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Review

The intricacies of isomiRs: from classification to clinical relevance

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MicroRNAs (miRNAs) and isoforms of their archetype, called isomiRs, regulate gene expression via complementary base-pair binding to messenger RNAs (mRNAs). The partially evolutionarily conserved isomiR sequence variations are differentially expressed among tissues, populations, and genders, and between healthy and diseased states. Aiming towards the clinical use of isomiRs as diagnostic biomarkers and for therapeutic purposes, several challenges need to be addressed, including (i) clarification of isomiR definition, (ii) improved annotation in databases with new standardization (such as the mirGFF3 format), and (iii) improved methods of isomiR detection, functional verification, and *in silico* analysis. In this review we discuss the respective challenges, and highlight the opportunities for clinical use of isomiRs, especially in the light of increasing amounts of next-generation sequencing (NGS) data.

Highlights

The commonly used isomiR format (mirGFF3) combined with classification guidelines has been proposed to facilitate communication within the field.

New technological and computational advances have validated certain isomiRs as biologically relevant. Various experimental studies have shown mediation of different regulation mechanisms through isomiRs as opposed to regulation mediated via archetypal miRNAs.

In cancer, isomiRs have been identified as promising biomarkers in solid tissue for prognosis, and in circulatory fluids for diagnosis. Additionally, an isomiR has been identified as a promising therapeutic target. Similar studies are under way in the context of neurodegenerative, cardiovascular, and metabolic diseases.

IsomiR research as an essential part of miRNA research

MiRNAs are short, widely conserved, non-coding RNA molecules that regulate mRNAs via complementary base pair binding; they therefore play a crucial role in various cellular and metabolic pathways [1]. For a long time the true complexity of miRNAs remained hidden. MiRNAs were grouped in families (**miRNA families** – see Glossary), precursors, and mature forms. However, with increasing sequencing data, mounting evidence suggested that the number of mature miRNA variations went beyond what one would expect from biological or technical variations, that is, spontaneous mutations or sequencing errors. While studying miRNA expression via deep sequencing in human embryonic stem cells, Morin *et al.* were the first to refer to miRNAs that vary from their archetype sequence as '**isomiRs**' in 2008 [2]. In 2012, a first comprehensive review was published highlighting the biogenesis and functional significance of isomiRs [3]. As of today, 402 articles are listed on PubMed containing the term isomiR; two thirds of these articles are associated with human research. Around 150 articles have been published about isomiRs in various human diseases – including cardiovascular, neurodegenerative, psychiatric, and chronic inflammatory diseases, and most notably cancer – highlighting their increasing importance in the context of disease. The most frequently published author (Guo Li) has published 28 papers in this field.

With the growing importance of isomiRs, reviews became available with respect to different aspects of isomiR research, from basic principles (such as biogenesis or targetome analysis) to very specific clinical applications (such as colorectal cancer). These reviews describe the current state of the art in the respective fields in detail: for example, in colorectal cancer isomiRs hold promise for new diagnostics and therapies, underscoring the need for comprehensive research in this specific cancer type [4]. Studies on miRNA variants reveal their significance in stem-cell regulation within colorectal cancer, suggesting that isomiRs could influence cancer stem cell phenotypes and patient outcomes [5]. Similarly, extensive reviews across diverse cancer types, including breast and prostate cancers, emphasize the potential of isomiRs in advancing oncogenic understanding and biomarker development [6]. Moreover, the role of isomiRs in

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neovascularization demonstrates their potential therapeutic impact in cardiovascular diseases, providing new avenues for enhancing vascular remodeling [7]. Likewise, emerging technologies have revealed isomiRs as key players in cancer and other diseases, highlighting their roles in cellular processes such as differentiation and homeostasis, and their potential as biomarkers [8,9]. Finally, review articles describe how the discovery of isomiRs enriches our understanding of the miRNome, offering insights into post-transcriptional gene regulation and the complexity of miRNA networks beyond humans in plant systems [10,11]. These review articles are great examples of the growing impact of isomiRs.

Nonetheless, the number of publications referencing isomiRs is still low compared with the publications on miRNAs. To allow researchers a convenient and broad access to this topic in general, in this review we provide an overview of the current knowledge on isomiRs with particular emphasis on their potential clinical relevance, as the interest in therapeutic application of miRNAs is increasing [12]. Starting with a brief introduction on the biogenesis and classification of isomiRs, we aim to generate a basic understanding of underlying challenges within the field. We then discuss technical advances and limitations of currently available analysis tools. Then we address comparative studies across species, and we describe the clinical importance of isomiR research and especially the relevance of isomiRs for cancer diagnosis/prognosis. Finally, we suggest future scenarios, with isomiR analysis being an essential, even a mandatory, part of NGS-based miRNA studies.

