REVIEW

One locus, several functional RNAs—emerging roles of the mechanisms responsible for the sequence variability of microRNAs

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Abstract

With the development of modern molecular genetics, the original "one gene-one enzyme" hypothesis has been outdated. For protein coding genes, the discovery of alternative splicing and RNA editing provided the biochemical background for the RNA repertoire of a single locus, which also serves as an important pillar for the enormous protein variability of the genomes. Non-protein coding RNA genes were also revealed to produce several RNA species with distinct functions. The loci of microRNAs (miRNAs), encoding for small endogenous regulatory RNAs, were also found to produce a population of small RNAs, rather than a single defined product. This review aims to present the mechanisms contributing to the astonishing variability of miRNAs revealed by the new sequencing technologies. One important source is the careful balance of arm selection, producing sequentially different 5p- or 3p-miRNAs from the same pre-miRNA, thereby broadening the number of regulated target RNAs and the phenotypic response. In addition, the formation of 5', 3' and polymorphic isomiRs, with variable end and internal sequences also leads to a higher number of targeted sequences, and increases the regulatory output. These miRNA maturation processes, together with other known mechanisms such as RNA editing, further increase the potential outcome of this small RNA pathway. By discussing the subtle mechanisms behind the sequence diversity of miRNAs, this review intends to reveal this engaging aspect of the inherited "RNA world", how it contributes to the almost infinite molecular variability among living organisms, and how this variability can be exploited to treat human diseases.

Introduction

One of the fundamental ideas that revolutionized biological science and was also considered to lay down the bases of molecular biology was the famous "one gene—one enzyme" hypothesis formulated by George Beadle and Edward Tatum in the beginning of the 1940s (Beadle and Tatum 1941). They proposed that enzymes carry out the metabolic functions in the cells, and all enzymes are determined by unique DNA segments called genes. With the advancement of biochemistry and genetics, and after the formulation of the "central dogma" of molecular biology (Crick 1958), the

hypothesis was refined as the "one gene—one polypeptide" statement; yet, it still turned out to be a simplified version of genetic complexity. Soon it was revealed that at the RNA level, the "message" is often a combinatorial output of the DNA sequence of a particular gene. The discovery of splicing and alternative splicing provided the first evidence that several mRNA isoforms can be generated from a single gene, potentially coding for several protein species (Berget et al. 1977; Chow et al. 1977; Baralle and Giudice 2017; Shenasa and Hertel 2019). Another important discovery was the phenomenon of RNA editing, showing that the nucleotide sequence of the transcribed mRNA can be functionally modified, which also results in the alteration of the encoded polypeptide sequence (Benne et al. 1986; Powell et al. 1987). Since these original observations, follow-up investigations revealed several other RNA modification pathways, further supporting the view that gene activity indeed produces a complex pool of transcripts, rather than a single RNA with

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a single determined function (reviewed in (Li and Mason 2014)).

The next important breakthrough in the RNA field was the discovery of small regulatory RNAs, later identified as microRNAs (miRNAs) (Lee et al. 1993; Wightman et al. 1993). Since their initial description, miRNAs were found to be important posttranscriptional molecular modulators, and apart from fungi, they represent an intracellular regulatory network of the RNA interference (RNAi) machinery in all other organisms examined so far (Lee et al. 2004; Ameres and Zamore 2013). Considering their transcription and maturation, the original assumption again was that one miRNA gene produces a single small RNA product of a discrete length. However, especially with the unforeseen developmental pace of next generation sequencing (NGS) technologies, more and more experimental data suggested that this is not the case and the miRNA repertoire is far more complex than previously thought. Once again in the history of RNA biochemistry, the "one gene—one RNA product" hypothesis needed to be revised by the data showing that the activity of miRNA loci produce numerous small RNAs, often a large population of isomiRs (Gebert and MacRae 2019; Trabucchi and Mategot 2019; Bofill-De Ros et al. 2020). In this review I attempt to present the molecular mechanisms behind this complexity: I will address the flexibility of the once underestimated pre-miRNA arm selection regulation, as well as the mechanisms resulting in 5' and 3' heterogeneity of mature miRNAs, producing numerous 5' and 3' isomiRs from a single miRNA gene. I will also discuss the functional consequences and the medical relevance of these carefully regulated miRNA maturation processes, and how the better understanding of these could contribute to the solution of current public health problems. The aim is to give an appreciative view on the unprecedented sequence diversity of small RNA species produced from a single miRNA gene, and through that, to demonstrate the complexity that is present in the existing "RNA world" of today.

