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Review:

Role of exosome-associated microRNA in diagnostic and therapeutic applications to metabolic disorders*

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Abstract: Metabolic disorders are classified clinically as a complex and varied group of diseases including metabolic syndrome, obesity, and diabetes mellitus. Fat toxicity, chronic inflammation, and oxidative stress, which may change cellular functions, are considered to play an essential role in the pathogenetic progress of metabolic disorders. Recent studies have found that cells secrete nanoscale vesicles containing proteins, lipids, nucleic acids, and membrane receptors, which mediate signal transduction and material transport to neighboring and distant cells. Exosomes, one type of such vesicles, are reported to participate in multiple pathological processes including tumor metastasis, atherosclerosis, chronic inflammation, and insulin resistance. Research on exosomes has focused mainly on the proteins they contain, but recently the function of exosome-associated microRNA has drawn a lot of attention. Exosome-associated microRNAs regulate the physiological function and pathological processes of metabolic disorders. They may also be useful as novel diagnostics and therapeutics given their special features of non-immunogenicity and quick extraction. In this paper, we summarize the structure, content, and functions of exosomes and the potential diagnostic and therapeutic applications of exosome-associated microRNAs in the treatment of metabolic disorders.

Key words: Metabolic disorders; Exosome; Exosome-associated microRNA; Non-alcoholic fatty liver disease; Obesity; Diabetes mellitus

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1 Introduction

Cells can generate and secrete a series of nanovesicles, which have biological structures similar to bilayer membranes. These nanovesicles have been described as extracellular vesicles (EVs) which may change the metabolic status of a target cell via fusion with its membrane, and transmit functional RNA or protein to distant sites within the body. Recent studies have shown that EVs are involved in multiple pathological processes, such as insulin resistance, lipid toxicity, dyslipidemia, endocrine disorders, hypercoagulable state, and chronic inflammation. Metabolic disorders are rapidly evolving into a global pandemic and pose a serious danger to human health. However, effective early detection methods and therapeutic strategies are lacking as patients are usually non-symptomatic until they progress to severe complications. EVs, especially exosomes, may provide a new non-invasive diagnostic tool and an effective treatment.

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According to their origin and status, cells can generate various types of EVs, such as apoptotic bodies, microvesicles, exosomes, oncosomes, and prostasomes (Vlassov et al., 2012), each named in accordance with their possible functions.

Apoptotic bodies, 50–2000 nm in diameter, are formed in the process of programmed cell death, which can be observed by light or electron microscopy. Nuclear chromatin comes together and disintegrates as the nucleus undergoes karyorrhexis and karyolysis. Fragmented chromatin pieces of variable size are secreted by budding directly from the plasma membrane. The shed vesicles, known as apoptotic bodies, contain cytoplasmic organelles and nuclear fragments (Hristov et al., 2004). Microvesicles, 100–1000 nm in diameter, are secreted into the extracellular environment by direct budding of cytoplasmic contents from the cell surface membrane. They contain protein, significant amounts of mRNA, microRNA (miRNA), and non-coding RNA, as well as some membrane receptors. Microvesicles have been reported to participate in the processes of intercellular communication, immune regulation, and metastasis (Thery et al., 2006; Lee et al., 2012). Exosomes are one of the most studied EVs, and are formed by invagination of the membranes of multivesicular endosomes (MVEs). Activated MVEs containing several exosomes become docked to the cell periphery and fuse with the plasma membrane. The exosomes are then released into the extracellular space for intercellular communication (Bobrie et al., 2011). However, there is controversy over the diameter of exosomes. Some studies have defined exosomes as vesicles with a diameter of 40–120 nm (El Andaloussi et al., 2013; Zaborowski et al., 2015), while others have applied the term to vesicles with a diameter of 40–100 nm (Raposo and Stoorvogel, 2013). Currently, popular EV isolation and purification methods cannot distinguish different types of EVs or demonstrate their cellular origin, especially exosomes and parts of microvesicles which are less than 200 nm in diameter.

While there is still much to learn about different kinds of EVs, recent research is helping us to better understand how different EVs, especially exosomes, are generated and secreted, and how they affect intercellular communication. Previous studies focused mainly on proteins carried by exosomes. Recently, more and more exosome-associated miRNAs have been found to be associated with different kinds of chronic diseases, especially metabolic disorders. Therefore, in this review, we aim to highlight the characterization of exosomes, current knowledge of their composition and biological functions, and the diagnostic and therapeutic potential of exosome-associated miRNAs for use in treating metabolic disorders.

2 Definition of exosomes

Trams et al. (1981) first observed nanovesicles in the supernatant of a tumor cell culture, denominating these vesicles “exosomes”. Subsequently, Johnstone et al. (1987) first isolated exosomes during a study of reticulocyte maturation and proved the presence of transferring receptors on their membranes. Later studies reported that exosomes could be generated and released by almost all animal cells, including blood cells such as mastocytes, T-lymphocytes, B-lymphocytes, and thrombocytes, and other cells like astrocytes, neurons, epithelial cells, dendritic cells (DCs), and hepatocytes (Laulagnier et al., 2004; Valadi et al., 2007; Conde-Vancells et al., 2008; Fei et al., 2015; Zhang et al., 2015b; Bosque et al., 2016). Early studies of exosomes were performed on supernatants of cultured cells, as well as human plasma or serum. Hawari et al. (2004) isolated exosomes from human serum and bronchoalveolar lavage fluid using ultracentrifugation and a sucrose density gradient. Besides plasma and serum, exosomes can be observed in and isolated from other body fluids, including urine, saliva, milk, amniotic fluid, ascites, and cerebrospinal fluid (Pisitkun et al., 2004; Runz et al., 2007; Asea et al., 2008; Ogawa et al., 2010; Liu et al., 2011; Street et al., 2012).