Classification and biogenesis of isomiRs

Classification of isomiRs

Currently, isomiRs are defined as heterogeneous variants of individual miRNAs that can differ in length or sequence from the **archetype miRNA** [13]. They arise mostly through variations of Dicer and Drosha cleavage processes, rather rarely through single-nucleotide polymorphisms (SNPs) within miRNA genes, or through shortening of mature miRNAs via exoribonucleases, non-templated nucleotide addition through nucleotidyl transferases, or miRNA editing [8]. Furthermore, chemical post-transcriptional modifications can occur [14]. These variations can affect miRNA stability, target selection, and loading of the RISC (RNA-induced silencing complex) via, for example, seed shifting (Figure 1A), while being expressed in a cell-type-specific manner [3]. We herein reference the initially defined miRNA sequence – also known as **canonical miRNA** or reference miRNA – as archetype sequence/miRNA. We choose the term archetype miRNA over canonical miRNA (Box 1) to avoid confusion, as there are also isomiRs of non-canonical miRNAs [15], such as isomiRs of miR-451a [16].

Historically, miRNAs were grouped in miRNA families such as the miR-29 family (Figure 1B), based on either ancestry/evolutionary conservation, similar motif usage, similar mature miRNA and therefore shared functional characteristics, and/or conserved mature miRNA–seed-target relationships [17]. Family annotations are stored in the miRBase [18] as well as Rfam [19], a reference database exclusively curated for RNA family identities. As for the miR-29 family, there are the three mature archetypes (miR-29a, miR-29b, and miR-29c) that are conserved between mouse, rat, and humans, sharing identical seed regions. Each of these archetype miRNAs has a 5p and a 3p mature miRNA form, arising from the 5′ and respectively from the 3′ end of the precursor miRNA (pre-miRNA) hairpin during miRNA maturation [20]. Precursors of these miRNAs in humans are encoded on chromosomes 1 and 7 [21]. As these archetype miRNAs have overlapping targetomes, they fulfill most criteria for a family annotation.

Since mature sequences within an miRNA family (e.g., within the miR-29 family) have high sequence similarities, there are several questions about how to correctly annotate isomiRs when using most common databases like MirGeneDB [22], miRBase [23] or miRCarta [24]. One

Glossary

Archetype miRNA: a historically defined mature miRNA sequence, also commonly referred to as a reference or canonical miRNA sequence.
Canonical miRNA: mature miRNA derived via the defined miRNA biogenesis pathway mediated by Drosha and Dicer cleavage.
EpisomiR: a variation of a mature archetype miRNA defined by chemical modification.
IsomiR: a mature miRNA with a sequence variation of the mature archetype miRNA; nucleotide addition, deletion, or variation at the 3′ and 5′ end can arise, or variation within the sequence, or all these variations are possible.
MiRNA family: an assembly of pre-miRNAs with common properties: a common seed sequence, motif usage, and therefore secondary structure, or common ancestry and shared functional characteristics, such as conserved miRNA-seed-target-relationship.

