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Review

Exosomal miRNAs and isomiRs: potential biomarkers for type 2 diabetes mellitus

Yong Ling Sou,[1] William M. Chilian,[2] Wickneswari Ratnam,[3] Shamsul Mohd Zain,[4] Sharifah Zamiah Syed Abdul Kadir,[4] Yan Pan,[1] Yuh-Fen Pung [1,*]

[1]Division of Biomedical Science, Faculty of Science and Engineering, University of Nottingham Malaysia, Semenyih, Selangor 43500, Malaysia
[2]Department of Integrative Medical Sciences, Northeast Ohio Medical University, Rootstown, OH 44272, USA
[3]Department of Biological Sciences and Biotechnology, Faculty of Science and Technology, Universiti Kebangsaan Malaysia, Bangi, Selangor 43600, Malaysia
[4]Department of Pharmacology, University of Malaya, Kuala Lumpur 50603, Malaysia
*Corresponding Author: Yuh-Fen Pung, E-mail: yuhfen.pung@nottingham.edu.my

Abstract

Type 2 diabetes mellitus (T2DM) is a metabolic disease that is characterized by chronic hyperglycaemia. MicroRNAs (miRNAs) are single-stranded, small non-coding RNAs that play important roles in post-transcriptional gene regulation. They are negative regulators of their target messenger RNAs (mRNAs), in which they bind either to inhibit mRNA translation, or to induce mRNA decay. Similar to proteins, miRNAs exist in different isoforms (isomiRs). miRNAs and isomiRs are selectively loaded into small extracellular vesicles, such as the exosomes, to protect them from RNase degradation. In T2DM, exosomal miRNAs produced by different cell types are transported among the primary sites of insulin action. These interorgan crosstalk regulate various T2DM-associated pathways such as adipocyte inflammation, insulin signalling, and β cells dysfunction among many others. In this review, we first focus on the mechanism of exosome biogenesis, followed by miRNA biogenesis and isomiR formation. Next, we discuss the roles of exosomal miRNAs and isomiRs in the development of T2DM and provide evidence from clinical studies to support their potential roles as T2DM biomarkers. Lastly, we highlight the use of exosomal miRNAs and isomiRs in personalized medicine, as well as addressing the current challenges and future opportunities in this field. This review summarizes how research on exosomal miRNAs and isomiRs has developed from the very basic to clinical applications, with the goal of advancing towards the era of personalized medicine.

Keywords: extracellular vesicles; insulin resistance; hyperglycaemia; obesity; precision medicine

Introduction

Type 2 diabetes mellitus (T2DM) is a chronic hyperglycaemic condition characterized by insulin resistance and impairment of pancreatic β cells to produce sufficient insulin levels. The coordination of the production and disposal of insulin is essential for maintaining glucose homeostasis and achieving normoglycemia. Such equilibrium is achieved by a complex network of physiological communications between insulin-secreting pancreatic β cells and other insulin-sensitive tissues, such as the liver, adipose tissue, and skeletal muscle. Under normal conditions, insulin is secreted into the blood circulation to facilitate glucose uptake into skeletal muscle and adipose tissue and to inhibit hepatic glucose production in response to raising blood glucose. Hence, the crosstalk communication between multiple organs plays a crucial role in regulating the insulin signalling network as disruption of the inter-organ crosstalk could result in the onset of T2DM [1, 2]. However, most research often focused on the roles of signalling molecules, such as proteins and metabolites between key insulin signalling sites, with less attention given to extracellular vesicles (EVs).

EVs are a heterogeneous population of lipid bilayer-enclosed vesicles. They are released by various types of cells into the extracellular matrices and biofluids such as plasma, urine, saliva, cerebrospinal fluid, and breast milk [3–5]. Based on their size, protein characteristics, and biogenesis pathway, EVs can be classified into three subpopulations: apoptotic bodies, microvesicles, and exosomes [6]. Exosomes, also referred to as small EVs, have diameters ranging between 30 to 150 nm [7, 8]. They carry distinct subtypes of protein markers such as CD9, CD63, CD81, TSG101, Alix, Annexin, and Flotillin, which distinguish them from other EV subpopulations [9]. Some studies also reported that their RNA profiles, such as microRNAs (miRNAs), are expressed differently from the other EV subpopulations [10, 11]. Initially, exosome was viewed as a mechanism for cells to dispose of their unwanted waste [12, 13]. Further research in this field revealed that these small vesicles can be a medium of communication between different cell types to transport functional biological compounds, even being considered as therapeutic carriers to transport nucleic acids for gene and cell therapy [14–16]. From the variety of cargo carried in exosomes, miRNAs have particularly garnered much attention due to their ability to alter gene expression in recipient cells, whether in direct vicinity or from distant origins when shuttled in exosomes [17–19].

miRNAs are one of the key regulators in post-translational regulation. In the past, many studies have associated the circulating miRNAs, which are freely floating in the bloodstream, with disease development. One of the main advantages of studying circulating miRNAs is that they often require a lesser starting material and skip the hassle of isolating exosomes from biofluids. There were also several studies reporting on the potential of circulating

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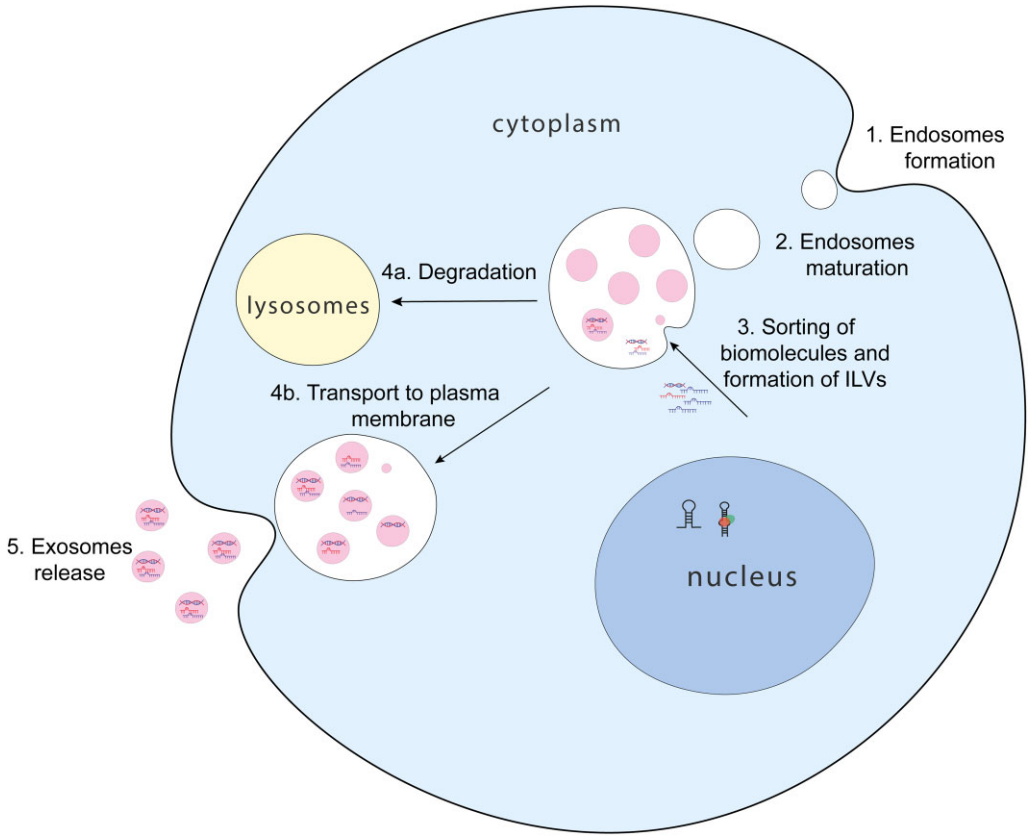

