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Opinion

A three-layer perspective on miRNA regulation in ß cell inflammation

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MicroRNAs (miRNAs) are noncoding RNA molecules that regulate gene expression post-transcriptionally and influence numerous biological processes. Aberrant miRNA expression is linked to diseases such as diabetes mellitus; indeed, miRNAs regulate pancreatic islet inflammation in both type 1 (T1D) and type 2 diabetes (T2D). Traditionally, miRNA research has focused on canonical sequences and offers a two-layer view – from expression to function. However, advances in RNA sequencing have revealed miRNA variants, called isomiRs, that arise from alternative processing or modifications of canonical sequences. This introduces a three-layer view – from expression, through sequence modifications, to function. We discuss the potential link between cellular stresses and isomiR biogenesis, and how this association could improve our knowledge of islet inflammation and dysfunction.

Highlights

Islet inflammation is a crucial factor in the pathogenesis of diabetes mellitus because it modulates pancreatic β cell function and survival.

MicroRNAs (miRNAs) play a central role in regulating inflammatory pathways. The current two-layer perspective (from expression to function) suggests that specific miRNAs are key modulators of islet inflammation but has yielded few translational benefits.

IsomiRs are post-transcriptional miRNA sequence variants and represent a consistent part of the intracellular miRNome; their existence justifies a three-layer perspective on miRNA biology that also includes miRNA modifications.

Inflammatory stresses can modulate isomiRs biogenesis and influence global miRNA function.

Understanding isomiR expression and function may help in the identification of novel therapeutic targets and biomarkers in diabetes.

Exploring miRNAs functionality in two layers: from reference genes to canonical sequence expression

Mammalian genomes harbor an abundant number of noncoding RNA genes that have emerged as crucial regulators of various physiological and pathological processes, including cell development, differentiation, growth and survival, and apoptosis [1]. Among these, miRNAs, first identified in *Caenorhabditis elegans* in 1993 [2], are pivotal rheostats of cellular functions and have been intensively studied in multiple contexts. Indeed, the discovery of miRNAs, recently honored with the 2024 Nobel Prize in Physiology or Medicine awarded to Victor Ambros and Gary Ruvkun, has revolutionized our understanding of gene regulation. miRNAs are small noncoding RNA molecules, ~22 nt in length, which regulate gene expression at the post-transcriptional level. Hence, altered expression of miRNAs leads to the dysregulation of multiple biological processes and can contribute to the development of various diseases including metabolic disorders such as diabetes mellitus.

In the human genome, most miRNA sequences are located in intergenic regions or in an antisense orientation with respect to annotated genes, indicating that they function as independent transcriptional units [3]. In addition, some miRNAs are found within intronic regions and may be transcribed as part of the annotated gene [3]. miRNA biogenesis occurs following specific stages, involving nuclear and cytoplasmic enzymes, and begins in the nucleus with the transcription of long stem-loop precursor molecules by RNA polymerases II or III [4,5]. The transcription of the miRNA gene by RNA polymerases represents the first layer of miRNA regulation. These precursors, known as primary miRNAs (pri-miRNAs), are polyadenylated and capped. The RNase III enzyme DROSHA, in complex with DGCR8 (forming the microprocessor complex), processes the pri-miRNA into a shorter precursor miRNA (pre-miRNA) of ~60-70 nt [6]. The pre-miRNA is then exported to the cytoplasm by Exportin 5, where the RNase III enzyme DICER processes the pre-miRNA into a 21–24 nt duplex RNA molecule with 3′ overhangs [7]. The miRNA duplex is then loaded into argonaute (AGO) proteins [8] with the help of HSP90 chaperone, forming the RNA-induced

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silencing complex (RISC) [9]. The maturation of precursor miRNA into a mature, functional, **canonical** (see Glossary) miRNA represents the second layer.

One of the most accessed miRNA sequence annotation databases, miRBase (https://www.mirbase.org/), currently reports a total of 1917 miRNA gene entries in humans, and 503 of these are classified as 'high confidence'. Similarly, MirGeneDB (https://mirgenedb.org/) reports 568 high-confidence miRNAs in human, based on rigorous criteria. These databases primarily rely on sequencing data submitted by authors and employ stringent criteria to define canonical miRNA sequences, typically one mature miRNA sequence per entry. However, the biogenesis of miRNAs is not linear, and various molecular mechanisms can influence maturation processes, leading to the generation of multiple sequence variants called **isomiRs** [10]. Specifically, imprecise cleavage by DICER/DROSHA, the activity of DICER-binding proteins, RNA editing, 3′ tailing by terminal nucleotidyl transferases (TENTs), and 3′ trimming by **exoribonucleases (exoRNases)** are crucial events in isomiR biogenesis. These post-transcriptional modifications, which affect miRNA maturation and contribute to isomiR production, represent a third layer in miRNA regulation.

At present, multiple online tools have been updated to offer the possibility to analyze isomiRs [11–13]. However, despite the increasing appreciation of their biological roles and the availability of technological tools to analyze them, these sequence variants are still often not considered in most small RNA sequencing experiments which primarily focus on canonical miRNA sequences. This leads to a missing piece of the puzzle in the relevant biological context and a 'streetlight effect' because they are exclusively guided by previously annotated miRNA canonical sequences. As a result, miRNA alterations in disease contexts are often studied from a limited **two-layer perspective** that focuses solely on the expression levels of the canonical reference sequence and its function. Undoubtedly, this approach fails to consider that a particular miRNA is expressed as a pool of heterogeneous sequences that differ from each other by single or multiple nucleotides, and that have divergent functions. This complexity could be more accurately represented by adding a third layer (**three-layer perspective**) through **isomiR analysis** [14].

