of the various processes involved. For a comprehensive description of current models, I refer interested readers to the reviews and primary literature references provided in this chapter and to the further contents of this book.

INTRODUCTION: PTGS IN PLANTS AND SMALL RNAs

In the early 90s a number of papers were published that revealed an activity in Tobacco and Petunia plants that was triggered by repetitive transgenic DNA and that resulted in the silencing of that DNA and any other DNA bearing significant homology to the trigger (cosuppression).[1,2] At least part of these phenomena acted downstream of transcription, through destabilization of mRNA and hence was named "Post-Transcriptional Gene Silencing" (PTGS). The molecular trigger of this phenomenon was not clear, although

*René F. Ketting—Hubrecht Institute-KNAW and University Medical Centre Utrecht, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands. Email: r.ketting@hubrecht.eu

Regulation of microRNAs, edited by Helge Großhans.

it was speculated that "aberrant" RNA or double stranded RNA (dsRNA) were good candidates for priming PTGS. Although aberrant RNAs still play an important role in many models on RNA-mediated silencing events in plants, for example as templates on which dsRNA is synthesized, we now know that dsRNA is indeed in most cases the primary trigger. Furthermore, in 1999 a landmark paper from David Baulcombe and colleagues identified small RNA molecules as potential "specificity determinants" in PTGS.[3] This hypothesis has turned out to be absolutely correct and the identification of this type of small RNA species helped to lay the basis for an outburst of research activity on RNA-based silencing processes in the years that followed.

RNAi

In 1998, double-stranded RNA (dsRNA) was first described as a very potent and specific agent for gene silencing in *C. elegans*. The term RNA interference was coined to refer to the described silencing effects, a term that is now usually abbreviated to RNAi.[4] This ground-breaking work, published by Craig Mello, Andrew Fire and their colleagues, was awarded the Nobel Prize in 2006. Mello and Fire noted that RNAi targets exonic regions in RNA and leads to decreased RNA levels, consistent with a model in which RNAi leads to sequence specific mRNA destabilization, as had been found for cosuppression in plants. It was also noted that dsRNA could very well be a trigger in plant cosuppression, since inverted repeat sequences had been described as very potent triggers of PTGS. Soon after this paper, RNAi-like processes were identified in numerous other systems.[5] Biochemical experiments in *Drosophila* started to reveal a mechanistic framework of RNAi,[6,7] while genetics in *C. elegans* was revealing endogenous functions for RNAi and genes required for it.[8,9] It appeared that RNAi could mechanistically be roughly divided into two steps: an initiation step and an effector step (Fig. 1).[10] In the initiation step small RNAs are generated from the dsRNA trigger; in the effector step these small RNAs guide an Argonaute protein-containing complex named RNA-Induced Silencing Complex (RISC) to cognate mRNAs. The realization that small RNA molecules (then named siRNAs, for short interfering RNAs), like those described by Baulcombe in Tobacco plants undergoing PTGS, rather than long dsRNA molecules provided the sequence specificity of the whole process,[7,11] enabled efficient RNAi also in mammalian cells.[12] This provided a highly efficient way to perform reverse genetic experiments in cell culture systems, a finding that has revolutionized research on mammalian cells.

DICER

Dicer, the enzyme that generates siRNAs from dsRNA, was identified in 2001.[13] This enzyme contains two RNase III active sites, a so-called PAZ domain (named after three proteins in which this domain was first recognized: Piwi, Argonaute and Zwille), a helicase domain and a dsRNA-binding domain. It binds to the ends of dsRNA substrates and introduces a staggered double-stranded break further along the dsRNA.[14] The catalytic activity is very characteristic and always leaves a 3′-hydroxyl group, a 5′-phosphate group and a two base overhang at the 3′ end. The length of the small RNA generated can vary, but usually is between 20 and 25 bases. Within one organism, different Dicer