Figure 1. The biogenesis and release of exosomes. Exosome biogenesis can be summarized in four steps: (1) The inward budding of the plasma membrane to form early endosomes; (2) Early endosomes mature into multivesicular bodies (MVBs) (also known as late endosomes); (3) The invagination of MVBs membrane to form intraluminal vesicles (ILVs) by the ESCRT-dependent or ESCRT-independent pathway and the sorting of biomolecules; (4a) Fusion of MVBs with lysosomes which results in degradation; (4b) Fusion of MVBs with the plasma membrane to (5) release ILVs as exosomes.

miRNAs as diagnostic biomarkers, but all of them have one common limitation, the lack of knowledge of the origin of these dysregulated miRNAs. Without the knowledge of origin, one cannot distinguish if these miRNAs in circulation are meant to be delivered into recipient cells, waste products of cells, or just by-products of apoptotic cells. On the other hand, the miRNAs encapsulated in exosomes provide more valuable information as their origin can be traced back. Furthermore, exosomes provide a more stable environment for miRNAs to prevent them from being degraded by RNase in the circulation [20–22]. In this review, we first give an overview of the exosome biogenesis pathway, then the formation of miRNAs and their isoforms (isomiRs). Next, we present evidence from cell culture experiments, animal studies, and clinical studies of how exosomal miRNAs play crucial roles in the development of T2DM. Subsequently, we discuss how the combination of exosomal miRNAs and their isomiRs can act as biomarkers to enhance the diagnosis of T2DM, as well as their future perspectives in personalized medicine. Finally, we address the current challenges and limitations, together with the research opportunities of exosomal miRNAs and isomiRs in T2DM.

Exosomes biogenesis

Currently, the classification of EVs still remains unclear due to their overlapping sizes and protein characteristics. Hence, one of the more precise ways to distinguish them is by the biogenesis pathway. For example, exosomes are formed via the inward folding of the plasma membrane, microvesicles are formed via the direct outward budding of the plasma membrane, whereas apoptotic bodies are formed as byproducts of cell apoptosis [19, 23, 24]. The biogenesis pathway of exosomes begins with an inward budding of the cell plasma membrane to form intracellular endosomes, which are also known as early endosomes (Fig. 1). These endosomes undergo acidification and other specific processes, along with the invagination of the endosomal membrane, to mature into multivesicular bodies (MVB) (also known as late endosomes) [25].

The production of MVB is governed by two pathways, the endosomal sorting complexes required for transport (ESCRTs)-dependent and ESCRTs-independent pathway. The ESCRTs are comprised of four protein complexes, ESCRT-0, -I, -II, and III, along with their auxiliary proteins, Vsp4 and Alix. ESCRT-0 complex is made up of ubiquitin-binding subunits that can recognise and bind to mono-ubiquitinated proteins [26]. When ESCRT-0 joins with the ESCRT-I and -II subunits, intraluminal budding of the MVB membrane is initiated [27]. ESCRT-III, together with Vsp4 protein, converged together with ESCRT-0, I, and II to facilitate membrane cleaving and final scission of MVB membrane [28]. As a result, small vesicles, also known as intraluminal vesicles (ILVs) are formed within MVB. Subsequently, small GTPases such as the RAB27a/b and RAB11 facilitate the transport of MVB towards the plasma membrane [29, 30]. Lastly, the SNARE proteins (Vamp7 and YKT6), Alix, Syndecan, and Syntenin work together to facilitate the fusion of MVB with the plasma membrane, releasing ILVs as exosomes into the extracellular matrix [31, 32]. Alternatively,

MVBs are subjected to lysosomal degradation if they carry ubiquitylated protein [33].

When exosomes are produced independent of the ESCRTs, lipid components such as ceramide and sphingomyelin play crucial roles. For example, during the early stage of endosome formation, activation of neural sphingomyelinase (nSMase) leads to ceramide formation, which, in turn, promotes the formation of a sphingomyelin-enriched lipid raft microdomain on the endosomal membrane. Subsequently, the lipid raft microdomain induces an inward budding of the MVB membrane, resulting in the formation of ILVs [34]. Further research demonstrated that nSMase also contribute to the packaging of proteins into exosomes [35]. Wei *et al.*, on the other hand, reported an interaction between flotillin and RAB31 which promoted the entry of EGFR protein into MVB and subsequently the formation of ILVs [30]. Furthermore, these proteins also inhibited the fusion of MVBs with lysosomes by inactivating RAB7, thereby promoting the secretion of exosomes [36]. The interaction between other tetraspanin proteins such as CD9, CD10, CD63, and CD81 was also reported to induce the formation of tetraspanin-enriched microdomains, which play fundamental roles in exosome biogenesis [37]. Recent evidence has also revealed that the inhibition of the ESCRT complex promotes the secretion of CD63-positive and CD9-enriched exosomes [38, 39]. However, the detailed roles of these tetraspanin proteins in exosome production remain unclear.

miRNA biogenesis and isomiR formation

miRNAs are single-stranded, small non-coding RNA that are approximately 22 nucleotides in length [40]. They bind to a target messenger RNA's (mRNA) 3′ untranslated region (UTR) to reduce mRNA translation or induce mRNA silencing and degradation [41]. The binding sites of miRNA are called the seed sequence, commonly located at positions 2 to 8 from the 5′ end of a miRNA. However, other nucleotide positions such as positions 8, or 13 to 16 are also involved in target binding [42, 43]. Due to their short-binding motif, the miRNA-target interaction is not specific to one binding site. Each miRNA has multiple binding sites that can either bind to different mRNAs or the same mRNA from different sites [44–46]. For example, although let-7, miR-48, and miR-241 have identical sequences in the first eight nucleotides, each targets the same mRNA from different sites [47]. Therefore, the silencing of an mRNA target could also be a combination effect of multiple miRNAs directly targeting its 3′ UTR [48].