The life of miRNAs—maturation and a "wobbling" mechanism of target recognition

miRNAs are the key actors in one of the RNA interference pathways, and have been evolved in parallel with the short interfering RNAs (siRNAs) and the Piwi-associated small RNAs (piRNAs) (Ghildiyal and Zamore 2009). The latter two represent two pathways that have still kept the common ancient function of protecting the organisms from invasive genetic elements such as viruses and transposons. siRNAs are the main defense guards against outside invaders, namely viruses, and represent a "cellular immune system" which is the major security line in organisms that lack an adaptive immune system (such as lower invertebrates or most plants) (Rosendo Machado et al. 2021; Kong et al. 2022). piRNAs, however, are found exclusively in the animal kingdom, and control the "resident aliens" of the genomes, eliminating endogenous viruses and transposons, especially in the germline (Onishi et al. 2021). Although recent studies revealed various functions of piRNAs in somatic cells, they are still predominantly considered as guardians of the germline, protecting stem cells and especially the gametes (Peng and Lin 2013; Wang et al. 2022). miRNAs, on the other hand, have evolved for a different functionality: they represent an elaborate network of posttranscriptional regulators, controlling endogenous mRNAs and thereby fine-tuning cellular gene expression patterns (Gebert and MacRae 2019). They are present in most eukaryotic groups of the tree of life, having a common origin in plants and animals, and current molecular evidence suggest that the loss of this pathway in some species (such as in fungi) rather represents a secondary loss during evolution (Moran et al. 2017).

microRNAs are short, ~20–24 nucleotide long, single-stranded RNA molecules encoded by the genome, and they may be relatively numerous in a given organism: in the human genome, for instance, approximately 2000 miRNAs have already been described, although some still require further validation (http://mirbase.org/). miRNAs can be regulated by their own promoter or they can be located in introns (or even in exons) of other, predominantly protein coding genes (Bartel 2018). Interestingly, in mammalian genomes they often form clusters where several miRNAs can be transcribed simultaneously (Altuvia et al. 2005; Kim and Kim 2007; Chan et al. 2012; Ramalingam et al. 2014; Kabekkodu et al. 2018). After transcription of the primary-miRNAs (pri-miRNA), their maturation requires two subsequent endonucleolytic cleavage steps (Fig. 1A). In animal cells, the first one occurs in the nucleus by the "Microprocessor" complex, containing the RNase III type enzyme Drosha, and its partner protein DGCR8 ('DiGeorge Syndrome Critical Region 8'). It generates a precursor-miRNA (pre-miRNA) with a hairpin-like structure that is transported to the cytoplasm where the second cleavage takes place by another endonuclease called Dicer. The so formed, usually mismatch containing short double-stranded RNAs are incorporated into an Argonaute (Ago) protein containing, RNA-induced silencing complex (RISC), where further processing eliminates one strand (the "passenger" strand), resulting in a single stranded miRNA-containing functional RISC (Iwakawa and Tomari 2022). The effector function of these ribonucleoprotein complexes is then elicited by the Watson–Crick base pairing complementarity between the miRNA and the target RNA, initiating RNA decay or translation inhibition (Huntzinger and Izaurralde 2011). However, in contrast to siRNAs and piRNAs, often only a small portion of the miRNA has complementarity to the target RNA. In most cases, the 2–8
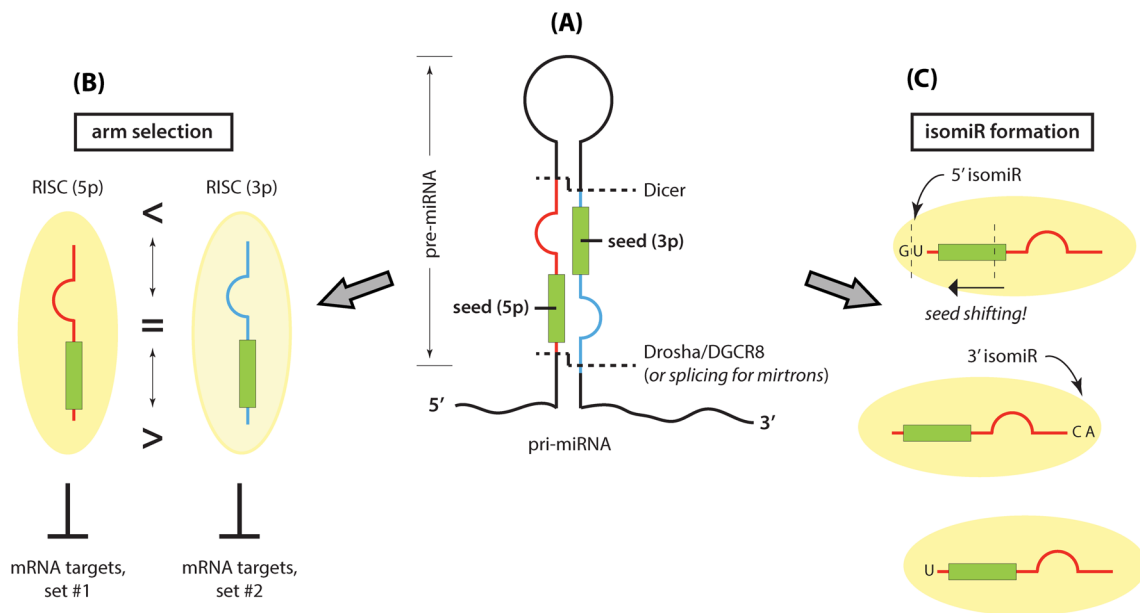
Fig. 1 MicroRNA maturation processes **A** Structure and processing of the pre-miRNA formed on the primary transcript (pri-miRNA). The staggered lines indicate the two consecutive endonucleolytic cleavages: the first is performed by the Drosha/DGCR8 (Microprocessor) complex in the nucleus or substituted by the spliceosome for the Drosha-independent mirtrons. The second cleavage occurs in the cytoplasm by Dicer, the other RNase III type enzyme involved in the maturation. Red and blue lines indicate the 5p or 3p arms, green rectangles depicting the seed sequences. **B** If they are functionally selected, the different arms are incorporated into distinct RNA-induced Silencing Complexes (RISCs). The ratio of such complexes may vary in cell types, or in different developmental or physiological states, and they target different sets of RNA molecules, although individual targets may overlap. **C** RISCs formed from a given miRNA locus may differ in their isomiR contents. As examples, distinct 5' and 3' isomiR-containing RISCs are shown above the one with the "canonical" or "reference" miRNA-containing complex. Note that for 5' isomiRs, a difference in target selection may occur due to "seed shifting" (depicted by an arrow)