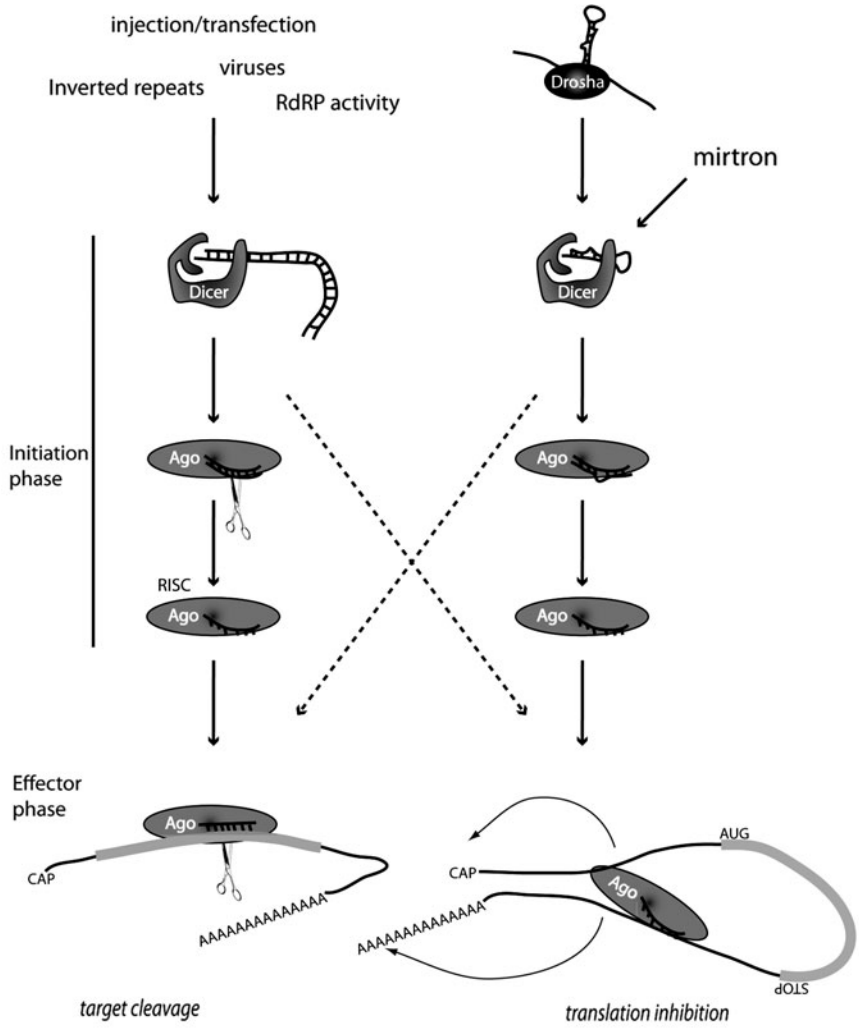

Figure 1. Schematic comparison between RNAi and miRNA mechanisms. For a more detailed scheme of miRNA action see Figure 2. "RdRP activity" refers to RNA-dependent RNA polymerase activity that in plants and yeast can turn ssRNA into dsRNA that is subsequently cleaved by Dicer. Likely, this is a major source for the dsRNA trigger in PTGS. The scissors indicate passenger strand and target cleavage. The dashed lines crossing from RNAi to miRNA and vice versa indicate that the separation between these pathways is not absolute: side effects from siRNAs in RNAi experiments can be triggered through miRNA like activities and miRNAs are capable of inducing target cleavage if presented with a properly matching target RNA. The type of silencing induced is also strongly dependent on the sub-type of Argonaute protein involved.

genes can be present, each encoding a protein generating rather specific subsets of small RNA products.[15] Mammals, however, only have one Dicer gene.

ARGONAUTE

Genetic experiments identified an Argonaute protein as a major player in RNAi in *C. elegans* soon after the discovery of RNAi itself.[16] Biochemical data supported this finding by identifying an Argonaute protein as an essential component of RISC.[17] Argonaute proteins had no known biochemical activities at that time. The only thing that was clear from Argonaute protein sequences is that they contained two characteristic domains: a PAZ domain (also found in Dicer) and a Piwi domain (named after the *Drosophila* Piwi protein, in which it was first recognized). It took a number of years before it became clear that Argonaute proteins actually form the catalytic center of RISC. The PAZ domain was shown to bind to the 3′ end of the small RNA,[18,19] while the Mid domain, in between the PAZ and Piwi domains, appeared to interact with the 5′ end of the small RNA.[20] Finally, the structure of the Piwi domain revealed an RNaseH-like structure,[21] consistent with the biochemical characteristics of RISC that had by then been well defined: endonucleolytic hydrolysis leaving a 5′-phosphate and a 3′-hydroxyl group.[22,23]

This RNA cleavage activity is first used for RISC activation: Initially Argonaute proteins are loaded with double-stranded siRNAs and in order to become active, one of the strands has to be removed. This can be done through endonucleolytic cleavage.[24-26] The discarded strand is referred to as the passenger strand, while the strand remaining bound to the Argonaute is known as the guide strand. It is this strand that guides the Argonaute protein to a target. At the target the very same catalytic activity used for RISC activation now can induce target RNA destabilization. However, this scenario is most likely an oversimplification, as there are indications that the catalytic activity of Argonaute proteins on small RNA duplexes differs from that on target RNA.[27,28]

It should also be noted that many Argonaute proteins contain a Piwi domain that is not compatible with nucleolytic activity. This has implications for both the mechanism of RISC activation as well as for the mechanism through which the targeted RNA is silenced. Passenger strand displacement in these Argonautes depends on weakened basepairing interactions within the small RNA duplex,[29] while target RNA silencing depends on additional cofactors recruited by the Argonaute (see below).

THE FIRST microRNAs AND LINKS TO RNAi

Already in 1993, years before the discovery of RNAi and siRNAs, two papers were published by the Ambros and Ruvkun labs describing a small RNA molecule in *C. elegans* that turned out to be the first microRNA ever to be described: *lin-4*.[30,31] *lin-4* was named as such because mutants display *lin*eage defects during development. The molecular basis of the lineage defects was the capability of *lin-4* to repress the activity of another gene, named *lin-14*, through imperfect basepairing interactions with the 3′UTR of the *lin-14* mRNA. It was also clear that *lin-4* came in two forms: a small 22 nucleotide version and a longer 61 nucleotide version that could fold into an imperfect hairpin structure. The small form of *lin-4* contained all the bases required for the basepairing interaction with *lin-14* and hence was likely the active, or mature form. In a follow-up study it was proposed that *lin-4* represses *lin-14* at the translational level.[32] The broader relevance of these findings remained unclear until 2000 when another small RNA gene was cloned: *let-7*.[33] This second small RNA had many features in common with *lin-4* but, in contrast to *lin-4*, turned out to be extremely well conserved across bilaterian animals.[34] This sparked

the idea that gene regulation through small RNAs could be a much more widespread phenomenon than was appreciated at the time. Ruvkun and colleagues also proposed that the biogenesis and/or function of *lin-4* and *let-7*-like small RNAs could relate to the phenomenon of RNAi for which many biochemical studies had by then also indicated that small RNAs were providing the sequence specificity of the process (see above).