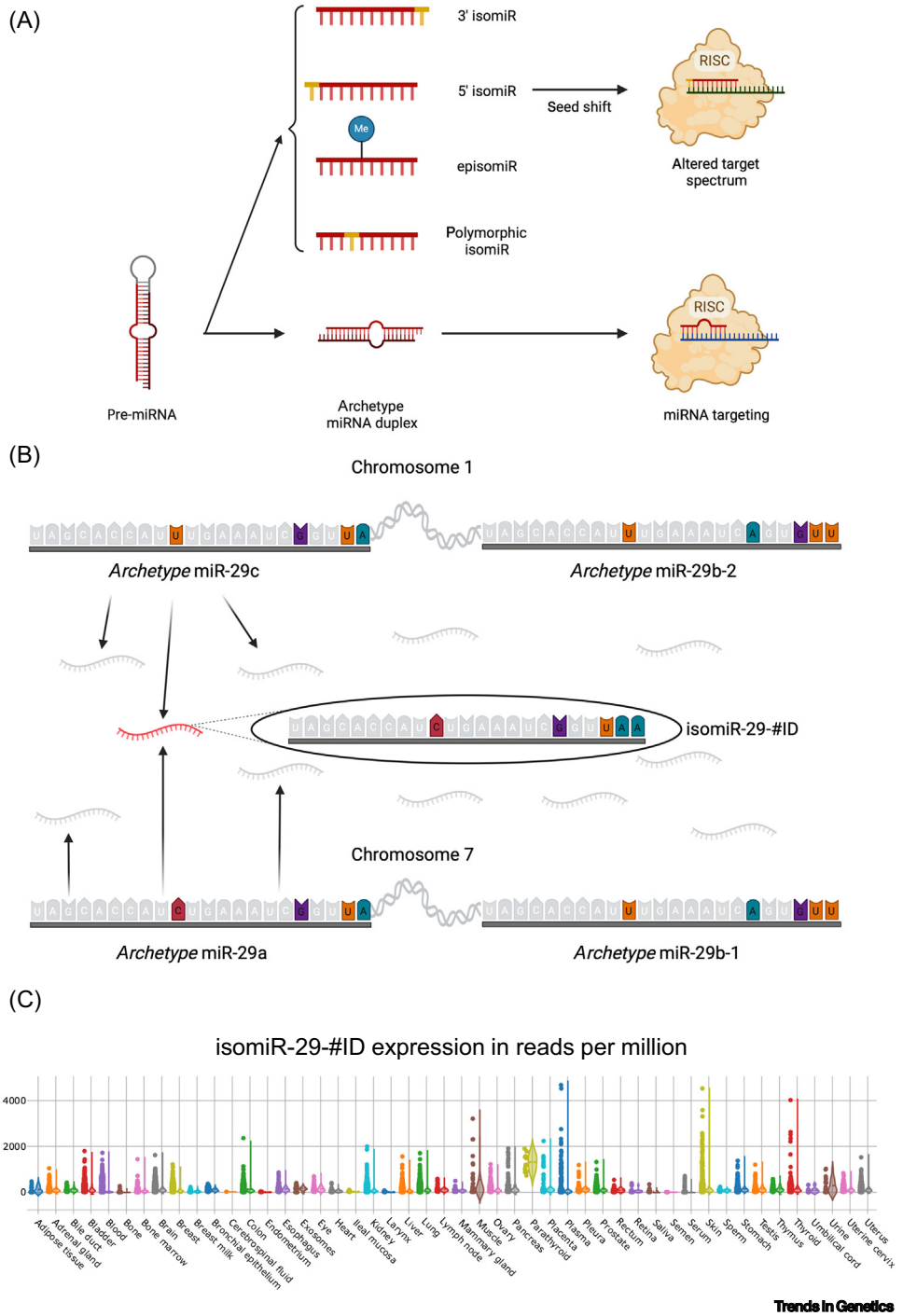

Figure 1. IsomiR annotation within microRNA (miRNA) families. (A) Examples of isomiR properties, such as 3′/5′ isomiRs, episomiRs, and polymorphic isomiRs, compared with archetype miRNA, and, respectively, mRNA targeting of archetype miRNA and altered target spectrum of (for example) 5′ isomiRs caused by seed shifting, created with BioRender.com. (B) Overview of chromosomal organization of miR-29 family in humans displaying the archetype sequence of miR-29a, miR-29b and miR-29c and their isomiRs, highlighting one example isomiR that could arise from

(Figure legend continued at the bottom of the next page.)

Box 1. Canonical and non-canonical miRNA biogenesis

Canonical miRNA biogenesis occurs through transcription via polymerase II, the formation of a pri-miRNA structure, followed by micro-processing via Drosha into the pre-miRNA hairpin. Exportin mediates transport from the nucleus to the cytoplasm, Dicer cleaves the hairpin into the miRNA duplex, and the mature sequence is loaded into the Argonaut (Ago) complex, which mediates mRNA silencing. By contrast with this process, non-canonical miRNAs arise via different biogenesis processes that are independent from Drosha and/or Dicer-catalyzed cleavage [129]. Canonical and non-canonical miRNAs can produce isomiRs [15].

question is whether to classify a sequence as either the archetype expression, for example, of miR-29a or an isomiR of miR-29b. This problem has not been much discussed in the community. In 2010, researchers addressed the challenge by dividing isomiR counts equally between homologous miRNA genes, such as hsa-miR-27a and hsa-miR-27b [25].

Another issue is: which archetype miRNA an isomiR is named after if the sequence could arise from different archetype miRNAs. In 2020, the mirGFF3 format was finally proposed to unify miRNA and isomiR research [13]. One possible solution in future research efforts might be to refer to isomiRs, for which a certain archetype miRNA cannot be defined without doubt, as isomiRs within the miRNA family with a unique identifier: for example, isomiR-29-#ID, extending the currently implemented miR-29a-3p|0|+1 format (Figure 1B). In our example, the isomiR could either arise from archetype miR-29c-3p (through an RNA edit combined with an adenylation) or from archetype miR-29a-3p (through an adenylation). Some isomiRs – such as isomiRs arising through the addition of a non-templated cytosine – are rather technical artifacts [26]. But this isomiR is an example for isomiRs expressed stably across various tissues and multiple datasets (Figure 1C) [27] that should rather be grouped with their miRNA family than one archetype miRNA. Implementing an isomiR family nomenclature within the miRGFF3 format and integrating it into common databases can mitigate ambiguity, given the lack of experimental methods to verify the original pri/pre-miRNA sequence of a mature miRNA [28]. Especially in the case of clinical applications, a unique name or ID might be superior to mentioning the variations of archetype miRNAs.

MiRNAs are categorized in five classes that are mutually exclusive, of which four are isomiRs. The archetype form describes the molecules that match the reference mature miRNA sequence defined in the miRNA database. The other four classes are composed of RNAs with sequence alterations from the archetype form, namely 5′ isomiRs (sequence changes at 5′ end of the archetype form), 3′ isomiRs (changes at the 3′ end), polymorphic isomiRs (changes within the sequence), and mixed type isomiRs (at least two of the above listed changes occur) [8,29]. Addition, deletion, and variation are the three subclasses into which 3′ and 5′ isomiRs can be divided [29].