nucleotides of the 5' sequence, the so-called "seed region" provides the base-pairing interactions but several validated examples have been shown where varying portions from the 3' end of the miRNA also contribute to target recognition which makes the mere bioinformatic target prediction a very difficult task (Berezikov 2011; Pasquinelli 2012). The lack of a long and strict sequence constraint also explains why one miRNA can often bind to several target mRNA molecules (in animals, usually in the 3' untranslated regions, 3' UTRs), and why a given mRNA may contain multiple miRNA-binding sites. The result of this is that similarly to the regulation by transcription factors, miRNAs and their targets represent a complex network of interactions, resulting in a post-transcriptional fine-tuning of gene expression (Arora et al. 2013; Gebert and MacRae 2019; Trabucchi and Mategot 2019).

For many years, it was believed that miRNAs are produced exclusively by the above outlined, well-defined "canonical" biogenesis pathway, with occasional differences only in certain organisms. However, later scientific data challenged this paradigm by the discovery of the Drosha- and Dicer-independent alternative maturation pathways (Winter et al. 2009; Miyoshi et al. 2010). The most prominent of these is certainly the mirtron pathway, with the spliceosome substituting for the Drosha/DGCR8 complex (Fig. 1A) (Berezikov et al. 2007; Okamura et al. 2007; Ruby et al. 2007). Although it was first considered to be a speciality of invertebrate genomes with a short average length of introns, later studies confirmed the existence of mirtrons also in higher organisms, including mammals (Havens et al. 2012; Schamberger et al. 2012; Sibley et al. 2012). The emergence of alternative pathways increased the potential sources of miRNA precursors; however, it soon turned out that the diversity of miRNA sequences is further increased by previously underestimated mechanisms acting not only on the precursors but also on the mature miRNA species.

miRNA arm selection—the "guide" or the "passenger"?

