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REVIEW

Many ways to generate microRNA-like small RNAs: non-canonical pathways for microRNA production

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Abstract MicroRNAs (miRNAs) are an abundant class of small non-coding RNAs that collectively regulate the expression of a large number of mRNAs by either promoting destabilization or repressing translation, or both. Therefore, they play a major role in shaping the transcriptomes and proteomes of eukaryotic organisms. Typically, animal miRNAs are produced from long primary transcripts with one or more of hairpin structures by two sequential processing reactions: one by Drosha in the nucleus and the other by Dicer in the cytoplasm. However, deviations from this paradigm have been observed: subclasses of miRNAs, which only partially meet the classical definition of a miRNA, are derived by alternative biogenesis pathways, thereby providing an additional level of complexity to miRNA-dependent regulation of gene expression.

Keywords miRNA · Drosha · Dicer · Argonaute · snoRNA · tRNA

Introduction

MicroRNAs (miRNAs) are a family of small regulatory RNAs that post-transcriptionally control the expression of specific genes by base-pairing, typically to the 3′-untranslated regions (3′-UTRs) of target messenger RNAs (mRNAs) to direct a reduction in their translation and/or stability (Filipowicz et al. 2008). They are involved in a broad range of developmental, cell differentiation and cell maintenance functions (Bushati and Cohen 2007), and miRNA perturbations are, therefore, closely linked to human diseases, such as cancer (Chang and Mendell 2007; Garzon et al. 2009). There are hundreds of unique miRNAs in a given species, and computational and experimental approaches indicate that individual miRNAs can base-pair and selectively regulate a plethora (probably several hundreds) of different target mRNAs (Bartel 2009; Friedman et al. 2009). Therefore, miRNAs greatly influence the spatio-temporal, global expression patterns in eukaryotic organisms (Baek et al. 2008; Bushati and Cohen 2007; Lim et al. 2005; Selbach et al. 2008). Importantly, recent data collectively suggest a correlation between miRNA diversity and morphological complexity as measured, for instance, by the total number of neurons in an organism (Grimson et al. 2008; Technau 2008).

miRNAs are approximately 22 nucleotides (nt) and originates from a precursor with a characteristic hairpin or stem-loop structure that does not contain large internal loops or bulges (Berezikov et al. 2006). miRNAs are 5′-phosphorylated and 2′-3′-hydroxylated and are typically processed through two sequential steps in animal cells: the first in the nucleus by an RNase III enzyme termed Drosha, and the second in the cytoplasm by another RNase III enzyme termed Dicer. These enzymes trim the genome-encoded stem-loop precursor into the mature miRNA (Carthew and Sontheimer 2009; Kim et al. 2009; Siomi and Siomi 2009). In principle, this process could be regulated at multiple levels. Indeed recent studies hint that each one of the miRNA processing steps is regulated, and that each

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miRNA is probably regulated independently, thereby providing an additional level of complexity to the miRNA-dependent regulation of gene expression (Siomi and Siomi 2010). Mature miRNA associates with a member of the Argonaute (AGO) family of proteins to form the core of the RNA-induced silencing complex (RISC: also referred to as the miRISC) and are then poised to interact and repress their target mRNAs.

Recent analyses of massive amounts of data produced by next generation sequencing technologies, also known as deep sequencing or high-throughput sequencing, from many animal cell types and tissues, have revealed subclasses of miRNA species that are derived by alternative biogenesis pathways and only partially meet the classical definition. It is important to bear in mind that in many of these analyses, sequences that met at least two operational criteria, the expression of a ~22-nt form and the presence of a hairpin precursor (Berezikov et al. 2006), were classified as non-canonical miRNAs. Here, we review recent progress in our understanding of the non-canonical mechanisms of miRNA biogenesis. Although much of the evidence discussed here refers to mammalian systems, it also includes a broader perspective from other systems.