Biogenesis of isomiRs

IsomiRs can arise through various processes. Deletions at the 3′ end are likely due to exonuclease trimming [30]. While addition and deletion relate, respectively, to addition and loss of nucleotides at the respective end, variation refers to a non-template change of the end nucleotide. Depending on whether the additional nucleotides match the precursor sequence, the subclass is partitioned into template (matching) and non-template (non-matching) RNAs [29]. Biogenesis of templated and non-templated isomiRs involves different steps. Templated isomiRs arise due to alternative or imprecise cleavage processes [31,32]. Structural differences between primary miRNA (pri-

precursor miRNA (pre-miRNA) sequences from either miR-29a or miR-29c. This isomiR displays either an isomiR arising through an adenylation of miR-29a (through e.g., nucleotidyl transferase activity) or through sequence editing combined with an adenylation of miR-29c. Sequence differences between miRNAs are highlighted in color, over identical sequences in gray, created with BioRender.com. (C) Expression data in reads per million from example miR-29–isomiR shown in (B) grouped and colored by tissue, created with isomiRdb [27]. Abbreviation: RISC, RNA-induced silencing complex.

miRNA) paralogs or pre-miRNA modifications, and therefore altered binding to RNA-binding proteins such as heterogeneous nuclear ribonucleoproteins (hnRNPCs), can generate different isomiR expression patterns through altered cleavage sites [33–35].

Non-templated isomiRs mostly exhibit a 3′ uridylation or adenylation of various lengths, which can respectively indicate altered stability and change the miRNA–mRNA network in a cell-type-specific manner. Current studies report insightful results, such as the stabilization of specific miRNAs through mono-adenylation in human primary fibroblast [36]. However, this was not reproducible in other cell types [37]. Furthermore, the results vary among cancer cell types [38], calling for further investigation to determine the role and linked opportunities of this complex regulation mechanism in a diagnostic and therapeutic context.

Polymorphic isomiRs can either occur due to SNPs or arise through post-transcriptional miRNA editing. ADARs (adenosine deaminases acting on RNA) can mediate editing of miRNAs [39] and thereby change several miRNA properties, such as the targetome [40].

The existence of another group of isomiRs, **episomiRs** (which fall outside of this classification) has been proposed recently [14]. EpisomiRs are isomiRs with chemical modifications – such as N6-methyladenosine (m6A) [41], 5-methylcytidine (5mC) [42], or 7-methylguanosine (m7G) [43] – not detected by standard sequencing methods. These modifications are also associated with altered functions [14,43,44].

Another important process involved in isomiR biogenesis and respective expression levels is miRNA arm selection and arm switching. MiRNA arm selection refers to the preferential expression of either 3p or 5p mature miRNA. Switching of the preference is highly dynamic and tissue-specific [45]. With arm selection and switching, isomiR expression varies accordingly [46]. Studies suggest that isomiR expression profiles were stable in the case of abundant expression of both mature 3p and 5p miRNAs [47]. However, recent studies suggest that a 3′ modification of pre-miR-324 led to arm switching and altered isomiR profiles [33]. Expression levels of isomiRs are, moreover, differentially expressed in various biological contexts. IsomiRs of miR-221 and miR-30a were detected at differing levels in male and female samples, amongst others [48,49], and gender-specific differing isomiR levels were detected in cancer samples [49]. IsomiR expression levels also vary between differing population groups [48] and within tissues [27,50,51].

Apart from classification of isomiRs based on biogenesis, agreeing on a common nomenclature is an important step to facilitate communication between research groups, as mentioned previously. In 2015, it was proposed that miRNA sequences be organized into three interconnected levels in databases: a reference mature miRNA sequence, herein referred as archetype miRNA (unchanging standard for reference), isomiR sequences (all variants of said miRNA plus an accession number for each isomiR), and the functional mature sequence (most highly expressed isomiR in a certain context, such as tissue, developmental stage, etc.) [30]. Other papers have raised concerns that the current archetype miRNA within miRBase is not the most abundant isomiR in certain cases, or is even incorrect [10,52]. However, this format including isomiRs was not implemented in the current miRBase update in 2019, which is the most used database for miRNA sequence annotations [23].

In total, over 90 000 isomiRs have been collected in recent databases from over 50 different tissues and almost 200 cell types [27]. To ease understanding between research groups, an incorporation of isomiRs as proposed previously into the miRBase would be rather beneficial. In that context, it is also crucial to address the issue of assigning isomiRs within a miRNA family, and

potentially defining the archetype miRNA as the most abundant isomiR within certain conditions to ensure comparability. We believe a commonly used database with isomiR annotations will facilitate usage of isomiR analysis tools and harvest all information generated by technological advances. This was one of the reasons for us to initiate the development of the isomiRDB [27]. Especially in the light of experimental advances in the past decades, with better computational tools leading to a higher resolution view on miRNAs, we might anticipate a still growing number of isomiRs.

Technological advances in isomiR detection and analysis

As isomiRs were first detected with NGS, this method is still the method of choice in isomiR research. It enables the least biased discovery of new miRNA variants [8]. Other methods, such as qRT-PCR, require prior knowledge of the sequences, and are not as specific for variation of one nucleotide and are therefore not ideal for isomiR detection. Recently a benchmarking paper compared different small-RNA sequencing protocols and showed that randomized adapter-based protocols outperform fixed adapter ones in isomiR analysis [26,53]. Specific isomiR detection is crucial, as isomiRs hold significance not only as potential biomarkers but also in understanding the miRNA targetome. With new miR-crosslinking and immunoprecipitation (CLIP) methods, the synergistic targeting effects of isomiRs and archetype miRNA forms were detected [54]. In bioinformatic prediction, incorporation of isomiRs is essential, as (for example) 3′ isomiR forms tune miRNA target specificity [55] or alter the entire targetome and mediate differential functions [56].

