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Versatile microRNA biogenesis in animals and their viruses

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MicroRNAs (miRNAs) are ubiquitous gene regulators that modulate essential cellular processes at the post-transcriptional level. In metazoans and their viruses, most miRNAs are produced from hairpin-containing primary transcripts that are sequentially cleaved by nuclear Drosha and cytoplasmic Dicer. In the last decade, alternative mechanisms that bypass either the Drosha or Dicer cleavage step have emerged, increasing the complexity of the miRNA regulatory network. Here, we highlight non-canonical pathways that generate miRNAs using a variety of molecular machineries that play fundamental roles in the biogenesis and processing of other classes of cellular RNAs.

Keywords: microRNA biogenesis, Microprocessor, Dicer, exportin-5, mirtron, m^{7}G-capped pre-miRNA, AGO2 slicer activity, viral microRNA, tRNaseZ, Integrator

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Introduction

The discovery of microRNAs (miRNAs) has profoundly changed our understanding of how gene expression can be regulated at the post-transcriptional level. These ~22-nucleotide (nt) RNA molecules associate with Argonaute (AGO) proteins to form the functional cores of RNA-induced silencing complexes (RISCs) and interact with target mRNAs (mRNAs) through imperfect Watson-Crick base-pairing. Specific targeting is most frequently conferred by the seed region at the 5′ end of the miRNA (nt 2–8) pairing with specific sequences in the 3′ untranslated region (UTR) of an mRNA.[1] RISC binding leads to translation repression and/or destabilization and degradation of the targeted mRNA.[2-4] More than half of human genes are estimated to be regulated by miRNAs and there are more than a thousand annotated miRNAs genes in the human genome.[5,6] Therefore, miRNAs contribute significantly to cellular pathways including differentiation, proliferation, and apoptosis, which are critical to human development and disease. It is not surprising that miRNA expression profiles are altered in a variety of disease states, including cancer.[7,8]

Given that miRNAs play essential roles in fine-tuning the gene-regulatory network, it should be possible to manipulate miRNA production to combat various diseases. To this end, substantial effort has been devoted to elucidating the molecular mechanisms by which these small molecules are generated. It is now clear that most animal miRNAs are produced from RNA hairpin structures embedded in long primary transcripts through sequential cleavage in the nucleus and cytoplasm by two different RNase III-class enzymes (detailed below). Yet, not long after the canonical miRNA biogenesis pathway was established, reports of a variety of alternative pathways that bypass one of the RNase III cleavage steps began to emerge. This versatility in miRNA biogenesis may permit expression of particular miRNAs in different developmental stages or altered cell states to achieve differential gene regulation. Recently, we discovered yet another novel miRNA biogenesis pathway that not only bypasses nuclear cleavage by Drosha, but also utilizes a different mode of nuclear-cytoplasmic transport; it produces mature miRNAs exclusively from one arm of the precursor (pre-)miRNA hairpin.[9]

