

Review

Biogenesis of Mammalian MicroRNAs: A Global View

Paul Graves¹, Yan Zeng^{2,*}

¹ Department of Radiation Oncology, New York Methodist Hospital, Weill Cornell Medical College, Brooklyn, NY 11215, USA

² Department of Pharmacology, Masonic Cancer Center, University of Minnesota, Minneapolis, MN 55455, USA

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Abstract

MicroRNAs (miRNAs) are approximately 22-nucleotide-long non-coding RNAs that are important regulators of gene expression in eukaryotes. miRNAs are first transcribed as long primary transcripts, which then undergo a series of processing steps to produce the single-stranded mature miRNAs. This article reviews our current knowledge of the mechanism and regulation of mammalian miRNA expression and points out areas of research that may enhance our understanding of how the specificity and efficiency of miRNA production is controlled *in vivo*.

Keywords: MicroRNA expression; MicroRNA processing; Primary microRNA; Drosha

The big picture: microRNAs as RNAs

MicroRNAs (miRNAs) are unique due to their small size, which is approximately 22-nucleotide (nt)-long; but as RNAs, they are still governed by the same mechanisms that apply to all the other RNAs in a cell. There are two well established principles that adeptly explain many findings regarding miRNA biogenesis, as detailed later, and, sometimes, regarding miRNA function as well.

The first principle is that most RNAs undergo processing before maturation. For example, ribosomal RNAs (rRNAs) are produced by cropping of their primary transcripts and contain many nucleotide modifications, and eukaryotic messenger RNAs (mRNAs) undergo 5' and 3' modifications as well as splicing. In fact, the only known class of RNAs in nature that forgo processing in general are mRNAs in bacteria. It is natural, therefore, that miRNA production also involves processing, which is carried out by proteins and enzymes with specialized activities.

The second principle is that cellular RNAs associate constantly with proteins, which can regulate RNA transcription, processing, localization, function and/or

degradation. There are hundreds of human proteins with recognizable RNA-binding motifs, and the actual number of RNA-binding proteins *in vivo* is likely much higher. Furthermore, additional proteins may be recruited to RNAs via interaction with RNA-binding proteins. Not surprisingly, a number of proteins have been identified that impact the processing or function of specific miRNAs. Nonetheless, because these proteins are quite often promiscuous, it is not trivial to ascertain the specific mechanism and function of such proteins in the miRNA pathway *in vivo*.

The production of mammalian miRNAs

Complex genomes encode hundreds of miRNA genes; *e.g.*, the human genome has over 1000 miRNA genes as categorized by miRBase [1]. miRNA genes are transcribed by RNA polymerase II (Pol II), or occasionally RNA polymerase III, to produce the primary miRNA transcripts (pri-miRNAs) [2]. As a result, pri-miRNAs are long and may contain 5' and 3' modifications identical to those present in mRNAs or pre-mRNAs. Indeed, many miRNA-coding sequences are located within or overlap with annotated genes for mRNAs or other RNAs, which are often referred to as the host genes for the miRNAs. However, miRNA

* Corresponding author.

E-mail: zengx033@umn.edu (Zeng Y).

genes are not well defined experimentally and pri-miRNAs are not as extensively characterized as other transcripts such as mRNAs. When DNA sequence coding for a mature miRNA is found situated within or near a known gene, it is often assumed that transcription of the known gene (host gene) produces a transcript that ultimately gives rise to the miRNA. Nevertheless, it is possible that the miRNA has its own promoter that has nothing to do with the “host” gene, or that the known host transcript(s) is just one of two or more possible pri-miRNAs that produce the same miRNA. There are also instances whereby miRNAs appear to form their independent transcription units, although it remains possible that they share transcripts with other genes as well.