In general, the miRNA sequences are identified based on their sequences listed in the miRBase data bank [49]. However, the advancement in high-throughput sequencing technologies such as next-generation sequencing (NGS) have resulted in the detection miRNA isoforms, also known as isomiRs [50]. Similar to protein isoforms, isomiRs are miRNA variants that arise from a single miRNA host gene. Subsequently, they underwent sequence modifications at the 3′ or/and 5′ end which made them different from their respective canonical miRNAs [51]. At first, they were interpreted as sequencing errors and artifacts [52]. Eventually, isomiRs were proven functional by working with Argonaute (Ago) protein to inhibit mRNA targets [53].

The biogenesis of human canonical miRNAs and isomiRs are interconnected. Like most of the gene transcription, miRNA's biogenesis first occurs in the nucleus (Fig. 2). The miRNA host genes are transcribed into long primary transcripts, known as the primary miRNAs (pri-miRNA) by RNA polymerase II [54]. Next, the double-stranded RNA binding protein DiGeorge Syndrome Critical Region 8 (DGCR8) interacts with Drosha to facilitate Drosha cleavage at approximately 22 nucleotides away from the terminal loop of pri-miRNA. The cleavage of pri-miRNAs by Drosha produces a hairpin structure precursor miRNA (pre-miRNA). Together, DGCR8, RNase III enzyme Drosha, and other associated proteins make up the Microprocessor complex [54, 55]. Pre-miRNAs are then transported out of the nucleus into the cytoplasm by the nucleocytoplasmic transporter complex consisting of exportin 5 and Ran-GTP [56]. In the cytoplasm, the RNA binding protein TRBP and Dicer facilitate the cleavage of pre-miRNAs to produce a 21nt in-length miRNA duplex, comprising a guide strand and a passenger strand. The duplex is then loaded onto the Ago protein to form the RNA-induced silencing complex (RISC) with the assistance of HSP70/HSP90 chaperone machinery. Subsequently, the passenger strand of the miRNA duplex is cleaved from the pre-RISC and removed by the endonuclease C3PO. However, some of the passenger strands tend to be retained due to the imperfect base pairing in the middle region of the miRNA duplex that increases passenger strand stability. Hence, on the occasion where both strands were loaded into RISC, the strand that produces from the 5′ end of the stem-loop will be referred to as '5p' while the strand that produces from the 3′ end of the stem-loop will be referred to as '3p' [54, 55]. Finally, the Ago-miRNA complex binds to a targeted region of mRNA and induces translational repression with assistance from GW182 proteins [56].

In most cases, the sequence variations in isomiRs are the results of Drosha and Dicer cleavage imprecisions [57]. Drosha commonly cleaves at approximately 22 nucleotides away from the terminal loop of pri-miRNAs [58]. Meanwhile, Dicer cleaves precisely at two sites within the stem-loop region of pre-miRNA, resulting in a miRNA duplex with a 3′ overhang of two-nucleotides. Warf *et al.* observed that some Drosha and Dicer did not cleave precisely at the predicted positions but rather at adjacent sites proximal to the target sites [59]. These cleavage imprecisions were somehow influenced by the nucleotide sequence or secondary structure motifs of miRNA precursors. For instance, distorted miRNA precursor structures caused by bulges or internal loops gave rise to alternative cleavage positions for Drosha and Dicer, leading to the formation of isomiRs of various lengths and altered seed sequences [60–63]. In the case of miR-142, a shift within its pri-miRNA sequence generated multiple 5′ isomiRs, each targeting different sets of mRNA genes [64]. Likewise, the presence of a sliding-bulge structure within the pre-miRNAs sequence of miR-203 resulted in the generation of various 5′ isomiRs [65]. Apart from that, Roslan *et al.* also observed that nucleotide sequences at the ribonuclease cleavage sites would determine both Drosha and Dicer cleavage positions. Drosha and Dicer cleavage sites rarely contain G-nucleotides but exhibit a strong preference for U-nucleotides [66, 67]. Therefore, these findings suggested that the Drosha and Dicer cleavage sites are not just located at a fixed position away from terminal loops but rather have a certain level of flexibility which gave rise to isomiRs.

After Drosha and Dicer cleavage, post-translational modification processes such as non-templated nucleotide addition (NTA) further contribute to the repertoire of isomiRs. The addition of nucleotides to the 3′ or 5′ end of a miRNA facilitated by nucleotidyl transferases has shifted the existing sequence of canonical miRNAs, which in turn alters the miRNA seed sequence [68]. The most prevalent form of NTA is the 3′ uridylation and adenylation [69, 70]. IsomiRs underwent these modifications not only exhibit different expression levels and stability as compared to their respective canonical miRNAs, but also have different miRNA targets [69, 71, 72]. Notably, this process also affects the sorting of miRNAs into exosomes in which the 3′ uridylated miRNAs were

