Review

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Update on non-canonical microRNAs

Abstract: Non-canonical microRNAs are a recently-discovered subset of microRNAs. They structurally and functionally resemble canonical miRNAs, but were found to follow distinct maturation pathways, typically bypassing one or more steps of the classic canonical biogenesis pathway. Non-canonical miRNAs were found to have diverse origins, including introns, snoRNAs, endogenous shRNAs and tRNAs. Our knowledge about their functions remains relatively primitive; however, many interesting discoveries have taken place in the past few years. They have been found to take part in several cellular processes, such as immune response and stem cell proliferation. Adversely, their deregulation has pathologic effects on several different tissues, which strongly suggests an integral role for non-canonical miRNAs in disease pathogenesis. In this review, we discuss the recently-discovered functional characteristics of non-canonical miRNAs and illustrate their principal maturation pathways as well as debating their potential role in multiple cellular processes.

Keywords: development; gene regulation; microRNA; non-canonical; stem cell.

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Introduction

The appreciation of RNA molecules' complexity is constantly increasing. Non-coding RNAs are now known to include several subtypes besides ribosomal and transfer RNAs. Among those, microRNAs (miRNAs) are small endogenous species of ~22 nucleotides that contribute to the carefully orchestrated post-transcriptional regulation of gene expression in a wide variety of eukaryotes (1). Ever since their discovery in 1993 by the Ambrose laboratory (2), our knowledge about miRNAs has increased exponentially. They have been deemed a prominent topic in the research of gene regulation. Over the course of the past few years, many studies have shown that miRNAs have cellular functions that are both numerous and various. They have been found to contribute immensely to a wide variety of cellular processes, mainly through regulation of gene expression. They are thought to exhibit their functions by binding to partially complementary sequences on the 3′-untranslated region (3, 4) or less commonly within protein-coding sequences (5), assisted by the Argonaute protein (Ago) component of the RNA-induced silencing complex (RISC) (6), and subsequently by inhibiting protein translation or promoting destabilization of the paired mRNA (1, 7, 8).

By fine-tuning gene expression, miRNAs are considered integral contributors to physiologic processes, such as cellular proliferation, differentiation, apoptosis, innate and adaptive immune responses (9–11), metabolism (12–14), and development (15, 16) including cardiogenesis (17, 18), neurogenesis (19, 20) and hematopoiesis (21, 22). In contrast, dysregulation of miRNAs is associated with diverse pathological effects such as cardiovascular disease (18, 23), neurological disorders (24) and neurodegeneration (25), and several types of cancers (26–31).

Most miRNAs are known to be synthesized by a canonical pathway. They pass through two sequential maturation steps. First in the nucleus by an RNase III enzyme called Drosha, and then in the cytoplasm by another RNase III enzyme called Dicer (32–35). Deep sequencing technology has, however, revealed subtypes of small RNA molecules that structurally resemble typical miRNAs, but follow alternative biogenesis pathways that bypass certain components of the aforementioned process. These newly-discovered miRNAs were classified as non-canonical miRNAs (36–39). In this review, we attempt to shed some light on recent discoveries in the biogenesis and

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functions of non-canonical miRNA species, and to highlight key similarities and differences between the canonical and non-canonical miRNA molecules.

Structure and biogenesis

Canonical miRNAs must undergo several steps of processing before evolving into a functional miRNA complex (Figure 1). Initially, miRNA genes are transcribed into relatively long primary transcripts (pri-miRNA) (33, 40). The next step in miRNA biogenesis still occurs inside the nucleus. The RNA binding protein Dgcr8 then recognizes the pri-miRNA and directs the nuclear RNase III enzyme Drosha towards it. Drosha subsequently cleaves at the base of the hairpin embedded within the pri-miRNA (33, 41), yielding a ~70-nt hairpin molecule termed precursor miRNA (pre-miRNA), which bears the characteristic 2-nucleotide 3′ overhang of RNase III-mediated cleavage. Dgcr8 and Drosha are collectively termed the microprocessor complex (33, 40, 41).

Pre-miRNAs are then bound to a high-resolution nuclear export factor Exportin-5, which then escorts them to the cytoplasm via a Ran-GTP-dependent mechanism (42, 43). Once in the cytoplasm, Dicer removes the terminal loop of the pre-miRNA to release the mature double-stranded ~22-nt miRNA molecule (34, 35). One strand of this duplex RNA molecule is introduced into a larger protein complex called RISC, which contains Ago and plays an important role in the execution of miRNA-based silencing. The miRNA's function at this time is to guide the silencing complex to complementary sequences on the target mRNA (44). It now clearly appears that without the collaboration between Drosha, Dgcr8 and Dicer, cells cannot produce canonical miRNAs.

Non-canonical miRNAs biogenesis

Cells have the ability to utilize a wide spectrum of mechanisms to produce functional miRNAs. Deviations from the classic canonical pathway have raised much speculation. Deep sequencing technologies have revealed several different classes of RNA molecules that structurally and functionally resemble miRNAs. They do, however, bypass one or more steps in the canonical biogenesis pathway. These miRNAs are therefore termed non-canonical miRNAs. It is important to mention that Dicer is almost always indispensable in the production of both canonical and non-canonical miRNAs, and without it almost all functional miRNAs are lost. Drosha and Dgcr8 are only needed to process canonical miRNAs, while non-canonical miRNAs can be generated in their absence. In other words, the deletion of Drosha or Dgcr8 causes the complete loss of canonical miRNA, but preserves non-canonical miRNA biogenesis (35–39).