Mammalian miRNAs can be divided into two broad classes, canonical and non-canonical, based on how the pri-miRNAs are processed leading to the production of mature miRNAs. In the canonical pathway (**Figure 1**), a pri-miRNA is cleaved by Drosha bound by its regulatory subunit DGCR8, to liberate a hairpin structured precursor, or pre-miRNA, of ~60–70 nucleotides (nt) in the nucleus [3–7]. The pre-miRNA often contains a 2-nt 3' overhang, as a result of Drosha's RNase III activity, and is exported to the cytoplasm by Exportin5 (Exp5) associated with its Ran cofactor coupled to GTP [8–10]. In the cytoplasm, GTP is replaced by GDP, inducing Exp5 to release its pre-miRNA cargo. The pre-miRNA is then cleaved by another RNase, Dicer, to produce a miRNA duplex intermediate of ~22 basepairs [11–16]. An Argonaute (Ago) protein binds the duplex and incorporates the mature, single-stranded miRNA into the Ago:RNA complex, while the other strand in the original duplex is discarded [17–19]. Which strand is retained depends on the relative thermodynamic stability of the two ends of the duplex intermediate [20,21]. Other RNA- and Dicer-binding proteins, such as TRBP and PACT, may facilitate the production of miRNA duplexes and/or the transfer of mature miRNAs to Ago proteins [22–28].

For non-canonical miRNAs, their processing does not require all of the protein factors mentioned above. For example, pre-miRNAs of mirtron genes are produced by splicing, not by Drosha cleavage [29–32]. Pre-miR-451 is cleaved by Ago2, bypassing Dicer [33,34]. Certain pri-miRNAs are small hairpin RNAs that probably serve as pre-miRNAs and Dicer substrates directly [35]. Such RNAs may also be defined as the endogenous small interfering RNAs (siRNAs). Canonical and non-canonical miRNAs can be distinguished by changes in their expression when a certain miRNA processing factor is knocked down or knocked out. Loss of Drosha, DGCR8 or Dicer should greatly reduce the expression of canonical miRNAs, while the non-canonical miRNAs would have variable responses depending on individual proteins [33,35]. Most ~22-nt long RNA species in mammals are canonical miRNAs [35]. It is unclear whether canonical miRNA genes outnumber non-canonical miRNA genes, as more and more miRNAs with low abundance are being identified by deep-sequencing experiments and deposited to miRBase, although how these RNAs are processed has rarely been examined.

RNA editing can introduce further variations in miRNAs. RNA adenosine deaminases convert adenosine into inosine residues that can form basepairs with cytosines or uracils [36–38]. Pri-miRNA, pre-miRNA and mature miRNA sequences might be modified by the deaminases, which potentially impact miRNA processing and target recognition.

What determines how fast and when a mammalian miRNA is degraded is poorly understood. A mammalian RNase called MCPI1 digests pre-miRNAs to block the Dicer processing step [39]. Once made, most miRNAs are believed to be stable, although the half-lives of miRNAs vary as a result of intrinsic stability or treatments [40–42]. It is unclear whether a mature miRNA can be stripped away from its Ago partner for degradation, or whether the decay of a miRNA must be coupled to that of the Ago protein. miRNAs also undergo tailing and trimming at their 3' end [43,44]. Such modifications are presumably coupled to miRNA function and degradation, although their significance in mammalian systems remains to be determined.

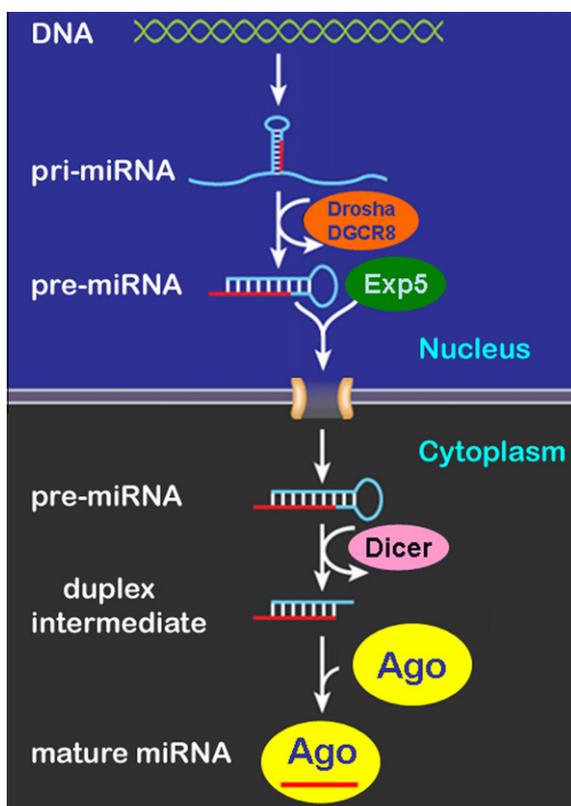


Figure 1 The canonical miRNA biogenesis pathway in animal cells. See text for explanations.