The mechanism for the generation and release of exosomes is very complex, involving many factors such as tetraspanins, ceramide and endosomal sorting complex responsible for transport (ESCRT). Exosomes are considered to originate from early MVEs, which have a bilayer structure similar to that of lysosomes without lysosomal enzymes (Fig. 1). Under certain stimulation conditions, such as cellular transformation, stress, and infection, the cell endocytosis system can be activated and followed by the

formation of early MVEs containing intraluminal vesicles (ILVs) (Raposo and Stoorvogel, 2013). Invagination of the mother cell membrane leads to the production of early MVEs covered with clathrin. As later MVEs bud from the early MVEs, variable sorting of their mRNA or miRNA contents can lead to later MVEs having different functions (Cha et al., 2015). Later MVEs can be transformed in one of two ways: some later MVEs containing cellular waste fuse with lysosomes, breaking down proteins on the surface of cells and the net structure of Golgi bodies (Fevrier and Raposo, 2004); other MVEs move to the cell periphery, dock with targeted sites, then release ILVs via fusion with the plasma membrane. These released ILVs or "exosomes" appear to have great physiological relevance in the human body (Harding et al., 1984; Lotvall and Valadi, 2007).

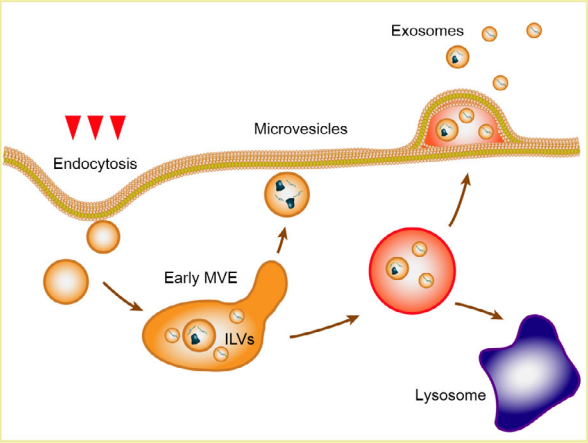

Fig. 1 Release of exosomes

Red triangles symbolize stimulations including cellular transformation, stress, or infection. Cellular endocytosis begins with some stimulation and is followed by the formation of early multivesicular endosomes (MVEs) containing intraluminal vesicles (ILVs). After a series of processes, early MVEs can be transformed into later MVEs which contain various RNAs and proteins. Exosomes are released via one of two pathways followed by later MVEs (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

3 Exosome structure, composition, and function

As stated above, exosomes originate from MVEs in almost all cells, and are secreted into the extracellular environment. Using standard negative staining methods for transmission electron microscopy (TEM), we can observe their typical lipid bilayer structure and cup-shaped morphology. Moreover, their orbicular structure can be observed by cryo-electron microscopy. van Niel et al. (2006) reported that the density of exosomes floating in sucrose solutions ranged from 1.13 to 1.19 g/ml. The process of exosome formation determines their composition which includes certain components of the mother cell cytoplasm. Previous studies reported that the main lipid composition of the membrane of exosomes included phospholipids, cholesterol, and a few glycolipids, which is similar to the composition of the plasma membrane (Vlassov et al., 2012). Unlike the plasma membrane, the membrane of exosomes was rich in cholesterol and sphingomyelin but had only small amounts of lecithin and phosphatidyl ethanolamine (Subra et al., 2010). These features of the exosome membrane, especially the high cholesterol levels, may enhance membrane fluidity thereby improving the stability of the exosomes. Lipid rafts are microdomains of a lipid bilayer membrane with a unique protein and lipid composition detected on the plasma membrane. Similar microdomains exist on the membranes of exosomes, and their collapse may be closely related to the formation of exosomes. Tan et al. (2013) showed that the formation of mesenchymal stem cell (MSC)-exosomes begins with the collapse of MVE-associated microdomains and the packing of annexins that mediate exosome transfer and assemblage.

Many proteins have been found to be carried by exosomes. Some, such as flotillin, which participate in the invagination of the bilayer structure, are related to the formation and movement of MVEs. Some protein complexes, like soluble *N*-ethylmaleimide-sensitive factor (NSF) attachment protein receptors (SNAREs), mediate the fusion of MVEs with the cell surface membrane of target cells. Other proteins, especially the Rab family, may regulate multiple steps in the transport of exosomes, including their formation, their movement (along with tubulin and actin), and fusion of their membranes (Mears et al., 2004). Some studies have found that suppression of the function of Rab35, a member of the Ras superfamily of monomeric G proteins, may promote the assemblage of MVEs and reduce the release of exosomes (Stenmark, 2009). Similarly, knockdown of Rab35 significantly inhibits the fusion of MVEs with the plasma membrane (Hsu et al., 2010). Using an RNA interference screen, Ostrowski et al. (2010) identified five members of Rab GTPase families which may

promote exosome secretion in HeLa cells. The silencing of Rab27a and Rab27b showed different effects on the exosome pathway. Silencing Rab27a led to an obvious increase in the size of MVEs, but reduced the total amount of exosomes secreted in the cell-culture supernatant. Silencing Rab27b resulted in the redistribution of MVEs without modification of their specific protein composition.