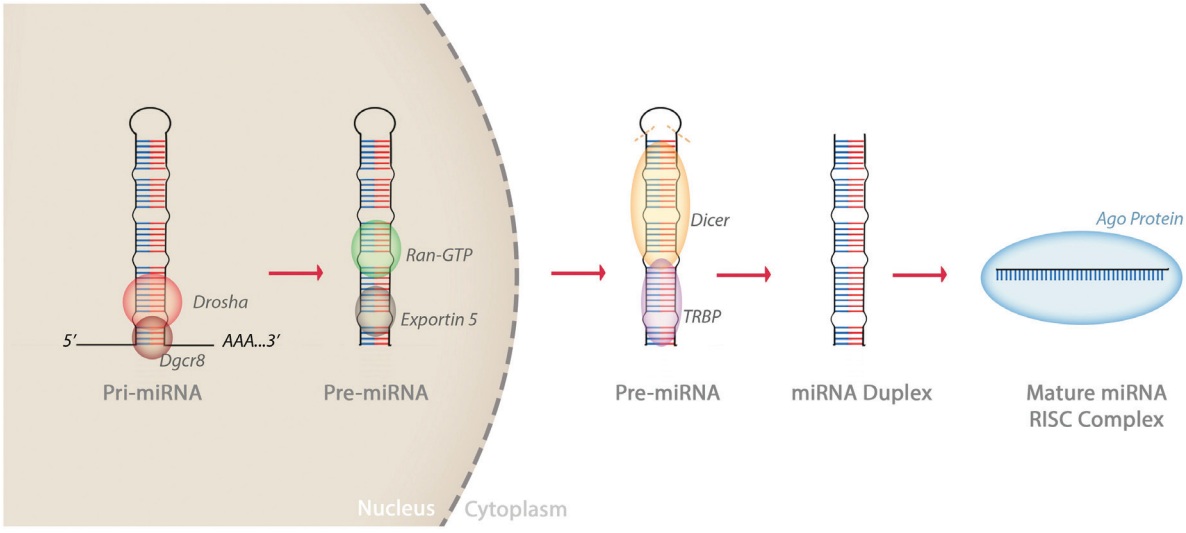

Figure 1 Canonical pathway of miRNA biogenesis.
After the production of primary miRNA transcript (pri-miRNA) by RNA polymerase II, pri-miRNA is cleaved by the microprocessor complex Drosha-Dgcr8 in the nucleus. The resulting hairpin (pre-miRNA) is then exported from the nucleus by the help of Exportin-5-Ran-GTP. In the cytoplasm, Dicer enzyme, in complex with the double stranded RNA-binding protein TRBP, cleaves the pre-miRNA into its mature length. Finally, the resulting mature structure is attached to Argonaute protein and incorporated into the RNA-induced silencing complex.

Drosha/Dgcr8-independent pathways

Mirtrons

The first non-canonical pathway to be discovered was the mirtron pathway. This parallel pathway utilizes Dicer in the cytoplasm, but does not need the Drosha/Dgcr8 complex in the nucleus to produce pre-miRNAs. Mirtrons were first discovered by examining the deep sequencing data of small RNAs from *Drosophila melanogaster* (38) and *Caenorhabditis elegans* (39), which revealed pre-miRNA-sized short introns. These introns are processed by spliceosomes and debranching enzymes in the nucleus to produce miRNA hairpins directly suitable for Dicer cleavage. This hairpin is then exported to the cytoplasm by Exportin-5 to be cleaved by Dicer. Thus, the mirtron pathway bypasses the microprocessor processing, or rather replaces it with splicing activity, and then merges with the canonical miRNA pathway at the Exportin-5-bound transport stage (38). After their discovery in flies and worms, pre-miRNA-sized hairpin introns that had mirtronic qualities were identified in humans and other mammals. Several candidate introns were later confirmed as mirtrons (45–47). Those mammalian mirtrons are thought to mature following the same pathway as their invertebrate counterparts. The mirtron-derived miRNA populations were found to persist in Drosha- or Dgcr8-deficient mice (45, 48, 49) as they did in *Drosophila* (50). Still, several dissimilarities were found between vertebrate and invertebrate mirtron species. Although vertebrates appear to generally have many more pre-miRNA-sized introns and mirtrons than flies and nematodes, their mirtron populations appear to exist in smaller ratios in comparison to flies and nematodes. Evidently, the relative ratios of pre-miRNA-sized introns in those species are flies > nematodes > mammals (46). Furthermore, in a typical mirtron-maturing fashion, both the 5′ and 3′ ends of the pre-miRNA are precisely defined by the spliceosome. Some exceptions to that have been discovered, however, where the RNA-generating hairpin resides towards one end of the intron (39) (Figure 2). An atypical locus, mir-1017, in *Drosophila* has been found to possess a 5′ hairpin terminus that coincides with the splice donor site, followed by substantial ~100 nt unstructured tail before the splice acceptor site (39). Mir-1017 was later found to exploit exosomes (the main eukaryotic 3′->5′ exonuclease complex) to trim it after splicing, in order to generate its Dicer substrate (51). Some vertebrate introns, in contrast, were found to have 3′ hairpin ends that coincide with splice acceptor sites but are preceded by unstructured tails following the splice donor site (45, 47). So far, 3′ tailed-mirtrons had only been found in *Drosophila*, while 5′ tailed mirtrons have only been described in vertebrates, suggesting that different animal clades adopt different methods of miRNA-yielding intron splicing (38, 39). In addition, mammalian mirtrons tend to have a higher GC content than invertebrate mirtrons, which helps to form more stable hairpins (46).