Canonical miRNA biogenesis

miRNA genes are transcribed typically by RNA polymerase II (pol II) to generate a long primary miRNA (pri-miRNA), which is characterized by a hairpin or fold-back structure with a ~32-nt long imperfectly base-paired stem and a terminal loop. The pri-miRNA is 5′-capped, spliced and polyadenylated, and can form two or more hairpins, each containing a distinct mature miRNA species (Carthew and Sontheimer 2009; Kim et al. 2009). Long pri-miRNAs driven by pol II can be either coding (mRNA precursors) or non-coding (Kim et al. 2009). Thus, maturation of miRNAs from pri-miRNAs involves the precise recognition and the accurate excision and release of functional mature miRNAs. In animal cells, pri-miRNAs undergo two-step processing: first in the nucleus and second in the cytoplasm (Carthew and Sontheimer 2009; Kim et al. 2009; Siomi and Siomi 2009) (Fig. 1a). Within the nucleus, hairpin structures within the pri-miRNA are recognized by a multiprotein complex called the Microprocessor, of which the core components are the RNase III Drosha and the double-stranded RNA-binding domain (dsRBD) protein DGCR8/Pasha (Denli et al. 2004; Gregory et al. 2004). DGCR8 directs Drosha to cleave the pri-miRNA at the base of the hairpin, resulting in a precursor miRNA (pre-miRNA) of 60–70 nt in length (Han et al. 2006). Exportin-5 recognizes the 2-nt 3′-overhang, characteristic of RNase III-mediated cleavage, and transports the pre-miRNA into the cytoplasm via a Ran-GTP-dependent mechanism (Kim et al. 2009; Okada et al. 2009).

Within the cytoplasm, a second RNase III, Dicer, which pairs with the dsRBD proteins TRBP/PACT/Loquacious (Loqs), cleaves the pre-miRNA to release a ~22 nt miRNA duplex, referred to as the miRNA/miRNA* duplex (Chendrimada et al. 2005; Förstemann et al. 2005; Jiang et al. 2005; Lee et al. 2006; Saito et al. 2005) (Fig. 1a). These are 5′-phosphorylated, 2′-3′-hydroxylated small RNAs, a consequence of the sequential processing by the RNaseIII enzymes Drosha and Dicer. One strand of the duplex, typically with a relatively lower stability of base-pairing at the 5′-end ("the thermodynamic asymmetry rule"), is destined to become the mature guide miRNA (Khvorova et al. 2003; Schwarz et al. 2003). This strand selectively associates with an AGO protein to form the core of the miRISC. The phosphorylated 5′-end of the miRNA guide strand is critical for the interaction with the AGO protein (Ma et al. 2005; Parker et al. 2005; Parker 2010). Once incorporated into the miRISC, the miRNA guides it to complementary sites typically but not always within the 3′-UTRs of the target mRNAs, to mediate repression of that target message (Bartel 2009). Most of the pairing specificity for target recognition is provided by the 5′-proximal "seed" region (nucleotides 2–8) of the miRNA (Bartel 2009). In some cases, miRNA repression can be reversed (Bhattacharyya et al. 2006), and the miRISC can even activate expression of the target (Vasudevan et al. 2007; Cordes et al. 2009). How the miRISC represses or activates the expression of specific genes appears to be context-dependent and is the subject of ongoing debate (Brodersen and Voinnet 2009; Filipowicz et al. 2008; Gu et al. 2009).

The passenger (opposite) strand of the mature miRNA, referred to as miRNA*, is excluded from the miRISC and is typically degraded. However, recent deep sequencing efforts indicate that a large number of miRNA*s are associated with AGOs and that the relative expression levels of the two strands vary widely among tissues (Czech et al. 2009; Hu et al. 2009; Okamura et al. 2008, 2009; Seitz et al. 2008). In flies, although miRNAs are typically loaded onto AGO1, the sorting of miRNAs and miRNA*s into different AGO complexes (AGO1 and AGO2 complexes) occurs in some cases. The 5′-ends of both miRNAs and miRNA* strands are typically more precisely defined than their 3′-ends, further implying that miRNA* sequences are under selective pressure to establish a unique seed sequence at the AGO loading step, implying that many miRNA* sequences are under evolutionary pressure to maintain their seed sequences (Seitz et al. 2008). In addition, miRNA*s seem to impact on the evolution of 3′ UTRs (Okamura et al. 2008), suggesting that they have a true regulatory function. Thus, these findings together suggest that, in addition to the thermodynamic asymmetry rule, additional