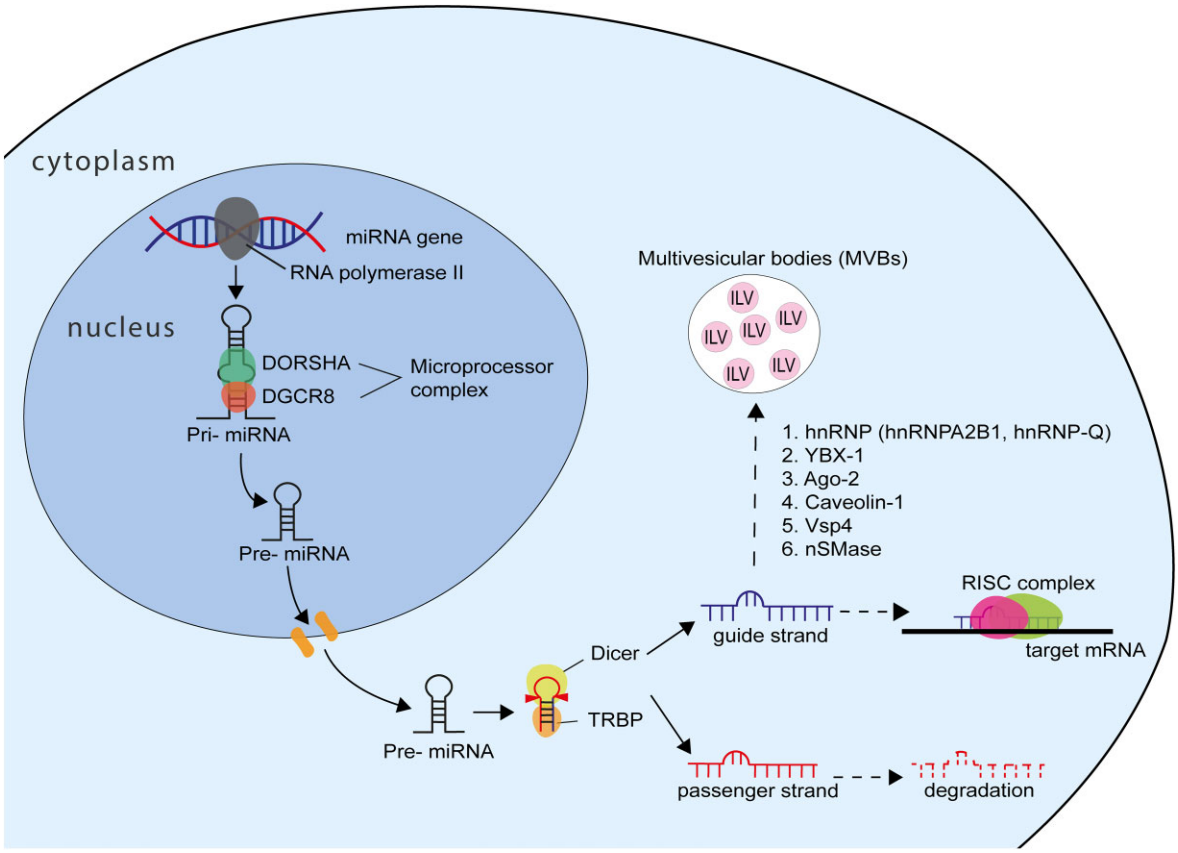

Figure 2. Canonical pathway of miRNA biogenesis and loading mechanism of mature miRNAs into exosomes. In the nucleus, miRNA genes are transcribed by RNA polymerase II into primary-miRNA (pri-miRNA). Pri-miRNAs are processed by the microprocessor complex, which consists of DORSHA, DGCR8, and RNA-binding proteins, into precursor miRNAs (pre-miRNA). Next, the pre-miRNA is exported out of the nucleus into the cytoplasm by exportin 5 and RanGTP. In the cytoplasm, the pre-miRNA is cleaved by Dicer and TRBP to form a miRNA duplex. The guide strand is loaded onto Argonaute (Ago) protein within the RNA-induced silencing complex (RISC) with the assistance of HSP70/HSP90 chaperone machinery, while the passenger strand is typically degraded. The proposed mechanisms involved in the sorting of miRNAs into exosomes include (1) heterogeneous nuclear ribonucleoproteins (hnRNP)-dependent pathway; (2) Y-box binding protein 1 (YBX-1)-dependent pathway; (3) the Ago-2-dependent pathway; (4) Caveolin-1-dependent pathway; (5) Vsp4-dependent pathway; (6) nSMase-dependent pathway.

preferentially sorted into exosomes, while 3′ adenylated miRNAs tend to be retained within parent cells [73].

Other alterations in miRNA precursor sequence such as RNA-editing and single nucleotide polymorphism (SNP) also occur in isomiR formation. The most abundant type of RNA-editing is the Adenosine-to-Inosine editing by the Adenosine deaminases acting on RNA (ADARs), which account for 90% of all RNA editing events [74, 75]. This type of RNA editing occurs primarily at the pri-miRNA level [76, 77]. Trontii *et al.* showed that 58.9% of miRNA undergo RNA editing, including those with modifications in the seed region were found to have different mRNA targets compared to their unedited counterparts [78]. On the other hand, SNPs have been demonstrated to affect the cleavage sites for Drosha and Dicer processors, thereby resulting in different miRNA isoforms [79, 80]. Taken together, the presence of isomiRs in human cells is not merely a misinterpretation of deep sequencing data.

The roles of exosomal miRNAs in T2DM pathogenesis

While the loading mechanisms of miRNAs into exosomes are not completely understood, the fact that miRNAs are discordantly distributed across tissues and exosomes indicates that it is not a random process [81]. In many cases, the biological condition of cells is often reflected in their exosomal miRNA profiles. Furthermore, the transfer of miRNAs via exosomes between cells appears to influence mRNA translation in recipient cells [82–86] (Fig. 3). As the pioneer of exosome research, Valadi *et al.* were one of the first to demonstrate the transfer of miRNAs from mouse exosomes to human mast cells in 2007 [87]. Since then, the intercellular transfer of miRNAs within exosomes between human tissues has been extensively studied for their roles in disease development [88, 89]. In this regard, the following sections will discuss how exosomal miRNAs are involved in the intercellular signalling between various insulin target sites relating to the onset of T2DM.

Adipocyte-derived exosomal miRNAs and T2DM

Obesity is a major risk factor of T2DM. In the context of obesity, there is a shift in adipocyte phenotypes, in which they undergo hypertrophy and hyperplasia in response to excessive calorie intake [90]. At the same time, these adipocytes are also known as the primary source of exosomal miRNA production [91]. They secrete miRNAs-enriched exosomes which not only remain within the adipose tissue but are also transported to other distant insulin-targeting tissues. Firstly, adipocyte-derived exosomal miRNAs affect adipogenesis and adipocyte differentiation through autocrine signalling. The upregulation of miR-122 in adipocyte-derived exosomes could promote adipogenesis by targeting the vitamin D3 receptor (*VDR*) gene, a negative regulator of the sterol