Those discrepancies underlying the structural features and biogenesis of mammalian and invertebrate mirtrons raise the possibility of distinct evolutionary origins. In fact, unlike canonical miRNAs, no mirtron was ever found to be shared between mammals, flies and worms. Although many questions remain unanswered, mirtrons have undoubtedly proven to be a rich, novel area of study. The discovery of hundreds of new mirtrons suggests that, collectively, these molecules play a significant role in gene silencing. The elucidation of their pathway has paved the way to uncovering additional non-canonical pathways.

Small nucleolar RNA-derived miRNAs

Small nucleolar RNAs (snoRNAs) are a class of noncoding RNAs found abundantly in the nucleolus of many organisms. They have been found to bind with Ago, in many cases interfering with the gene expression process at the levels of mRNA stabilization and translation. They are now theorized to be rRNA, tRNA and snRNA modifiers, as well as processors of precursor rRNA (52).

snoRNAs are classified according to the presence of sub-motifs into C/D box or H/ACA box classes (Figure 3). The C/D box snoRNAs are in charge of 2′-*O*-methylations, while the H/ACA box snoRNAs are responsible for pseudouridylation of their respective rRNA targets (52). There are also many snoRNA molecules that lack any apparent rRNA or snRNA targets, thus potentially following distinct pathways and possessing discrete functions. These snoRNAs have been termed ‘orphan snoRNAs’ (53, 54).

Analysis of deep sequencing data of selected snoRNAs associated with Ago 1 and 2 identified snoRNAs as another source of miRNA that may follow the canonical pathway in some cases (55–58). However, a particular human snoRNA (ACA45) was found to be independent of Drosha/Dgcr8 processing, only requiring Dicer. It has a precursor structure that resembles two pre-miRNA-like hairpins linked by a hinge. A potential mRNA target for ACA45 was identified using bioinformatic prediction algorithms and

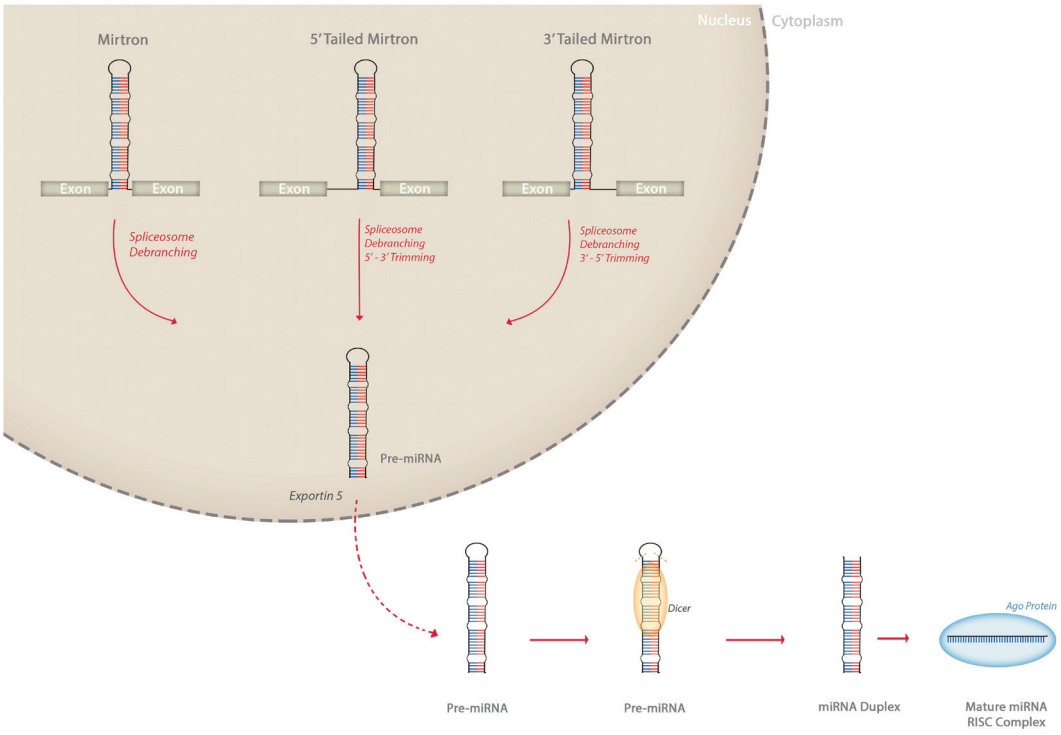

Figure 2 Mirtron pathway.
The 5′ and 3′ ends of pre-miRNA are usually defined by spliceosomes, unless the miRNA-generating hairpin resides towards one end of the intron. The resulting pre-miRNA hairpin is subsequently exported out of the nucleus to the cytoplasm, where it is processed by Dicer to generate the mature miRNA that complexes with Argonaute protein.