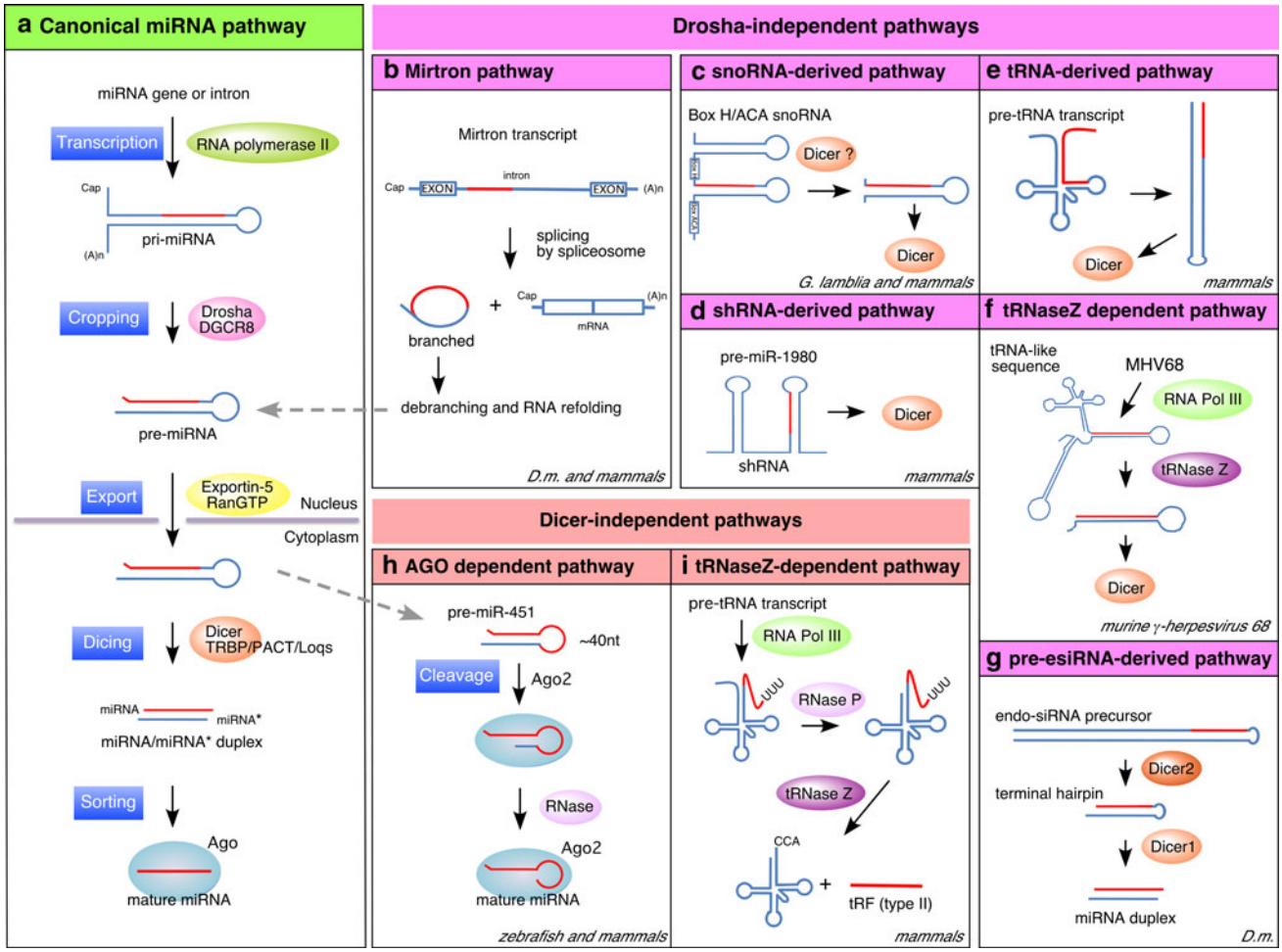

Fig. 1 Canonical and non-canonical pathways of miRNA biogenesis. **a** Canonical pathway of miRNA biogenesis in animals. miRNAs are transcribed from the genome as long primary transcripts. Pri-miRNAs form hairpin structures, and are processed to about 60–70 nt precursor microRNAs (pre-miRNAs) by the Microprocessor (Drosha-DGCR8/Pasha complex) in the nucleus. After being exported by Exportin-5, pre-miRNAs are further processed to a ~22 nt miRNA/miRNA* duplex by the Dicer1-TRBP/PACT/Loqs complex in the cytoplasm. Finally, mature miRNAs are loaded onto Argonaute proteins. **b** Mirtron-derived pathway. Short introns with hairpin potential, termed mirtrons, can be spliced and debranched into pre-miRNA hairpin mimics that bypass Drosha cleavage. Debranched mirtrons access the canonical miRNA pathway during nuclear export, and are then cleaved by Dicer and loaded onto Argonaute proteins. **c** snoRNA-derived pathway. The processing of snoRNAs to miRNAs requires Dicer activity but is independent of Drosha/DGCR8. **d** shRNA-derived pathway. The predicted secondary structure of the presumed mir-1980 precursor is shown. The processing may be similar to that of the snoRNA-derived pathway. **e** tRNA-derived pathway. Predicted secondary structures for the tRNA cloverleaf structure and alternative shRNA hold. **f** tRNase Z-dependent pathway. miRNAs encoded by MHV68 derived from one arm of one out of two proposed pre-miRNA-like stem loops located 3′ of the tRNA-like structure. The processing of the pre-miRNA is dependent on tRNase Z but is independent of Drosha in the nucleus. After nuclear export, pre-miRNA is processed by Dicer. **g** miRNAs derived from a terminal hairpin of an endo-siRNA precursor. Endo-siRNA precursors are processed by the Dicer2 complex to yield pre-miRNA-like terminal hairpins, which are then processed by the Dicer1 complex. Then, these small RNAs are loaded onto AGO1 to form the core protein of the miRISC. **h** Dicer-independent pathway. Although Drosha cleavage proceeds normally, the Dicer step is skipped