This notion appeared correct, as in 2001 a number of papers demonstrated that Dicer mediates both RNAi and *let-7* function[35-38] by processing the approximately 70 bases long *let-7* precursor into the mature small RNA and the longer dsRNA molecules used in RNAi into siRNAs. What's more, specific Argonaute proteins were shown to be essential for *let-7* function and processing,[35] just like another type of Argonaute protein had been shown to be required for RNAi.[16] Finally, *let-7* was found to trigger an RNAi-like reaction on target RNAs to which *let-7* could basepair perfectly.[39] Together, these findings made a very strong case for intimate mechanistic connections and similarities between *lin-4*-and-*let-7*-mediated gene regulation on the one hand and RNAi on the other (also see Fig. 1).

miRNAs: ANCIENT MEDIATORS OF GENE REGULATION

Not long after the finding that *let-7* was evolutionarily conserved, a number of reports appeared describing numerous endogenous small RNA genes from different animals, including *C. elegans*, *Drosophila* and human.[40-42] Like *let-7*, some of these were evolutionarily well conserved. They also derived from potential double stranded RNA structures, much like *let-7* and *lin-4,* and later studies indeed verified that for most of these novel endogenous small RNAs Dicer is required for the conversion of a longer precursor RNA into a mature small RNA.[43] Apart from their huge scientific impact, these findings also started to illustrate the need for a unifying name for this type of regulatory small RNA molecules: microRNAs (or miRNAs).[44]

Given all these results, it was absolutely clear that mRNA silencing by small RNA molecules was no exceptional feature of either *C. elegans* or plants, but represented a widespread and likely ancient mode of gene regulation. Indeed, miRNAs in the animal kingdom date far back, as far as the very base of the metazoan tree, suggesting that miRNAs have played an important role in the evolution of all animal life on our planet.[45] Notably, the complexity of the encoded miRNA repertoire correlates with the apparent complexity of the animal, raising the possibility that miRNAs have played a role in the growing complexity of multi-cellular metazoans. As in the animal kingdom, very primitive members of the plant kingdom produce miRNAs, as for example in the single celled "green yeast" chlamydomonas,[46] suggesting that also during plant evolution miRNAs have been around from very early-on. Interestingly however, while both plants and animals have a well-developed miRNA system, fungi do not appear to contain an equivalent of miRNA-mediated gene silencing, although a basic RNAi machinery is often present. This, in combination with the fact that plant and animal miRNA biogenesis and silencing mechanism appeared quite distinct in the first instance has been taken as an indication that miRNA-like pathways have independently evolved from the basic RNAi machinery in plants and animals.[47] However, recent discoveries on the mechanism of miRNA action in plants have started to unveil many more similarities between plant and animal miRNA systems, suggesting that despite the many differences, miRNA-type silencing may have started to evolve from the basic machinery even before plant and animal lineages split.[48]

Table 1. Factors involved in the various stages of miRNA biogenesis and function

	Arabidopsis	Drosophila	*C. elegans*	Mammals
First miRNA cleavage	DCL1	Drosha	DRSH-1	Drosha
	HYL1	Pasha	PASH-1	DGCR8
Second miRNA cleavage	DCL1	Dicer-1	Dcr-1	Dicer
	?	Loqs	?	TRBP, PACT
Nuclear export	HASTY	Exportin-5	?	Exportin-5
miRNA Argonaute	Ago1, Ago10	Ago-1	ALG-1, ALG-2	Ago1, 2, 3, 4
Silencing effector	?	GW182	AIN-1, AIN-2	TNRC6
Decapping	Varicose	Ge-1	?	EDC4
	?	Dcp-1, 2	?	Dcp1, 2
Deadenylation	?	?	?	Pan2-Pan3
	?	CAF1-NOT1	?	Ccr4-Caf1
	?	?	?	PABP

PRIMARY miRNA PROCESSING BY DROSHA

Already from the first description of *lin-4* it was clear that miRNAs are derived from RNA hairpin structures formed by RNAs of roughly 65-75 bases in length. As this hairpin RNA is the direct precursor for a mature miRNA it is now referred to as the pre-miRNA. However, it soon became clear that pre-miRNAs are only an intermediate between a long primary RNA transcript (known as pri-miRNA) and the mature form.[49] Pri-miRNAs are generated through transcription by RNA polymerase II and in most cases resemble regular genic transcripts in that they are capped, poly-A tailed and spliced.[50,51] In fact, many pre-miRNAs are generated from protein-coding loci, in which cases they are usually embedded in intronic sequences.[52]

While in Arabidopsis DCL1, one of the four available Dicer-like proteins, is the only RNaseIII-type nuclease required for the generation of most miRNA duplexes[53] and the whole process takes place in the nucleus, in animal systems Dicer only processes the pre-miRNA into the mature form and does so in the cytoplasm. The step from pri- to pre-miRNA occurs in the nucleus and is catalyzed by another RNase III-type enzyme, named Drosha[54] (also see Table 1). This enzyme, together with the RNA-binding protein Pasha, or DGCR8, and other components that together are named the microprocessor,[55-57] binds to the open-ended region of the miRNA-containing hairpin.[58] It then releases the pre-miRNA from the pri-miRNA by a double strand cleavage, leaving a two-base 3′ single-stranded overhang. After transport to the cytoplasm, mediated by a specialized nuclear export factor named exportin 5,[59-61] Dicer recognizes the 3′ overhang on the pre-miRNA through its PAZ domain, after which it cleaves off the loop, generating again a two-basepair 3′ overhang. This product is then loaded into an Argonaute protein.

A minority of animal miRNAs can be processed without involvement of Drosha. In these cases, pre-miRNAs are directly derived from spliced introns, which are processed by a lariat-debranching enzyme into a suitable Dicer substrate. These microRNAs go by the name mirtrons.[62-64]

SMALL RNA SELECTIVITY OF ARGONAUTE PROTEINS

In most species, multiple Argonaute proteins are present and in many cases, specific Argonaute proteins have a strong preference for specific types of short RNA molecules. Two well-described determinants of this effect are the identity of the most 5′ base of the future guide strand[65] and the thermodynamic properties of the small RNA duplex.[66,67] Regarding miRNAs in mammals however, these all seem to go into each of the four Argonaute proteins known as Ago1, 2, 3 and 4[68] and functional differences between these four proteins regarding miRNA function has so far remained unclear. Only Ago2 has a unique role in target mRNA cleavage during RNAi in mammalian cells.