luciferase reporter assays, suggesting a role in post-transcriptional gene silencing similar to other miRNAs (56). Similarly, another study characterized *Giardia lamblia*'s GlsR17 snoRNA as a source of a Dicer-dependent, Drosha/Dgcr8-independent miRNA that takes part in translational repression (57).

These studies have broadened our knowledge about the biological roles of snoRNA and supplied evidence that this family of small RNAs is a source of functional non-canonical miRNAs that contribute to RNA silencing. Despite this, our understanding of snoRNAs' contribution to miRNA generation remains primitive.

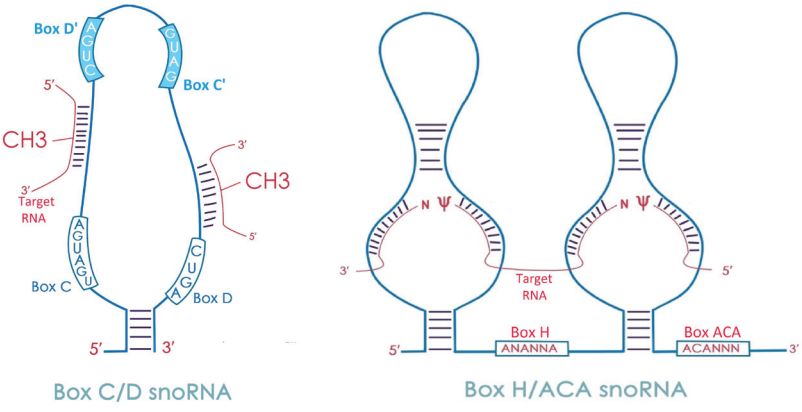

Figure 3 Anatomical features of C/D and H/ACA snoRNAs.
(A) The secondary structure of the C/D snoRNA box entails conserved sequences (box C and box D) that base pair with target RNA. (B) H/ACA snoRNAs are formed of two hairpin units separated by a single-stranded hinge that contains box H. Each hairpin structure is formed of a lower stem, pseudouridylation pocket, upper stem and an apical loop. Target RNA is contained within the pseudouridylation pocket.

miRNAs from endogenous short hairpin RNAs

Several siRNAs were found to follow the non-canonical miRNA processing pathway. This was elucidated after analyzing non-mirtronic genomic loci to find any sign of microprocessor independence. The analysis gave rise to Dicer-dependent, Dgcr8-independent reads for *mir-320* and *mir-484*, whose transcripts have the capability to form hairpin structures; thus they are characterized now as endogenous short hairpin RNAs (44, 45).

Further inspection of these genes showed that they differ structurally from canonical miRNA in the sequences flanking the pre-miRNA hairpin. In canonical miRNA, positions flanking their pre-miRNA hairpin are highly conserved to maintain its ability to be recognized properly by the microprocessor complex (59). However, those short hairpin RNA (shRNA)-derived miRNAs lack that microprocessor-binding sequence, which further demonstrates its Dgcr8 independence. The most prolific shRNA-derived miRNA loci, *mir-320* and *mir-484*, were found to be highly expressed in embryonic stem cells (ESCs) suggesting their role in the stem cells, which will be discussed further on in the article. The *mir-320* locus seems to have a characteristic structural feature; the vast majority of the small RNAs from its locus mapped towards the 3′ end of the hairpin, while its 5′ species were strongly under-represented. RACE amplification of the 5′ end of the hairpin detected a processed end corresponding to the 5′ end. This feature was also highlighted in the mir-484 locus (45). This common complexity in the precursor structure of these shRNA-derived miRNAs might indicate the presence of an unknown processing step that turns the precursor into a Dicer-suitable substrate (45).

Another interesting endogenous short hairpin RNA locus corresponding to the *isoleucine tRNA* gene was shown to produce a long 110-nt hairpin that acts as an alternative secondary structure for a Dgcr8-independent, dicer-dependent miRNA, and again, the majority of the abundant, Dicer-dependent small RNAs mapped to the 3′ end of this extended hairpin. Interestingly, data also showed that this gene retained its ability to generate mature tRNA, suggesting an unknown mechanism by which it is differentially processed into either the cloverleaf tRNA structure or the hairpin precursor structure (45). In contrast, *mir-1980*, another shRNA locus, has more reads generated from its 5′ end, in contrast to the previously mentioned examples (45).

In retrospect, these data and analysis report a genome-wide analysis of small RNA molecules in the hope of finding distinctions between small RNA biogenesis pathways. In turn, studies have discovered and identified genomically-clustered endogenous siRNAs, and have also classified a group of endogenous shRNAs. This categorization has also allowed the differentiation of canonical miRNAs from shRNA-derived miRNAs as well as mirtron-derived miRNAs, further expanding the scope of small RNA research.