and the pre-miR-451 is loaded directly into Ago2. **i** Dicer-independent, tRNase Z-dependent pathway. A 3′-trailer fragment of a precursor tRNA is released by a cleavage by tRNase Z. Organisms that represent each pathway (**b–i**) are indicated at the bottom of the panels. D.m. *Drosophila melanogaster*, G. lamblia *Giardia lamblia*

strand selection mechanisms operate on the miRNA duplex in animal cells, and in such cases both strands are likely to be functional (Okamura and Lai 2008). Other determinants for strand selection and partitioning into different AGO complexes include the structure of the miRNA/miRNA* duplex (e.g., the positions of mismatches) (Tomari et al. 2007; Czech et al. 2009; Ghildiyal et al. 2010; Okamura et al. 2009), the 5′ nucleotide identity (Chen 2009; Czech et al. 2009; Okamura et al. 2009), and factors other than sequence features (Hu et al. 2009).

Non-canonical pathways of miRNA biogenesis

We now have a good understanding of the basic process of miRNA biogenesis, and current interest is turning toward

variations on the canonical mechanism. The analysis of biogenesis of miRNAs has benefited from deep-sequencing technologies. Indeed, the lengths of miRNAs make next generation sequencing instruments ideal for genome-wide discovery of miRNA genes (Mardis 2008). The sequencing of small RNA fragments is followed by an extensive bioinformatics-based characterization that examines the genomic locations and the potential for secondary structure formation, to identify putative miRNAs. This approach has identified several classes of miRNA that bypass a key step(s) involved in the canonical miRNA biogenesis pathway. The challenge for these studies is to distinguish whether the identified miRNA-like small RNA sequences are merely non-functional degradation products or bona fide miRNAs of functional significance. It is important to bear in mind that the functionality of only a small number of these non-canonical miRNAs has been demonstrated, mostly using artificial reporter constructs. However, this is also the case for canonical miRNAs.