Besides proteins participating in the transport, docking, and release of exosomes, studies have also reported the presence of a whole family of transmembrane proteins-tetraspanins, also known as the transmembrane 4 superfamily (TM4SF). These proteins have four transmembrane domains, short N- and C-hydrophobic termini within the cells, and two extracellular loops. TM4SF proteins, enriched on the membrane of exosomes and MVEs, are highly relevant to the formation of MVEs (Yauch and Hemler, 2000). Escola et al. (1998) first proved the existence of CD63, CD81, and CD82 on the exosomes released by B-lymphocytes. These are now used as exosome protein markers. In addition, the ESCRT consists of ESCRT-0, ESCRT-I, ESCRT-II, and ESCRT-III, and accessory proteins such as Alix and VPS4, which play an important role in the fusion of MVEs with the cellular membrane. Interestingly, some studies have reported that tetraspanins, particularly CD63 and CD81, may be associated with the functions of ESCRT (Raiborg and Stenmark, 2009; Gan and Gould, 2011) (Fig. 2).

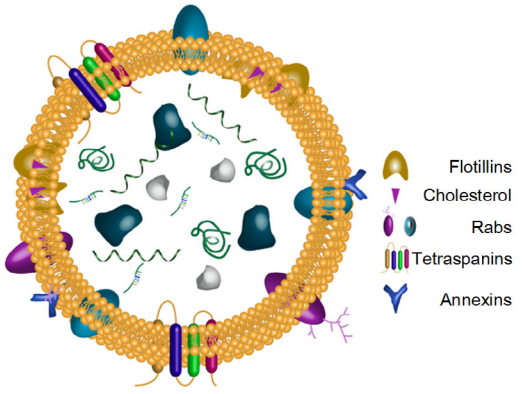

Fig. 2 Molecular structure of exosomes
Exosome membranes consist of a lipid bilayer, similar to that of the cell plasma membrane, and some functional proteins, symbolized as shown in the key. The cargo carried by exosomes contains proteins (shown as blobs) and RNAs (shown as green ribbons) (Note: for interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

Owing to their special structure and enriched composition, exosomes play an important role in a bewildering array of biological processes, which suggests that exosomes may be useful as critical markers in the initial diagnosis, treatment, and prognosis of different diseases. In the following paragraphs, we will discuss the known biological functions of exosomes.

3.1 Exosome-mediated miRNA transfer

Most cells of the human body can secrete exosomes containing mRNA, miRNA, and other non-coding RNA. For example, about 100 different miRNAs have been detected in exosomes secreted by mast cells (Valadi et al., 2007; Cortez et al., 2011). Exosome-associated miRNAs can be stabilized in circulation under the protection of the vesicular structure, and then be transferred into target cells to inhibit the expression of certain genes (Zhang et al., 2010; de Jong et al., 2012). Long non-coding RNA (lncRNA) is a newly discovered non-protein coding RNA comprising transcripts longer than 200 nucleotides. Interestingly, lncRNAs are found in exosomes. Ahadi et al. (2016) reported that certain lncRNAs, which contain miRNA seeds such as let-7 family members as well as miR-17, miR-18a, and miR-20a, were enriched in exosomes derived from prostate cancer, and probably participated in tumor initiation and progression.

3.2 Exosome-mediated regulation of tumor microenvironments

Cancer-associated exosomes are considered as messengers for communicating signals to local and remote cells and tissues, thereby remodeling tissue matrices. Exosomes released by melanoma cells can launch a proangiogenic program, which might initiate preparation for lymphatic metastasis of cancer cells (Hood et al., 2011). Further studies have found that miRNAs carried by exosomes can enter the tumor microenvironment to accelerate angiogenesis and metastasis of the tumor. Felicetti et al. (2016) isolated miR-222 from exosomes secreted by human melanoma cells. This miRNA may enhance the proliferation and invasion of cancer cells by suppressing the tumor suppressor gene $p27^{Kip1}$. Moreover, Au Yeung et al. (2016) pretreated ovarian cancer cells with exosomes from adipocytes and fibrocytes, and found that apoptosis was inhibited, indicating that exosomes

probably participated in the mechanisms of tumor immune escape.

3.3 Messengers of cell–cell communication

Exosomes are also involved in spreading pathogens such as Epstein-Barr virus (EBV) from an infected cell to uninfected neighboring cells (Pegtel et al., 2011). Further studies have suggested that exosomes play a role in intercellular communication. Since exosome cargos are protected by their lipid bilayer and are stable in the circulation, they could target specific locations to be mediators of biological processes. Sun et al. (2016) reported that exosomes from activated human brain microvascular endothelial cells may promote the intercellular transfer of antiviral factors to macrophages, which could protect against human immunodeficiency virus (HIV) neuroinvasion.

4 Isolation and authentication of exosomes

Studies of the function and composition of exosomes have many aspects, including the isolation, identification, and compositional analysis of exosomes. Considering the sources of exosomes and relative controllability of the chemical composition of culture media, scientists have tended to use supernatants of cultured cells to isolate exosomes at an early stage. Differential centrifugation is considered an optimum way to isolate exosomes secreted by cultured cells, and consists of two steps: first, low speed centrifugation (300*g* for 10 min, 2000*g* for 10 min, 4 °C) designed to remove cells and fragments, and second, ultracentrifugation (100000*g* for 24 h, 4 °C) to collect exosomes in the samples (Thery et al., 2006). Note that using ultrafiltration between the two steps can concentrate exosomes and at the same time filter out impurities and EVs greater than 220 nm. To assist studies aimed at improving our understanding of the role of exosomes in mediating pathophysiological processes, numerous commercial kits for gathering exosomes are widely used, which can harvest exosomes from different body fluids and make purification simpler. For example, the ExtraPEG method enriches exosomes from cell culture supernatants rapidly and at low cost (Rider et al., 2016). Exosomes are abundant in biological fluids, especially plasma and serum, and the isolation methods from body fluids are roughly similar to those described above (Table 1). However, exosomes obtained after differential centrifugation have been mixed with nucleic acid protein complex and other small molecules. In some studies, small molecular impurities have been removed by size exclusion chromatography (Kim et al., 2005). Recently, isolation of exosomes from plasma and other body fluids has become more popular. However, it is more difficult than using supernatants of cultured cells for many reasons, such as the uncertainty about the origin of the exosomes and the difficulties in their purification.