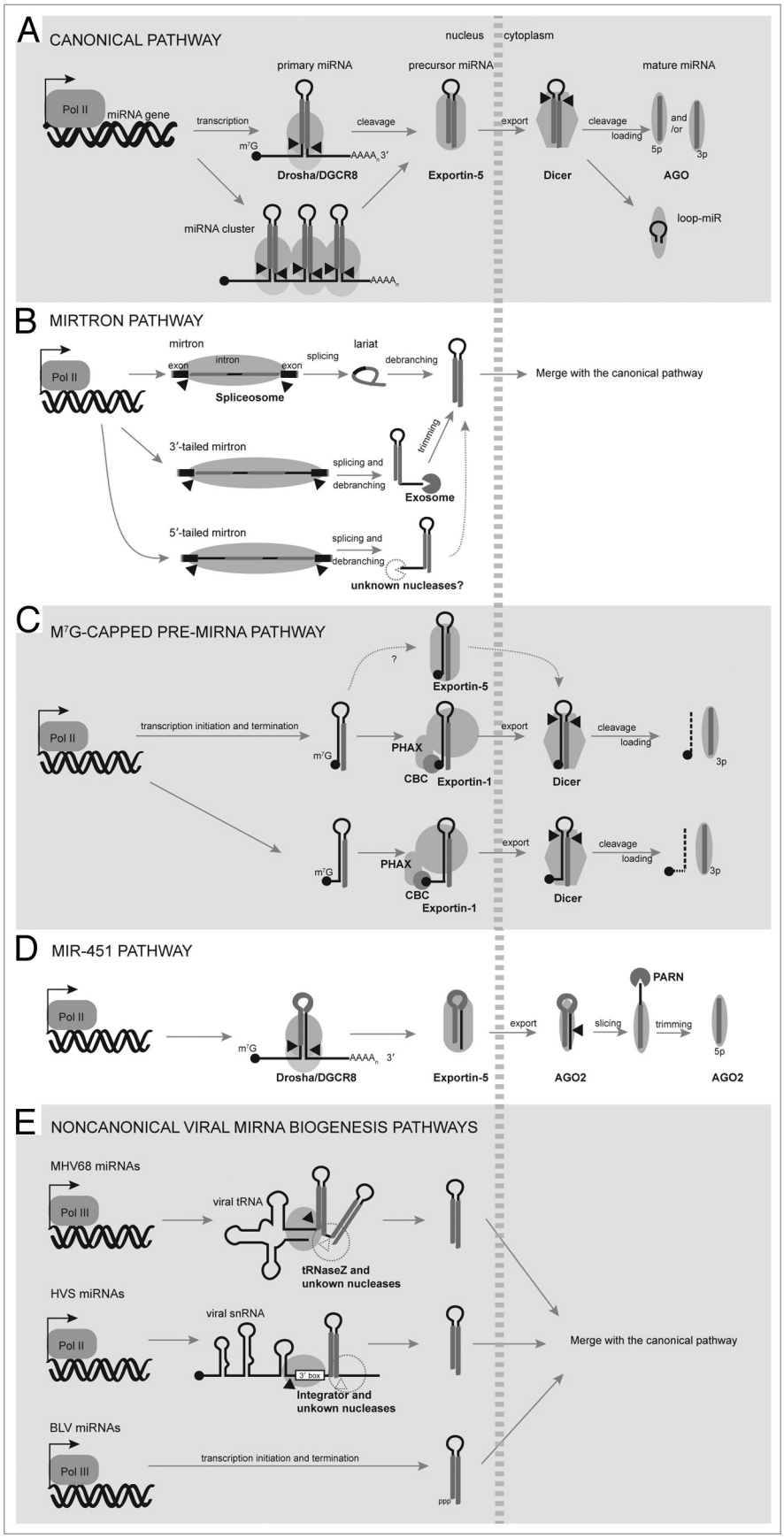

Figure 1. MiRNA biogenesis pathways in animals and their viruses. (**A**) The canonical pathway. Pri-miRNAs containing a single or a cluster of hairpins are typically transcribed by Pol II, 5′-capped, and 3′-polyadenylated. The Microprocessor complex (Drosha and DGCR8) cleaves pri-miRNAs to release the pre-miRNA hairpins, which are exported by XPO5 and processed by Dicer into mature miRNA duplexes in the cytoplasm. One miRNA strand is preferably selected by AGO to form RISC. In certain cases, the loop of the pre-miRNA hairpin may be incorporated into RISC. (**B**) The mirtron pathway. Microprocessor-independent mirtrons are directly generated by splicing of short introns and the resulting lariats are debranched to form pre-miRNA hairpins. Mirtrons could have tails on either end of the pre-miRNA hairpins. 3′-tailed mirtrons are further trimmed by the exosome, while the nuclease that processes 5′-tailed mirtrons remains elusive. (**C**) The m^{7}G-capped pre-miRNA pathway. M^{7}G-capped pre-miRNAs are directly transcribed by Pol II, bypassing Microprocessor cleavage. The presence of the 5′ cap directs pre-miRNAs to the PHAX-XPO1 export pathway, known to function in the export of snRNAs. However, except for the 5′-tailed variants, the m^{7}G-capped pre-miRNAs retain the ability to be exported by XPO5. After Dicer cleavage, the 5′-capped 5p-miRNA is unable to associate effectively with AGO, resulting in the production of only 3p-miRNPs. (**D**) The miR-451 pathway. Drosha cleavage of pri-miR-451 releases an unusual pre-miRNA that is too short to be processed by Dicer. Instead, AGO2 cleaves the 3p-arm of pre-miR-451 and PARN trims to generate a 5p-miRNA. (**E**) Drosha-independent miRNA biogenesis in animal viruses. MHV68 pri-miRNAs are tRNA-pre-miRNA chimeras that are processed by tRNaseZ at the 5′ end of the first pre-miRNA hairpin. The enzyme that separates the two pre-miRNAs is unknown. HVS pri-miRNAs are snRNA-pre-miRNA chimeras that are processed by Integrator to release the pre-miRNA. The 3′-end formation mechanism for HVS pre-miRNAs remains elusive. BLV miRNAs are derived from pre-miRNAs that are directly transcribed by Pol III as endogenous shRNAs. All viral pre-miRNAs described here are exported by XPO5 and processed by Dicer.