Drosha-independent pathways

Mirtrons

Approximately 40% of animal miRNAs are encoded in the introns of protein-coding genes and, therefore, many pri-miRNA transcripts are simultaneously pre-mRNAs and pri-miRNAs (Cai et al. 2004; Kim et al. 2009). In *Drosophila*, the distribution of intron length has a sharp peak at around 60 nt (Lim and Burge 2001), which is conspicuously similar to the size of pre-miRNAs. Examination of deep sequencing data of small RNAs from *Drosophila* has revealed small RNAs that originate from pre-miRNA-sized short introns with hairpin potential (Okamura et al. 2007; Ruby et al. 2007). This class of short introns, termed "mirtrons", can be spliced and debranched into pre-miRNA hairpins that are suitable for Dicer cleavage, thus bypassing the Microprocessor (Fig. 1b). The mirtron biogenesis pathway converges in the nucleus with the canonical miRNA pathway during hairpin export, which is mediated by Exportin-5 (Okamura et al. 2007) (Fig. 1b). Thus, mirtron maturation is initiated by splicing and intron lariat debranching, which contrasts with the splicing-independent biogenesis of canonical miRNAs found within introns, whose cleavage by Drosha occurs prior to host intron splicing (Kim and Kim 2007).

In vertebrates, the majority of introns extend to several kilobases or even megabases in length. However, a considerable fraction of vertebrate introns have a minimal size (50–150 nt), with the peak at the lower end of the size distribution (Yu et al. 2002). Some of these short introns are predicted to form hairpin structures, and miRNA-sized RNAs corresponding to these introns are present in collections of mammalian small RNAs (Berezikov et al. 2007; Babiarz et al. 2008). Thus, mirtrons comprise a small fraction of miRNAs in mammals as well as in *Drosophila*.

miRNAs derived from snoRNAs

Some small nucleolar RNAs (snoRNAs) also provide a secondary source of pre-miRNAs that is independent of the Microprocessor-mediated processing (Ender et al. 2008; Saraiya and Wang 2008) (Fig. 1c). snoRNAs are evolutionarily conserved small RNAs approximately 70–200 nt long that function as small nucleolar ribonucleoprotein (snoRNP) complexes to guide the enzymatic modification of selected ribosomal RNA (rRNA) nucleotides (Matera et al. 2007). Analysis of deep sequencing data of small RNAs associated with immunopurified human Ago1 and Ago2 identified miRNA-like molecules originating from particular snoRNAs, whose secondary structure is characterized by two pre-miRNA-like hairpins linked by a hinge (Ender et al. 2008). Processing of these snoRNAs into miRNAs requires Dicer activity but is independent of Drosha. These snoRNAs represent functional snoRNAs, because they associate with specific snoRNP proteins such as GAR1. Thus, some snoRNAs are both the source of miRNAs and the component of snoRNPs that modifies rRNAs. Similar snoRNA-derived miRNAs have been identified in *Giardia lamblia,* which is a unicellular protozoan (Saraiya and Wang 2008).

These findings raise questions as to how snoRNPs normally present in the nucleolus are exported to the cytoplasm for Dicer processing, and how in the cytoplasm Dicer is recruited onto snoRNAs that are packed with several specific proteins. Finally, the identification of miRNAs generated from snoRNAs in both humans and the unicellular eukaryote *Giardia* and likely many other organisms, suggests that snoRNAs may have an ancient link with miRNAs (Scott et al. 2009; Taft et al. 2009).

miRNAs derived from endogenous short-hairpin RNAs (endo-shRNAs) and tRNA precursors

The analysis of miRNA biogenesis has benefited from classic mutation analyses combined with deep sequencing. Mouse embryonic stem cells (mESCs) that lack Dgcr8, the partner of Drosha in the Microprocessor, have less severe phenotypes than mESCs lacking Dicer, suggesting a physiological role for Microprocessor-independent, Dicer-dependent small RNAs (Babiarz et al. 2008). Analyses of small RNA populations identified from wild-type, *dgcr8* and *dicer* knockout mESCs uncovered a diverse population of Dgcr8-independent, Dicer-dependent small RNAs. These included miRNAs from mirtrons. In addition, a

fraction of miRNA-like molecules was derived from endo-shRNAs.