This diversity in the structural and functional features of shRNA-derived miRNA probably underlies unperceived pivotal processing and regulatory steps. In addition, further examination of the shRNA-derived miRNA population will likely require them to be sub-classified into distinct groups to cope with the growing functional and structural divergence as the database grows. This has already been manifested as regards to tRNA-derived miRNA, as the discovery of the isoleucine tRNA-derived miRNA prompted more in-depth studies of tRNA-derived RNA fragments.

miRNAs from tRNAs

Not long after the discovery of the isoleucine tRNA-derived miRNA, several studies followed reporting the discoveries of more tRNA-derived RNA species. Those species are believed to be byproducts of the tRNA maturation pathway. They differ from classical miRNA but still take part in RNA silencing. The discovery of tRNA-derived RNA fragments set a precedent that tRNA-processing enzymes (such as tRNase Z) can release functional miRNA-like species, as was later identified (Figure 4).

In the first of a series of studies, RNAs derived from tRNA were reported in HIV-infected cells. RNA fragments corresponding to a portion of the host cell's tRNA-Lys3 (known to bind to a site in the genomic HIV to serve as a primer for reverse transcriptase) were detected. This ~20-nt RNA was found to bind to Ago2 proteins. It was also found to be expressed in proportion to HIV expression level. The study reported that the identified RNA fragment has the ability to silence a luciferase reporter designed to be a target. A duplex of that RNA fragment was also found to be a substrate for Dicer cleavage *in vitro* (60).

Only months later, another team analyzing deep sequencing data of human prostatic carcinoma cells reported finding 17 RNA fragments corresponding to tRNA sequences. The discovered fragments were 18–22 nt in length and were found to be derived from either the 3′ and 5′ ends of mature tRNAs, or from the 3′ trailer region of precursor tRNAs. One of the RNAs (tRF-1001) discovered was found to correspond to the 3′ trailer of the pre-tRNA-Ser-TGA. It was also found to be highly expressed in various

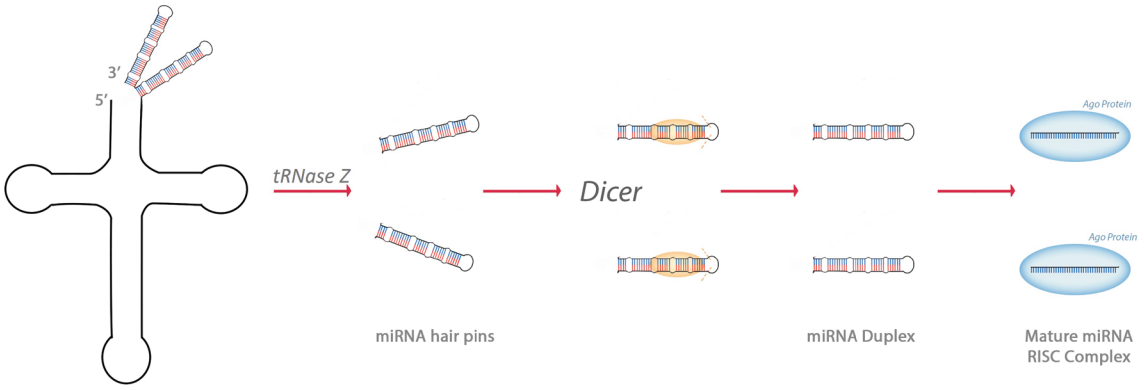

Figure 4 tRNase Z-dependent miRNA.
These species of miRNA are believed to be byproducts of the tRNA maturation pathway. The generated pre-miRNAs are then processed by Dicer into mature miRNAs.

organs. Its expression rate was correlated with the cell's proliferation rate. Knocking down tRF-1001 was interestingly found to slow proliferation rate (61).

Another study showed that miRNAs and small RNA fragments derived from tRNAs were the major sources of small RNAs in the immortal HeLa cells (62). The four most abundant tRNAs in HeLa cells (tRNA-Lys, tRNA-Val, tRNA-Gln and tRNA-Arg) were found to be cleaved at their 5′ ends by Dicer to produce abundant ~19-nt-long RNA fragments. The study went on to identify certain important features of the tRNA-Gln-derived RNA fragments that separated them from miRNAs (62). Although those RNA fragments were generated by Dicer cleavage and bound to Ago1 and Ago2 to some extent, they were found to be more sensitive to Dicer knockdown than other miRNAs. In addition, their association with Ago was less stable than conventional miRNA-Ago complexes. Moreover, these tRNA-derived RNAs had blocked 2′-OH ends (62).

In all of these respects, tRNA-derived RNAs differed from typical mammalian miRNAs. Nonetheless, their discovery in human cells immediately provoked theories on their biological function. It has not yet been fully elucidated whether they take part in RNA silencing or not. If not, then the question still remains: why do tRNAs act as substrates for Dicer in HeLa cells? Their abundance as RNA species in HeLa cells and their competition with pre-miRNA for Dicer might suggest a role for them in the regulation of miRNA generation rather than exhibiting silencing functions themselves. Or, adversely, they could be responsible for dysregulation of miRNA levels and Dicer activity in HeLa cells. With all these queries unanswered, the overall picture remains incompletely understood.