Protocols for identification of exosomes include TEM, cryo-electron microscopy, and nanoparticle tracking analysis (NanoSight). When the exosomes are treated with negative stain, their lipid bilayer structure with its characteristic cup-shape morphology can be observed. The collapse of vesicle structure may be caused by the drying process (Raposo et al., 1996). Nanoparticle tracking analysis can compute the size and concentration of exosomes in the solution through recording the trace of Brownian motion and particle diffusion of the exosomes, which measure 40–120 nm in diameter (Dragovic et al., 2011).

Further biological characterization of exosomes demands Western blot analysis, enzyme-linked immuno sorbent assay (ELISA), high-performance liquid chromatography-mass spectrometry (HPLC-MS), and other modern biochemical techniques. Exosomes from different cells contain endosomal proteins such as Alix, Tsg101, and tetraspanins (CD37, CD53, CD63, CD81, CD82), which are used as markers of exosomes (Wieckowski et al., 2009). However, little is known about the cargos of exosomes or the relationships among their components. With the development of exosome analysis technology, a "quantum leap" was the discovery that exosomes contain mRNA, miRNA, small interfering RNA (siRNA), and exosome-associated mRNAs, which can be translated into bioactive proteins by specific cells. In particular, some studies reported that exosome-associated miRNAs play a significant part in human immunoregulation. For example, miR-148a and miR-451 contained in exosomes released by DCs may affect post-translational control among DCs (Montecalvo et al., 2012). Nevertheless, little is known about how RNA segments are sorted into exosomes.

Table 1 Methods for isolating exosomes from different sources

Source	Differential centrifugation and filtration steps	Additional purification	Reference
Cell culture supernatant	(1) 300*g*, 10 min, 4 °C (2) 2000*g*, 10 min, 4 °C (3) Filtration (0.1, 0.22, or 0.8 μm) (4) 100000*g*, 70 min, 4 °C	(1) Resuspend pellet in PBS and centrifugation at 100000*g*, 70 min, 4 °C (2) Continuous sucrose density gradient	Thery et al., 2006
Urine	(1) 17000*g*, 30 min (2) 200000*g*, 3 h, 0 °C	(1) Suspend in DTT and heat at 37 °C, 30 min (2) Centrifugation at 200000*g*, 1.5 h, room temperature (3) Continuous sucrose density gradient	Bourderioux et al., 2015
Plasma/serum	(1) 2000*g*, 10 min, 4 °C (2) 10000*g*, 10 min, 4 °C (3) Filtration (0.22 μm)	(1) Size-exclusion chromatography (2) Ultracentrigufation at 105000*g*, 2 h, 4 °C (3) Resuspend pellet in PBS and continuous sucrose density gradient	Muller et al., 2014

PBS: phosphate buffer saline; DTT: dithiothreitol

5 Exosome-associated miRNAs and metabolic disorders

The biological function of exosomes was first reported in studies of tumor microenvironments and the packing proteins, lipids, and nucleic acids carried by these vesicles. Research was driven by the need for information about interactions between tumor cells and normal cells. Further studies showed that exosomes may be involved in a variety of fields, including the immune, cardiovascular, and nervous systems. Wan et al. (2008) showed that exosomes released from DCs in mice could raise the proliferation and enhance the function of B-lymphocytes. Major histocompatibility complex (MHC) II carried by exosomes from DCs was found to be bound directly to $CD4^+$ T-lymphocytes to trigger an immunological cascade (Naslund et al., 2013). In the field of cardiovascular disease, aorta endothelial cell-derived exosomes contain various kinds of miRNAs, which have been implicated as "messengers" to facilitate the exchange of cellular information. Notably, under high glucose conditions, exosomes from aorta endothelial cells were reported to carry 1354 proteins and about 2000 mRNAs, which play a vital role in the regulation of many pathophysiological processes, such as angiogenesis, inflammatory response, and myocardial ischemia (de Jong et al., 2012; van Balkom et al., 2013). Recent studies have detected more and more exosome-associated miRNAs associated with different kinds of chronic diseases, especially metabolic disorders such as diabetes mellitus, fatty liver, obesity, and atherosclerosis. Below, we discuss the effects that miRNAs carried by exosomes may have on the functioning of several endocrine tissues and organs.

5.1 Liver

The liver is an organ that has a major metabolic function. Normally, it plays a central role in the metabolism of glucose, lipids, proteins, vitamins, hormones, and other substances in the human body. Moreover, it has a lot of physical functions such as bioconversion, secretion, and excretion. Exosomes generated by normal liver cells have been reported to carry various cargos, like proteins (glycoprotein, lipoprotein, and other substances) and a great number of miRNAs. Lewis and Jopling (2010) and others verified that miR-122 is primarily synthesized by liver cells, and takes part in the metabolism of lipids and cholesterol. This miRNA could be used as a new biological marker in the serum of patients who are suffering from liver cancer (Conde-Vancells et al., 2008; Sohn et al., 2015). The use of antisense oligonucleotide to block the combination of miR-122 and the target gene *HMGCR* (3-hydroxy-3-methylglutaryl-coenzyme A reductase) led to a dramatic decline in the serum total cholesterol level (Krutzfeldt et al., 2005). According to Pirola et al. (2015), the main source of miR-122 detected in the serum of non-alcoholic fatty liver disease (NAFLD) patients is in the form of exosomes.