To harvest the entire information provided within an miRNA sequencing dataset, it is essential to have tools that account for the multidimensional structure of isomiRs. Identification and quantification of isomiRs in datasets is possible using tools such as isomiRmap [57], sRNAbench and sRNAtoolbox 2022 [58], and miRMaster 2.0 [59], amongst others [60–67]. A detailed overview of current isomiR tools was published in 2021 [68]. Furthermore, a standard data format called mirGFF3 has been proposed to facilitate downstream analysis and comparison between tools [13]. In recent updates of previous tools, new tools, and databases, this format has been implemented or is now supported [57,59,69–71].

Most tools offer different additional analysis functionalities, and a few examples are listed as follows. IsomiRex has the option to identify *de novo* miRNA and isomiRs [72]. MiFrame can perform control/case studies [73]. MiR-isomiRExp enables analysis of expression patterns at miRNA/isomiR level [74]. IsomiR-SEA additionally provides miRNA–mRNA interaction sites [75]. Several downstream analyses-only tools have been developed and tested within the last years, for example: multivariate differential expression by Hotelling's T2 test (MDEHT) to identify differentially expressed miRNAs and isomiRs between control and disease samples [76]. Benchmarking efforts in different settings have been published for isomiR tools [77,78], but no gold standard tool for all applications has been established to date.

Several isomiR web services and databases are available: for example, miR-isomiRExp [74], isomirDB [27], Tumor IsomiR Encyclopedia [50], and IsomiR Bank [79], in which isomiR expression patterns over various studies were collected. However, concerns have been raised that a certain amount of isomiRs detected so far are results of sequencing errors rather than being true biological (and functionally relevant) molecules. Using a pipeline comparing small-RNA paired end with single-sequencing reads, a systematic difference between these sequencing data forms has been detected. This especially manifests in the putative internally edited isomiRs and terminal-length-changed isomiRs to a lesser degree [80]. During a batch correction study of The Cancer Genome Atlas (TCGA), the usage of different sequencing platforms is named as a relevant source of bias. A

significant platform-dependent isomiR length difference, GC content at first position, and general GC content were reported.

Another challenge – apart from technical bias identification – is determining whether detected isomiR expression levels have functional consequences or are by-products of other processes. Therefore, it is crucial to be able to associate isomiR expression changes with changes in isomiR-generating proteins, such as RNA-binding proteins, or variations in miRNA degradation. Detected isomiRs can be products of target-directed miRNA degradation (TDMD), a mechanism of miRNA decay [81]. Alternatively, differing expression of TAR RNA-binding protein (TRBP) and protein activator of PKR (PACT), RNA-binding proteins modulating Dicer cleavage [82] as well as increased ADAR expression, impact isomiR expression levels [39] with unclear functional consequences.

To advance the isomiR field from mere detection of putative functionally relevant isomiRs in health and disease towards diagnostic biomarkers and therapeutic targets, studies that validate isomiRs are necessary [83]. Few studies to date have been published validating isomiR–mRNA interactions, compared with archetype miRNA–mRNA interaction, supporting functional importance of detected isomiRs (Figure 2, Key figure) [52,84–86]. As an example, sequence-specific miRNA sponges were used to specifically suppress the effect of the archetype miRNA and confirm the differing isomiR targetome within a luciferase assay [84]. Furthermore, it has been shown that uridylated isomiRs can regulate non-canonical miRNA targets [87]. Studies validating the functional role of isomiRs must be completed by studies exploring the mechanisms mediating these properties to generate a thorough understanding of isomiRs. For 5′ isomiR altered target genes compared with the archetype miRNA are explained mainly by the occurring seed shifting [34,84]. In isomiRs with a 3′ uridylation, alternative targeting can be achieved via tail-U-mediated repression [87]. However, generally for 3′ isomiRs and other possible modification patterns detailed studies are needed to understand the mechanisms mediating their differing functions. Additionally, it is important to extend previous studies on how these isomiR/archetype miRNA unique functions can act cooperatively [88]. In another study, the impact of isomiRs on global gene regulatory networks was assessed [89].

These studies can be used as example workflows for systematic isomiR validation benchmarking efforts. Another possible option to investigate biological functionality and importance of isomiRs are cross-species analyses of conserved expression patterns.