Here, we present additional data supporting our recent finding of a variant miRNA biogenesis pathway. We also review presently known miRNA biogenesis pathways in animals and their viruses. In plants, miRNA biogenesis also relies on RNase III-family enzymes but is substantially different from animal miRNA biogenesis. Readers are referred to recent reviews for details.[10,11]

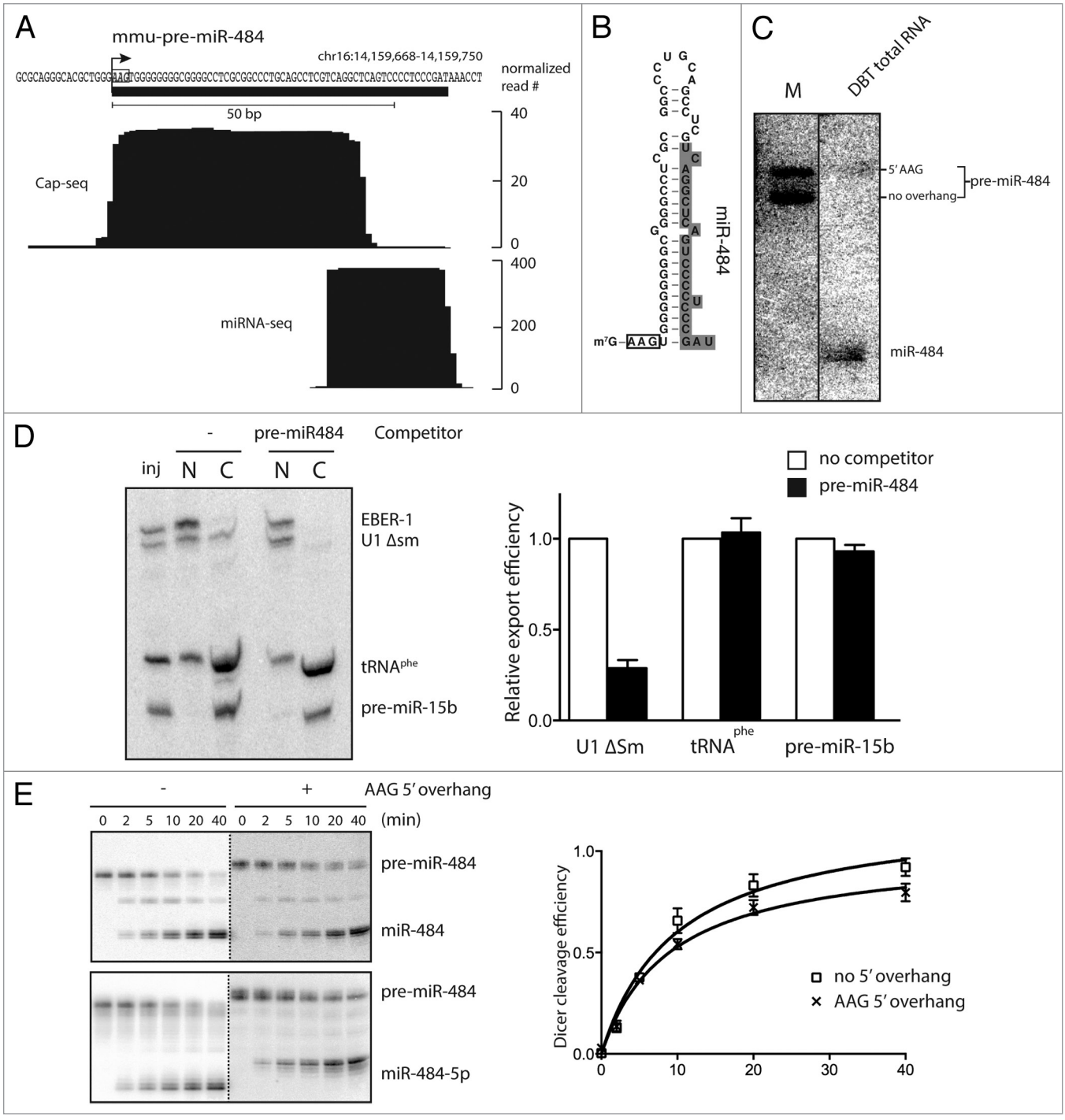

Figure 2. Mouse pre-miR-484 is a 5′-tailed m^{7}G-capped pre-miRNA. (**A**) The histogram shows cap-seq reads mapping to the mmu-miR-484 locus. TSS and transcription directionality are indicated by a black arrow. The height of each bar is proportional to the normalized read number (raw read number per million mapped reads). Cap-seq and miRNA-seq data were taken from refs. 9 and 66, respectively. (**B**) Mouse pre-miR-484 is illustrated with miR-484 highlighted in gray. (**C**) Northern blot probed for miR-484 of 10 µg total RNA isolated from mouse astrocyte DBT cells. M, in vitro-transcribed m^{7}G-capped pre-miR-484 markers with or without a 3-nt AAG 5′-end extension. (**D**) *Xenopus* oocyte microinjection assay. A mixture of 1–10 fmoles ^{32}P-labeled EBER-1, U1ΔSm, tRNA^{phe}, and pre-miR-15b with or without 1 pmole of unlabeled pre-miR-484 (m^{7}G-capped and containing 5′-AAG) was injected into the nuclei of *X. laevis* oocytes. After 2-h incubation, six oocytes were manually dissected, and RNAs from the nucleus (N) and cytoplasm (**C**) equivalent to one oocyte were extracted and analyzed on an 8M urea-15% polyacrylamide gel. inj, injected material. The bar graph shows relative RNA export efficiency (cytoplasmic/total) of the indicated RNAs with pre-miR-484 competition (black bars) compared with no competition (white bars). Error bars represent standard deviation from two experiments. (**E**) In vitro-transcribed pre-miR-484 with or without a 3-nt AAG 5′-end extension was incubated with recombinant human Dicer (a generous gift from Dr Jennifer Doudna) for various times and analyzed by northern blot to detect miR-484 (top panel) or miR-484-5p (lower panel), using ^{32}P-labeled DNA oligos complementary to miR-484 or miR-484-5p, respectively. The blot shows cleavage of pre-miR-484 without the 5′-end extension (□) or pre-miR-484 (x) by purified human Dicer at each time point. Error bars represent standard deviations in two experiments. The Materials and Methods used here were essentially the same as described in reference 9.

The Canonical miRNA Biogenesis Pathway

In metazoans, canonical miRNA biogenesis starts with the transcription of long primary (pri-)miRNA transcripts that contain one or a cluster of hairpin structures harboring mature miRNA sequences (**Fig. 1A**). Pri-miRNAs are typically transcribed by RNA polymerase (pol) II,[12,13] with a few exceptions that may be transcribed by RNA pol III.[14-16] Pol II transcription generally allows more elaborate regulation and tissue- or cell type-specific expression. Following Pol II transcription, pri-miRNAs are 5′ 7-methylguanosine (m^7G)-capped and polyadenylated at their 3′ ends, similar to mRNAs. In fact, many miRNA hairpins are embedded in the introns or exons of mRNAs or other long non-coding (lnc) RNAs, conferring dual functions on these primary transcripts.[17,18] Other miRNA hairpins are located in intergenic regions, driven by independent RNA pol II promoters.

In the nucleus and often co-transcriptionally, pri-miRNA hairpins are recognized and cleaved by the Microprocessor complex, minimally composed of Drosha, an RNase III enzyme, and DGCR8 (DiGeorge critical region 8), an RNA binding protein that is also known as Pasha (Partner of Drosha) in invertebrates.[19-23] The Microprocessor counts 10–11 base pairs from the base of the pri-miRNA stem-loop and cleaves to release the 55–70 nt pre-miRNA hairpin. Both the lower stem and the flanking single-stranded region are required for Drosha cleavage.[24,25] The terminal loop of the pri-miRNA also influences Drosha cleavage in some cases.[26,27] This cleavage yields a pre-miRNA with a 5′ monophosphate group and a 2-nt 3′-end overhang, characteristic of RNase III enzyme processing products.