Jordan et al. (2011) explained a possible mechanism for obesity-induced insulin resistance based upon their studies of the liver of obese mice models. They discovered that an increase in miR-143 lowered the expression of oxysterol-binding-protein-related protein (ORP) 8, and then induced inactivation of the insulin-induced AKT pathway in the liver. Upregulation of miR-103 and miR-107 expression could also be detected in the livers of these models. Silencing of

miR-103 and miR-107 may increase the insulin sensitivity of liver and fat tissues (Mittelbrunn et al., 2011). In the area of lipid metabolism, miR-122, miR-33, and sterol regulatory element-binding protein (SREBP)-2 may regulate the cell cholesterol level. SREBP is a key transcription factor, and has two subtypes: miR-33a/b. Both subtypes play an important role in the transportation of free cholesterol and synthesis of high-density lipoprotein (HDL) (Najafi-Shoushtari et al., 2010). Although miR-33 has been reported in exosomes associated with liver cancer (Huang et al., 2013), there have been no reports of miR-33 in exosomes released by liver cells.

5.2 Pancreas

The pancreas is a glandular organ that has a role in both the endocrine and digestive systems. According to research on exosomes from the pancreas, miR-375 may act on pancreatic β-cells to regulate insulin secretion and the formation of islets. miR-375 is one of only a few miRNAs isolated from exosomes in the serum or plasma whose tissue specificity has been verified (Kloosterman et al., 2007; Li et al., 2015). Knockout mice lacking miR-375 (375KO) show the characteristics of hyperglycemia, an increase in total pancreatic α cell numbers, gluconeogenesis, and output of hepatic glucose (Poy et al., 2009). Furthermore, Poy et al. (2004) reported that overexpression of miR-375 suppressed insulin secretion induced by high glucose, whereas inhibition of miR-375 could promote insulin secretion. Subsequently, they mimicked the effects of miR-375 with siRNA to inhibit the expression of myotrophin (MTPN), considered to be a target of miR-375. Therefore, miR-375 carried by exosomes from the pancreas may reduce insulin secretion via inhibition of MTPN, which can regulate the function of actin.

Unlike miR-375, miR-200 family members, including miR-200a, miR-200b, and miR-200c, were recently reported to have potential in the diagnosis of cancers, such as breast cancer and ovarian cancer, but less is known about their possible role in metabolic disorders (Taylor and Gercel-Taylor, 2008; Rupp et al., 2011). miR-200 may suppress the function of β cells, but overexpression of miR-200 may result in an increase of β cell apoptosis, or even death from type 2 diabetes mellitus (T2DM) (Belgardt et al., 2015). In addition, miR-103 and miR-107 found in exosomes from plasma were verified to participate in the immune responses of T-lymphocytes and have an important role in cancer diagnosis.

Another study showed miR-103/107 isolated from plasma could adjust the sensitivity of hepatocytes to insulin and the balance of body glucose (Wilfred et al., 2007; Mittelbrunn et al., 2011; Trajkovski et al., 2011). However, the association between exosome-associated miR-103/107 and glycometabolism needs further investigation.

5.3 Adipose tissue

Adipose tissue is not only an inert tissue for storing excess energy, but also an active endocrinal organ which can secrete substantial amounts of adipokines. Moreover, it is able to maintain energy homeostasis and modulate the equilibrium of glucose and lipid metabolism in the human body. Adipose tissue can be divided into brown adipose tissue (BAT) and white adipose tissue (WAT) according to differences in its structure and function. BAT can consume energy to release heat, and has remarkable potential for the diagnosis and treatment of metabolic disorders. Many kinds of miRNA play important roles in the formation and differentiation of BAT. For example, the combination of miR-455 and the hypoxia-inducible factor 1-alpha inhibitor (*HIF1an*) gene can activate 5'-adenosinemono-phosphate-activated protein kinase α1 (AMPKα1) and peroxisome proliferator-activated receptor γ coactivator 1α (PGC1α), thereby promoting the formation of BAT as well as the synthesis of mitochondria (Zhang et al., 2015a).

Chen et al. (2016) discovered that miR-92a obtained from exosomes either released by BAT cells or separated from mice serum would change with fluctuations in BAT activity. This research indicated that exosome-associated miR-92a could serve as a serological marker reflecting BAT activity in the human body. Other reports about miRNAs from serum-separated exosomes suggested that these miRNAs might play a part in the formation of BAT and in converting BAT into WAT. For example, the miR-193b-365 mRNA cluster can modulate BAT formation. Blocking miR-193b and/or miR-365 in brown adipocyte precursors can induce the formation of BAT and enhance the expressions of runt-related

transcription factor 1 (RUNX1) and RUNX1 translocated to 1 (RUNX1T1), which might be a possible mechanism of BAT conversion (Sun et al., 2011; Huang et al., 2013). There have been very few studies of miRNAs derived from WAT-released exosomes. WAT or metabolic function-related miRNAs have not been detected from serum-separated exosomes.

5.4 Heart and artery

MiRNAs derived from exosomes play an important role in the physiological and pathological processes of the cardiovascular system. We can see the importance of these miRNAs, especially in aspects of angiopoiesis and atherosclerosis. miR-214 carried by exosomes secreted from vascular endothelial cells may be able to provide resistance to vascular aging and promotion of angiopoiesis by inhibiting expression of the ataxia telangiectasia mutated (ATM) protein kinase of adjacent cells. Angiopoiesis could be reduced dramatically by blocking endothelial miR-214 specifically on its way to exosomes (van Balkom et al., 2013). Hergenreider et al. (2012) discovered that endothelial cells and smooth muscle cells could realize information interaction via exosomes rich in miR-145 and miR-143 when the two kinds of cells were co-cultivated. Krüppel-like factor 2 (KLF2) has the capacity to induce endothelial cells to release exosomes containing miR-143/145, and these specific exosomes can fuse with co-cultivated smooth muscular cells to reduce the expression of atherosclerosis-related genes, thereby providing protection for the vascular endothelium.