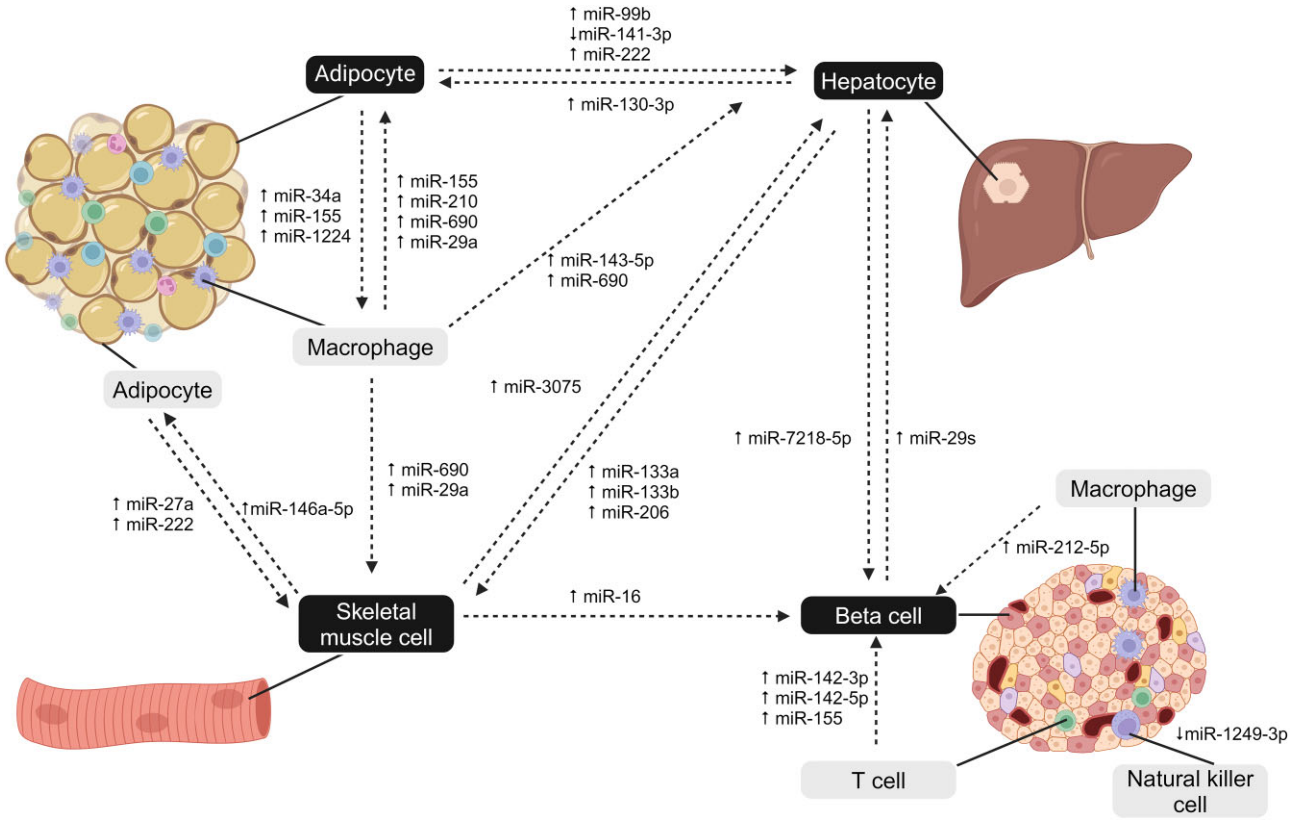

Figure 3. The roles of exosomal miRNAs in facilitating interorgan crosstalk between the primary sites of insulin action. miRNAs are differentially regulated in exosomes produced by different cell types. Exosomal miRNAs are shuttled between the primary sites of insulin action, thereby contributing to the development of T2DM by inducing obesity, insulin resistance, and β cell dysfunction. Created with Biorender.com.

regulatory element-binding transcription factor 1 (SREBF1) [92]. Ojima *et al*. demonstrated that during the adipocyte differentiation stage, they release miRNAs-enriched exosomes to promote their own differentiation, however, these exosomes were found to suppress skeletal muscle differentiation [93]. Next, adipocyte-derived exosomes are shown to either induce or suppress macrophage polarization, depending on their miRNA contents through paracrine signalling. For example, the upregulation of miR-155 in adipocyte-derived exosomes promotes M1 macrophage polarization by targeting the Suppressor of cytokine signalling 1 (*SOSC1*) gene [94]. On the other hand, the high levels of miR-34a and miR-1224 in adipocyte-derived exosomes suppress M2 macrophage polarization by targeting the Krüppel-like factor 4 (*Klf4*) and musashi RNA binding protein 2 (*MSI2*) genes, respectively [95, 96]. As a result, the accumulation of M1 macrophages triggers the release of pro-inflammatory cytokines within the adipose tissue environment, contributing to the occurrence of insulin resistance.

Lastly, adipocyte-derived exosomes modulate the gene expressions of recipient cells in other organs through endocrine signalling. The effects, however, are influenced by their miRNA content and the pathophysiological state of the adipocytes when these exosomes are produced. In β cells, exosomes produced by healthy adipocytes promoted cell survival and insulin secretion, while exosomes produced by inflamed adipocytes induced β cell death and insulin resistance [97]. In skeletal muscle cells, adipocyte-derived exosomal miR-27a induces insulin resistance by targeting Peroxisome proliferator-activated receptor gamma (*PPARγ*) gene [91]. Further studies using animal models also confirmed the upregulation of serum exosomal miR-27a in obese mice, and these exosomal miR-27a were significantly reduced followed by exercise intervention as a result of white adipose tissue browning [98]. Moreover, exosomal miR-222 produced by adipocytes suppress the insulin receptor substrate 1 (*IRS1*) gene, one of the upstream proteins of the PI3K/Akt signalling pathway, thus reducing the glucose uptake ability of skeletal muscle cells and hepatocytes [99]. In hepatocytes, miR-99b-enriched exosomes were found to reduce the fibroblast growth factor 21 (*FGF21*) gene, leading to impaired insulin sensitivity in hepatocytes [100]. On the contrary, miR-141-3p-deficient exosomes in hepatocytes show association with impaired insulin action through the upregulation of its direct target, phosphatase and tensin homolog (PTEN), a negative regulator of the PI3K/Akt pathway that is involved in glucose uptake [101]. Finally, despite the mechanisms remaining elusive, there has been evidence that adipocyte-derived exosomal miRNAs could induce insulin resistance even in cardiac muscle cells. The uptake of miR-802-5p-enriched exosomes into cardiac muscle cells suppress the heat-shock protein 60 (HSP60), a mitochondrial chaperone protein that plays crucial role in maintaining mitochondrial function [102].

Immune cell-derived exosomal miRNAs and T2DM

The immune cells play a crucial role in the development of T2DM by producing various pro-inflammatory cytokines that contribute to low-grade tissue inflammation and insulin resistance. Macrophages, the most abundant immune cells in the adipose tissue, released miRNAs enriched exosomes delivered into adipocytes to facilitate the insulin action and glucose uptake

into the adipocytes. For instance, the adipose tissue macrophages (ADMs)- derived exosomal miR-155 suppressed *PPAR-γ*, causing a reduction in the glucose 4 transporter (GLUT4) expression and glucose uptake into the adipocytes [103]. Further study suggested that these miR-155-enriched exosomes could even be transported to distant sites such as the pancreas and taken up into β cells, exerting an inhibitory effect on the glucose-stimulated insulin secretion in β cells [104]. Remarkably, miR-155 was upregulated in exosomes secreted by M1-polarized islet-resided macrophages [105]. Additionally, Tian *et al*. reported that exosomal miR-210 produced by ADMs also targeted the NADH dehydrogenase ubiquinone 1 alpha subcomplex 4 (*NDUFA4*) gene in adipocytes and reduced their glucose uptake ability as well as mitochondrial function [106]. Subsequently, a follow-up study demonstrated an upregulation of serum exosomal miR-210 in obese individuals, and the increased exosomal miR-210 level shown a positive association with body mass index (BMI) and T2DM risk [107]. Exosomal miR-29a from ADMs, on the other hand, was found to reduce the glucose uptake ability of adipocytes and myocytes by targeting the peroxisome proliferator-activated receptor delta (*PPAR-δ*) gene, leading to the development of insulin resistance [108].