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Interactions between the universal miRNA processing factors and miRNA transcripts

Biochemical and structural studies have provided significant insights into how various miRNA transcripts are recognized by Drosha, Exp5, Dicer and Ago proteins. The

Drosha/DGCR8 holoenzyme (Drosha in short) recognizes an extended hairpin structure in a pri-miRNA. Preferably, the structure contains a large terminal loop (>10 nt), a mostly double-stranded RNA moiety of ~ three helical turns long, and flanking single-stranded RNA [45–47] (Figure 1). Structures of several putative RNA-binding domains in Drosha have been solved [48,49], although it remains unknown how they specify pri-miRNAs. Determining the structure of an active Drosha complexed with a substrate will be necessary to understand the mechanism of pri-miRNA recognition and cleavage.

Exp5 is a member of the karyopherin family, which mediates macromolecular nucleocytoplasmic transport, and is similar in structure to other karyopherins [50]. Exp5 cargoes are minihelix-containing RNAs with a short 3' overhang [51], such as the human Y1 RNA, adenovirus VA1 RNA, and pre-miRNAs. In addition to mediating pre-miRNA export, Exp5 further protects RNAs from degradation [52]. These properties are explained by the extensive interactions between human Exp5 and the duplex and 3' overhang of human pre-miR-30a in a crystal structure [50].

The structure of a simple, *Giardia* Dicer has been determined [53]. *Giardia* Dicer has a PAZ domain that preferentially binds the single-stranded 3' ends of double-stranded RNAs [54], and as a result, Dicer acts as a molecular ruler to cleave the RNAs at a set distance from one end. In more complex organisms such as flies and humans, Dicer has a pocket around the PAZ domain that binds the 5' end of RNAs [55]. It is probable that Dicer coordinately recognizes the end structure formed by both the 5' and 3' ends of a pre-miRNA. In addition, the duplex region and the terminal loop of a pre-miRNA modulate Dicer activity [56]. Accessory Dicer cofactors such as TRBP appear to enhance Dicer activity without significantly impacting its substrate specificity [57,58].

A number of Ago proteins have been studied by X-ray crystallography. Ago proteins consist of four globular domains: the amino-terminal N domain, PIWI, PAZ and MID domains [59]. The PIWI domain folds like an RNase H, explaining the potential endonuclease activity [60]. The PAZ domain, like that in Dicer, binds the 3' end, while the MID domain binds the 5' monophosphate of a small RNA such as a miRNA [61–64]. In perhaps the only instance whereby a universal miRNA processing factor interacts with RNA in a nucleotide-dependent manner, the MID domain exhibits preferences for certain residues; *e.g.*, human Ago2 binds UMP and AMP much tighter than CMP and GMP [62]. This finding correlates with the fact that human miRNAs often have 5' uridine residues. The binding of a miRNA protects human Ago2 from proteolytic cleavage, *i.e.*, enhances the structural stability of the protein [64].

Regulating the expression of specific miRNAs

miRNA expression levels vary spatially and temporally *in vivo* and alter in response to internal and external signals. In addition, miRNA dysregulation is often associated with disease states such as cancers. Once a miRNA is produced,

it presumably associates with an Ago protein to regulate the expression of target genes downstream. Hence, the presence of a miRNA implies its activity. Consequently, the regulation of miRNA expression is an active area of research. As in the case of mRNA expression, a number of transcription factors have been identified that stimulate or inhibit the transcription of specific miRNA genes, either constitutively or under specific conditions. An example is the mammalian miR-34a/b/c genes, which are transcriptionally activated by p53, a widely expressed and inducible transcription factor as well as a master tumor suppressor. The regulation of miR-34 expression by p53 places the miRNAs under the p53 signaling pathways that have major implications in normal physiological processes as well as tumorigenesis [65–69]. More examples of transcription factors for miRNA genes are categorized in another review [70].