This opinion article explores the importance of adding an extra layer of complexity to miRNA research by analyzing isomiRs in the context of pancreatic islet inflammation. Our perspective addresses a crucial gap by moving beyond mechanistic models that focus only on a limited subset of miRNA sequences. Instead, we propose a more comprehensive approach that considers the full spectrum of sequence variants involved in the functional behavior of miRNAs, thereby offering a more accurate representation of miRNA functionality. By expanding beyond the traditional two-layer model, we can gain a more comprehensive understanding of miRNA biology, revealing how different isomiR species contribute to cellular processes and disease mechanisms in diabetes. The three-layer perspective addresses the limitations of the traditional model by introducing a novel view of the molecular mechanisms regulating cellular functions and providing insights into miRNA post-transcriptional modifications which serve as fingerprints of cell status and health.

Insights from two-layer miRNA analysis of β cell inflammation in diabetes

Pancreatic islet inflammation represents a crucial element in the pathogenesis of both T1DM and T2DM. Inflammatory processes induce pancreatic β cell dysfunction and death, both of which are key factors in the development and progression of T1DM and T2DM [15].

In T1DM, interferon γ (IFN-γ) and IFN-α play a major role in the initial stages of β cell dysfunction and death by promoting autoreactive $CD8^+$ T lymphocytes islet infiltration, thus enhancing cell death and, consequently, a proinflammatory milieu [16,17]. Type I IFNs in pancreatic islets

Glossary

Adenosine to inosine modification (A-to-I): a post-transcriptional modification mainly driven by adenosine deaminases acting on RNA (ADARs), resulting in deamination of adenosine (A) to inosine (I).
Canonical: the sequence of a mature microRNA (miRNA).
Classical miRNA analysis: the standard workflow in which all sequences mapping to a particular miRNA are assigned to the canonical miRNA.
EpisomiR: a variation of isomiR that harbors specific chemical modifications of single or multiple nucleotides of its sequence.
Exoribonucleases (exoRNases): enzymes involved in the exonucleolytic degradation of miRNAs from the 3′ end.
IsomiR: a sequence assigned to an miRNA that shows variations with respect to the canonical miRNA sequence.
IsomiR analysis: the alternative analysis in which each sequence mapping to a particular miRNA is analyzed as a distinct entity.
3′ Length variant (Lv3p): a length variant that affects the 3′ end of a miRNA. This class of modification includes both the loss of nucleotides (trimming) and the addition of nucleotides concordant with the precursor sequence (extension).
5′ Length variant (Lv5p): a length variant that affects the 5′ end of a miRNA; this class of modification includes both the loss of nucleotides (trimming) and the addition of nucleotides concordant with the precursor sequence (extension).
Multiple length variant (Mv): a length variant that affects both ends of a miRNA.
Non-templated addition (NTA): the addition of nucleotides at the 3′ end of a miRNA such as by terminal nucleotidyl transferases (TENTs).
Nucleotide variant (NucVar): an miRNA that contains an internal sequence variation as introduced by enzymes such as ADARs.
Seed sequence: nucleotides from position 2 to position 7 at the 5′ end of the miRNA that are involved in binding to the target RNA sequence.
Three-layer perspective: expands the repertoire of sequences attributed to a single miRNA after miRNA transcription (first layer), precursor

mediate the overexpression of HLA class I molecules, as well as of other proinflammatory molecules. They promote endoplasmic reticulum (ER) stress and induce β cell apoptosis synergistically with interleukin-1β (IL-1β) [18].

In T2DM, IL-1β and/or tumor necrosis factor α (TNF-α), primarily produced by islet macrophages and/or by other innate immune cells, can lead to impaired insulin secretion and β cell exhaustion and/or apoptosis mainly through activation of the NF-κB inflammatory pathway [19]. Hence, although the nature of islets inflammation differs between T1DM and T2DM, the effects of downstream signaling pathway activation could result, in both cases, in oxidative and/or ER stress induction [15,20,21]. Exposure of pancreatic islets to proinflammatory molecules and the activation of downstream signaling pathways lead to crucial changes in their transcriptome [22–24], proteome [25], and lipidome [26]. In addition, several studies demonstrated the involvement of miRNAs in pancreatic islet inflammation (Figure 1), thus contributing to β cell dysfunction and death [27] or in a compensatory response [28,29]. Table 1 summarizes miRNAs associated with proinflammatory stimuli in pancreatic islets or β cells, focusing on those with at least two independent observations. As a result, increased expression of miR-146a-5p, miR-146b-5p, miR-21, and miR-155-5p can be recognized as a hallmark of β cell inflammation in the murine and/or human context [30–35] (Table 1).

Upregulation of miR-146a/b impairs insulin release, alters mitochondrial function [34], and induces β cell apoptosis [30]. Notably, miR-146a-5p silencing protects β cells from cytokine-induced dysfunction and apoptosis [30,34]. By contrast, miR-21 upregulation induces insulin release defects [30] but protects β cells against inflammation-induced apoptosis, showing an opposite effect respect to miR-146a [29,30]. miR-155-5p upregulation, initially observed in macrophages exposed to inflammatory triggers including IFN-β and Toll-like receptor (TLR) ligands [36], is induced by lipopolysaccharide (LPS), whereas its expression is downregulated by the anti-inflammatory cytokine interleukin-10 (IL-10) [37]. A proinflammatory milieu in pancreatic islets leads to increased expression of miR-155-5p. Notably, in murine autoimmune diabetes, miR-155-5p is transferred from pancreas-infiltrating lymphocytes to β cells through secreted extracellular vesicles (EVs) [35,38], whereas in humans it is upregulated in pancreatic islets upon exposure to classical T1D cytokines (IL-1β + IFN-γ) [31,33], but there are no data verifying its transfer via EVs in the human setting. Nevertheless, it is plausible that both mechanisms coexist and contribute to the inflammatory response in pancreatic islets. Similarly miR-153 is upregulated by IL-1β in rodent islets, resulting in impaired insulin secretion and apoptosis [39,40], although its role in human context remains controversial.