Besides all these, it should also be noted that ADMs produce exosomes of distinct miRNA content depending on their polarization state. For example, the M1-polarized ADMs produced exosomes with high levels of miR-503-5p, altering adipocytes gene expression [109]. In contrast, when polarized towards the M2 state, the macrophages secrete exosomes enriched in miR-690, improving insulin sensitivity in adipocytes, hepatocytes, and skeletal muscle cells [110]. Further to this, in the liver, the M1-polarized macrophages secrete exosomes enriched in miR-143-5p and induce insulin resistance by targeting the mitogen-activated protein kinase phosphatase-5 (*MKP5*) [111]. In the pancreas, islet-resided macrophages secrete exosomes enriched in miR-212-5p, targeting the *SIRT1* in β cells, which subsequently inhibit insulin secretion through inactivating the AKT signalling pathway [112].

Besides macrophages, overexpression of miR-142-3p, miR-142-5p, and miR-155 in T cells-derived exosomes was also shown to induce cell death by stimulating the production of chemokines [113]. However, the effect of immune cell-derived exosomal miRNAs is not entirely detrimental. miR-1249-3p in exosomes released by natural killer (NK) cells was found to suppress the release of pro-inflammatory cytokines in adipocytes and hepatocytes by targeting the SKI family transcriptional corepressor 1 (*SKOR1*) gene, leading to the alleviation of insulin resistance [114].

Skeletal muscle cell-derived exosomal miRNAs and T2DM

The skeletal muscle, as one of the largest tissues, contributes significantly to the production of exosomes. However, research on the skeletal muscle cells-derived exosomes is relatively limited, with only a handful of studies reported on this subject. Jalabert *et al*. reported that skeletal muscle cells of mice fed with a high palmitate diet produced exosomes with upregulated levels of miR-16. These exosomes were transported to the pancreas and promoted β cell proliferation through the downregulation of Protein patched homolog 1 (*Ptch1*) gene, which could be a potential mechanism causing β cell hyperplasia [115]. Besides that, Qin *et al*. reported that skeletal muscle cells-derived exosomal miR-146a-5p suppressed the differentiation of pre-adipocytes into mature adipocytes by inhibiting PPAR-γ signalling. Therefore, exosomal miR-146a-5p shows great potential as a therapeutic target for the treatment of obesity-induced T2DM [116].

On a side note, physical exercise is widely known to enhance insulin sensitivity in muscle cells. With respect to this, Castaño *et al*. reported the beneficial effects of skeletal muscle-derived exosomes from trained animals. The three miRNAs that were enriched in these exosomes, namely miR-133a, miR-133b, and miR-206, were demonstrated to reduce gluconeogenesis and improve glucose tolerance in hepatocytes by targeting the Forkhead box protein O1 (*FoxO1*) gene [117]. However, the intricate mechanisms of these miRNAs still require further elucidation.

Hepatocyte-derived exosomal miRNAs and T2DM

The liver, as a major organ for glucose metabolism and lipid synthesis, plays a pivotal role in the development of T2DM. Within the liver, hepatocytes are specifically involved in several important metabolic processes such as gluconeogenesis, glycogenesis, and lipogenesis. Similar to other metabolic organs, hepatocytes also produce exosomes with distinct profiles of miRNAs where they could modulate gene expressions when uptake by recipient cells. For instance, miR-130-5p is transported in the exosomes from hepatocytes to adipocytes, affecting the GLUT4 receptors translocation in recipient cells by targeting the PH domain leucine-rich repeat protein phosphatase 2 (*PHLPP2*) in a cell culture study [118]. Additionally, miR-7218-5p is transported in the exosomes from hepatocytes to β cells, promoting β cell proliferation [119]. Lastly, hepatocytes secrete exosomes enriched in miR-3075 during the early stage of obesity, which are either kept in the liver, or transported to adipocytes and skeletal muscle. Bioinformatics analyses confirmed that miR-3075 bind to the fatty acid 2-Hydroxylase (*FA2H*) gene, however, the downstream regulatory mechanisms remain unclear [120].

Pancreatic β cell-derived exosomal miRNAs and T2DM

Apart from insulin secretion, pancreatic β cells also function as a glucose sensor to facilitate the balance between insulin and glucagon production [121]. In the initial phase of hyperglycaemia, β cells increase proliferation to compensate for insulin secretion in order to maintain normoglycaemia [122]. As the condition progresses, their proliferation capacity deteriorates as a result of endoplasmic reticulum (ER) stress [123]. Consequently, the progressive loss of β cells reduces insulin production, further accelerates the development of T2DM. In this context, Xu *et al*. has identified that miR-26a in β cell-derived exosomes can target the cell proliferation pathways in β cells, thereby reducing the compensatory hyperplasia of β cells. These exosomes can also be released into the circulation and delivered to hepatocytes, leading to improved insulin sensitivity [124]. Conversely, the miR-29 family (consists of miR-29a, miR-29b, and miR-29c) in β cell-derived exosomal miRNAs were shown to reduce insulin sensitivity in hepatocytes through the inhibition of p85 subunit of PI3K signalling pathway in hepatocytes.

Besides regulating cell functions, many studies also suggest the use of β cell-derived exosomal miRNAs as biomarkers for islet damage. Fu *et al*. reported that the chronic exposure of β cells to proinflammatory cytokines such as tumour necrosis factor alpha (TNF-α), interleukin 1 beta (IL-1β), and interferon gamma (IFN-γ) induced changes in the β cells exosome contents, leading to the overexpression of miR-375-3p and miR-129-3p. Subsequent experiments also confirmed that exosomal miR-375-3p expression was significantly increased in the serum exosomes of early-stage

Table 1. Differentially expressed exosomal miRNAs in T2DM patients compared to healthy control, as evidenced by clinical studies.

miRNAs	Sample type	Expression change	Sample size	References
miR-375-3p	Serum	Upregulated	N/A	Fu *et al.* [125]
miR-20b-5p, miR-150-5p	Serum	Upregulated	21(T2DM)/16(IGT)	Katayama *et al.* [127]
miR-20b-5p	Serum	Upregulated	10(T2DM)/10(CG)	Chen *et al.* [128]
miR-23a-5p, miR-6087	Serum	Upregulated	13(T2DM)/18(CG)	Kim *et al.* [83]
miR-6751-3p	Serum	Downregulated	13(T2DM)/18(CG)	Kim *et al.* [83]
miR-551b-3p	Serum	Downregulated	15(T2DM)/7(CG)	Dracheva *et al.* [129]

CG: control group; IGT: impaired glucose tolerance; T2DM: type 2 diabetes mellitus.