The levels of pri-miRNAs or pre-miRNAs do not always correlate with those of the mature miRNAs (*e.g.*, [71–73]), suggesting additional post-transcriptional regulation. Several RNA-binding proteins have been reported to control the processing of specific miRNAs. The most thoroughly characterized example is Lin-28 and its close homolog, Lin-28B, which inhibit let-7 maturation by binding to the terminal loop region to inhibit pri-miRNA cleavage by Drosha, to inhibit pre-miRNA cleavage by Dicer and/or to induce the modification and degradation of let-7 transcripts [74–79]. Interestingly, Lin-28 and Lin-28B have an exclusive expression pattern in human cells and inhibit let-7 biogenesis through different mechanisms due to their diverse subcellular localization [80]. Lin-28 or Lin-28B is often overexpressed in several human cancers, consistent with the tumor suppressing function of let-7 [80].

hnRNP A1, a common RNA-binding protein, enhances the processing of miR-18a and perhaps other miRNAs as well, by inducing conformational changes in the pri-miRNAs to favor Drosha cleavage [81,82]. The KH-type splicing regulatory protein can activate the maturation of numerous miRNAs such as let-7 and miR-155, by facilitating Drosha and Dicer recruitment to the miRNA substrates [83,84]. Other proteins that have been implicating in specific miRNA processing include the p72 and p68 RNA helicases, SMAD, p53, the estrogen receptor, Ars2 and SF2/ASF [85–90]. Compared to the Lin-28/B example, these proteins are recruited to RNA via diverse mechanisms and have a broader range of substrates. Because some of these proteins are known to have many mRNA ligands or interact with many proteins, it is not surprising that they would associate with certain miRNA transcripts. With relatively weak interactions with miRNA or miRNA processing enzymes, however, whether their mechanisms and effects on miRNA processing extend definitively beyond their chaperone-like properties remains to be seen.

Regulating miRNAs, globally and specifically

Some miRNAs are highly tissue-specific, such as miR-1 in muscles and miR-122 in the liver, while many miRNAs are

largely ubiquitously expressed, albeit at different levels *in vivo*. Imagine one could sample miRNA expression in every cell at all of its developmental times in the body of an organism, one would find that miRNAs differ in their expression in individual cells, at specific time points, and as a whole in the organism [38,91]. Why miRNAs differ in their relative expression is an issue that has been mostly overlooked. An analogous question can be posed for many fundamental issues in biology: the expression of mRNAs and proteins, levels of protein modifications, etc. Although transcriptional activity is commonly assumed to play a deciding role in determining the overall expression pattern of miRNAs, the direct evidence is lacking. The identification of specific transcription factors for individual miRNAs in itself does not address the question of why a particular miRNA is more or less abundant than another.

A simple test of the contribution of transcription to global miRNA expression is to examine the correlation between cellular miRNA levels and the occupancy of Pol II on miRNA genes, based on CHIP-seq experiments. Because CHIP-seq experiments sample only partial Pol II occupancy, a less straightforward alternative is to use mRNA profiling data from RNA-seq or microarray studies. We have performed such analyses in a number of human cell lines using publicly-deposited GEO datasets and obtained variable results. As shown in Table 1, Pol II occupancy and host gene mRNA expression positively correlate with mature miRNA expression in K562 cells. The same is true in HepG2 and human embryonic stem cells (data not shown). On the other hand, HeLa (Table 1) and 293 cells (data not shown) do not exhibit such a significant correlation. Even when a clear correlation exists, the correlation coefficient is relatively small (~ 0.3 , Table 1). These results suggest that the data available contain a significant amount of noise and/or that transcription shapes global miRNA expression only modestly or in a complex manner.

The other mechanism that could regulate global miRNA expression is the miRNA processing pathway. Expression changes (*e.g.*, reductions) of the canonical miRNA processing factors have been reported in certain human conditions, resulting in a loss of miRNAs [93]. Drosha

and Dicer do not cleave their substrates precisely, leading to the generation of multiple miRNA isoforms from a single pri-miRNA, thereby increasing the complexity of miRNA biogenesis and function [38,94]. More importantly, however, there are two general considerations or problems regarding miRNA biogenesis. First, a haploid human genome has three billion basepairs of DNA, and assuming that only 50% in one strand is transcribed at any time or any place in a human body, we will have 1.5 billion nt of RNAs. If any 150-nt-long RNA is subject to secondary structure prediction, it almost certainly contains at least a stem-loop structure. In other words, the human transcriptome encodes likely millions of hairpin elements, while the miRBase currently documents only ~ 1000 human miRNA genes. So the miRNA processing machinery must discriminate against a vast majority of hairpin-containing structures, with potential help from other mechanisms. Secondly, these ~ 1000 human miRNA genes produce transcripts with diverse structures that are unlike to interact with the miRNA processing machinery identically. In other words, there are both a challenge and an opportunity to ensure the specificity and efficiency of miRNA production. A key mechanism is likely through Drosha action.