Furthermore, exposure of human islets to IL-1β and IFN-γ decreases the expression of miR-23a-3p, miR-23b-3p, miR-211-5p, and miR-204-5p, leading to increased expression of their target genes, including those encoding the proapoptotic factors DP5 and p53 upregulated modulator of apoptosis (PUMA), as well as the ER stress pathway components protein kinase RNA-like ER kinase (PERK) and C/EBP homologous protein (CHOP). Therefore, this dysregulation elevates apoptosis [31,41], highlighting the importance of maintaining miRNAs homeostasis to mitigate cellular stress.

It is important to emphasize that most studies on miRNA expression in pancreatic islets or β cells exposed to proinflammatory stressors have relied on murine samples and none of them employed sequencing technologies (Table 1) [117–122], although one recent exception evaluated small RNA expression in human pancreatic islets exposed to cytokines using next-generation sequencing (NGS) [33]. Intriguingly, although these data highlight the regulatory role of miRNAs in proinflammatory pathways within β cells, we still lack a comprehensive understanding of miRNAs sequence

maturation (second layer), and further sequence modifications (third layer).
Two-layer perspective: a view that exclusively considers the single canonical reference sequence that results from miRNA transcription (first layer) and subsequent precursor maturation (second layer).

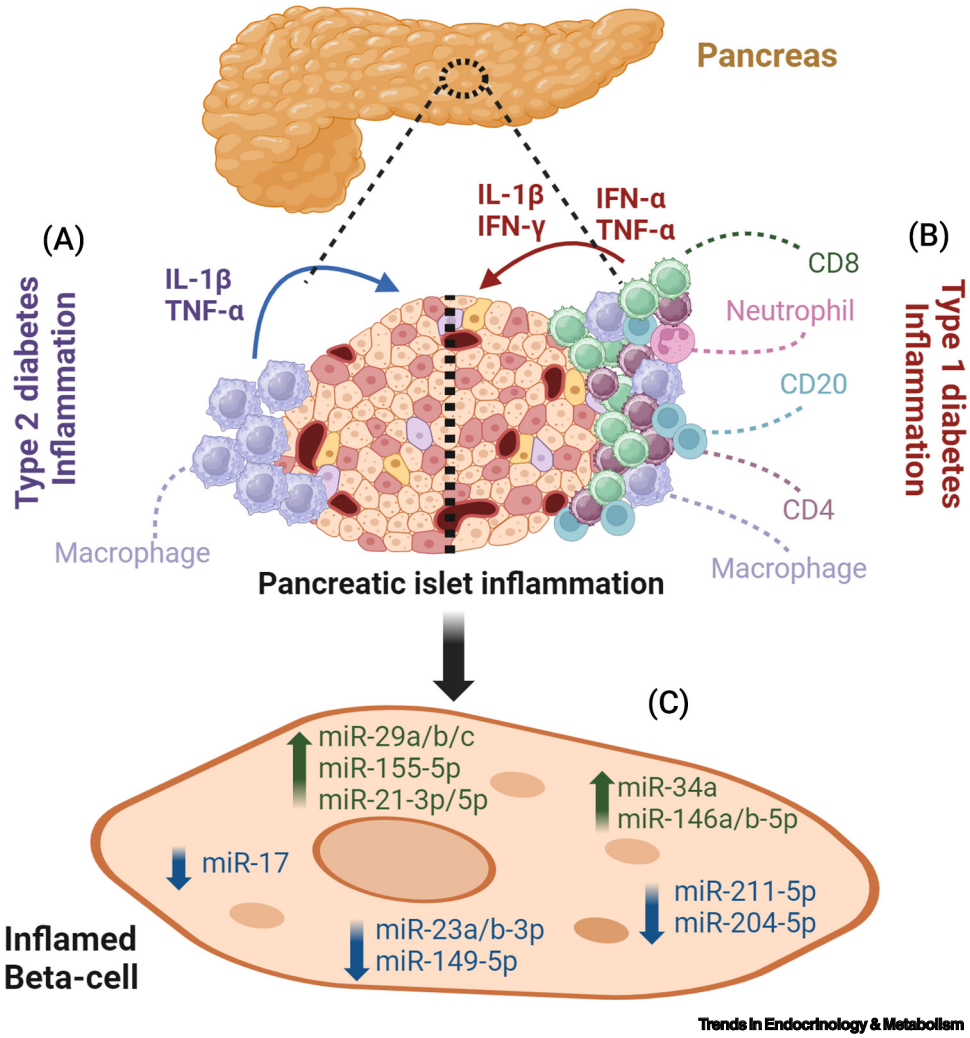

Figure 1. Insights into the expression of microRNAs (miRNAs; two layers of complexity) in pancreatic islets inflammation typical of type 1 and type 2 diabetes (T1D and T2D). Pancreatic islets are inflamed in diabetes, and distinct immune responses characterize T1D and T2D. (A) In T2D there is an intra-islet accumulation of macrophages, leading to the production of the proinflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β. (B) In T1D various immune cells infiltrate pancreatic islets, including T lymphocytes ($CD8^+$, $CD4^+$), B lymphocytes ($CD20^+$), neutrophils, and macrophages, which collectively contribute to the islet infiltrate. These immune cells produce a set of proinflammatory cytokines such as interferon (IFN)-α, IFN-γ, TNF-α, and IL-1β. (C) This proinflammatory environment in β cells, that is present in both T1D and T2D, affects the expression of several miRNAs, resulting in their differential regulation. Specifically, some miRNAs are downregulated, including miR-211-5p, miR-204-5p, miR-23a/b-3p, and miR-17, whereas others are upregulated, such as miR-29a/b/c, miR-21a-3p/5p, miR-34a, miR-146a/b-5p, and miR-155-5p. Figure created using BioRender.

dysregulation under proinflammatory stresses in diabetes. Therefore, we do not currently have information about the post-transcriptional modifications of the miRNAs listed in Table 1 whose expression is altered during β cell inflammation because most studies did not account for isomiRs. This highlights the urgent need to explore this additional layer of complexity and move from a two-layer to a three-layer perspective which views miRNAs not merely as single canonical sequences but as a spectrum of sequences that include isomiRs (Figure 2A,B). Considering these sequence variations in future miRNA research could unveil novel regulatory mechanisms and therapeutic targets in the context of diabetes and β cell inflammation.