Argonaute loading occurs in the context of the so-called RISC-Loading Complex, or RLC.[69-71] In general, this complex consists of a Dicer protein, an Argonaute and an RNA-binding protein. The human version of this complex consists of three subunits: Dicer, Argonaute and the RNA-binding protein TRBP.[72] In addition, this core complex can interact with additional RNA-binding proteins, such as the TRBP-related protein PACT.[73] Structural studies have begun to reveal how the core Dicer-Ago2-TRBP complex may enable the transition of a processed double-stranded siRNA from Dicer to Argonaute,[74] although no data at atomic resolution is yet available for the RLC.

Apart from a molecular understanding of how siRNAs can be transferred between two proteins, such structural studies will likely shed light on another intriguing observation regarding the maturation of miRNA:Argonaute complexes. This relates to the finding that for most miRNAs, one of the two strands is found in vast excess over the other. The dominating strand is named "mature miRNA" while the other strand is usually called the "miRNA star" (miR*) strand. Interestingly, this is not unique to endogenous miRNAs, as clear directional strand loading can be observed with synthetic, fully basepaired siRNA duplexes where the chance of each strand to become either a guide or a passenger strand is often not random. The physical basis behind this observation has been shown to relate to the thermodynamic properties of the basepairing in the small RNA duplex: in thermodynamically asymmetric duplexes the strand whose 5′ end is basepaired least strongly will remain associated with the mature Argonaute complex (e.g., will become the mature miRNA or the siRNA guide strand).[75,76] The other strand is usually discarded and degraded, although so far no study has directly addressed the fate of the discarded strand in detail.

TARGET RECOGNITION

As described above, miRNAs guide the Ago protein to homologous mRNAs. However, not the complete sequence of the miRNA is relevant in this step: a region in the 5′ part of the miRNA is the main determinant when it comes to target recognition by miRNAs.[77-81] This region, spanning from base two to seven or eight is also known as the "seed" region and structural studies have shown that precisely these bases are projected away from the Argonaute protein allowing efficient basepairing to other RNA molecules.[82-84] Surprisingly, in animal systems, this region alone is sufficient for regulation of the targeted RNA molecules, although more extensive basepairing of the target to regions more 3′ in the miRNA can have an effect on target recognition.[79]

Although mRNAs can in principle be recognized along their complete length, meaning either in their 5′ untranslated region (UTR), coding region or 3′UTR, most naturally occuring miRNA regulatory sites in animals have so far been found in the 3′UTR.[81] This may relate to their mode of action, which often does not include direct target cleavage but rather relates to processes affecting translation (see below), as target RNA cleavage by extensively base-paring miRNAs, which is a common event in plants, often occurs within the coding region.[85]

Given the small amount of sequence information used in target recognition by miRNAs and the fact that target site accessibility also plays an important role in the interaction between RISC and target RNA,[86] in silico target predictions are not easy to make. Just searching for so-called "seed-matches" results in enormously long lists of potential miRNA targets and more sophisticated algorithms had to be developed to generate more meaningful outputs. These algorithms for example take into account the conserved nature of a predicted miRNA target site and apply scores to certain base compositions directly flanking the miRNA-binding site and/or the complete secondary structure of the 3′UTR. Many of these programs produce very useful information to people studying individual miRNAs or protein-coding genes, but one should keep in mind that many of the applied rules are based on correlations and not on mechanistic studies. The output of these programs is therefore highly enriched in genuine miRNA targets, but at the gene-by-gene level one still has to take good care and validate predicted interactions through experiments. For a more in-depth review on this topic please see.[81]

MECHANISMS OF miRNA-MEDIATED SILENCING

As described above, some miRNAs regulate their mRNA target through direct target cleavage, analogous to the mRNA degrading activity observed during RNAi. As already described above, this cleavage event is a very specific one: nucleolytically active Argonaute proteins cleave targeted RNA molecules between the bases that pair to bases 10 and 11 of the small RNA, as counted from its 5′ end,[11] leaving a 5′ phosphate and a 3′ hydroxyl group on the cleavage products.[22,23] These signatures have been used to demonstrate direct target RNA cleavage induced by miRNAs, as it is quite distinct from any other indirect RNA decay pathway. In order to facilitate such direct, Argonaute-mediated cleavage events, the miRNA requires significant basepairing beyond the seed region, including perfect basepairing in the region surrounding the site to be cleaved, i.e., bases 9-11 of the miRNA. This type of regulation is mainly found in plants, although recent work has shown that in plants translational inhibition, as discussed below, makes a major contribution to miRNA-mediated silencing in plants as well.[87,88]

In cases where miRNAs do not induce direct target cleavage, they lead to translational inhibition and mRNA destabilization through mechanisms other than Argonaute-mediated cleavage. The mechanism of translational inhibition by miRNAs has been and still is heavily debated with various models being proposed, ranging from effects directly on the initiation step, shortening of the polyA tail, effects on translation elongation and proteolysis of the nascent poly-peptide chain.[89] However, without going into the many details that can be discussed regarding this topic, many laboratories seem to converge on a model in which miRNAs interfere in some way with the initiation phase of translation, through recognition of the initiation complex or the cap structure[90-92] and in which the Argonaute protein needs to interacts with a GW-repeat-containing protein named GW182,