Comparative studies of isomiRs across species

IsomiRs have been detected in various species: plants (*Arabidopsis thaliana, Oryza sativa*, and *Ricinus communis L.*), human, cow, pig, rat, mouse, zebra fish, fruit fly, and worm) [90–97]. Functionalities of isomiRs are at least partially conserved: the influence on miRNA stability and efficiency of target repression occurring during 3′ addition of nucleotides, generating isomiRs, is evolutionarily conserved between species, ranging from *Caenorhabditis elegans* to humans [95]. Moreover, 5′-isomiRs were produced for over 70% of all miRNAs in human, mouse, fruit fly, and worm [91]. In humans and the mouse, a 5′-isomiR preference was proposed [91]. This 5′ isomiR preference can change during evolution, which is called ‘seed shifting’. This process of creating a new archetype miRNA is one mechanism during evolution for duplicated miRNA genes to acquire new functionalities. Mostly between distant species, seed shifting in miRNA orthologs was detected [98]. For example, miR-100, the most ancient metazoan miRNA, experienced seed shifting, as at the 5′ end of the mature archetype miRNA experienced a one-base shift in *Nematostella vecetensis* (sea anemone) compared with all bilaterians [99]. Another study also claims that isomiRs are important players in adjusting to evolutionary pressure, as they observed complex patterns across the animal kingdom. These researchers observed that

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Key figure

Important features of isomiRs across species in health and disease via differential regulation

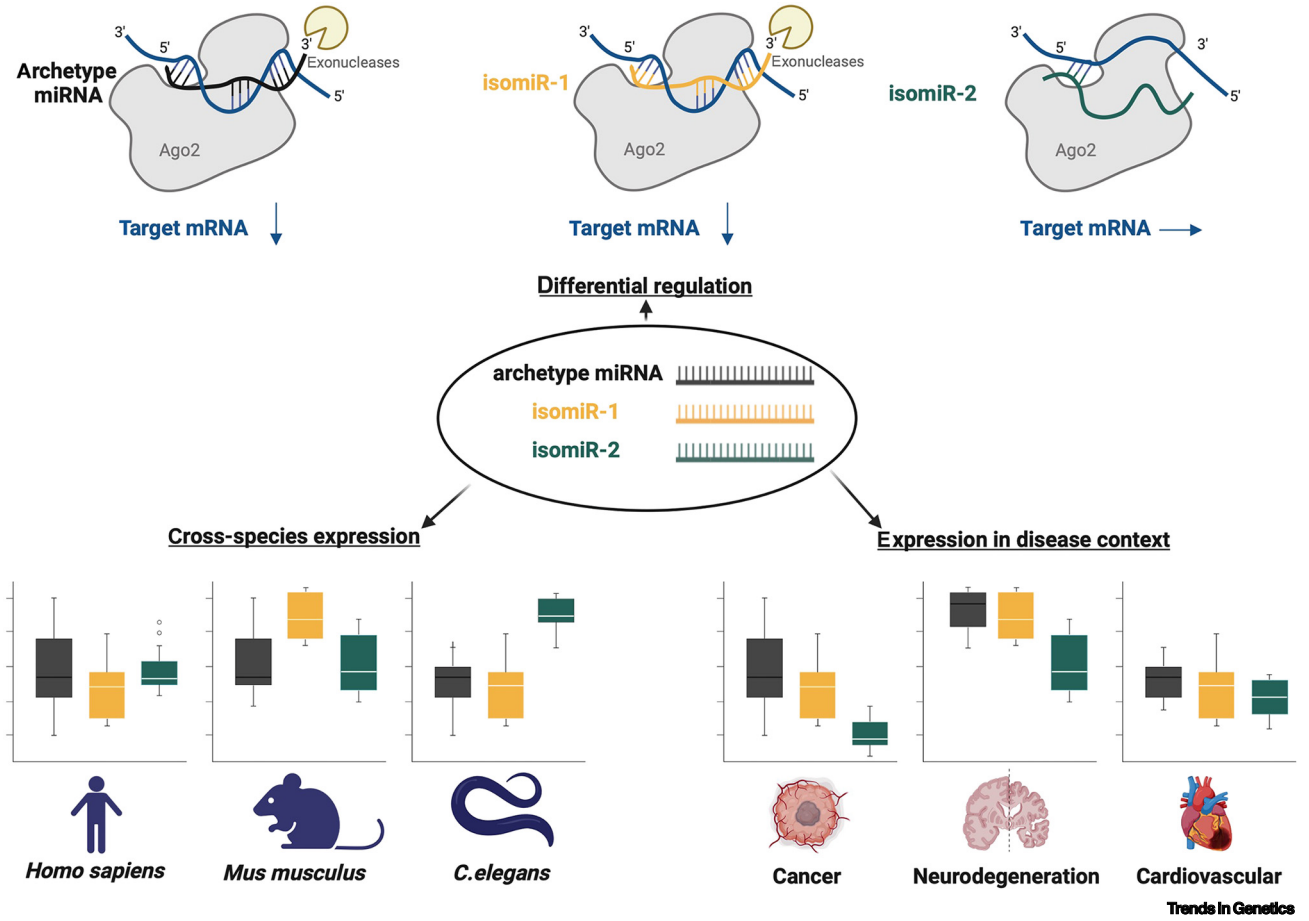

Figure 2. Schematic overview of main topics. (i) Differential regulation of mRNAs mediated by isomiRs of one archetype microRNA (miRNA). Incorporation of isomiRs within Argonaut (AGO) can vary due to sequence alterations. (ii) Differential expression across various species of archetype miRNA and its isomiRs. (iii) Expression changes of archetype miRNA and its isomiRs in different diseases. Depending on the cell type or state and disease, different expression patterns of archetype miRNA and isomiRs have been reported, identifying isomiRs as important diagnostic and therapeutic targets, created with BioRender.com. Abbreviation: *C. elegans, Caenorhabditis elegans*.

dominant isomiRs were consistent across species, but the most abundant isoform varied between species [100] (Figure 2). The conservation of miR-27 across different species and its isoforms have been studied in detail, revealing that the diversity of isomiRs exhibited patterns comparable to the diversity of homologous miRNA genes [101].