IsomiRs: adopting a three-layer perspective on the intracellular miRNA sequence landscape

What is an isomiR?

Emerging evidence suggests that, during the maturation process, a single miRNA gene can give rise to a range of sequence variants known as isomiRs [42,43]. These variants can differ from the reference sequences at their 5′ ends, 3′ ends, internal regions, or a combination of these. Numerous studies, taking advantage of advances in NGS technologies and bioinformatic analyses, have shown that isomiRs are not sequencing artifacts. Instead, they have a biological origin and functional significance that impact on their abundance and distribution across cells and tissues. Nevertheless, it is important to note that, although isomiR biogenesis has been validated as a physiological event, some false isomiRs may arise due to the sequencing library preparation kit used. Therefore, it is crucial to implement stringent bioinformatic filtering analyses to exclude false miRNA variants [44,45].

In light of these sequence variants generated from a single miRNA, a specific classification of isomiRs is necessary to allow nonredundant annotation and to understand their possible biogenesis and function. A specific isomiR classification based on miRNA sequence features has been proposed [46,47] that classifies isomiRs as canonical, **nucleotide variants (NucVars)**, variants generated through **non-templated addition (NTA)**, **3′ length variant (Lv3p)** and **5′ length variant (Lv5p)** isomiRs**,** and **multiple length variants (Mvs)**. Consequently, a single miRNA locus can give rise to many distinct sequences.

What is the process of isomiR biogenesis?

IsomiR biogenesis is closely related to miRNA biogenesis. Post-transcriptional sequence modifications, resulting into isomiR formation, can occur at any step of the miRNA maturation through various processes (Figure 3). Lv3p and Lv5p isomiRs can originate early in the biogenetic process through alternative cleavage by DROSHA or DICER. These variations are defined as 'templated' owing to their matching to the pre-miRNA sequence, and can be influenced by the presence of single-nucleotide polymorphisms (SNPs) [48] and/or by alternative conformational structures of the pri- or pre-miRNA [49]. For instance, the distortion and flexibility of the lower stem sequence of pri-miR-9 results in alternative DROSHA cleavage, leading to the biogenesis of an isomiR with a 5′ end modification and a consequent shift in the **seed sequence** [50].

Alternative cleavage by DROSHA alters the subsequent processing by DICER [51]. Indeed, structural/sequence modifications in the pre-miRNA sequence can lead to alternative DICER processing [52], thereby generating isomiRs. Moreover, DICER cleavage activity is also influenced by its interaction with multiple proteins such as transactivation response element RNA-binding protein (TRBP) and protein activator of protein kinase R (PKR) (PACT). These proteins cooperate with DICER in the recruitment, orientation, and structural arrangement of pre-miRNA [53], thus modulating isomiR production.

RNA editing plays a pivotal role in post-transcriptional regulation of miRNAs and in the biogenesis of isomiRs. The major RNA-editing event is **adenosine-to-inosine (A-to-I) modification**, mediated by adenosine deaminases acting on RNA (ADARs) [54–56]. ADAR-sensitive sites can be present in up to 80% of pri-miRNAs and at a much lower rate in mature miRNAs, indicating that most editing occurs at the pri-miRNA stage [57]. Although many of these events lead to suppression or enhanced processing by DROSHA [58,59], some result in the predominant expression of isomiRs over canonical sequences [60] or in the modification of the seed sequence [61]. Moreover, miRNA editing can also alter the strand selection process, thus affecting the ratio between the Lv3p and Lv5p sequences [57].

Table 1. Studies using two-layer analysis of miRNAs in pancreatic β cell inflammation[a]

Cells-Tissue for miRNA profiling/ validation	Pro-inflammatory exposure tested	NO. of miRNAs studied	miR-146a-5p	miR-21-3p/-5p	miR-146b-5p	miR-29a-b-c	miR-155-5p	miR-34a	miR-211	miR-153	miR-375	miR-299-5p	miR-204	miR-23a-3p	miR-23b-3p	miR-17	Refs.
MIN6/ MIN6, HI	IL-1β; IL-1β+IFN-γ+TNFα	379	↑	↑	↑			↑									[30]
Rat islets/ Rat islets	IL-1β+IFN-γ+TNFα	279		↑		↑				↑			↑	↑	↑	↑	[40]
NOD mouse islets/ MIN6, mouse islets, HI	IL-1β+IFN-γ+TNFα	627	↑	↑	↑	↑	↑	↑									[35]
HI/ HI	IL-1β+IFN-γ	378	↑		↑		↑		↓			↑		↓	↓		[31]
INS-1E/ Rat islets, HI	IL-1β; IL-1β+IFN-γ+TNFα	758	↑		↑	↑					↑						[32]
HI/ HI	IL-1β+IFN-γ	378							↓				↓				[41]
EndoC-βH1/ N/A	IL-1β+IFN-γ+TNFα	391										↑					[117]
N/A /MIN6	IL-1β	1								↑							[39]
N/A / mouse islets, MIN6	IL-1β+IFN-γ+TNFα	1	↑														[34]
HI/ HI	IL-1β+IFN-γ	1110; 890	↑	↑			↑										[33]
HFD mouse islets/ MIN6 (Evs)	IL-1β	2083				↑											[118]
N/A / media from mouse islets	IL-1β+IFN-γ+TNFα / STZ	1									↑						[119]
N/A / MIN6, EndoC-βH1, HI	IL-1β+IFN-γ+TNFα	1		↑													[120]
N/A / INS-1, HI	IFN-γ	1														↓	[121]
N/A / INS-1 832/13	IL-1β+IFN-γ+TNFα	1		↑													[122]

[a]Key and abbreviations: ↑, increased; ↓, decreased; EndoC-βH1, human β cell-derived line; N/A, not applicable; NOD, non-obese diabetic; HFD, high-fat diet; INS-1, rat insulinoma; MIN6, mouse insulinoma.