T2DM patients [125]. Besides that, the miRNA contents of β cells-derived exosomes undergo changes from the initial stage of cell stress to later stage of cell apoptosis. Saravanan *et al.* reported that miR-29b-3p and miR-216-5p were first increased in exosomes at the initial stage of cell stress, followed by the increase of miR-148-3p and miR-375 in β cells-derived exosomes after 24hr of cytokine exposure that indicates induction of β cells apoptosis [126].

Exosomal miRNAs in clinical studies of T2DM

Building on the groundwork of experimental findings from both cell culture and preclinical studies, a few clinical studies have demonstrated the distinct miRNA expression in exosomes between T2DM subjects and healthy controls (Table 1). In 2018, Fu *et al.* published the first paper to identify the differences in serum exosomal miRNA profiles of individuals with newly diagnosed T1DM and T2DM. The results showed a 2.8-fold increase of exosomal miR-375-3p in serum of early-stage T2DM patients as compared to individuals with normal glucose tolerance. Further *in vitro* model study discovered that the miR-375-3p was primarily released by β cells in response to injury factors and cytokines [125]. Next, Katayama *et al.* reported that 1.52-fold upregulation of serum exosomal miR-20b-5p and 1.65-fold upregulation of miR-150-5p in T2DM patients as compared to individuals with normal glucose tolerance. At the same time, these upregulations were positively correlated with an increase in the oral glucose tolerance test and body fat mass. Interestingly, these changes were observed only with serum exosomal miRNAs, not total serum miRNAs. Subsequent *in vitro* studies using human skeletal muscle cells showed that miR-20b-5p repressed several mRNA genes such as AKT-interacting protein (*AKTIP*) and signal transducer and activator of transcription 3 (*STAT3*), which explained the impaired insulin signalling pathway observed in T2DM patients [127]. Additionally, these findings were supported by Chen *et al.*, who similarly demonstrated that miR-20b-5p was upregulated in exosomes of T2DM patients. In addition, they also reported another target of miR-20b-5p, the vascular endothelial growth factor (*VEGF*) gene, in which miRNA directly bound and led to diabetic foot ulcers [128]. In a prospective study using NGS, Kim *et al.* reported a list of dysregulated miRNAs in exosomes of obese and T2DM subjects. Interestingly, most of these exosomal miRNAs found in T2DM overlap with those found in obesity, confirming the epigenetic association between obesity and T2DM. In particular, there were 4-fold upregulation of miR-23a-5p, 2.03-fold upregulation of miR-6082, and 2.86-fold downregulation of miR-6751-3p in exosomes of obese and T2DM subjects as compared to control subjects [83]. Lastly, Dracheva *et al.* reported that miR-551b-3p was downregulated in serum exosomes of obese subjects. However, compared to patients with only obesity, those with T2DM as comorbidity had significantly higher levels of miR-551b-3p in their serum exosomes. The ROC analysis showed AUC value of miR-551b-3p was 0.770 (95%CI 0.678–0.963), with a sensitivity of 0.60 and a specificity of 0.889. In addition, miR-551-3p was positively correlated to HbA1c values [129]. Collectively, these above findings provide evidence that the changes in exosomal miRNA expression take place not only in cell culture studies and animal models, but also in humans, further highlighting the great potential of exosomal miRNAs in T2DM diagnosis.

Prospects in personalized medicine: combining exosomal miRNAs and isomiRs as biomarkers for diagnosing T2DM

With the advancement of NGS, exosomal miRNAs have been extensively studied for their roles as disease biomarkers, including for the diagnosis of T2DM, since they are implicated in the disease development and act as mediators of intercellular communication across various insulin-targeting sites. Recently, several studies have discovered that isomiRs too are differentially expressed within exosomes [130]. In other words, the formation of certain isomiRs is disease-specific [131]. As such, many researchers have suggested that it should be mandatory to include isomiRs analysis in all NGS studies [132]. The information is extremely relevant to exosomal miRNA research because when the abundance of precursor miRNA remains constant, changes in canonical miRNAs can be reciprocated in isomiRs expression [133, 134].

In the context of T2DM, findings from Baran-Gale *et al.* revealed that the 5′ shifted isomiRs of miR-375 in human β cells had an even stronger correlation to T2DM genes than their canonical miRNAs. It is noteworthy that miR-375 was previously found to be upregulated in the serum exosomes of T2DM patients, as well as β cells in response to injury factors. However, the evidence of isomiRs was lacking to provide a more comprehensive understanding of how 5′-shifted isomiRs contributed to the development of T2DM [135]. Nonetheless, given their unique expression profiles, these isomiRs still hold great potential to be further explored as T2DM biomarkers. Furthermore, the receiver operating characteristic (ROC) analysis, which is often used to evaluate diagnosis performance, also demonstrated high discriminative power of isomiRs signatures between T2DM patients and healthy subjects. In addition to that, the isomiR expression profiles can also be highly distinguished between T1DM and T2DM patients (AUC = 0.75, 95% CI 0.65–0.84, $p < 0.001$) [136]. All these findings have suggested the importance of incorporating isomiRs into analysis as differential expression results might be misinterpreted by canonical miRNA sequences alone.

Another useful approach to interpret exosomal miRNAs and isomiRs data would be to integrate them with the existing T2DM parameters, such as HbA1c and fasting blood glucose (FBG), which could further improve the diagnostic precision. Previously, several studies demonstrated that by incorporating exosomal miRNAs into the ROC curve, the diagnostic power increased signifi-

cantly when compared to using conventional parameters alone. For example, by including exosomal miR-223 in the diagnostic model for epithelial ovarian cancer, the diagnostic value increased from 0.928 to 0.944 when compared to using the conventional parameter CA125 alone [137]. The addition of exosomal miR-1258 together with neutrophil counts and neutrophil to lymphocyte ratio also increased the diagnostic model to predict chronic obstructive pulmonary disease (COPD) from 0.900 to 0.983 [138]. These findings suggest that using exosomal miRNAs and isomiRs, in conjunction with the conventional T2DM biomarkers such as HbA1c and FBG, will be an approach to enhance T2DM diagnosis.