Overall, few studies have addressed isomiRs across species, even though studies in different organisms have lately created new insights [102–104]. This might be due to previously discussed isomiR nomenclature issues even within one species, and the lack of incorporation in commonly used databases hinders comprehensive cross-species studies. This underlines the importance of facilitating communication within the field to enable further research on the evolution and conservation of isomiRs.

In turn, these results – especially by comparing model organisms – would facilitate the translation of research findings to humans. Easy translation is crucial, as most studies of disease and all clinical therapies are first tested in mice as model organisms. Cancer was amongst the first diseases in which isomiR expression patterns were studied. Already early in isomiR research in 2012, the first study was published investigating isomiR expression patterns in breast cancer [105].

IsomiRs in cancer diagnosis and prognosis

IsomiR expression has been extensively studied over the last decade, and databases for comparable analysis and easy access of large datasets have been created [50,106]. In malignant cutaneous melanoma (CM), for instance, an isoform of miR-125 (hsa-miR-125a-5p|0|–2) was enriched tenfold over the archetype form (Figure 2). Moreover, only this isoform of the miRNA, and not the archetype form, is dysregulated in multiple melanomas [107]. This isomiR is just one example of many isomiRs distinctly expressed in certain cancer types and deregulated between cancer and healthy tissues [108]. In 2017, a paper was published using over 10 000 cancer datasets to build a classifier that could distinguish among 32 cancer types, as well as between cancer and healthy samples, just based on isomiR expression. The classifier was based on a binary labeling of isomiRs as either present or absent [109], which underlines – in combination with other similar studies – the strongly specific isomiR expression in cancer [110]. Furthermore, identification of cancer subtypes was possible through isomiR expression in breast cancer patients. This classification method even outperformed the previous classification based on published gene expression profiling [111].

Of note, the distinct expression of isomiRs is not limited to solid tissue samples, its potential as a diagnostic biomarker extends also to non-invasive methods. To detect prostate cancer in a non-invasive manner, miRNA expression profiles of urine extracellular vesicles were analyzed, and isomiRs of miR-21, miR-204, and miR-375 were found to be discriminatory between patients and healthy controls. However, expression analysis of archetype miRNAs was not sufficient to identify diseased individuals [112]. In lung cancer, early detection is crucial for prognosis. Using the expression of isomiRs in serum amongst other RNAs enabled researchers to detect lung cancer up to 10 years before manifestation of disease symptoms in smokers [113]. These findings highlight the importance of isomiRs as potent biomarkers for cancer diagnostics.

The usage of isomiR expression data is not limited to diagnostics alone; isomiR expression can be a useful tool for prognosis of cancer patients and can offer novel therapy targets. For instance, survival of liver cancer patients was significantly associated with expression of two isomiRs of miR-21-5p|+/–1. Another isoform of this miRNA targets and suppresses the growth hormone receptor because of the shifted seed sequence. In mouse models, a treatment with an antagomir specifically against this isomiR showed promising results, as tumorigenesis was inhibited [35]. IsomiRs of miR-21-5p, amongst others, were also identified to be influential for survival of lung cancer patients, and likely even have opposite effects compared with the archetype miRNA expression [114]. Other studies focused on understanding the specific regulation mechanisms of isomiRs in cancer development. As an example, researchers identified that isomiR miR-183-5p|+2 is involved in a negative feedback loop by targeting E2F1 to prevent uncontrolled cell proliferation [115]. Another study identified an miR-451a isomiR as a tumor suppressor in melanoma, acting through retardation of cell migration and invasion [16]. These in-depth studies to understand the specific mechanisms of isomiRs in cancer are crucial first steps in developing therapeutics using them as targets or targeting the regulated pathways [108,116]. In addition to cancer, other fields have also gained rapid traction in considering the role of isomiRs.

IsomiRs in non-cancer diseases

Aside from cancer, most patients nowadays die because of metabolic, neurodegenerative, or cardiovascular disease. IsomiR research within these diseases is rather sparse, but also provides a promising outlook on their potential as diagnostic tools or even as therapeutic targets. Three 5′-shifted isomiRs were identified as highly expressed in beta cells, and they likely impact gene regulation in type 2 diabetes through their differing targeting mechanisms [117]. Another study identified circulating isomiRs as potential biomarkers between diabetes patients and non-diabetes controls [118].

In neurodegenerative diseases (e.g., in Alzheimer's disease) significant changes in isoform levels have been observed between early and late disease stages [119]. In Parkinson's disease, differential isomiR expression could be used to separate controls from early- and late-stage patients [120]. Deregulated isomiRs were also detected in Huntington's disease and causally linked to aberrant gene expression based on their putative targets [121]. Another study explored the possibility of 5′-isomiRs regulating the HTT transcript [122]. Likely there are several polymorphic miRNAs with functional roles in metabolic and neurodegenerative diseases. These edited miRNAs arise through activities of ADAR proteins, and their impact via RNA editing has already been found to play an important role in these pathologies [123]. Therefore, more detailed studies of isomiR expression within these diseases are of utmost importance. In tuberous sclerosis complex (TSC), another neuropsychiatric disorder, circulating isomiRs have been identified as potential early risk biomarkers [124].