After cleaving the terminal loop of pre-miRNA by Dicer, an important step is the selection of the appropriate strand (referred to as "arm") of the short double-stranded RNA which will be acting as a guide for the Ago protein in the RISC (Fig. 1A, B). A similar process occurs during siRNA generation (also catalyzed by Dicer) therefore in early studies, the selection mechanism was investigated in parallel in

the two RNAi pathways. Analyzing a high number of siRNA or miRNA containing complexes, a clear bias between the strands was found and was shown to be associated with the thermodynamic properties of the arm sequences. It was revealed that the strand having the less stable 5' end is favorable over the other, and it is selected for incorporating into the Ago-complex by unknown mechanisms (Khvorova et al. 2003; Schwarz et al. 2003). This "thermodynamic asymmetry rule" seemed to explain the formation of several mature miRNAs, and the selected arm was named as the "guide" strand, as opposed to the other "passenger" strand (or miRNA*) which was considered to be functionless and therefore destined to be degraded (Hutvagner 2005). Although this rule was clearly helpful in designing effective siRNAs or shRNAs for genetic knock-down experiments, large-scale sequencing data already indicated the functional presence of miRNA* species for some miRNA loci (Ruby et al. 2006), including their presence in human embryonic stem cells (Morin et al. 2008). Meanwhile, a systematic study on miRNA expression profiles from several species indicated that the thermodynamic selection rule is not universal and for various loci, both arms can be expressed simultaneously. Moreover, if expression abundance is considered for defining the dominant arm, than a tissue-specific total switch from miRNA to miRNA* was also revealed for a number miRNA genes (such as for mouse mmu-miR-30e-5p and -3p arms) (Ro et al. 2007). Analyzing NGS data, another group also identified an "outlier" from the rule: in some cancer samples, they detected an almost 1000-fold expression difference of the hsa-miR-223* species, and they proposed that this pattern is likely to be used as a biomarker for leukemia (Kuchenbauer et al. 2008). Nevertheless, the thermodynamic-based selection rule seemed to be working for most examined miRNA loci, still supporting the "one miRNA gene—one mature small RNA product" hypothesis.

But why would one consider the importance of the passenger strand at all? The first answer would be that even if the two strands in the pre-miRNA were fully complementary to each other, they would still be sequentially different: selecting the miRNA* would open up the possibility of targeting other mRNA species by the RISC, and thereby increasing the regulatory repertoire of a given miRNA locus. On the other hand, as shown in Fig. 1A, the two arms of the pre-miRNA stem-loop structure are very often not fully complementary and contain one or more bulges with varying length. This structural feature, however, provides an excellent platform for distinct sequence selection and evidence were soon given for the evolutionary independence of the two arms. In David Bartel's laboratory, when investigating the origin and the evolution of miRNAs, they found that both arms of the aqu-miR-2015 in sponges are expressed in a cell type-dependent manner, and this regulated arm switching was found to be important not only in

sponge development but also in the evolution of sponge species (Grimson et al. 2008). In a parallel study, in Eric Lai's laboratory they revealed the high conservation and therefore the evolutionary importance of several miRNA* species, and their connection to the evolution of 3' UTR sequences of the targeted protein coding genes (Okamura et al. 2008). Several subsequent studies showed that the guide and the passenger arms show tissue-specific expression patterns in various vertebrate species, including humans, and these all indicated the active regulation of arm selection, despite the thermodynamic stability differences between the two strands (Cloonan et al. 2011; Yang et al. 2011; Marco et al. 2012; Schamberger et al. 2012; Zhou et al. 2012).

The widespread occurrence of arm switching was followed by studies where the phenomenon was connected to physiological regulations and to medical importance. An early example was the work on the expression from the hsa-miR-9/hsa-miR-9* locus in neuronal development: both strands were detected in the samples and the misregulation of their expression profiles was not only associated with Huntington disease but they were also found to target mRNAs from the same biochemical pathway, even sharing common targets (Packer et al. 2008). This was followed by various examples where the miRNA guide/passenger ratio was shown to influence pathways of medical relevance in human diseases, such as the association of the miRNA arm usage pattern with the prognosis and the therapy of macroglobulinemia (Roccaro et al. 2009). There were particularly numerous examples in different cancer types (Zhang et al. 2013; Kuo et al. 2015; Mitra et al. 2015a, 2015b), indicating that the functional expression of the miRNA* strand is much more frequent than previously thought (Table 1).