Once produced, the pre-miRNAs are bound by Exportin-5 (XPO5) in the presence of its Ran-GTP co-factor and exported into the cytoplasm through the nuclear pore complex.[28-30] The association between pre-miRNA and XPO5 requires recognition of the mini-helix structure and the 2-nt 3′-end overhang.[29,31-33] Conversely, 5′ overhangs on pre-miRNAs are inhibitory for XPO5 binding, and hence, prevent pre-miRNA nuclear-cytoplasmic export.[29,32] Interestingly, XPO5 also protects pre-miRNAs from nuclease attack, as knocking down XPO5 does not result in pre-miRNA accumulation in the nucleus.[28,32] Structural work has confirmed an extensive interaction between pre-miRNA and XPO5 that would be expected to counteract nuclease digestion.[33]

Once in the cytoplasm, GTP hydrolysis leads to dissociation of the pre-miRNA from XPO5. Subsequently, another RNase III enzyme, Dicer, cleaves off the loop of the pre-miRNA hairpin to produce a ~22 bp mature miRNA duplex, with 2-nt 3′-end overhangs on both strands.[34-37] Dicer protein recognizes structural features to ensure accurate and efficient cleavage: counting ~22 nt from both the 5′ monophosphate and the 3′-end overhang of the pre-miRNA hairpin.[38-40] Recently, it was reported that the distance from the Dicer cleavage site to the pre-miRNA loop is also critical for accurate processing.[41] Different from Drosha cleavage, human Dicer alone is sufficient to carry out accurate and efficient cleavage reactions in vitro. Although not essential for activity, RNA binding proteins including TRBP (TAR RNA binding protein) and PACT (protein activator of PKR) associate with Dicer in vivo, facilitating assembly of the miRNA into RISC (see below).[42-44] In contrast, *Drosophila* Dicer-1 requires the RNA binding protein Loquacious (Loqs) for pre-miRNA processing.[45-47]

The miRNA duplex resulting from Dicer cleavage is incorporated into AGO proteins dependent on the RISC loading complex, which contains Dicer, AGO, and RNA binding proteins such as TRBP/PACT in humans and Loqs in *Drosophila*.[42,43,48,49] In humans, there are four AGO proteins (AGO1–4), all of which can bind miRNAs and regulate gene expression. However, only AGO2 possesses catalytic slicer activity. Flies have two AGO proteins, with AGO1 mediating miRNA function.[50] After the miRNA duplex is loaded, one strand is preferentially selected to form the functional miRNA–RISC complex (miRISC), while the other strand is displaced and degraded.[51-53] The duplex strand with relatively weak base-pairing at its 5′ end is preferentially selected and designated as the guide strand or miRNA;[54,55] the other strand is termed the passenger strand or miRNA*. However, because strand selection is not a stringent process, recent deep sequencing studies have identified substantial numbers of functional miRNA* strands in miRISCs.[56-58] Moreover, since equal numbers of miRNAs can theoretically be derived from either arm of the miRNA duplex, it is difficult to distinguish miRNA from miRNA* and miRNAs are also named depending on their stem-loop arm of origin (miR-5p or -3p). Recently, it was demonstrated that the loop region of specific pre-miRNAs can also be incorporated into AGOs and direct target repression, suggesting that endogenous loading of AGO proteins does not occur exclusively via miRNA duplexes.[59,60] Mutational analysis revealed that effective loop-miR loading is determined by a combination of features including the pre-miRNA stem and loop sequences, as well as length (~21 nt).[59]

The Mirtron Pathway

The first example to challenge the paradigm that all miRNAs are synthesized in the same way came from a class of Drosha-independent pre-miRNAs called "mirtrons" (pre-miRNAs/introns) in *Drosophila* and *C. elegans* (**Fig. 1B**).[61,62] As their name implies, these pre-miRNAs are directly generated by the splicing machinery. Mutating the 5′ and 3′ splice sites abolishes both pre-miRNA and miRNA production from a mirtron,[61,62] demonstrating a requirement for splicing. Importantly, mirtron hairpins are ~10 bp shorter than canonical pri-miRNA hairpins and can therefore bypass Drosha cleavage.[62] Once an intron is spliced out by the spliceosome, it requires the debranching enzyme (Ldbr) to resolve the lariat and fold into the hairpin secondary structure needed for downstream steps in miRNA biogenesis. The pre-miRNA then undergoes XPO5-dependent export and merges with the canonical pathway.