TENTs generate isomiRs by adding non-templated nucleotides to the 3′ end of the pre-miRNA, a process called 3′ end tailing [62], leading to the generation of NTA variants via diverse mechanisms. For example, TENT2 mediates adenylation and guanylation [63], whereas TUT4 and TUT7 add uridines either through mono-uridylation or oligo-uridylation. However, 3′ tailing operated by TENTs does not play a general role in miRNA turnover. Indeed, although TENT2 stabilizes a specific set of miRNAs in a context-dependent manner

[64,65], TUT4 and TUT7 regulate their expression levels via multiple mechanisms, including their degradation [66–68].

IsomiRs are also generated by the activity of 3′-to-5′ exoRNases that are involved in the biogenesis of Lv3p isomiRs via trimming of nucleotides at the 3′ end. In *Drosophila*, the Nibbler exoRNase is responsible for the generation of Lv3p isomiRs [69,70]. In human, the activity of multiple exoRNases was confirmed to play a pivotal role in the biogenesis of Lv3p isomiRs, including poly(A)-specific ribonuclease (PARN) [71], polyribonucleotide nucleotidyltransferase 1 (PNPT1) [72], three prime repair exonuclease 1 (TREX1), interferon-stimulated exonuclease gene 20 (ISG20), and exoribonuclease 1 (ERI1) [73].

In addition, a significant emerging process known as target-directed miRNA degradation (TDMD) has been recently linked to the generation of Lv3p isomiRs. This process involves the binding of specific mRNA targets to the miRNA of interest that induce its decay by exposing the 3′ end from argonaute RISC catalytic component 2 (*AGO2*) to still unidentified enzymes [74,75].

Finally, isomiRs can undergo chemical modifications, such as through base methylation to generate *N*6-methyladenosine, leading to the formation of **episomiRs** [76]. These chemical modifications can influence their maturation process or alter their interactions with target molecules, thereby expanding their functional roles and regulatory potential [76].

What is the function of an isomiR?

The function of a miRNA is dependent on the degree and type of its binding to the mRNA target [77]. Hence, variations in the miRNA sequence may lead to changes in its function. Therefore, the function of an isomiR is strictly related its sequence variation (Figure 2C,F). Because target recognition between miRNA and mRNA is primarily mediated by the seed sequence [78] that is located in the 5′ region of the miRNA, Lv5p isomiRs exhibit the most significant changes in their functions. Nucleotide modifications at the 5′ end cause a shift in the seed sequence, thus altering their targetome and function [79–81]. Notable examples of miRNA functional variations due to isomiRs have been identified in studies involving ischemic endothelial cells and in breast cancer. For instance, the Lv5p of isomiR-411 is upregulated in chronically ischemic human blood vessels and has been shown to target different transcripts compared with its canonical miRNA counterpart, resulting in decreased cell migration and impaired wound healing [79]. Similarly, increased expression of Lv5p miR-140-3p in breast cancer is associated with reduced cell proliferation and migration because it targets a distinct set of genes compared to the canonical form [80].

The functional relevance of Lv3p isomiRs is particularly intriguing. The binding of miRNA 3′ regions to target mRNA is known as supplementary or compensatory binding [82]. Supplementary binding can stabilize the miRNA–mRNA interaction, thereby enhancing the regulatory effect of miRNAs on their targets or shifting the targetome [83]. Hence, Lv3p isomiRs may diversify miRNA functions by enabling the recognition of a broader/different range of targets, thereby expanding the functional repertoire of miRNAs. In addition, miRNAs with different 3′ end sequences may exhibit different rates of loading into AGO2 [84,85], which can affect their functionality (Figure 2F). Lv3p isomiRs also exhibit specific temporal expression patterns during physiological development. For instance, miR-122 Lv3p and its uridylated isoforms accumulate in the liver at later timepoints after birth, whereas levels of the canonical miRNA form decrease. This pattern occurs because the canonical miRNA (22 nt) is first converted into a 21 nt Lv3p isomiR, which then undergoes poly-uridylation, leading to its degradation. Understanding the dynamics of isomiR expression provides deeper insights into miRNA turnover and regulation [86].