But if not only the thermodynamic rule then what else regulates pre-miRNA arm selection? Can those other mechanisms override the stability-based assortment? The first indication for a distinct and elaborate mechanism was revealed when the loading of miRNA duplexes into different Ago proteins was studied in Drosophila. In that model organism, two proteins, Ago1 and Ago2 can incorporate these double-stranded small RNA species: it was shown that Ago2 is sensitive to central bulges which can take the priority over thermodynamics and leads to the active selection of the formerly considered passenger strands (Okamura et al. 2009). Similarly in human cells, where four Agos compete for the targets, Ago3 preferentially selects for the passenger strands of certain miRNA duplexes, such as the let-7a-3p arm. It was also shown that hemin can selectively induce Ago3 expression in the K562 cell line, a widely used cellular model of myeloid leukemia, so the selective incorporation of let-7a-3p into this Argonaute protein may have relevance in that particular cancer type (Winter and Diederichs 2013). In line with these observations, an earlier study revealed that the human Ago2 has a clear bias for the first 5'-nucleotide

Table 1 Selected examples on the functional role of miRNA* species in cancers

miRNA* species (arm)	Main target genes	Cancer type	Reference
miR-146a* (-3p)	NTRK2 proto-oncogene	Thyroid cancer	Jazdzewski et al. (2009)
miR-199a* (-3p)	MET proto-oncogene; ERK2	Several primary and metastatic	Kim et al. (2008)
miR-126* (-5p)	Prostein	Prostate cancer	Musiyenko et al. (2008)
miR-18a* (-3p)	K-Ras proto-oncogene	Skin and colon cancer	Tsang and Kwok (2009)
miR-29c* (-5p)	DNMT1, DNMT3A	Mesothelioma	Pass et al. (2010)
miR-136* (-3p)	TRIAP1[a]		
miR-361* (-3p)	RASL10 proto-oncogene	Gastric cancer	Li et al. (2012)
miR-423* (-3p)	RAP2C proto-oncogene		

[a] *TP53 regulated inhibitor of apoptosis 1*

identity, selecting mature miRNAs with either uracil (U) or adenine (A) base in that position (Frank et al. 2010). In a systematic follow-up study, this U and A preference was also shown for all human Agos and the authors proposed that the miRNA arm selection asymmetry is determined by a combination of an "analog pattern" (the thermodynamic instability preference) and a "digital code" (the U or A preference at the 5' end) (Suzuki et al. 2015). Nevertheless, this model still does not give a reassuring answer for all experimental results, nor does it explain the difference in Ago3 selectivity described above (Nakanishi 2022).

In parallel with investigating the roles of Agos in arm selection, various studies revealed that asymmetry "sensors" already influence the process before the double-stranded small RNAs are loaded into the pre-RISC. In Drosophila, Dicer-2 and its binding partner, R2D2 are responsible not only for loading the siRNA duplexes into the Ago2-containing complex but also influencing strand selection; however, it was also shown that human Dicer is dispensable for the asymmetry (Betancur and Tomari 2012). Quite contrary, in Jennifer Doudna's lab, they provided evidence that human Dicer, in complexed with either the TRBP or the PACT protein, strongly influence miRNA arm selection of Agos (Noland and Doudna 2013), which aspect was further supported by the crystal structure of the Dicer-TRBP complex (Wilson et al. 2015). In addition, a recent study revealed that 3' uridylation of the pre-miRNA could also actively influence arm selection: in case of the pre-miR-324, it causes an alternative (re-positioned) Dicer cleavage which results in a switch from 5p to 3p usage in miRNA maturation (Kim et al. 2020). On the other hand, other mechanisms also exist which can ensure the strict usage of one miRNA arm. One prominent example is the Dicer-independent alternative maturation pathway: in such cases, illustrated by miR-451, the short stem length of the pre-miRNA excludes Dicer binding, and the Ago2 cleavage of the stem-loop structure already determines the exclusive presence of the 5p arm in the RISC-loading complex (Cheloufi et al. 2010; Cifuentes et al. 2010). Interestingly, this alternative pathway is also utilized for shRNA vector design to eliminate off-target effects caused by the expression of the passenger strand (Herrera-Carrillo et al. 2015).