In cardiovascular disease, researchers identified circulating isomiRs as discriminatory markers opposed to corresponding archetype miRNAs. Furthermore, due to altered seed sequences, different targets were predicted, which were enriched in disease-related pathways [125]. In human blood vessels in ischemia, a 5′-isomiR of miR-411 is upregulated. This isomiR has a widely differing targetome from the archetype miRNA, which influences vascular cell migration and results in decreased *in vitro* wound healing and a different response in acute ischemia in mouse models [85]. Furthermore, consideration of isomiR changes is pivotal, especially in studying miRNA roles in immune responses, where systematic shifts in isomiR proportions have been observed, such as upon viral challenge [126]. Widespread isomiR expression alterations have also been observed in psoriasis [127]. In rheumatoid arthritis, a long-term auto immune disorder, miR-22-3p isomiRs were positively associated with disease-related parameters [128].

Concluding remarks and future perspectives

While extensively studied in cancer, insights into isomiR biology suggest significant potential in other disease contexts. Several issues need to be addressed to advance the field (see Outstanding questions). Currently, several isomiR databases exist, but no isomiR annotation is incorporated within the miRBase, the most used database within the miRNA research field. The inclusion of isomiR references and the critical evaluation of currently annotated archetype miRNAs within the database plays a crucial role in advancing the isomiR field. It will be highly beneficial for isomiR research to add information on frequently detected isomiRs in differing biological settings into this database. Furthermore, adding information on expression of different isomiRs in special biological settings will raise awareness of isomiRs and their biological function, and hopefully encourage people to analyze isomiR expression within their studies.

It is equally important to settle for a common nomenclature. Incorporating isomiR family identity ensures accurate naming, preventing misattribution to a single archetype miRNA and instead associating it with the miRNA family it belongs. We also propose the usage of archetype miRNA as a standard nomenclature to avoid confusion, as canonical miRNA nomenclature excludes miRNAs

Outstanding questions

What could be a widely accepted and used isomiR nomenclature that accounts for all possible variations of mature archetype miRNA, including episomiRs? And how can this complexity of miRNA and isomiRs be incorporated into miRBase and other widely used databases in an easily understandable and accessible manner? How can even further information of cross-species conservation/expression and expression patterns within different tissues be provided?

How can nomenclatures like mirGFF3 account for the uncertainty from which archetype miRNA certain isomiRs arise? What could be a standard way for analysis tools to deal with such uncertainties if miRNAs are not separated into isomiRs without introducing bias into the data?

How can further technological advances, together with computational tools, facilitate the identification of true biologically functional isomiRs with clinical relevance? Which previously identified validation experiment to identify functional isomiRs and their targets can be scaled for benchmarking studies? Which computational tools will become the gold standard tool for isomiR quantification in NGS datasets?

How can isomiR research advance into clinical application? How conserved are mechanisms and expression patterns of isomiRs between humans and mice, the mostly used model organisms? Which isomiRs are useful only as diagnostic biomarkers and which have the potential to become therapeutic targets? Is differential isomiR expression as important in diseases other than cancer (e.g., neurodegenerative, cardiovascular, or metabolic diseases)? How do population and gender differences in isomiR expression arise, and how can we account for these differences in clinical applications?

with different biogenesis pathways. These efforts – alongside established guidelines in nomenclature and annotation – will facilitate comparative studies across species and help to translate functional findings between species, thereby also easing clinical research.

While the functional relevance of certain isomiRs has been demonstrated in comparison with their respective archetype miRNAs, there is a lack of comprehensive comparative studies to validate functional isomiRs across a wide range of archetype miRNAs, or for all detectable isomiRs of a single archetype miRNA. The functional relevance of isomiRs has been proved for few isomiRs of certain archetype miRNAs. However, there are no comparative studies to validate functional isomiRs over a broad number of archetype miRNAs, or for all detectable isomiRs of one archetype miRNA. These future studies could bring insight into properties of functional isomiRs and ease the distinction between biological functional isomiRs and sequences without any functional implication or even 'isomiRs' detected due to sequencing errors. Previously mentioned studies used different approaches to validate functionality of isomiRs and can be used as a blueprint for workflows of benchmarking studies. Results of such studies would also advance studies of isomiRs in the disease context, because functional isomiRs could be easily distinguished from non-functional ones and enable the usage of isomiRs as therapy targets.

In conclusion, isomiR research represents an evolving field with discoveries such as episomiRs, emphasizing the need for a clear cross-species nomenclature and the incorporation of isomiRs into miRBase to enhance communication within the research field. Overcoming these challenges will facilitate the usage of isomiRs as biomarkers and therapeutic targets in clinical applications. Given the varying modes of action and biological activities of isomiRs, we advocate for mandatory isomiR analysis in all current and future miRNA studies, particularly those with potential clinical applications.

Declaration of interests

A.K. is a member of the scientific advisory board of Firalis. The remaining authors declare no competing interests.

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