Another study in the tRNA-based wave of research reported finding two types of Ago-associated tRNA-derived RNA fragments in the adenovirus-transformed human embryonic kidney 293 cell line. The first type was Dicer-dependent while the second type was interestingly generated by RNase Z cleavage downstream from a tRNA gene. The modulation of the level of those RNA fragments was found to have an effect on the abundance and silencing activities of miRNAs (63).

These revelations were the cornerstone for other discoveries involving tRNase Z-derived miRNAs. An alternative non-canonical pathway was found in murine gamma herpes virus 68. MHV68 was found to posses several different miRNAs. Each of those miRNAs was derived from one arm of a pre-miRNA hairpin that was originally linked to the tRNA molecule in the pri-miRNA stage (64, 65). Intriguingly, that pri-miRNA was processed in the nucleus by the tRNA-processing enzyme 'tRNase Z' instead of the Drosha/Dgcr8 complex used in the canonical pathway (Figure 4). The tRNase Z defines the 5′ end of the MHV68 hairpins, while the mechanism by which the 3′ end is defined has not been elucidated, but it may involve an endonuclease enzyme that divides the tandem hairpins. These newly-generated pre-miRNAs are then processed by Dicer into mature miRNAs. This exposes a novel Drosha-independent, tRNase Z and Dicer-dependent pathway to generate mature functional miRNAs (64–66).

Dicer-independent pathways

As opposed to all of the aforementioned pathways, a few astonishing Dicer-independent pathways have been revealed. Unlike all the non-canonical subclasses discussed above, miR-451 in human, mouse and zebrafish

(37, 67, 68) was shown to mature via an unprecedented microprocessor-dependent, Dicer-independent pathway. Pri-miR-451 is initially cleaved in the nucleus by Drosha/Dgcr8 to generate a very short pre-miRNA (~18 bp), too short to act as a substrate for Dicer. It is then either loaded directly onto a non-slicing Ago1, or cleaved by an unknown endonuclease at the 3′ end and then loaded on an Ago2, to subsequently join its designated RISC complex. Furthermore, although miR-451 was generated in the absence of Dicer, its generation was significantly reduced with the functional loss of Ago2 (67). These observations suggest that Ago2 regulates Dicer-independent miR-451 in the post-transcriptional stage by affecting its processing ability.

Interestingly, several other miRNAs have been produced in the absence of Dicer, but unlike miR-451, have shown to be independent of Dgcr8, Dicer, Exportin-5 or Ago2 in their biogenesis (69). As mentioned earlier, mirtrons are processed by spliceosomes and debranching enzymes to generate suitable Dicer substrates, bypassing the microprocessor step. Remarkably, in another study on human mirtrons, miR-1225 and miR-1228 were found to be splicing-independent mirtron-like miRNAs or 'simtrons' (69). Simtron biogenesis seemed to be unaffected by the deletion of Dgcr8, Dicer, Exportin-5, or Ago. Their biogenesis was reduced only after knockout of Drosha. Nonetheless, both simtrons and mirtrons are involved in the gene-silencing mechanisms of target transcripts and are found in the RISC complex, in which they interact with Ago. Collectively, this information reveals simtrons to be a distinct Drosha-dependent pre-miRNA class that has the ability to produce functional miRNAs (69).

The recent study of simtrons and other Dicer-independent miRNAs has revealed new, non-canonical pathways that were not only unknown before, but are also integral to our understanding of miRNA biogenesis independent of Dicer. Collectively, the results show that Dicer, which is commonly known to be pivotal in the canonical as well as most non-canonical pathways, is not needed for some miRNAs that have yet to be analyzed.

Functions

The miRNAs were found to contribute immensely to a wide variety of cellular processes. As we discussed in the introduction, the functions of canonical miRNAs are diverse and well established. However, the functions of non-canonical miRNAs in particular still seem unclear. Many of their roles in cellular processes and disease pathogenesis have yet to be identified. The increasing interest in non-canonical miRNAs has led to several efforts to determine their function. To achieve that, numerous studies have examined cells with conditional knockout of Drosha, Dgcr8, Ago and Dicer, anticipating phenotypic differences between the resulting cells. These studies have demonstrated that non-canonical miRNA loss results in major changes to cellular phenotype. However, this hypothesis has not been consistently supported. In some comparisons, conditional knockout of Drosha and Dicer generated seemingly indistinguishable phenotypes. This was the case with T cells lacking Drosha or Dicer. Both knockouts seemed to produce fatality due to inflammatory disease within a short period of time (70). Knockout of either enzyme (Drosha or Dicer) in developing skin produced similar disease as well: defects in the hair follicle, rough flaky skin, and early lethality after birth (49).

As comparative studies have grown in number, certain cell lines have shown phenotypic differences after manipulation of their miRNA population by deleting Drosha or Dgcr8 versus their apparent phenotype after Dicer deletion (71–79). This suggests underlying roles of non-canonical miRNAs in the phenotype and/or function in those selected cell lines.