Thorough investigations in recent years eventually changed our original model of pre-miRNA maturation and the exclusive arm usage determined by the thermodynamic stability differences between the two arms. From sequence constraints to the discriminating action of regulatory proteins, several mechanisms were uncovered which can influence miRNA strand selection and although the molecular details are not always completely understood, it is clear that this process is actively regulated in a cell-type dependent manner, presumably by at least the cell-type specific expression level of the identified regulatory factors. This shift in the model is also noticeable in the nomenclature (https://mirbase.org/), as we are not talking about guide and passenger (*) miRNAs any more but rather 5p or 3p species matured from a given miRNA locus. Although understanding this aspect already challenged the 'one locus—one miRNA' belief, the real paradigm shift came with the discovery of miRNA isomiRs.

Formation of isomiRs—substantially increasing the miRNA repertoire

After the discoveries that miRNAs are wide-spread posttranscriptional regulators in most eukaryotic genomes, scientist started annotating and placing miRNA gene sequences into databases (such as "mirbase", https://mirbase.org/), analogously to protein coding genes with their mature mRNA sequences. However, these annotation very often ran into discrepancies concerning the mature miRNA end sequences, detecting not only alternative ends and internal sequences but also non-templated 5' or 3' extensions, the latter ones being end nucleotides different from those present in the flanking genomic sequences (Azuma-Mukai et al. 2008). When several studies reported such miRNA sequence variations in various plant and animal species, the idea of these

representing merely sequencing errors were becoming less and less favorable (Ebhardt et al. 2009; Wu et al. 2009; Lee et al. 2010; Zhang et al. 2010; Wyman et al. 2011). It was even revealed that the tissue-specific distributions of these miRNA variations are significantly different, again arguing for the functional presence of these species, which were subsequently referred to as "isomiRs" (Cloonan et al. 2011; Li et al. 2011; Zhou et al. 2012). Their presence was first connected to the miRNA arm switching or the RNA editing phenomena but they were soon shown to represent a distinct miRNA modifying pathway (Ebhardt et al. 2009; Cloonan et al. 2011; Zhou et al. 2012).

Following many years of uncertainty, it finally became accepted that isomiRs exist and are functional, and they were eventually considered as "the hitherto overlooked repertoire of the microRNAome" which potentially influence target selection, miRNA stability, or affect miRNA loading into the RISCs (Burroughs et al. 2011; Neilsen et al. 2012). This again lead to a "paradigm shift", since from that on, the "reference miRNA" sequence became very hard to define, and a miRNA gene could not be considered as a locus producing a single RNA product with a defined length and sequence, but rather a locus producing a population of related small RNAs with considerable sequence variability (Fig. 1C). According to a recent classification, at least the following categories of isomiRs can be distinguished beyond the canonical (the "reference") miRNA species: (i) 5' isomiRs, having a different length at the 5' end; (ii) 3' isomiRs, having a different length at the 3' end; (iii) polymorphic isomiRs, with the same length but having internal sequence variations; and (iv) "mixed type" isomiRs, containing differences in any combinations of the first 3 types (Tomasello et al. 2021). However, several questions and problems arose, including the mechanisms leading to isomiR formation, their functional versatility, as well as the problem of how to reliably detect and measure the level of a single isomiR.

Concerning their production, templated and non-templated isomiRs should be discussed separately. Templated 5' and 3' isomiRs can be considered as alternative or imprecise cleavage products from the precursor transcripts, sometimes referred to as Drosha or Dicer "promiscuity" (Wu et al. 2009; Burroughs et al. 2011). As discussed above for miRNA arm selection, theses enzymes are sensitive to sequential and structural differences, and especially for Dicer, structural motifs in the precursor hairpins influence cleavage site selection and therefore the length diversity of the small dsRNA products (Starega-Roslan et al. 2011, 2015a, 2015b). For miR-203, for example, it was found that a "sliding-bulge structure" can cause the formation of two differently folded pre-miRNA structures which are then processed differently by Dicer (Ma et al. 2016). In addition, as TRBP and PACT proteins are important regulatory partners of Dicer, they were also shown to influence cleavage site selection,

thereby the production of isomiRs (Lee et al. 2013; Wilson et al. 2015). Finally, it was shown that miRNA arm selection and isomiR production are interconnected: systematically analyzing NGS data it was revealed that the more stable the 5p or the 3p arm of a miRNA is, the more stable its corresponding isomiR profile is (Guo and Chen 2014). It is overall not surprising, as similar sequence motifs and the same processing enzymes are responsible for both miRNA maturation processes.