Over the past years, research on exosomal miRNAs and their isomiRs has progressed from fundamental research to clinical studies. Thus, the next step forward would be to explore their potential from the perspective of personalized medicine. One of the main aspects of personalized medicine is the accurate diagnosis of disease. Diabetes is not a singular disease but rather divided into different subtypes with varying conditions. As expressions of miRNAs and their isomiRs in exosomes are specific to T2DM, hence, they can be used to distinguish between T2DM from T1DM [136]. Furthermore, the prevalence of T2DM varies with age, sex, and living lifestyle. Currently, the standard diagnostic criteria of T2DM are FBG and HbA1c [139]. However, many researchers have raised concerns about depending solely on FBG and HbA1c to diagnose T2DM as these parameters are influenced by various factors. For instance, HbA1c was reported to correlate less with FBG in men than in women, leading to under-diagnosis of T2DM in men [140]. Yet, in certain ethnic groups such as African Americans, Hispanics, and Asians, the HbA1c values are overestimated [141]. In this respect, exosomal miRNAs and isomiRs could be wider used in diagnosis of T2DM apart from FBG and HbA1c.

Given the distinctive profiles of miRNAs and isomiRs in various diseases, they can also contribute to the advancement of personalized medicine in another aspect, preventive medicine. Recent studies have reported the changes in exosomal miRNA expression profiles as an individual develops complications from T2DM. Hence, the use of exosomal miRNAs and isomiRs for T2DM should not be just limited to diagnosis alone. For example, Zhao *et al.* reported that patients with diabetic kidney disease had significant upregulation of miR-4687-3p in urinary exosomes compared to T2DM patients. ROC analysis indicated that miR-4687-3p had an AUC value of 0.786 (95% CI 0.607–0.965, $p = 0.01$) [142]. Jiang *et al.* reported that patients with diabetic macular oedema had significant upregulation of miR-377-3p in serum exosomes compared to T2DM patients. ROC analysis indicated that miR-377-3p had an AUC value of 0.778 (95% CI 0.638–0.918, $p = 0.001$) [143]. Wang *et al.* reported that miR-181b-5p was upregulated in serum exosomes of patients with diabetic foot ulcer as compared to T2DM [144]. As a result, the changes of exosomal miRNAs, and potentially isomiRs profiles of T2DM patients, can be useful tools to predict and track the onset of T2DM complications.

Challenges and future work

Despite the bright prospects, there are still a number of challenges that limit the clinical applications of exosomal miRNAs and isomiRs. Firstly, the fundamental aspects of EV biology such as its biogenesis and the sorting of biomolecules into exosomes are still subjects of ongoing research. Currently, EVs are distinguished by their surface tetraspanin proteins such as CD9, CD63, and CD81. However, studies showed that tetraspanin proteins alone are not sufficient to distinguish between exosomes from other types of EVs in blood samples, as they were also detected in endosome and platelet surfaces [145]. Furthermore, since miRNA profiles in exosomes are influenced by the conditions of their parent cells, the loading mechanism may vary with the parent cell environment. Therefore, unless the intracellular formation of exosomes can be clearly demonstrated by video microscopy or novel tracking techniques, it remains a great challenge to distinguish them from other EV subpopulations and to study the underlying mechanisms of how miRNAs and isomiRs are loaded into exosomes.

Secondly, despite the findings from several clinical studies that reported systemic dysregulations of exosomal miRNAs in disease conditions, the cellular origins of such circulating exosomes are not sufficiently studied. Without the knowledge of origin, there is a lack of clarity on whether exosomes are produced in response to certain conditions. In this case, multi-omics approaches may be useful in investigating the parental origins of exosomes. Since exosomes are products of the inward budding of cell plasma membranes, conducting lipidomics and proteomics analyses on the plasma membrane of various cell types alongside their respective exosomes may potentially identify cell-type specific markers on the exosome surface. In addition, single vesicle analysis methods, such as fluorescent or radiolabelling using flow cytometry and super-resolution microscopy, could provide deeper insights into the structural dynamics of exosomal membranes across varied conditions [146].

Thirdly, the experimental workflow to incorporate exosomal miRNAs and isomiRs into clinical settings has not been fully established and standardized. The pre-analytical steps such as isolating contaminant-free exosomes from biofluid remain a key challenge. Furthermore, the choice of exosome isolation method is mostly dependent on the downstream experiments. Therefore, more research and comparative studies are needed for standardizing exosome isolation methods to minimize results discrepancies due to different isolation methods. To date, most studies use unbiased sequencing methods such as NGS, instead of microarray, to do exosomal miRNA profiling. However, problems have always existed such as lacking standardized reference genes during the downstream PCR validation step [147]. Apart from the methods normalization, the selection of endogenous reference genes should be clearly justified as the use of unstable reference genes across groups may affect the reliability and reproducibility of gene expression results. Notably, using a combination of reference gene sets instead of only one reference gene to normalize the differential expression results better ensures a stable expression across biological samples [147, 148]. Alternatively, newer technologies such as digital PCR (dPCR) that allow absolute quantification of gene expressions could be used to mitigate the issues of unsuitable reference genes. As isomiR research is currently an emerging field, the existing validation techniques using RT-qPCR are not precise enough to discriminate isomiR variants from their canonical sequences [149, 150]. Appropriate methods must first be developed to ensure validity of isomiRs before they can be utilised as biomarkers. Moreover, most clinical studies that investigated the differential exosomal miRNA expression between control and disease groups lacked functional mRNA-miRNA interaction studies, or only validated single miRNA-mRNA interaction. In biological contexts, multiple miRNAs interact in a coordinated way to regulate a single mRNA expression. Thus, studying interactions between multiple miRNAs and a mRNA gene may be important to provide a better overview of the underlying mechanisms that govern disease development and progression.

Conclusion

During the developing stage of T2DM, miRNAs are selectively packaged into exosomes to facilitate crosstalk between the primary insulin target sites, such as the adipose tissue, liver, skeletal muscle cells, and pancreas. These miRNA-enriched exosomes are taken up by their target recipient cells and regulated a series of pathways leading to obesity, insulin resistance, and β cells dysfunction, particularly through the PI3K/Akt signalling pathway. As evidenced by existing clinical studies, exosomal miRNAs are differentially expressed in T2DM patients as compared to healthy controls. Therefore, exosomal miRNAs and their isomiRs hold great potential as biomarkers to enhance T2DM diagnosis, at the same time they can be used to predict and track the onset of T2DM and its complications. Overall, exosomal imRNAs and isomiRs can contribute to the advancement of personalized medicine and offer opportunities for tailored therapeutics for T2DM.

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Authors' contributions

YLS and YFP made substantial contributions to the conceptualization of the paper. YLS drafted the manuscript and figures. YFP, WMC, WR, and YP provided supervision support. YFP and WR involved in funding acquisition. WMC, WR, SMZ, SZSAK, YP, and YFP reviewed and edited the manuscript. All authors gave their approval of the version to be published.

Conflict of interests

All authors declared that there are no conflict of interests.

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