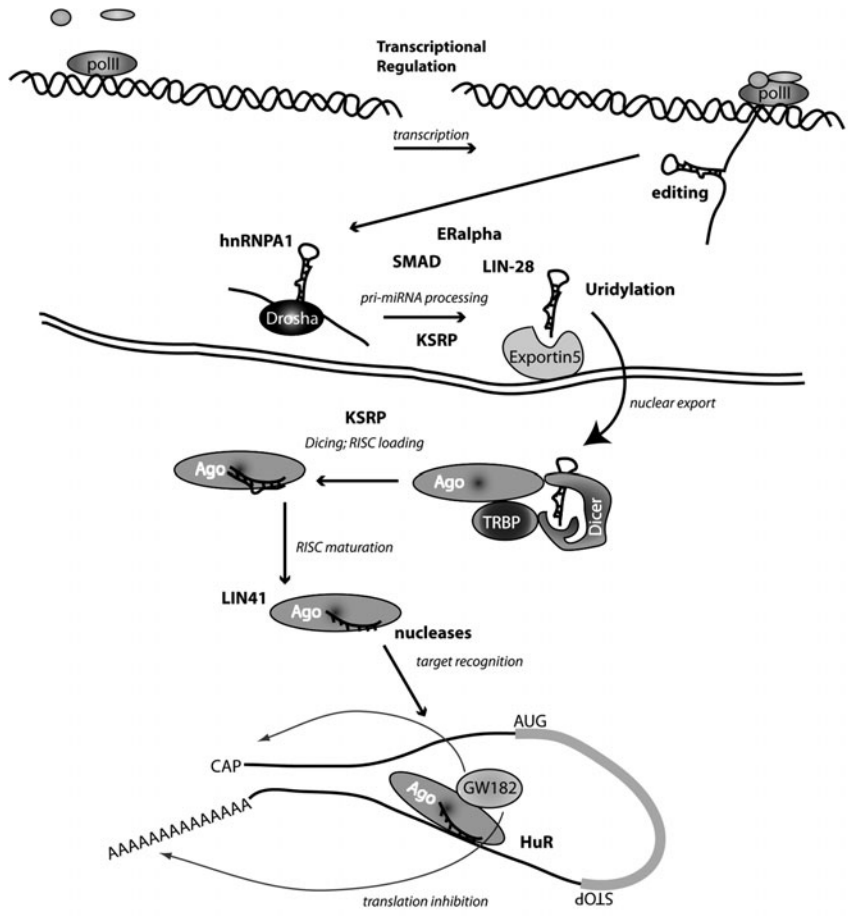

Figure 2. A schematic overview of miRNA biogenesis and function is presented. Important events are indicated in italics. Regulatory activities and/or factors are indicated in bold. The various factors/activities are described in detail in the main chapters of this book.

also known as TNRC6, to achieve this.[93-97] Interestingly, miRNA-targeted mRNAs appear to be separated from the bulk of the active mRNA pool by sequestration into cytoplasmic foci, named P-bodies.[98] While initially P-bodies were considered mechanistically related to the miRNA silencing event, it has now been clearly shown that these structures do not to play an active role in the translational silencing process,[99] but rather might function as a storage place from which silenced mRNA can sometimes be reactivated.[100]

While none of the above interactions directly degrade the targeted mRNA, miRNA-mediated silencing often correlates with decreases in mRNA abundance.[80] Most likely this is the result of mRNA de-capping and de-adenylation processes, known to be associated with P-bodies and these processes do contribute to the overall silencing response,[87,95,101-105] suggesting that while P-body localization is not the main trigger in miRNA silencing, it may still be relevant to the total silencing effect imposed by miRNAs. In fact, large-scale proteomics experiments seem to indicate that mRNA destabilization

may be even more important for general miRNA silencing than effects on translation.[106,107] Clearly, the way miRNAs impose their effects is still open to much debate.

REGULATING miRNAs

Many recent reports have illustrated that miRNA activity itself is subject to regulation. The main focus of the following chapters in this book will discuss in detail a number of mechanisms affecting miRNA-mediated gene regulation. Consequently, I limit myself to the notion that already at this point in time almost all levels in the miRNA pathway have been found to be subject to regulation: miRNA transcription, Drosha, Dicer, Argonaute, 3′UTR and miRNA molecules themselves are all affected by the action of additional proteins (Fig. 2). Furthermore, miRNA pathway genes themselves tend to be strongly regulated by miRNAs, suggesting that negative feed-back loops are essential for proper miRNA-mediated gene regulation. Although it may not be surprising that such a general gene regulatory mechanism is not allowed to operate unchecked, these findings emphasize that the rather simplistic way in which miRNA-mediated gene regulation has so far been approached will surely not suffice to completely understand the how, when and why of miRNA-mediated gene silencing.

CONCLUSION

In a very short time, the research field of small RNAs has grown enormously. The fact that a book can now be written on the mere regulation of just one of the small RNA pathways, the miRNA pathway, is by itself already a clear illustration of that notion. Less than 10 years after the realization that miRNAs represent a significant and widely conserved way of gene regulation, we know many of the core players (Table I) and we are starting to develop a sense of how this small RNA machinery is interwoven with the rest of the cell's processes (Fig. 2 and further chapters). Given the rapid pace of discovery of regulatory steps in the miRNA pathway at present, it seems inevitable that the findings described in this book will soon turn out to describe only our first encounters with miRNA regulatory steps. It appears likely that the miRNA pathway is manipulated in many different ways and a good understanding of these processes will be of great value not only for understanding miRNA function but also for a better appreciation for the many noncoding RNA molecules, other than RNAi-related small RNAs, that are presently being identified. Personally, I look forward to the many discoveries to be made in these fields in the coming years.

REFERENCES

1. Jorgensen RA, Atkinson RG, Forster RL et al. An RNA-based information superhighway in plants. Science 279 1998; 5356:1486-1487.
2. Kooter JM, Matzke MA, Meyer P et al. Listening to the silent genes: transgene silencing, gene regulation and pathogen control. Trends Plant Sci 4 1999; 9:340-347.
3. Hamilton AJ, Baulcombe DC. A species of small antisense RNA in post-transcriptional gene silencing in plants. Science 286 1999; 5441:950-952.