Analyzing the constantly growing volume of NGS datasets, a significant amount of non-templated isomiRs were also discovered. The majority of them represent 3' uridylated or adenylated species of various lengths which were connected to miRNA stability processes and decay intermediates: it was suggested that 3' uridylation marks miRNAs for degradation, whereas 3' adenylation is a sign for stability (Scheer et al. 2016). On the other hand, there were indications that 3' mono- and oligo-uridylation of pre-miRNAs could play opposing roles in the biogenesis of certain miRNAs (Heo et al. 2012), whereas another study revealed that terminal uridylation of mature miRNAs is physiologically significant and it is especially pervasive in the neonatal period (Jones et al. 2012). But this raised an important question: can an isomiR with a seemingly *genomic* 3' end sequence be in fact a non-templated isomiR? In principle yes, it could be a mono-uridylated miRNA, even if by coincidence the Dicer-cleaved mature miRNA 3' end is followed by a U nucleotide in the genomic sequence. For certain miRNA groups, such as mirtrons, these cases may be easier to test: the 5' and the 3' ends of a mirtronic pre-miRNA is determined the stringency of splicing, therefore they represent better candidates for studying posttranscriptional modifications, such as non-templated 3' uridylation (Westholm et al. 2012). However, for general miRNA molecules, these cases are both technically and biologically challenging to verify. And this also leads to a very important aspect of isomiR investigations: how can functional isomiRs be reliably identified?

The first problem is the methodology: although carefully optimized NGS methods can detect all isomiRs in a sample (Smith and Hutvagner 2022), this approach is rather expensive and cumbersome when a single locus is to be studied. Traditional RNA detection methods may be applied—but Northern blots, although detecting all isomiRs expressed above a certain threshold, cannot be used to identify new species. The more sensitive real-time qPCR methodologies can increase detectability of single known miRNA variants but the canonical assays cannot reliably distinguish among closely related isomiR sequences (Lee et al. 2010; Schamberger and Orban 2014). However, newly developed and optimized qPCR platforms could provide the desired selectivity in isomiR detection but they still require preliminary NGS data to start with, followed by careful adjustments

when a new miRNA locus is to be investigated (Honda and Kirino 2015; Franco et al. 2022).

With the technological improvements, the detection problem seems to be settled. Nevertheless, the real biological problem still remains: can the functional role of a detected isomiR be verified? How can we distinguish miRNA degradation products from *bona fide* isomiRs? As a hint, there are examples where global changes in the NGS-determined isomiR profiles are associated with a certain phenotype or a disease. As examples, these include the drastic change in the mosquito vector isomiR distribution after Dengue virus infection (Etebari et al. 2015), the dysregulation of 5'-isomiR levels in connection to the Alzheimer disease (Wang et al. 2016), or the global changes in isomiR profiles in certain cancer types (Li et al. 2012; Kuo et al. 2015; Saiselet et al. 2015). However, these results cannot be attributed to single miRNA variants and the real challenge is to assign distinct function(s) to different individual isomiRs. For animal miRNAs, where target selection is strongly based on the seed sequence, the shift at the 5' terminus can clearly influence silencing function (Fig. 1C); however, the potential effect of changes in the length or the sequence of the 3' end could be more problematic to validate. If a new population of isomiRs is to be examined, only a rigorous pipeline of analysis could exclude potential decay intermediates and provide evidence for functional importance of the detected miRNA variants. These include investigating at least (i) the tissue-specific expression patterns, (ii) the association with Ago proteins, (iii) in vitro functional activities (in luciferase assays or on predicted targets), and (iv) the potential evolutionary conservations, although this latter one may exclude species-specific isomiRs (Tan et al. 2014). Despite such methodological challenges, many examples are already known where the concrete functions of selected isomiRs have been proven, which provides evidence that this process of miRNA regulation is actively regulated and physiologically important (Table 2).