Under some pathological conditions, exosomes containing specific miRNAs are released from cardiac myocytes and later swallowed by adjacent cells. This process might cause either cardiovascular lesion or protection. Abnormal expressions of miR-1, miR-92a, miR-92b, and miR-25 were found in the cardiac disease models established by Dirkx et al. (2013), and inhibiting the expression of miR-25 can result in cardiac dysfunction. In a clinical study by Kuwabara et al. (2011), patients with acute myocardial infarction (AMI) showed markedly increased levels of miR-1 and miR-133 in serous exosomes compared with a control group. These findings indicate the potential of exosomes as a serous marker for myocardial damage at an early stage.

6 Exosomes in the diagnosis of metabolic disorders

Recent research has proved that exosomes can be isolated from almost all body fluids, including serum, plasma, urine, milk, and saliva. The cargos carried by exosomes, including specific proteins, RNAs, and lipids, have recently become appreciated for their potential as indicators of different kinds of disease. With the development of new technologies for isolating exosomes from body fluids, exosomes may enable disease diagnosis to enter a brand new phase. Below, we will discuss the clinical value of some reported exosome-associated miRNAs found in body fluids and cell culture supernatants (Table 2) for the treatment of metabolic disorders.

6.1 Non-alcoholic fatty liver disease (NAFLD)

NAFLD has already become a global metabolic disorder with high prevalence along with obesity and diabetes mellitus, yet there are no definite serum diagnosis criteria or effective treatments for it. In recent years, research on exosomes in respect of metabolic disorders has provided possibilities for the assessment as well as treatment of fatty liver disease. Lots of researchers have been trying to find specific markers to reflect the function of the liver by separating exosomes from serum or plasma. For instance, Povero et al. (2014) separated exosomes from high-fat-diet NAFLD mice models and found that the proteins carried by exosomes were different from those in control groups, and that levels of miR-122 and miR-192 had increased in the blood yet decreased in the liver, indicating that these miRNAs might be prospective markers for reflecting the development of fatty liver disease. miR-122 has also played an important role in research on hepatic fibrosis. Reducing its expression may induce the down-regulation of some hepatic reconstruction regulator factors such as mitogen-activated protein kinase kinase kinase 3 (MAP3K3), which could reflect the degree of liver regeneration under pathological conditions (Csak et al., 2015).

Exosomes secreted by normal liver cells are different from those of lysophosphatidylcholine (LPC)-stimulated liver cells. Exosomes secreted by LPC-stimulated liver cells can carry tumor necrosis

Table 2 Important functional microRNAs in exosomes

Source	MicroRNA	Target cell/tissue	Function	Target gene	Reference
Body fluids					
Urine	miR-130a/145	GMS	A marker for DN	*AMBP*, *VDAC1*	Zubiri et al., 2014
	miR-320c	Kidney	A marker in type II DN	*TSP-1*	Delic et al., 2016
Serum/plasma	miR-29a/b	Pancreas	Insulin secretion	*MCT1*	Taylor et al., 2008; Pullen et al., 2011
	miR-33a/b	Liver macrophage	Cholesterol metabolism and hepatocellular carcinoma	*SREBP-2*	Horie et al., 2010; van Balkom et al., 2015
	miR-92a	BAT	A marker for BAT activity	*E2F2*	Chen et al., 2016
	miR-103/107	Liver, adipose, insulin sensitivity	Adipogenesis	*HIF-1β*	Taylor et al., 2008
	miR-133a	Myocardium	A marker for cardiomyocyte death		Kuwabara et al., 2011
	miR-193b/365	BAT	Brown fat differentiation	*Runx1t1*	Sun et al., 2011; Huang et al., 2013
	miR-199	Liver	A marker for NAFLD		Povero et al., 2014
	miR-214	Recipient endothelial cell	Angiogenesis	*ATM*	van Balkom et al., 2013
Cell culture supernatant					
Myocyte and cardiac progenitor cell	miR-29	Heart	Myocardial fibrosis	*MMP9*	Hergenreider et al., 2012; Chaturvedi et al., 2015
Endothelial cell; liver cell	miR-122	Liver	Cholesterol metabolism and hepatocellular carcinoma		Taylor et al., 2008; van Balkom et al., 2015
Liver cell; endothelial cell; adipocyte	miR-143	Liver	Insulin resistance; angiogenesis	*ORP*	Balkom et al., 2015
Adipocyte; dendritic cell	miR-155	Macrophages	Intercellular communication	*PPARγ2*	Aoki et al., 2010; Ogawa et al., 2010

GMS: glomerular mesangial cell; BAT: brown adipose tissue; DN: diabetic nephropathy; NAFLD: non-alcoholic fatty liver disease; *AMBP*: α-1-microglobulin/bikunin precursor; *VDAC1*: voltage-dependent anion-selective channel protein 1; *TSP-1*: thrombospondin 1; *MCT1*: monocarboxylate transporter 1; *SREBP-2*: sterol regulatory element-binding protein 2; *E2F2*: E2F transcription factor 2; *HIF-1β*: hypoxia-inducible factors 1β; *Runx1t1*: runt-related transcription factor 1 translocated to 1; *ATM*: ataxia telangiectasia mutated; *MMP9*: matrix metalloprotease 9; *ORP*: oxysterol-binding-protein-related protein; *PPARγ2*: peroxisome proliferator-activated receptors γ2

factor related apoptosis-inducing ligand (TRAIL), which might activate macrophages and induce hepatic inflammation (Hirsova et al., 2016). Earlier research compared the level of exosomes released by T-lymphocytes (mainly by $CD14^+$ T-lymphocytes and natural killer (NK)-T cells) in the serum of NAFLD and non-alcoholic steatohepatitis (NASH) patients. The quantity of exosomes was positively correlated to the serum level of alanine aminotransferase (ALT), indicating the possibility of progression from NAFLD to NASH (Kornek et al., 2012).