Pathological effects of dysregulation of non-canonical miRNAs

Attempts have been made to identify the roles of non-canonical miRNAs in physiologic processes or development of pathological states. Strong evidence has linked dysregulation of non-canonical miRNAs with various diseases. Emerging studies have associated certain snoRNA-derived and tRNA-derived types of non-canonical miRNAs to psoriatic skin disease. These miRNAs are differentially expressed in psoriatic versus normal skin, which suggests that they function as regulators of gene expression in skin and potentially have a role in psoriasis pathogenesis (71).

In another experiment, miR-320 was also found to be dysregulated in ischemic hearts *in vivo* and *ex vivo*, associating miR-320 with ischemic/reperfusion injury in the murine heart. Furthermore, knockdown of endogenous miR-320 expression by anti-miR-320 led to reduction in *in vivo* cardiac infarction size (72). The very same miR-320 was also found to be one of a group of miRNAs that were highly expressed in the pancreatic islets of Langerhans cells that were unresponsive to glucose. Its expression was 50-fold higher in insulin-resistant adipocytes, suggesting a role in diabetes pathogenesis and regulation of insulin resistance (73).

In a different experiment of the kind, miR-320 was hypothesized to possess a role in the tumorigenesis of mammary epithelial malignancies. It was found to be down-regulated in Pten-deleted stromal fibroblasts, acting as a component of the Pten tumor suppressor axis which contributes to the reprogramming of the tumor stromal microenvironment. This provides epithelial tumors with an environment conducive to tumor progression via an unclear mechanism (74).

Non-canonical miRNAs in neuronal tissue

Another recent study has detected elevated expression levels of certain subclasses of miRNAs in the adult mouse brain in relative to other tissues. Utilizing deep sequencing technology of small RNAs from the hippocampus and cortex of conditional Dgcr8-null, Dicer-null and control mice, the study concluded that deletion of either Dgcr8 or Dicer in post-mitotic neurons resulted in a lethal phenotype (75). Nevertheless, the Dicer-null mice consistently showed earlier lethality than the Dgcr8-null mice did. Dicer-knockout mice's brains showed an increase in the number of apoptotic cells, with cortical thinning, enlarged ventricles and smaller hippocampi, reminiscent of changes seen in the brains of humans with schizophrenia and autism (76, 77), supporting a possible role for non-canonical miRNA deregulation in the pathogenesis of these neurologic diseases.

Another observation made by the latter study was that the majority of the Dgcr8-independent and Dicer-dependent miRNA population in post-mitotic neurons was found to be predominantly derived from mirtrons and snoRNAs. Such miRNAs are expressed in significantly lower levels in ESCs (45), whose non-canonical miRNA populations are mainly derivatives of endogenous shRNAs (miR-320 and miR-484). The mechanism by which different cells choose to deploy their preferred miRNA type is unknown, but it may underlie the significant phenotypic differences between each cell type.

Non-canonical miRNA involvement in immune response

Similar to their contribution to all the aforementioned cellular processes, miRNAs were also found to participate in the regulation of immune response. Pioneering endeavors to elucidate miRNAs' effect on the immune system have focused on the examination of Dicer-deleted T-cells, which proved to be defective in development and function (78–80). Later on, studies started to focus on identifying the role of specific miRNAs in the maturation, function and regulation of the immune system, such as miR-146 (81), miR-223 (82), miR-150 (83) and many others (84–87).

Most of the miRNAs important in immunity seemed to follow the canonical maturation pathway. However, a recent study also suggested that non-canonical miRNAs may be involved in the basal immune response. Certain Drosha/Dgcr8-independent miRNAs were found to be differentially expressed in MHV68-infected murine cells. In that study, the authors prepared and sequenced small-RNA libraries of cells infected with MHV68 versus mock-treated cells. They searched for canonical, non-canonical miRNAs and endo-siRNAs in MHV68 and mouse genomes. They identified a total of 30 novel miRNAs, which included two antisense miRNAs in the MHV68 genome, as well as three canonical and 25 non-canonical miRNAs derived from tRNAs, snoRNAs and introns of the mouse genome. These up-regulated viral and murine miRNAs were thought to have several target genes involved in gene transcription and protein phosphorylation. They were also localized to the cellular membrane, where the majority of signaling receptors function. The viral miRNAs appeared to target many transcripts in the virus and/or host. Twenty-six open reading frames in the viral genome were identified as putative targets for the up-regulated viral miRNAs. Meanwhile, in the host, 4653 protein-coding genes in the mouse were identified to be potential targets for the viral miRNAs, reflecting the large number of host genes that could be manipulated by the infecting virus's miRNAs (88). Interestingly, eight of those target genes were previously found to be down-regulated in the MHV68-infected cells (89).

Among the up-regulated mouse miRNAs, one particular non-canonical miRNA in the mouse called miR-142-3p was most prominently up-regulated (35 folds) in the infected cells in comparison to the mock-treated ones. This miR-142-3p was found to have a total of 240 mRNA putative gene targets in the mouse genome, while it had no open reading frame targets in the viral genome. This could indicate a method that the virus developed to evade the host's immune counterattack.