These examples underline the relevance isomiR formation and distribution in human physiology and diseases, and the most prominent examples come from cancer biology. An earlier attempt to classify 32 cancer types using isomiR profiling was surprisingly successful: even if a "binary" (present or absent) approach was applied, this method could label different tumor types with high (90%) sensitivity and with a very low (3%) false discovery rate (Telonis et al. 2017). A recent meta-analysis of available cancer datasets and publications not only supported this approach but could identify specific miRNA isomiRs that can be used as biomarkers for certain tumor types (Zelli et al. 2021). A further deep analysis of isomiR datasets could differentiate even among breast cancer subtypes (Nersisyan et al. 2022), whereas a recent study provided evidence that the heterogeneous nuclear ribonucleoprotein C (hnRNPC) induced 5' isomiR shift of miR-21-5p could lead to liver cancer (Park et al. 2022). All these studies show that isomiR-based tumor profiling seems to be a very encouraging approach for cancer diagnostics but isomiR-based tumor targeting also holds promise for future therapy applications.

Nowadays, after many years of intensive research, it is widely accepted that miRNA isomiRs represent an actively regulated and physiologically important layer of gene regulation. The mechanisms behind isomiR formation have, in general, been revealed: beside the subtle regulation of the main miRNA processing enzymes (Drosha and Dicer), the action of terminal nucleotidyl transferases, exoribonucleases and RNA editing enzymes are also responsible for the existence of certain isomiR groups (Tomasello et al. 2021). On the other hand, the regulation of cell-type specific isomiR distribution is still not completely understood and this aspect is currently in the focus of intensive investigations (Zelli et al. 2021; Aparicio-Puerta et al. 2023). Nevertheless, isomiRs should definitely be considered in miRNA target predictions (Ahmed et al. 2014), as well as in the development of new diagnostic tools and in planned therapy applications (Nikolova et al. 2021; Scheper et al. 2022).

Table 2 Selected examples of miRNA isomiRs with experimentally verified functions

miRNA locus	IsomiR type	Function(s)	Reference
hsa-miR-101-3p	5' isomiRs	Selective and age specific silencing in brain tissues	Llorens et al. (2013)
hsa-miR-142-3p	5' isomiRs	Hematopoietic lineage regulation; selective viral mimicry	Manzano et al. (2015)
hsa-miR-124-3p	5' isomiRs	Roles in human retina development	Karali et al. (2016)
hsa-miR-140-3p	5' isomiRs	Tumor suppression in breast cancer	Salem et al. (2016)
hsa-miR-34-5p; hsa-miR-449-5p	5' isomiRs	Controlling multiciliogenesis in airway epithelial cells	Mercey et al. (2017)
hsa-miR-122-5p	3' isomiRs	Influencing Hepatitis C virus replication	Yamane et al. (2017)
mmu-miR-27-3p	3' isomiRs	Differences in regulation of metabolic genes	Ma et al. (2019)

Conclusions for future biology

After more than a decade of intensive research, RNA scientists started to appreciate the unforeseen complexity in the hidden layers of small RNA regulation. From the once described "interesting but seemingly unique examples", we have moved to discover the complex regulation of miRNAs and certainly reached "beyond the one-locus-one-miRNA" paradigm (Telonis et al. 2015). The development and the continuous refinement of new sequencing technologies provide an unprecedented resolution to reveal regulatory mechanisms even at the level of single autonomous cells (Smith and Hutvagner 2022). By revealing the subtle molecular mechanisms of miRNA arm selection and isomiR formation, we have started to understand both the evolutionary and the physiological importance of the elicited posttranscriptional fine-tuning of gene regulation. The discussed phenomena influencing the formation of dynamic miRNA populations have become important for diagnostic tools in several human diseases, presenting useful biomarkers especially in various cancer types, and furthermore also providing potential therapeutic targets for future biomedical research. In addition to the already described functional roles, with the constantly developing detection technologies, new important aspects of miRNA arm selection and isomiR formation can and should also be closely investigated, such as the population- and gender-specific expression profiles which were already described earlier (Loher et al. 2014). Moreover, with the current challenges of new viral threats to human populations, this unexplored population-dependency of miRNA and isomiR repertoire may have a strong influence on the response to viral infections, and therefore this medical aspect of small RNAs should not be neglected either (Rotival et al. 2020).

And the journey has certainly not stopped yet, as similar complexities are expected to be deciphered not only in the other RNAi pathways but most likely in other, RNA-related regulatory mechanisms. All these provide evidence that members of the former "RNA world" are not simple relics of our evolutionary past but they are still active contributors to the current life on Earth. Moreover, they should be considered and utilized also for human medical applications, especially in times of unexpected threats of new potent human infecting viruses.

Declarations

Conflict of interest The author declares that he has no conflict of interest.

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