Drosha initiates the irreversible pri-miRNA cleavage, so it may not only differentiate whether a transcript encodes a miRNA or not, but also dictate how much a mature miRNA is ultimately produced by controlling how efficiently a pri-miRNA is cleaved. It has indeed been shown that Drosha cleaves hundreds of human pri-miRNAs differentially, at least *in vitro*, which positively and significantly correlates with the expression levels of mature miRNAs *in vivo* [95]. Certain secondary structures in pri-miRNAs, *e.g.*, a flexible terminal loop region and flanking single-stranded RNAs, predicts how well a pri-miRNA is cleaved and how well the mature miRNA is expressed in humans [95]. Obviously, such structural requirements likely preclude most cellular hairpin-containing RNAs from entering the miRNA processing pathway. There are Drosha-independent miRNAs, but their expression is typically weaker than that of canonical miRNAs, so their

Table 1 Correlation between miRNA expression and Pol II occupancy or mRNA expression in K562 and HeLa cells

Range (kb)	K562						HeLa					
	Pol II			mRNA			Pol II			mRNA		
	<i>n</i>	ρ	<i>P</i>									
1	124	0.12	0.17	244	0.33	***	85	0.11	0.31	122	0.13	0.15
5	187	0.13	0.07	292	0.32	***	142	0.05	0.55	147	0.05	0.51
10	227	0.19	0.003	327	0.28	***	179	0.03	0.65	171	0.04	0.57
20	284	0.23	***	399	0.27	***	225	0.07	0.27	217	0	1.0

Note: The expression of a miRNA is defined as the sum of the expression of the mature miRNA and miRNA*. Range: genomic DNA flanking the mature miRNA sequences, ranging from 1, 5, 10, and 20 kb at both ends, according to miRBase release 18 (November 2011). Pol II: total Pol II chip-seq reads within the indicated range of genomic DNA; mRNA: expression of mRNAs whose transcripts overlap with the indicated range of genomic DNA; *n*: number of the overlapping genes in the miRNA expression dataset and Pol II or mRNA dataset (we did not add additional zeros to genes not already in these datasets); ρ : Spearman rank correlation coefficient; *P*: *P* value calculated by permutation (Graphpad). As stated in the text, we also examined such correlation in human embryonic stem cells (hESCs), HepG2 and HEK293 cells. The datasets used for these cell lines are provided in Table S1 and Ref [92]. *** *P* < 0.0001.

presence, in a sense, supports the checkpoint or regulatory role of Drosha. Dicer also cleaves human pre-miRNAs with different efficiencies, although such relative specificity does not significantly correlate with miRNA expression (Zeng Y, unpublished data). These data suggest that Drosha action is more discriminatory and rate-limiting than Dicer action in setting differential miRNA expression *in vivo*.

How much Exp5 and Ago proteins contribute to the regulation of miRNA biogenesis is unclear, due to the lack of large-scale *in vitro* data. Human Ago2 prefers a 5' uridine residue in a miRNA [62], although how that transpires to miRNA expression is unknown. Abundance in Exp5 and Ago proteins may impinge on the expression of specific miRNAs, either directly through protein:RNA interactions, or indirectly by altering the expression of other proteins and miRNAs [96,97].

Summary: a microcosm of the RNA universe

Studies of the miRNA pathway have shed new light on how gene expression is regulated. At the same time, they also reinforce our notion of how cellular RNAs are produced and function. miRNAs undergo processing like any other eukaryotic RNAs do, and miRNA transcripts interact with proteins with specialized functions as well as proteins with more generalized functions in a cell. Moreover, the hundreds of miRNA genes in humans and the small number of proteins required to process these miRNAs provide us with an opportunity to investigate how the universal processing pathway can differentiate the expression of specific miRNAs. Thus, a general processing factor such as Drosha can regulate miRNA expression both globally and specifically [95], and this further illustrates an example to interrogate the regulation of other complex biochemical systems *in vivo*. Finally, as an added bonus, studying miRNA biogenesis enables us to design vectors to express small interfering RNAs to suppress gene expression in cells or *in vivo*, which greatly facilitates gene functional studies and disease modeling in suitable systems [98–100].

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Supplementary material

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.gpb.2012.06.004>.

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