It was hypothesized that *Drosophila* and *C. elegans* contain relatively short introns that are suitable for evolving of

pre-miRNA-like molecules. Later, hundreds of mirtrons were found in the short introns of vertebrates and even rice genomes.[63-69] Moreover, variant mirtrons that contain either a 5′- or 3′-end extension were discovered.[61,64,65] In other words, pre-miRNA hairpins can reside at either end of an intron, with the 5′ or 3′ end the pre-miRNA hairpin directly generated by splicing. In the case of a 3′-tailed mirtron in *Drosophila*, the tail of miR-1017 is ~100 nt long and removed by the exosome, a major 3′-5′ exonuclease acting in RNA turnover in eukaryotic cells.[70] In contrast, 5′-end extended mirtrons are predominantly found in vertebrates, with the identity of the nucleases that process this class of mirtrons into the pre-miRNA molecules remaining elusive. It is not yet known whether XRN-1 or XRN-2, the major 5′-3′ exonucleases, is responsible for this trimming. Curiously, one report indicates that two predicted human mirtrons (miR-1225 and 1228) are splicing-independent, despite the fact that both ends of the predicted pre-miRNAs match splice sites.[71] These so called "simtrons" (splicing-independent mirtrons) are also independent of DGCR8, XPO5, Dicer, and AGO2 for their biogenesis, but the production of mature miRNAs is reduced upon overexpression of a dominant negative Drosha. Further investigation is required to define this pathway.

The m^{7}G-capped Pre-miRNA Pathway

Another group of Drosha-independent pre-miRNAs is directly transcribed by RNA pol II and contains a m^{7}G cap at its 5′ ends (**Fig. 1C**).[9] Initial indications that these miRNAs might be derived directly from stand-alone pre-miRNAs came from their insensitivity to knockdown of core components of the Microprocessor complex (Drosha or DGCR8) in mouse cells.[65,72] In addition, 5p-miRNAs derived from these pre-miRNAs are significantly underrepresented in miRNA sequencing (miRNA-seq) analyses, which require a 5′ monophosphate on the RNA molecule for inclusion in the miRNA-seq library. These results implied that the 5′ ends of these pre-miRNAs were generated by transcription initiation and lack 5′ monophosphate. Therefore, together with other pre-miRNAs whose ends are defined by transcription initiation/termination, these miRNA precursors were named endogenous short hairpin RNAs (endo-shRNAs), analogous to exogenously expressed shRNAs driven by the U6 (RNA pol III) promoter.[65]

We recently characterized a subset of endo-shRNAs, designated m^{7}G-capped pre-miRNAs, which are directly transcribed by RNA pol II (**Fig. 1C**).[9] Their cap structures are added and co-transcriptionally bound by the cap binding complex (CBC) and PHAX (phosphorylated adaptor for RNA export), which was previously assigned to the export of small nuclear RNAs (snRNAs).[73] Therefore, like snRNAs, m^{7}G-capped pre-miRNAs are exported by the Exportin-1 (XPO1) pathway. Interestingly, *Xenopus* oocyte microinjection assays showed that saturating amounts of m^{7}G-capped pre-miRNAs not only inhibit XPO1-dependent U1 snRNA export, but also canonical pre-miRNA export, indicating that capped pre-miRNAs can alternatively interact with XPO5. Thus, if a particular m^{7}G-capped pre-miRNA is not efficiently bound by CBC/PHAX, it could perhaps be exported by XPO5 through the canonical pathway. Another unique feature of m^{7}G-capped pre-miRNAs is that they produce only 3p-miRNAs. This is due to the inefficient incorporation of an m^{7}G-capped 5p-miRNA into AGO. Hence, devising Pol II-driven shRNA constructs to deliver single (3p-) small interfering RNAs (siRNAs) for targeted gene silencing should minimize the off-target effects of undesired 5p-siRNAs.