Research on exosomes and their contents provides a possibility for the early treatment of metabolic disorders such as NAFLD that are difficult to diagnose and treat. However, there is still a lack of affordable and effective techniques for us to determine the histological origin of the exosomes separated from body fluids.

6.2 Obesity

The morbidity of obesity has been increasing year by year. Fat tissues play a key role in modulating energy homeostasis and metabolic equilibrium. The dysfunction of adipokine secretion and exosome release are crucial aspects of obesity. The fat content of tissues can increase by increasing either the size or the number of adipocytes. The processes involved are closely related to intercellular communication. Some recent research has verified that adipocytes can release nanovesicles that can be regarded as exosomes specific to adipocytes (also called adiposomes). These exosomes carry glycosylphosphatidylinositol-anchored (c)AMP-degrading phosphodiesterase (Gce1) and are secreted by larger adipocytes and later phagocytosed by smaller adipocytes, during which free fatty acid is made available to accelerate the synthesis of triglyceride (Muller, 2011).

In the high-fat-diet obese mice models molded by Aoki et al. (2010), adipocytes were mainly in a condition of dysfunction and abnormal secretion. In particular, exosomes and microvesicles released at that time promoted angiogenesis by reducing the adipocyte content of leptin, tumor necrosis factor-α (TNF-α), and fibroblast growth factor-γ. Subsequent studies have reported that adiposomes consist of about 7000 mRNAs and 140 miRNAs, among which miR-143, miR-155, and other miRNAs play a vital role in inflammation, angiogenesis, and other aspects of disease (Ogawa et al., 2010).

When comparing *ob*/*ob* mice with wild-type (WT) mice fed under the same conditions, we found that exosomes released from *ob*/*ob* mice had increased. They expressed adiponectin on their surface, but lacked the cargo of leptin or resistin. Adiponectin may be regarded as a marker of adipose-derived exosomes (Phoonsawat et al., 2014). There is a possibility that source-cleared exosomes could become a kind of valid marker or therapeutic tool for obesity. These exosomes can be loaded with designed contents (specific proteins, miRNAs, or even chemical medicines) which can be transferred automatically to the target cells to activate intracellular signal pathways to achieve a therapeutic effect (Milbank et al., 2016).

6.3 Diabetes mellitus

Many recent studies have provided helpful information for diagnosing and treating diabetes mellitus by using exosomes from serum or plasma. In animal experiments, exosomes released from MSCs could stimulate the autoimmune responses in non-obese diabetic (NOD) mice, a spontaneous disease model for type 1 diabetes. Interestingly, serum exosome levels and exosome-induced interferon-γ production were positively correlated with the progression of the early prediabetic stage (Rahman et al., 2014). Scientists' eyes were focused not only on the pathogenesis of diabetes, but also on the early diagnosis and differential diagnosis of diabetic complications. As previously mentioned, miRNAs carried by exosomes from body fluids could be expected to be efficient biomarkers for diabetic complications, such as diabetic nephropathy (DN), diabetic neuropathy, and diabetic vascular complications.

There are many challenges in the early diagnosis and therapeutic treatment of DN, which shows poor clinical outcomes. Zubiri et al. (2014) isolated urine exosomes from patients with DN and reported that their cargo included about 352 proteins. The protein of urine exosomes from DN patients was extracted using two-dimensional gel electrophoresis and studied using mass spectrometry. They proposed that the levels of α-1-microglobulin/bikunin precursor (AMBP), histone-lysine *N*-methyltransferase (MLL3), and voltage-dependent anion-selective channel protein 1 (VDAC1) might reflect the earliest biological abnormalities in the DN patients. MiRNAs were enriched in the urine-associated exosomes, and therefore were considered to be indicative of the development of DN. For example, exosomes from DN patients' urine contained a lot of miR-130a and miR-145, which were confirmed to be packaged in the exosomes from glomerular mesangial cells (GMSs). Furthermore, there were positive correlations between the glucose concentration (treatment condition) and the quantity of exosomes as well as the expression of miR-145 in the cell experiment.

Several studies have indicated that diabetes patients are at a greater risk of Alzheimer's disease, which probably results from a high-glucose microenvironment in the central nervous system. The CA1 regions of the hippocampus are damaged by changes in the micro-environment, usually showing as degeneration of neurons and loss of astrocytes and synapses. Nakano et al. (2016) showed that exosomes secreted from rat bone marrow (BM)-MSCs could repair the damaged neurons and astrocytes and reverse the dysfunction. These results indicate that exosomes might be a promising treatment tool for diabetic nervous impairment.

Diabetic cardiovascular complications are the main cause of crippling and death of diabetic patients. These complications are closely related to insulin resistance and lipid disorders. Cardiosomes are the exosomes released from cardiomyocytes, and contain miR-455, miR-29b, miR-323-5p, and miR-466. These miRNAs can bind to the 3' region of matrix metalloprotease 9 (MMP9) and downregulate its function. Chaturvedi et al. (2015) observed that these miRNAs could reduce myocardial fibrosis and inhibit myocyte uncoupling, thus promoting myocardial regeneration.

Further studies showed that exosomes released from endothelial cells contained miRNAs (miR-214, miR-143/145) that had a momentous role in angiogenesis and anti-atherosclerosis (Hergenreider et al., 2012; van Balkom et al., 2013).