4. Fire A, Xu S, Montgomery MK et al. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391 1998; 6669:806-811.
5. Hutvagner G, Zamore PD. RNAi: nature abhors a double-strand. Curr Opin Genet Dev 12 2002; 2:225-232.
6. Hammond SM, Bernstein E, Beach D et al. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404 2000; 6775:293-296.
7. Zamore PD, Tuschl T, Sharp PA et al. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 101 2000; 1:25-33.
8. Tabara H, Grishok A, Mello CC et al. RNAi in C. elegans: soaking in the genome sequence. Science 282 1998; 5388:430-431.
9. Ketting RF, Haverkamp TH, van Luenen HG et al. Mut-7 of C. elegans, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell 99 1999; 2:133-141.
10. Hannon GJ. RNA interference. Nature 418 2002; 6894:244-251.
11. Elbashir SM, Lendeckel W, Tuschl T et al. RNA interference is mediated by 21- and 22-nucleotide RNAs. Genes Dev 15 2001; 2:188-200.
12. Elbashir SM, Harborth J, Lendeckel W et al. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411 2001; 6836:494-498.
13. Bernstein E, Caudy AA, Hammond SM et al. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 409 2001; 6818:363-366.
14. Zhang H, Kolb FA, Jaskiewicz L et al. Single processing center models for human Dicer and bacterial RNase III. Cell 118 2004; 1:57-68.
15. Ramachandran V, Chen X. Small RNA metabolism in Arabidopsis. Trends Plant Sci 13 2008; 7:368-374.
16. Tabara H, Sarkissian M, Kelly WG et al. The rde-1 gene, RNA interference and transposon silencing in C. elegans. Cell 99 1999; 2:123-132.
17. Hammond SM, Boettcher S, Caudy AA et al. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 293 2001; 5532:1146-1150.
18. Ma JB, Ye K, Patel DJ et al. Structural basis for overhang-specific small interfering RNA recognition by the PAZ domain. Nature 429 2004; 6989:318-322.
19. Song JJ, Liu J, Tolia NH et al. The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nat Struct Biol 10 2003; 12:1026-1032.
20. Ma JB, Yuan YR, Meister G et al. Structural basis for 5′-end-specific recognition of guide RNA by the A. fulgidus Piwi protein. Nature 434 2005; 7033:666-670.
21. Song JJ, Smith SK, Hannon GJ et al. Crystal structure of Argonaute and its implications for RISC slicer activity. Science 305 2004; 5689:1434-1437.
22. Martinez J, Tuschl T. RISC is a 5′ phosphomonoester-producing RNA endonuclease. Genes Dev 18 2004; 9:975-980.
23. Schwarz DS, Tomari Y, Zamore PD et al. The RNA-induced silencing complex is a Mg2+-dependent endonuclease. Curr Biol 14 2004; 9:787-791.
24. Matranga C, Tomari Y, Shin C et al. Passenger-strand cleavage facilitates assembly of siRNA into Ago2-containing RNAi enzyme complexes. Cell 123 2005; 4:607-620.
25. Leuschner PJ, Ameres SL, Kueng S et al. Cleavage of the siRNA passenger strand during RISC assembly in human cells. EMBO Rep 7 2006; 3:314-320.
26. Rand TA, Petersen S, Du F et al. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell 123 2005; 4:621-629.
27. Steiner FA, Okihara KL, Hoogstrate SW et al. RDE-1 slicer activity is required only for passenger-strand cleavage during RNAi in Caenorhabditis elegans. Nat Struct Mol Biol 16 2009; 2:207-211.
28. Wang B, Li S, Qi HH et al. Distinct passenger strand and mRNA cleavage activities of human Argonaute proteins. Nat Struct Mol Biol 2009.
29. Kawamata T, Seitz H, Tomari Y et al. Structural determinants of miRNAs for RISC loading and slicer-independent unwinding. Nat Struct Mol Biol 16 2009; 9:953-960.
30. Lee RC, Feinbaum RL, Ambros V et al. The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 75 1993; 5:843-854.
31. Wightman B, Ha I, Ruvkun G et al. Post-transcriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75 1993; 5:855-862.
32. Olsen PH, Ambros V. 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; 2:671-680.
33. Reinhart BJ, Slack FJ, Basson M et al. The 21-nucleotide let-7 RNA regulates developmental timing in Caenorhabditis elegans. Nature 403 2000; 6772:901-906.
34. Pasquinelli AE, Reinhart BJ, Slack F et al. Conservation of the sequence and temporal expression of let-7 heterochronic regulatory RNA. Nature 408 2000; 6808:86-89.