Analysis of small hairpin RNAs selected by the m^{7}G cap-binding protein eIF4E (small RNA cap-seq) revealed that the 5′ ends of most m^{7}G-capped pre-miRNAs map to the base of the hairpin. An exception is miR-484, whose pre-miRNA contains a 4-nt extension (counting the m^{7}G cap, **Fig. 2A and B**). To confirm these extensions, we performed northern blot analysis of total RNAs extracted from the mouse astrocyte DBT cell line and detected endogenous pre-miR-484 migrating at the position of a 5′-extended hairpin marker (**Fig. 2C**). Several lines of evidence suggest that pre-miR-484 is exported and processed as a 5′-extended m^{7}G-capped pre-miRNA. First, *Xenopus* oocyte injection confirmed that pre-miR-484 export is XPO1-dependent, as saturating amounts of pre-miR-484 inhibit XPO1-dependent U1 snRNA export (**Fig. 2D**). However, unlike m^{7}G-capped pre-miRNAs that do not contain a 5′-tail, pre-miR-484 does not compete with canonical pre-miRNA export (**Fig. 2D**). This is consistent with the fact that a 5′-end extension on a pre-miRNA inhibits its binding to XPO5.[29,32] Second, miR-484-5p is underrepresented in miRNA seq data, suggesting that it retains the m^{7}G cap.[65] Finally, the 5′-tailed pre-miR-484 is a Dicer substrate, albeit the dicing efficiency is slightly lower than that of a pre-miRNA hairpin without a 5′-end extension (**Fig. 2E**). Dicer cleavage generates a ~25-nt m^{7}G-capped 5p miRNA, which is expected to be inefficiently bound by AGO proteins.

Interestingly, a new group of miRNAs derived from the transcription start sites (TSS-miRNAs) of protein coding genes has been identified recently.[74] Similar to miRNAs derived from m^{7}G-capped pre-miRNAs, the synthesis of TSS-miRNAs is Drosha/DGCR8-independent but Dicer-dependent, and 3′-end formation of the pre-miRNAs is linked to RNA pol II promoter proximal pausing. Although TSS-pre-miRNAs appear comparable to 5′-tailed m^{7}G-capped pre-miRNAs, the capped 5′-end extension may be removed by exonuclease activities, allowing these intermediates to merge with the canonical biogenesis pathway. This model is supported by the fact that many TSS-5p-miRNAs contain 5′-monophosphates. What is not clear is whether all TSS-miRNA precursors share exactly the same start site with annotated protein-coding mRNAs. Because polynucleotide phosphorylase (PNPase) treatment in our small RNA cap-seq procedure preferentially selects shRNAs over mRNAs, it would be interesting to examine TSS-miRNA loci by small RNA cap-seq to see if a subset of TSS-miRNAs is derived from m^{7}G-capped pre-miRNAs.

The miR-451 Pathway

To date, miR-451, an erythropoietic miRNA conserved in vertebrates, is the only Dicer-independent miRNA identified (**Fig. 1D**).[75-77] Pri-miR-451 is cleaved by the Microprocessor as in the canonical pathway. However, the resulting pre-miRNA is only 17 base-pairs long and is therefore too short to be recognized by Dicer. Moreover, the mature miR-451 sequence includes the loop region of the pre-miRNA, arguing against a Dicer cleavage model. Indeed, Dicer knockout in either mouse ES cells or zebrafish diminished overall miRNA production, while levels of miR-451 remained unaffected.[75,76] Intriguingly, AGO2 slicer activity is required for miR-451 biogenesis, as introduction of catalytically inactive AGO2 could rescue neither miR-451 expression in AGO2-knockout cells nor related developmental defects in AGO2-null animals.[75,76] In lieu of Dicer cleavage, pre-miR-451 (after Drosha cleavage and XPO-5-mediated export) is directly loaded into AGO2, which cleaves between base pairs 10 and 11 on the 3p arm. The resulting ~30-nt AGO2-cleaved pre-miR-451 can then undergo poly(A)-specific ribonuclease (PARN)-mediated trimming of the remaining 3p arm, yielding a ~23 nt 5p-miRNA.[78] However, trimming seems to be dispensable for target silencing in vivo, suggesting that a miRNA longer than 22 nt is functional in RISC. Similar to m^{7}G-capped shRNAs, shRNA vectors designed to have features of the pre-miR-451 pathway could be used to deliver a single 5p-siRNA for gene silencing.

Drosha-Independent Pathways in Viruses

Given that miRNAs play fundamental roles in gene regulation, it is not surprising that many viruses express their own miRNAs to regulate host and viral genes during the viral life cycle. Like the majority of host miRNAs, most viral miRNAs utilize the canonical host pathway for biogenesis. However, some viruses are very creative in inventing their own ways of making miRNAs.