One of the identified endo-shRNAs gives rise to a transcript that forms a secondary structure with two potential pre-miRNA-like hairpins linked by a hinge (Babiarz et al. 2008). However, only the second hairpin produces a substantial number of sequenced small RNAs (mir-1980) (Fig. 1d). This is reminiscent of snoRNAs producing miRNAs (Ender et al. 2008). The Microprocessor independence in both cases suggests that an unknown nuclease removes the first hairpin up to the 5′-end, or cleaves at the 5′-end of the second hairpin to produce a suitable Dicer substrate. Other miRNAs including mir-320 and mir-484, also originate from endo-shRNAs. However, these appear to be transcribed directly as short hairpins.

An endo-shRNA locus corresponds to the isoleucine tRNA (tRNA-Ile) gene (Babiarz et al. 2008). Like other tRNA genes, this locus is transcribed by RNA polymerase III (pol III) and the transcription is terminated in a poly-U stretch. Unlike other tRNAs, which typically form cloverleaf secondary structures, the primary transcript from this gene has the potential to form a long 110 nt hairpin as an alternative secondary structure (Fig. 1e). The cluster of Dgcr8-independent, and Dicer-dependent small RNAs mapped to the 3′-end of the hairpin. The primary transcript is also processed into a mature tRNA. Thus, this transcript is a dual function molecule with both miRNA and tRNA precursors, as is the case for snoRNAs that also give rise to miRNAs.

tRNase Z-dependent pathways

miRNAs encoded by murine γ-herpesvirus 68 (MHV68) are transcribed by pol III to give rise to a pri-miRNA consisting of a tRNA linked to a pre-miRNA hairpin (Pfeffer et al. 2004, 2005; Bogerd et al. 2010). All nine miRNAs encoded by MHV68 are derived from one arm of one of two proposed pre-miRNA-like stem loops located 3′ of tRNA-like structures (Bogerd et al. 2010). The pri-miRNAs are not processed by Drosha but instead by the cellular tRNA processing enzyme tRNase Z (a.k.a ELAC and 3′ tRNase), which cleaves 3′ of the tRNA to liberate pre-miRNA hairpins that are then processed by Dicer to yield the mature viral miRNAs (Fig. 1f). Therefore, the biogenesis of MHV68 miRNAs is Drosha-independent but is dependent on the action of both a distinct endonuclease, i.e., tRNase Z, and Dicer. Therefore, the MHV68 miRNAs are distinct from other Drosha-independent miRNAs and are also distinct from other tRNA-derived small RNAs (see below). tRNase Z is thought to function in the nucleus (Lund and Dahlberg 1998); thus, this biogenesis pathway likely converges in the nucleus with the canonical miRNA pathway during hairpin export.

miRNAs derived from a terminal hairpin of an endo-siRNA precursor

Endogenous siRNAs (endo-siRNAs or esiRNAs) are small RNAs derived from long endogenous dsRNAs. In *Drosophila*, endo-siRNAs derive from transposons, heterochromatic sequences, intergenic regions, long RNA transcripts with extensive structure and, in some cases, from mRNAs (Okamura and Lai 2008). Although miRNAs are processed by Dicer1 and are loaded onto AGO1, endo-siRNAs are generated by Dicer2 and are loaded onto AGO2 (Siomi and Siomi 2009). However, many endo-siRNAs depend on Loqs, the canonical partner of Dicer1 in the miRNA pathway, but not on R2D2, the partner of Dicer2. Recent studies have shown that Dicer1 associates with Loqs isoforms, Loqs-PA and -PB, whereas Dicer2 interacts with another isoform, Loqs-PD. The production of endo-siRNAs strongly depends on Loqs-PD (Hartig et al. 2009; Miyoshi et al. 2010; Zhou et al. 2009). These different Dicer complexes recognize the stem length of precursor hairpins (Miyoshi et al. 2010). This suggests that, besides the intrinsic substrate specificity of the individual Dicer proteins, the identity of Dicer-interacting cofactors is also crucial for the biogenesis of different classes of small RNAs.