35. Grishok A, Pasquinelli AE, Conte D et al. Genes and mechanisms related to RNA interference regulate expression of the small temporal RNAs that control C. elegans developmental timing. Cell 106 2001; 1:23-34.
36. Ketting RF, Fischer SE, Bernstein E et al. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in C. elegans. Genes Dev 15 2001; 20:2654-2659.
37. Hutvágner G, McLachlan J, Pasquinelli AE et al. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 293 2001; 5531:834-838.
38. Knight SW, Bass BL. A role for the RNase III enzyme DCR-1 in RNA interference and germ line development in Caenorhabditis elegans. Science 293 2001; 5538:2269-2271.
39. Hutvagner G, Zamore PD. A microRNA in a multiple-turnover RNAi enzyme complex. Science 297 2002; 5589:2056-2060.
40. Lagos-Quintana M, Rauhut R, Lendeckel W et al. Identification of novel genes coding for small expressed RNAs. Science 294 2001; 5543:853-858.
41. Lau NC, Lim LP, Weinstein EG et al. An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. Science 294 2001; 5543:858-862.
42. Lee RC, Ambros V. An extensive class of small RNAs in Caenorhabditis elegans. Science 294 2001; 5543:862-864.
43. Ambros V. microRNAs: tiny regulators with great potential. Cell 107 2001; 7:823-826.
44. Ambros V, Bartel B, Bartel DP et al. A uniform system for microRNA annotation. RNA 9 2003; 3:277-279.
45. Grimson A, Srivastava M, Fahey B et al. Early origins and evolution of microRNAs and Piwi-interacting RNAs in animals. Nature 455 2008; 7217:1193-1197.
46. Molnár A, Schwach F, Studholme DJ et al. miRNAs control gene expression in the single-cell alga Chlamydomonas reinhardtii. Nature 447 2007; 7148:1126-1129.
47. Jones-Rhoades MW, Bartel DP, Bartel B et al. MicroRNAS and their regulatory roles in plants. Annu Rev Plant Biol 57 2006:19-53.
48. Voinnet, O, Origin, biogenesis and activity of plant microRNAs. Cell 136 2009; 4:669-687.
49. Lee Y, Jeon K, Lee JT et al. microRNA maturation: stepwise processing and subcellular localization. EMBO J 21 2002; 17:4663-4670.
50. Cai X, Hagedorn CH, Cullen BR et al. Human microRNAs are processed from capped, polyadenylated transcripts that can also function as mRNAs. RNA 10 2004; 12:1957-1966.
51. Lee Y, Kim M, Han J et al. microRNA genes are transcribed by RNA polymerase II. EMBO J 23 2004; 20:4051-4060.
52. Kim YK, Kim VN. Processing of intronic microRNAs. EMBO J 26 2007; 3:775-783.
53. Park W, Li J, Song R et al. CARPEL FACTORY, a Dicer homolog and HEN1, a novel protein, act in microRNA metabolism in Arabidopsis thaliana. Curr Biol 12 2002; 17:1484-1495.
54. Lee Y, Ahn C, Han J et al. The nuclear RNase III Drosha initiates microRNA processing. Nature 425 2003; 6956:415-419.
55. Han J, Lee Y, Yeom KH et al. The Drosha-DGCR8 complex in primary microRNA processing. Genes Dev 18 2004; 24:3016-3027.
56. Denli AM, Tops BB, Plasterk RH et al. Processing of primary microRNAs by the Microprocessor complex. Nature 432 2004; 7014:231-235.
57. Landthaler M, Yalcin A, Tuschl T et al. The human DiGeorge syndrome critical region gene 8 and Its D. melanogaster homolog are required for miRNA biogenesis. Curr Biol 14 2004; 23:2162-2167.
58. Han J, Lee Y, Yeom KH et al. Molecular basis for the recognition of primary microRNAs by the Drosha-DGCR8 complex. Cell 125 2006; 5:887-901.
59. Lund E, Güttinger S, Calado A et al. Nuclear export of microRNA precursors. Science 303 2004; 5654:95-98.
60. Yi R, Qin Y, Macara IG et al. Exportin-5 mediates the nuclear export of pre-microRNAs and short hairpin RNAs. Genes Dev 17 2003; 24:3011-3016.
61. Bohnsack MT, Czaplinski K, Gorlich D et al. Exportin 5 is a RanGTP-dependent dsRNA-binding protein that mediates nuclear export of pre-miRNAs. RNA 10 2004; 2:185-191.
62. Berezikov E, Chung WJ, Willis J et al. Mammalian mirtron genes. Mol Cell 28 2007; 2:328-336.
63. Okamura K, Hagen JW, Duan H et al. The mirtron pathway generates microRNA-class regulatory RNAs in Drosophila. Cell 130 2007; 1:89-100.
64. Ruby JG, Jan CH, Bartel DP et al. Intronic microRNA precursors that bypass Drosha processing. Nature 448 2007; 7149:83-86.
65. Mi S, Cai T, Hu Y et al. Sorting of small RNAs into Arabidopsis argonaute complexes is directed by the 5′ terminal nucleotide. Cell 133 2008; 1:116-127.
66. Steiner FA, Hoogstrate SW, Okihara KL et al. Structural features of small RNA precursors determine Argonaute loading in Caenorhabditis elegans. Nat Struct Mol Biol 14 2007; 10:927-933.