In two Herpesviruses, viral pre-miRNA hairpins reside directly downstream of another class of non-coding RNAs (ncRNAs) to form ncRNA-pre-miRNA chimeras (**Fig. 1E**). These chimeric pri-miRNAs are cleaved first by the 3′-end processing machinery of the ncRNA to separate the pre-miRNA from the ncRNA, replacing Drosha cleavage.[79,80] In murine γ-herpesvirus 68 (MHV68), host tRNaseZ cleaves transfer RNA(tRNA)-pre-miRNA chimeras;[79] in Herpesvirus saimiri (HVS), the host Integrator complex cleaves snRNA-pre-miRNA chimeras.[80] In the MHV68 tRNA-pre-miRNA chimeras, there are usually two pre-miRNA hairpins, with the yield of miRNAs from the first hairpin being greater.[79,81,82] The presence of a poly-U stretch at the end of the second pre-miRNA suggests that the 3′ end is defined by Pol III termination. However, the enzymatic activity that separates the two pre-miRNAs remains elusive. Similarly, the detailed 5′- and 3′-end formation mechanisms for HVS pre-miRNAs are uncharacterized: the Integrator complex potentially cleaves upstream of the 3′ box, a conserved sequence essential for snRNA 3′-end processing,[83] but the resulting 5′-extended pre-miRNA may be trimmed by either the Integrator itself or other exonucleases. In both cases, viral pre-miRNAs are exported by XPO5 and undergo canonical miRNA biogenesis thereafter.[79,80] Intriguingly, similar tRNA-pre-miRNA structures were identified in the mouse genome by computational analysis and confirmed by northern blotting, suggesting that the virus has acquired this unusual miRNA biogenesis pathway from the host.[82] However, no snRNA-pre-miRNA chimeric RNAs have been identified in primates.

In a retrovirus, bovine leukemia virus (BLV), a subgenomic region produces several RNA pol III-dependent miRNAs (**Fig. 1E**).[84] Such an arrangement avoids detrimental Drosha-mediated cleavage of the BLV genomic RNA. BLV pre-miRNAs appear to be directly transcribed as endo-shRNAs, mimicking U6 promoter-driven exogenous shRNAs. Such pre-miRNAs are structurally similar to canonical pre-miRNAs generated by Drosha cleavage and should be exported by XPO5 and cleaved by Dicer.

Conclusion and Perspectives

Here we have summarized the current understanding of both canonical and alternative miRNA biogenesis pathways that produce miRNAs from dedicated miRNA genes (**Fig. 1**). Although also dependent on Dicer action, the biogenesis of endogenous small interfering RNAs (endo-siRNAs), which are derived from long endogenous double-stranded RNAs or from long hairpin RNAs in *Drosophila* or mouse oocytes, are beyond the scope of this review.[65,85-89] In addition, Dicer-dependent or -independent miRNA-like small RNAs are reported as being derived from other ncRNAs, such as snoRNAs, tRNAs, and vault RNAs, thereby bypassing Drosha cleavage.[65,90-94]

Although noncanonical miRNAs make up only a small fraction of the total miRNAs, their biogenesis pathways are conserved in different organisms. For example, the mirtron mechanism is found in almost all animals examined and one candidate was even found in rice, although not a single mirtron-derived mature miRNA is conserved in sequence across different metazoan lineages.[63] The Dicer-independent pathway for generating the blood-specific miR-451 is conserved from zebrafish to human.[75,76] Finally, m^{7}G-capped pre-miRNAs can be found in all mammals.[9] The conservation of mechanisms for the noncanonical miRNA pathways points to important biological significance. One possible advantage is to allow spatial and temporal expression of specific miRNAs in the absence of canonical miRNA biogenesis factors under certain conditions. It will be interesting to ask if there are consequences of forcing the production of noncanonical miRNAs through the canonical pathway.

Remarkably, Pol III- (U6) or Pol II- (U1) promoter driven exogenous shRNAs and tRNaseZ-dependent shRNAs were artificially constructed preceding the discovery of their natural counterparts.[95-97] Recently, several other artificial miRNA biogenesis pathways have been devised, providing proof-of-principle evidence that yet additional pathways may exist in nature. For example, RNase III-independent miRNA biogenesis was achieved in mammalian cells by combining the tRNaseZ- or

Integrator-dependent pathway with the AGO2-dependent pathway.[98] Another example is the discovery that cytoplasmic RNA viruses, such as Sindbis virus and tick-born encephalitis virus, can be engineered to produce functional miRNAs.[99,100] Artificially inserted pri-miRNAs are successfully processed despite the fact that these viruses replicate exclusively in the cytoplasm, perhaps suggesting a noncanonical miRNA processing pathway. Therefore, many other unexpected miRNA biogenesis mechanisms may be discovered in the years to come.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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