A subset of endo-siRNAs is derived from long RNA hairpins containing multiple mismatches, which are processed to mature endo-siRNAs by the Dicer2-Loqs-PD complex. Although mature endo-siRNAs are loaded onto AGO2, small RNAs derived from the pre-miRNA-sized terminal hairpin of the endo-siRNA precursor were found associated with AGO1. This result suggests that the Dicer 2-Loqs-PD complex processes the precursors to produce endo-siRNAs but cannot process the terminal hairpins, which instead are recognized as miRNA precursors and processed to miRNAs by the Dicer1-Loqs-PA/PB complex (Miyoshi et al. 2010) (Fig. 1g). These miRNAs are a unique subset of miRNAs, and are referred to as ‘siblings of esiRNAs’ or semiRNAs.

Dicer-independent pathways

Argonaute proteins are the core components of RISCs that modulate gene expression, regulate chromosome structure and function and provide an innate immune defense against viruses and transposons (Hutvagner and Simard 2008). AGO proteins consist of an amino-terminal domain, the Mid domain and their signature PAZ and PIWI domains (Parker 2010). The PAZ and Mid domains help to anchor the small RNA guide, with PAZ binding the 3′-end of the RNA and the Mid domain providing a binding pocket for the phosphorylated 5′-end. The PIWI domain forms an RNase H-like fold in the tertiary structure (Song et al. 2004).

Loading of a highly complementary target into an AGO protein brings the scissile phosphate, opposite nucleotides 10 and 11 of the small RNA guide, into the endonuclease (Slicer) active site, which consists of a catalytic DDH triad that serves as a metal-coordinating site (Elbashir et al. 2001; Liu et al. 2004; Schwarz et al. 2004; Song et al. 2004). Of four AGO proteins in mammals, only Ago2 has retained both the DDH motif and demonstrable Slicer activity (Azuma-Mukai et al. 2008; Liu et al. 2004; Meister et al. 2004).

The Hannon group has produced mice with a catalytically inactive Ago2 that disrupts RNA cleavage without impeding small RNA binding (Cheloufi et al. 2010). Mice lacking catalytic Ago2 died shortly after birth with anemia. Ago2 catalytic activity is important for postnatal development. Deep sequencing of miRNAs in the liver of the Ago2 mutants revealed that miR-451 was markedly reduced in the mutants, although other miRNAs were present at nearly normal levels. The miR-451 precursor has an unusual feature: although canonical miRNAs are defined as mapping to the stem only, the six terminal nucleotides of the 23-nt long mature miR-451 span the loop region and extend into the complementary strand of the hairpin precursor. In *drosha*$^{-/-}$ mouse embryonic fibroblasts, mature miR-451 was reduced by 20-fold compared with wild-type levels, and in vitro processing assays using purified Drosha complexes with miR-451 primary transcripts revealed that the substrate was processed to a pre-miRNA only 41-nt long, which is also unusual. These experiments provide both genetic and biochemical support for Drosha catalyzing the excision of pre-miR-451 from its primary transcript. The levels of miR-451 were unaffected in *dicer* null cells, indicating that the conversion of pre-miR-451 into a mature miRNA proceeds independently of Dicer. Thus, the maturation of miR-451 is Drosha-dependent and Dicer-independent (Fig. 1h).