67. Tomari Y, Du T, Zamore PD et al. Sorting of Drosophila small silencing RNAs. Cell 130 2007; 2:299-308.
68. Farazi TA, Juranek SA, Tuschl T et al. The growing catalog of small RNAs and their association with distinct Argonaute/Piwi family members. Development 135 2008; 7:1201-1214.
69. Chendrimada TP, Gregory RI, Kumaraswamy E et al. TRBP recruits the Dicer complex to Ago2 for microRNA processing and gene silencing. Nature 436 2005; 7051:740-744.
70. Gregory RI, Chendrimada TP, Cooch N et al. Human RISC couples microRNA biogenesis and post-transcriptional gene silencing. Cell 123 2005; 4:631-640.
71. Tang G. siRNA and miRNA: an insight into RISCs. Trends Biochem Sci 30 2005; 2:106-114.
72. MacRae IJ, Ma E, Zhou M et al. In vitro reconstitution of the human RISC-loading complex. Proc Natl Acad Sci USA 105 2008; 2:512-517.
73. Lee Y, Hur I, Park SY et al. The role of PACT in the RNA silencing pathway. EMBO J 25 2006; 3:522-532.
74. Wang HW, Noland C, Siridechadilok B et al. Structural insights into RNA processing by the human RISC-loading complex. Nat Struct Mol Biol 16 2009; 11:1148-1153.
75. Schwarz DS, Hutvágner G, Du T et al. Asymmetry in the assembly of the RNAi enzyme complex. Cell 115 2003; 2:199-208.
76. Khvorova A, Reynolds A, Jayasena SD et al. Functional siRNAs and miRNAs exhibit strand bias. Cell 115 2003; 2:209-216.
77. Lewis BP, Shih IH, Jones-Rhoades MW et al. Prediction of mammalian microRNA targets. Cell 115 2003; 7:787-798.
78. Kloosterman WP, Wienholds E, Ketting RF et al. Substrate requirements for let-7 function in the developing zebrafish embryo. Nucleic Acids Res 32 2004; 21:6284-6291.
79. Brennecke J, Stark A, Russell RB et al. Principles of microRNA-target recognition. PLoS Biol 3 2005; 3:e85.
80. Lim LP, Lau NC, Garrett-Engele P et al. Microarray analysis shows that some microRNAs downregulate large numbers of target mRNAs. Nature 433 2005; 7027:769-773.
81. Bartel, DP. microRNAs: target recognition and regulatory functions. Cell 136 2009; 2:215-233.
82. Wang Y, Juranek S, Li H et al. Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. Nature 456 2008; 7224:921-926.
83. Wang Y, Sheng G, Juranek S et al. Structure of the guide-strand-containing argonaute silencing complex. Nature 456 2008; 7219:209-213.
84. Wang Y, Juranek S, Li H et al. Nucleation, propagation and cleavage of target RNAs in Ago silencing complexes. Nature 461 2009; 7265:754-761.
85. Jones-Rhoades MW, Bartel DP. Computational identification of plant microRNAs and their targets, including a stress-induced miRNA. Mol Cell 14 2004; 6:787-799.
86. Ameres SL, Martinez J, Schroeder R et al. Molecular basis for target RNA recognition and cleavage by human RISC. Cell 130 2007; 1:101-112.
87. Brodersen P, Sakvarelidze-Achard L, Bruun-Rasmussen M et al. Widespread translational inhibition by plant miRNAs and siRNAs. Science 320 2008; 5880:1185-1190.
88. Lanet E, Delannoy E, Sormani R et al. Biochemical evidence for translational repression by Arabidopsis microRNAs. Plant Cell 21 2009; 6:1762-1768.
89. Pillai RS, Bhattacharyya SN, Filipowicz W et al. Repression of protein synthesis by miRNAs: how many mechanisms? Trends Cell Biol 17 2007; 3:118-126.
90. Pillai RS, Bhattacharyya SN, Artus CG et al. Inhibition of translational initiation by Let-7 microRNA in human cells. Science 309 2005; 5740:1573-1576.
91. Thermann R, Hentze MW. Drosophila miR2 induces pseudo-polysomes and inhibits translation initiation. Nature 447 2007; 7146:875-878.
92. Zdanowicz A, Thermann R, Kowalska J et al. Drosophila miR2 primarily targets the m7GpppN cap structure for translational repression. Mol Cell 35 2009; 6:881-888.
93. Behm-Ansmant I, Rehwinkel J, Doerks T et al. mRNA degradation by miRNAs and GW182 requires both CCR4:NOT deadenylase and DCP1:DCP2 decapping complexes. Genes Dev 20 2006; 14:1885-1898.
94. Eulalio A, Huntzinger E, Izaurralde E et al. GW182 interaction with Argonaute is essential for miRNA-mediated translational repression and mRNA decay. Nat Struct Mol Biol 15 2008; 4:346-353.
95. Chen CY, Zheng D, Xia Z et al. Ago-TNRC6 triggers microRNA-mediated decay by promoting two deadenylation steps. Nat Struct Mol Biol 16 2009; 11:1160-1166.
96. Zipprich JT, Bhattacharyya S, Mathys H et al. Importance of the C-terminal domain of the human GW182 protein TNRC6C for translational repression. RNA 15 2009; 5:781-793.
97. Eulalio A, Helms S, Fritzsch C et al. A C-terminal silencing domain in GW182 is essential for miRNA function. RNA 15 2009; 6:1067-1077.

98. Liu J, Valencia-Sanchez MA, Hannon GJ et al. microRNA-dependent localization of targeted mRNAs to mammalian P-bodies. Nat Cell Biol 7 2005; 7:719-723.
99. Eulalio A, Behm-Ansmant I, Schweizer D et al. P-body formation is a consequence, not the cause, of RNA-mediated gene silencing. Mol Cell Biol 27 2007; 11:3970-3981.
100. Bhattacharyya SN, Habermacher R, Martine U et al. Relief of microRNA-mediated translational repression in human cells subjected to stress. Cell 125 2006; 6:1111-1124.
101. Eulalio A, Rehwinkel J, Stricker M et al. Target-specific requirements for enhancers of decapping in miRNA-mediated gene silencing. Genes Dev 21 2007; 20:2558-2570.
102. Fabian MR, Mathonnet G, Sundermeier T et al. Mammalian miRNA RISC recruits CAF1 and PABP to affect PABP-dependent deadenylation. Mol Cell 35 2009; 6:868-880.
103. Zekri L, Huntzinger E, Heimstädt S et al. The silencing domain of GW182 interacts with PABPC1 to promote translational repression and degradation of microRNA targets and is required for target release. Mol Cell Biol 29 2009; 23:6220-6231.
104. Giraldez AJ, Mishima Y, Rihel J et al. Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. Science 312 2006; 5770:75-79.
105. Eulalio A, Huntzinger E, Nishihara T et al. Deadenylation is a widespread effect of miRNA regulation. RNA 15 2009; 1:21-32.
106. Baek D, Villén J, Shin C et al. The impact of microRNAs on protein output. Nature 455 2008; 7209:64-71.
107. Selbach M, Schwanhäusser B, Thierfelder N et al. Widespread changes in protein synthesis induced by microRNAs. Nature 455 2008; 7209:58-63.