In addition to cardiovascular complications, diabetic peripheral angiopathy combined with ocular complications accounts for 41% of severe cases of diabetic retinopathy. In a clinical test, Tokarz et al. (2015) filtered 48 diabetic patients with various degrees of ocular complications to form an experimental group. This group was then divided into controlled diabetic (CD) patients and uncontrolled diabetic (UD) patients. Using enzyme-linked immunosorbent assay (ELISA), the expressions of the cytokine RANTES (regulated on activation, normal T-cells expressed and secreted) and angiopoietin-2 (Ang-2) were assayed in the exosomes from all patients. They showed that the expressions of exosome-associated RANTES and Ang-2 were increasing in the UD patients, which provided strong evidence of how exosomes could regulate diabetic prognosis and how cytokines in exosomes might be related to disease duration.

In studies of special types of diabetes mellitus, such as gestational diabetes mellitus (GDM), exosomes from body fluids also show enormous potential for diagnosis and treatment. Salomon et al. (2016) retrospectively analyzed the quantity of exosomes in plasma from patients with GDM and those with normal pregnancies at three-time points (11–14, 22–24, and 32–36 weeks' gestation). Interestingly, both gestational age and pregnancy status influenced the concentration of plasma exosomes. Furthermore, as the gestation time increased, the plasma concentration of exosomes increased gradually, and the increase was more apparent in GDM pregnancies (2.2-fold, 1.5-fold, and 1.8-fold greater than in normal pregnancies at three-time points, respectively) (Mitchell et al., 2015). Though less is known about the role of exosomes during GDM, they may become an effective diagnostic tool for screening susceptible populations.

7 Conclusions

In the past decade, we have witnessed many breakthroughs in the study of exosomes, and learned much about their biology and their value in diagnosis and treatment. Escudier et al. (2005) treated melanoma patients with self-exosomes released by DCs via back transfusion. They proved that these self-exosomes contained MHC proteins that could improve the quality of T-lymphocyte-associated immune responses. However, to our knowledge, studies of the biological functions of exosomes are still in the initial stages, and further studies are needed to reveal how they interact with target cells, how they sort their cargo, and to answer other questions. They created a manufacturing process for mass production of exosomes, though the effects of pharmaceutical-grade exosomes fell short of expectations. Subsequent studies suggested that exosomes could be used as a new drug delivery carrier. Attempts to find such carriers had become a focus of research in the field of medicine. Alvarez-Erviti et al. (2011) showed that self-exosomes loaded with exogenous miRNA by electroporation could pass the blood-brain barrier to deliver miRNA specifically to neurons, microglia, and oligodendrocytes, with the inhibition of β-secretase 1 (BACE1). Recently, the value of exosomes in the treatment of metabolic disorders, particularly some refractory diseases, has attracted the eye of scientists. In a novel study of diabetic ulcers, Geiger et al. (2015) found that specific exosomes could be released by fibroblasts when stimulated by fibroblast growth factor 2. The exosomes contained HSP90α and miRNAs (including miR-124a/125a/126/130a/132) that were confirmed to promote angiogenesis and induce anti-inflammatory response. Thus, the use of fibrocyte-derived exosomes for the treatment of diabetic ulcers shows their potential for treating metabolic disorders.

To summarize, after significantly expanding our understanding of exosomes isolated from cell supernatants in early studies, scientists and physicians have shifted their focus to studies of exosomes from body fluids. Compared with exosomes from cell supernatants, those from body fluids contain abundant exosome-associated miRNAs, which can directly reflect the body's health status. Furthermore, isolation and purification of self-exosomes could be a brand new type of therapy for patients with metabolic disorders. However, the isolation and purification of exosomes from body fluids are still complicated. The baffling problems are how to separate exosomes

derived from specific cells and how to determine the origin of exosomes in body fluids. We are learning more about the mechanisms of their formation and secretion, and the sorting of their contents every day, but there are many problems associated with studying exosomes from body fluids in theory and in practice. We hope that the use of exosome-associated miRNAs goes beyond fundamental studies into applications in the diagnosis and treatment of metabolic disorders, and that further studies can demonstrate the feasibility of each type of EV for therapeutic strategies applied to different diseases.

Compliance with ethics guidelines

Zhen-yu YAO, Wen-bin CHEN, Shan-shan SHAO, Shi-zhan MA, Chong-bo YANG, Meng-zhu LI, Jia-jun ZHAO and Ling GAO declare that they have no conflict of interest.

This article does not contain any studies with human or animal subjects performed by any of the authors.

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中文概要

题　目：外泌体相关的 microRNA 在代谢性疾病诊断和治疗中的作用

概　要：代谢性疾病是指包括代谢综合征、肥胖和糖尿病在内的一系列复杂疾病。其中脂毒性、慢性炎症和氧化应激可以通过改变细胞功能，从而在代谢紊乱的病理进程中发挥重要作用。近期研究发现细胞可以分泌含有蛋白质、脂质、核酸的纳米级微小囊泡，介导相邻和远处细胞间的信号传导和物质转运。外泌体作为这类囊泡的一种，参与包括肿瘤转移、动脉粥样硬化、慢性炎症和胰岛素抵抗等多种病理过程。外泌体的研究大多集中于其所含的蛋白质，而近期关于外泌体相关 microRNA 的功能研究也日益受到关注。尤其是，现已证明外泌体相关 microRNA 参与了机体代谢的诸多生理、病理进程，为代谢性疾病的诊断和治疗提供了新的方向。本文总结了外泌体的结构、内容物及产生的机制（图 1 和图 2）；体液和细胞培养液中外泌体所含 microRNA 的种类、靶器官及其功能（表 2）；外泌体相关 microRNA 在代谢性疾病中的作用，以及在诊断和治疗方面的潜能。

关键词：代谢性疾病；外泌体；外泌体相关microRNA；非酒精性脂肪肝；肥胖；糖尿病