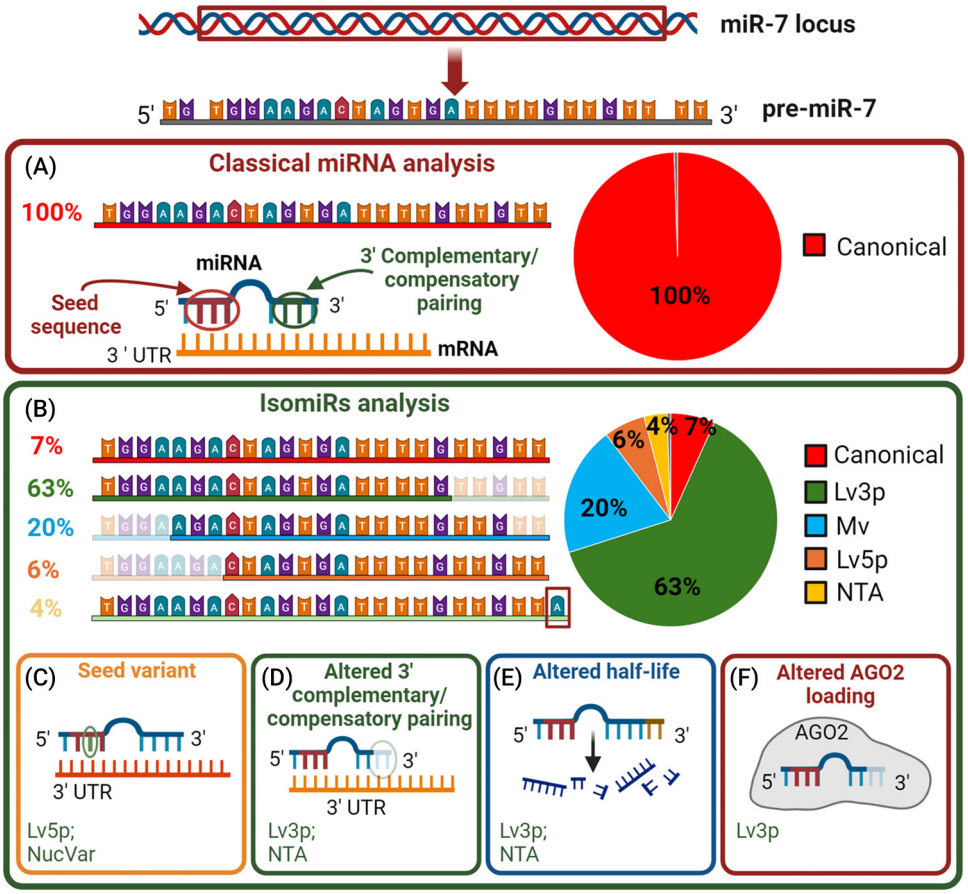

Figure 2. Expression of microRNAs (miRNAs; two layers of complexity) versus isomiRs sequence composition analysis (three layers) and function(s): the example of hsa-miR-7 in β cells. Analysis using the IsomiRdb database (https://ccb-compute.cs.uni-saarland.de/isomirdb) reveals that the canonical miRNA sequence of miR-7 represents only a minor portion of the total miR-7 expression in β cells. (A) Traditional miRNA analysis (two layers) assumes that a single miRNA locus produces one mature miRNA, typically the canonical miRNA, which interacts with the 3′ untranslated region (UTR) of target mRNAs through the complementarity of its 5′ seed sequence and an eventual 3′ complementary/compensatory pairing. However, this simplistic view does not capture the true complexity of miRNA biogenesis. (B) By contrast, isomiR analysis (three layers) considers each sequence mapping to the precursor as a distinct entity, revealing greater complexity. Indeed, most of the expression of miR-7 in β cells resulted from different entities of 3′ length variant (Lv3p) isomiRs, whereas the canonical sequence accounts only for a low percentage of total miR-7 expression. This enhanced complexity offers new insights into post-transcriptional modifications and alternative processing events during miRNA biogenesis. Furthermore, isomiRs may exhibit different functional activities. (C) Nucleotide variant (NucVar, in the seed sequence) and 5′ length variant (Lv5p) isomiRs result in a different seed sequences, modifying the miRNA targetome. (D) Lv3p variants can affect the 3′ compensatory/complementary, altering the affinity of the interaction between the isomiR and the mRNA. (E) Lv3p variants, in particular those created by non-templated addition (NTA), can influence isomiR stability and alter the half-life of the molecule. (F) Shorter 3′ ends are associated with reduced AGO2 loading and, consequently, decreased functional activity of the isomiR. Abbreviation: Mv, multiple length variant. Figure created using BioRender.

Interestingly, isomiRs may also exhibit distinct sorting patterns into EVs compared with their canonical miRNA counterparts because their loading in EVs is sequence-dependent. Indeed, some miRNA short sequence motifs (defined as EXOmotifs) are enriched in EVs compared with the intracellular compartment, highlighting that even minor sequence variations can influence the selective loading of isomiRs [87]. Notably, nucleotide tailing further impacts on miRNA sorting:

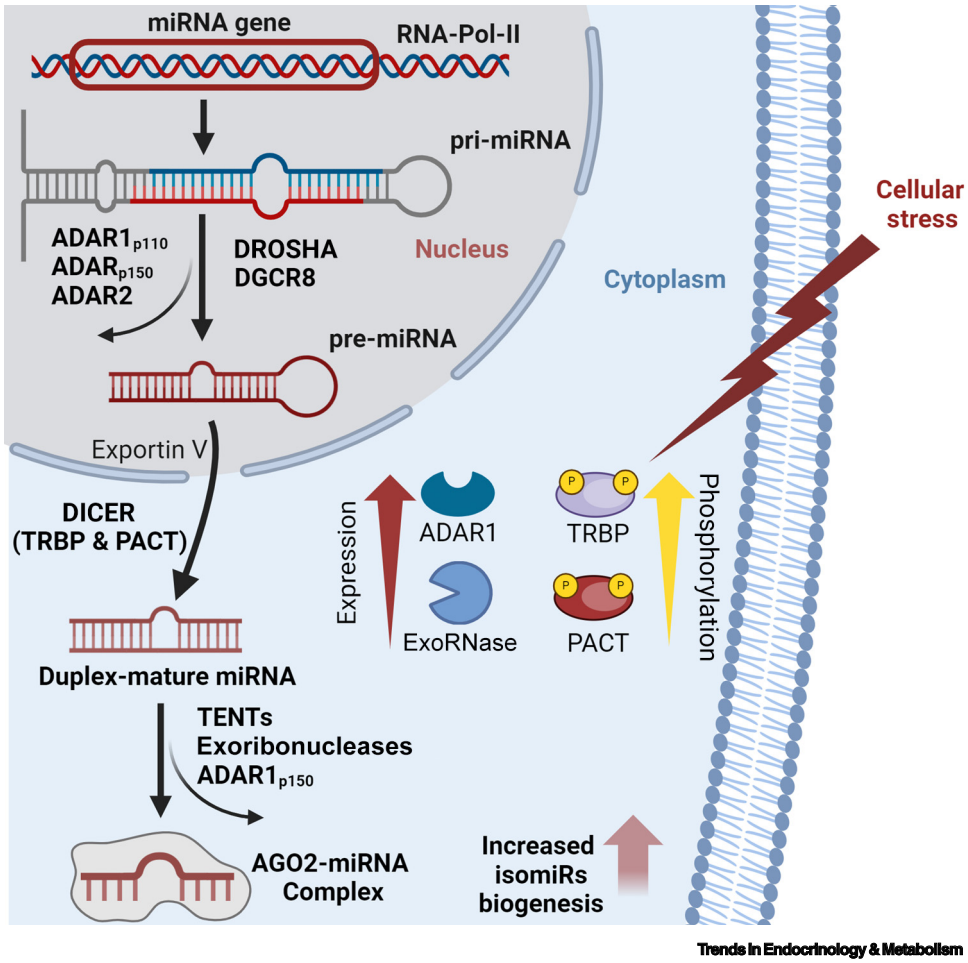

Figure 3. Molecular mechanisms involved in isomiR biogenesis. The biogenesis of microRNAs (miRNAs) and of isomiRs are closely related processes. Alternative cleavage of the pri-miRNA by DROSHA/DGCR8 and of the pre-miRNA by DICER can result in the production of isomiRs with length variants. RNA editing, particularly through deamination of adenosine to inosine (A to I) by adenosine deaminases acting on RNA (ADARs), occurs at both the pri-miRNA and pre-miRNA stages, contributing to isomiRs diversity. DICER-binding proteins transactivation response element RNA-binding protein (TRBP) and protein activator of protein kinase R (PKR) (PACT) also play roles in isomiR biogenesis. Furthermore, post-transcriptional modifications at the mature miRNA stage, such as 3′ end trimming by exoribonucleases (exoRNases) and 3′ end tailing by terminal nucleotidyl-transferases (TENTs), increase isomiR diversity. The activity and the expression of enzymes involved in their biogenesis can be regulated by cellular stress and inflammation. For instance, ADAR enzymes and various exoRNases are upregulated by type I interferon, and stress signals increase the phosphorylation (P) of TRBP and PACT, linking isomiR biogenesis to cellular stress and inflammation. Figure created using BioRender.

adenylated isoforms tend to be enriched within the cellular compartment, whereas uridylated isoforms are preferentially sorted into EVs [88]. This adds a further layer of complexity, and expands the role of isomiRs to cell–cell communication.

The link between isomiRs and inflammation in diabetes

Evidence from cancer research highlights the significance of isomiRs and their potential link with inflammation. Proinflammatory stresses affect various aspects of cancer biology [89] including development [90] and disease progression [91]. Numerous dysregulated miRNA isoforms have been identified in cancer studies [92] and several isomiR-based cancer databases such as isomiR-eQTL [93], isomiRTar [94], and TIE [95] assist in identifying new therapeutic targets and

biomarkers. Notably, inflammation can influence isomiR biogenesis through multiple mechanisms, many of which are shared between cancer and inflammatory and metabolic diseases.

ExoRNases that degrade RNA in the 3′-to-5′ direction are modulated by proinflammatory molecules. PNPT1 is stimulated by type I IFN and post-transcriptionally regulates and degrades miR-221 [72]. An increase in shorter isoforms of miR-222 is induced in human fibroblasts treated with IFN-β, corroborating the link between 3′ end trimming and type I IFN stimulation [96]. IFN-induced, manganese-dependent exoRNase ISG20 has been shown to lead to 3′ trimming of AGO-loaded miRNAs [73]; of note, ISG20 is increased in pancreas of T1D donors with higher levels of inflammation [97].

TNF-α, IL-1β, and hypoxia (which induces oxidative and ER stress) can lead to changes in isomiR biogenesis and expression [79,98,99]. Interestingly, these stimuli are involved in pancreatic islet dysfunction and β cell apoptosis in diabetes [100,101]. In addition, exposure to IFNs, mainly IFN-α and IFN-γ, promotes ADAR1 expression and increases A-to-I RNA editing in pancreatic β cells [102] and, potentially, increases the biogenesis of isomiRs. Similarly, ADAR2, which is primarily associated with the central nervous system [103], is also expressed in pancreatic islets. Its expression in these cells is regulated by the stress-activated protein kinase JNK1 (mitogen-activated protein kinase 8) in response to chronic inflammation [104]. Notably, chronic activation of the JNK pathway contributes to proinflammatory β cell dysfunction and apoptosis in diabetes [105]. Under physiological conditions, RNA editing regulates β cell function; disruption of the RNA editing machinery in β cells, as reported in an *Adar1* β cell-specific knockout mouse model, triggers a massive IFN response, islet inflammation, and β cell failure that resemble the early stages of T1D [106]. Nonetheless, it remains to be directly assessed whether these changes in pancreatic β cells involve major dysregulation of isomiRs. Interestingly, multiple isomiRs in β cells have been identified as key regulatory hubs of their function. Three isomiRs (Lv5p miR-375+1, Lv5p miR-375-1, and Lv5p miR-183-5p+1) exhibited variations in their seed sequences, leading to different regulation of the targets tested and suggesting a potential involvement also in inflammation-mediated modulation [107].

Finally, inflammation-mediated modulation and post-translational modifications (PTMs) of DICER and/or DROSHA can alter their processing activity toward pri-, pre-, and mature miRNAs. The activities of DICER-binding proteins, TRBP and PACT, are intricately linked to an IFN-induced inflammatory pathway through PKR [108,109]. Consequently, modulation of their activity may impact on pre-miRNA processing, leading to alternative cleavage and biogenesis of isomiRs. DROSHA is subjected to various PTMs that modulate its enzymatic activity. Specifically, protein arginine methyltransferase 1 (PRMT1) methylates multiple sites on DROSHA [110] and is associated with inflammation and oxidative stress [111].