Pre-miR-451 was detected in Ago2 immunoprecipitates. In processing assays using affinity-purified Ago2 and pre-miR-451, mature miR-451 was produced depending on Ago2 catalytic activity. Together these findings suggest that pre-miR451 produced by Drosha is directly loaded onto Ago2 without the Dicer step, and the Ago2 catalytic centre helps to catalyze the maturation of this miRNA (Fig. 1h). This is at odds with earlier reports indicating a coupling of Dicer cleavage and RISC loading (Chendrimada et al. 2005; Wang et al. 2009). However, the miR-451 pathway is conserved in zebrafish (Cifuentes et al. 2010). miR-451 has a defined 5′-end created by a Drosha cut, but a variable 3′-end that extends over the loop region and ranges between 20 and 30 nt long. Reads stopped at nucleotide 30 measured from the 5′-end of the pre-miRNA, which is the Ago2 cleavage site, and longer reads contained 1–5 non-templated uridines. This suggests a model whereby Ago2-mediated cleavage produces an intermediate that is uridylated and is then further trimmed by a cellular nuclease to generate mature miRNA sequences protected by Ago2 (Fig. 1h). These findings also link the conservation of Ago2's Slicer activity to a conserved mechanism of miRNA biogenesis and suggest the possibility that additional miRNAs may well rely on the Ago2-dependent pathway for maturation.

Several studies have identified a large number of miRNA-sized small RNAs derived from human tRNAs (Cole et al. 2009; Kawaji et al. 2008; Lee et al. 2009; Haussecker et al. 2010). At least a fraction of these tRNA-derived small RNAs is associated with Argonaute proteins (Cole et al. 2009; Haussecker et al. 2010). These tRNA-derived small RNAs are largely subdivided into two groups: Dicer-dependent and Dicer-independent but tRNase Z-dependent. Pre-tRNAs produced by pol III are trimmed by RNase P at the 5′-end (Altman 2000) and by tRNase Z at the 3′-end (Weiner 2005). The phosphorylated 5′-ends of these tRNA-derived small RNAs are generated from pre-tRNAs by RNaseZ cleavage (Mayer et al. 2000), while the 3′-end results from transcription termination by pol III. Those derived in a Dicer-independent and tRNase Z-dependent manner are 3′-trailer sequences from pre-tRNAs, released by a cleavage by tRNase Z (Fig. 1i). They do not even partially meet the classical definition of a miRNA and are, therefore, called either tRNA-derived RNA fragments (tRFs) (Lee et al. 2009) or type II tRNA-derived small RNAs (tsRNAs; type I tsRNAs being Dicer-dependent) (Haussecker et al. 2010). Because the 3′-end of almost all eukaryotic RNAs including polyadenylated mRNAs and non-polyadenylated histone mRNAs are generated by specific nucleolytic cleavage (Weiner 2005), 3′-trailer sequences from these precursors could be another source of small RNAs. Although the precise function of these tRNA-derived small RNAs is not known, it is tempting to speculate that tRNAs, as an abundant class of RNAs, compete with pre-miRNAs for Dicer, and tRNA-derived small RNAs compete with miRNAs for Argonaute proteins, thereby regulating the abundance of miRISCs and maintaining miRNA homeostasis.

Conclusions

Cells take advantage of a wide variety of mechanisms to generate miRNAs and miRNA-like molecules, and have evolved the means to use a variety of transcripts as sources of miRNAs. Non-canonical pathways of miRNA biogenesis demonstrate the ability and flexibility of cells to generate pre-miRNA-like hairpins that are routed to Dicer and/or 5′-phosphorylated small RNAs that bind directly to AGOs. On the other hand, current data also raise the possibility that any cellular RNAs with potential pre-miRNA-like

hairpins could be substrates for Dicer. Thus, there must be systems in eukaryotic cells to protect transcripts with potential hairpins against Dicer and/or regulate their availability to Dicer.

There are many future challenges. How many classes of non-canonical miRNAs exist? How are these miRNAs generated? How is the processing of these non-canonical miRNAs used to regulate the activity of canonical miRNAs? What are their biological functions? Some snoRNAs and tRNAs are dual function molecules with both miRNA and snoRNA or tRNA capabilities. This already hints that other abundant non-coding RNAs such as rRNAs and U snRNAs among others (Hüttenhofer and Schattner 2006) may well provide sources of non-canonical miRNAs.

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