This overall pattern suggests a potential contribution of those differentially up-regulated viral and viral-response miRNAs to the manipulation of the host cell's immune response during a lytic infection. In particular, the significant differential expression of murine miR-142-3p

during the MHV68 lytic infection makes its targets subjects of particular interest for further investigation (88).

Non-canonical miRNA role in stem cells

Another clearly identified function of miRNAs is their participation in regulating stem cell differentiation. Several miRNAs are specifically expressed in ESCs and contribute to maintaining a pluripotent state (90, 91). This feature was highlighted in Dicer-deficient mice, in which the number of stem cells within the blastocyst cell mass was markedly reduced (92), as well as in Dicer-mutant *Drosophila*, whose germline stem cells showed a reduced capacity to pass through the G1/S transition phase in the cell cycle (93).

miRNAs were found to play an active role in the reprogramming of somatic cells into induced pluripotent stem cells (90, 91, 93). Several miRNAs were found to be highly expressed in mouse ESCs and their rapid proliferation was enabled by expediting the G1/S transition of the ESC cycle (94). Introducing those miRNAs into mouse embryonic fibroblasts (MEFs) together with the three transcription factors Klf4, Oct4 and Sox2 facilitated dedifferentiation of fibroblasts into induced pluripotent stem cell colonies (95). Conversely, suppression of miRNA processing was found to have a profound effect on reprogramming efficiency (96).

Finally, several studies have shown that miRNAs alone can be used to efficiently reprogram mouse and human somatic cells to pluripotency even in the absence of exogenous transcription factors (97, 98). In fact, miRNAs also proved indispensable for reprogramming MEFs into induced stem cell-like cells. In a recent study, Dicer-null MEFs failed to dedifferentiate, even after overexpressing a combination of transcription factors (Oct4, Sox2, Klf4, cMyc, and Lin28). Furthermore, dedifferentiation capacity was restored when the human Dicer homologue was introduced into Dicer-null MEFs before the dedifferentiation step, suggesting the essential role of miRNA in dedifferentiation reprogramming (99).

Although the stem cell studies mentioned above demonstrate the importance of miRNAs in general, none of them show that non-canonical miRNAs specifically have a function in stem cells. Interestingly, a recent study demonstrated the role of non-canonical miRNAs in stem cell function for the first time (100). Two non-canonical miRNAs (miR-320 and miR-702) were identified that promote proliferation in Dgcr8-deficient ESCs. They were found to target the cell cycle inhibitors p57 and p21, respectively, and thus release them from G1 arrest. miR-320 and miR-702 were initially found to be significantly expressed in Dgcr8-deficient ESCs, although they were absent in Dicer-null ESCs. Their potential role in stem cell proliferation was suggested given that the proliferation defect of Dicer-null ESCs is much worse than that of Dgcr8-null ESCs. This hypothesis was further supported when introducing them to the slowly-proliferating Dicer-deficient ESCs (lacking both canonical and non-canonical miRNAs) enhanced the proliferation of these cells by down-regulating p21 and p57 and transitioning from the G1 to the S phase. Furthermore, a set of reverse experiments were carried out by introducing inhibitors of miR-320 and miR-702 into Dgcr8-deficient ESCs. These cells showed higher p21 and p57 expression levels and a slower proliferation rate than those without the aforementioned anti-miRNAs. These experiments provided solid evidence of the non-canonical miRNAs' role in regulating ESC proliferation (100).

Future directions

Presently, relatively little is known about non-canonical miRNAs. Deep sequencing technology has enabled us to learn much more about miRNAs in general and specifically, the mechanisms and functions of non-canonical miRNA. Separately-conducted studies have demonstrated that the same miRNA might play different roles in different cell types. For example, miR-320 has been implicated in mammalian neuronal development, stem cell proliferation, several metabolic functions, and pathologic associations, as discussed above. This eclectic group of studies underscores the importance of cellular context that likely influences the role of any miRNA. Furthermore, they demonstrate that a miRNA has the ability to function in a diverse manner, given that each miRNA is known to target multiple mRNAs. Despite this, still very little is known about the mechanisms of regulating non-canonical miRNA expression, transport from the nucleus to the cytoplasm, activation, and degradation. These issues need to be addressed in order to better understand the roles of non-canonical miRNAs in various cellular contexts, as well as to identify ways to manipulate them for clinical use in humans, either as diagnostic markers or therapeutic agents.

In this review, we have highlighted the principal non-canonical pathways that have been discovered in the past decade. Given the mounting evidence that miRNAs play a crucial role in regulating gene expression, it is more important than ever to classify the thousands of miRNAs

already discovered and identify their distinct functions in gene regulation. Furthermore, the diversity of non-canonical miRNA types and the likely existence of multiple mRNA targets for each miRNA portend many functions yet to be discovered.

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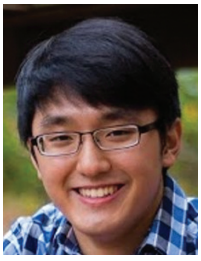

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