Supporting this concept, the connection between diabetes, inflammation, and isomiR biogenesis is further highlighted by the dysregulation of isomiR expression observed in human umbilical vein endothelial cells (HUVECs) undergoing replicative senescence and after long-term exposure to metformin. This glucose-lowering drug, which is known for its anti-senescence effects, leads to the differential expression of 133 isomiRs [112], suggesting complex interplay between diabetic inflammatory stress, isomiR biogenesis, and therapeutic interventions.

Transitioning from two-layer to three-layer analyses in miRNA research

In our view, the traditional two-layer approach used in **classical miRNA analysis** provides an oversimplified mechanistic model because it overlooks miRNA sequence variants, their biogenesis, and related functions, and thus misses important physiological changes which may be crucial in fully understanding some phenomena. As mentioned earlier, the example of Lv3p miR-122 in the

liver demonstrates how the observed increase in overall miR-122 levels is primarily driven by its isoforms, whereas the canonical form decreases [86]. Similarly, during mouse brain development, a three-layer analysis revealed a decrease in the canonical form of miR-338-3p over time, accompanied by an increase in its Lv3p isoforms [113]; notably, the increase of miR-338-3p Lv3p isoforms was associated with more potent inhibition of its target genes [113]. Consequently, elucidating the mechanisms leading to the biogenesis of isomiRs might offer novel targets to modulate biological pathways and new opportunities to treat multiple diseases, including autoimmune and inflammatory disorders. Hence, given the significance of this approach in uncovering novel mechanisms to potentially design innovative therapeutic strategies – such as modulating the biogenesis of specific isomiRs in particular disease conditions – we propose several action points to improve miRNA analysis.

(i) Advanced RNA-sequencing technologies and improved downstream bioinformatic pipelines should be widely employed as initial screening strategies to capture the full spectrum of miRNA and isomiR populations, thereby providing more comprehensive datasets.

(ii) Several online tools and algorithms, designed for miRNA quantification in small RNA-sequencing experiments, should be adopted to facilitate the analysis of isomiRs as distinct sequences [11,12,47,114].

(iii) Following quantification, rigorous classification of isomiRs based on their post-transcriptional modifications, as previously described, is important. Accurate classification and quantification provides essential information about the molecular mechanisms that regulate isomiR biogenesis. By adopting this approach, miRNA analysis can shift from a simple expression-based perspective to a more nuanced representation in which the expression of each miRNA is depicted as the proportion contributed by different isomiR classes.

This perspective is particularly informative in experimental settings that involve comparing two conditions (e.g., cytokine-treated cells versus controls). In such scenarios, researchers can evaluate how a specific perturbation affects the sequence composition of each miRNA, and could potentially uncover common patterns across miRNAs that point to molecular mechanisms induced by the experimental conditions. Unlike the traditional differential expression pipeline, this differential proportion analysis focuses on miRNA composition rather than on total expression and can only be performed using a three-layer approach.

In diabetes research, this novel perspective could be implemented to investigate the effects of T1D- and T2D-related inflammatory cytokines on isomiR class proportions. Experiments could be conducted in cellular models (e.g., EndoC-βH1 or MIN6 cell lines), or in primary pancreatic islets from human donors or mouse models, to determine whether these stress-inducing stimuli trigger specific post-transcriptional modifications in miRNA sequences.

Concluding remarks and future perspectives

Based on the current evidence, it is necessary to reassess the intracellular pool of miRNAs and transition from a two-layer to a three-layer view of miRNAs in β cell inflammation and diabetes. Given the functional variations resulting from isomiR biogenesis, traditional miRNA expression analysis may fail to capture the full spectrum of molecular mechanisms activated during stress-induced inflammation; this could lead to omission of key therapeutic targets for reducing β cell inflammation, dysfunction and death in diabetes. Instead, we propose that isomiR biogenesis, potentially modulated by inflammatory stresses in pancreatic islets during diabetes, should be considered when examining the effects of inflammation on the composition and function of

Outstanding questions

How do specific inflammatory signals regulate the biogenesis and function of isomiRs in pancreatic islets?

How do variations in isomiR sequences affect their targetome and overall function in pancreatic β cells?

How does dysregulation of isomiRs directly impact on β cell insulin secretion, survival, and overall function in diabetes?

Can modulation of isomiR pathways provide therapeutic benefits in managing inflammation and β cell dysfunction?

miRNA sequence pools. This approach has the potential to identify mechanisms of isomiR biogenesis which could be therapeutically targeted using novel drugs (e.g., innovative compounds or biomolecules that can restore the physiological biogenesis of isomiRs). Indeed, shifting the analytical focus from miRNA expression levels to a proportional representation of sequence variants not only provides deeper insights into the enzymatic processes that drive miRNA modifications but also unravels the biological mechanisms that characterize the disease. In addition, the introduction of isomiR analysis in the context of inflammation can be also beneficial to the discovery of novel circulating biomarkers, given the promising advances made in the association of circulating miRNAs with the severity and progression of T1D or T2D (see Outstanding questions) [115,116].

In conclusion, isomiRs introduce a new layer of complexity to miRNA analysis, and offer novel perspectives on the role of post-transcriptional modifications. IsomiRs may be pivotal players in governing multiple cellular processes and allow us to gain more insights into the molecular mechanisms that regulate miRNA biogenesis and functions. Investigating this novel class of molecules could significantly enhance our understanding of inflammatory and metabolic diseases and broaden the repertoire of available potential therapeutic targets.

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Declaration of interests

The authors declare no competing interests.

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