THE ROLE OF EXOSOMES IN METABOLIC AND ENDOCRINE DISEASE

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THE ROLE OF EXOSOMES IN METABOLIC AND ENDOCRINE DISEASE

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EDITORIAL
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Editorial: The Role of Exosomes in Metabolic and Endocrine Disease

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Keywords: exosomes, bone, diabetes, metabolic syndrome, endocrine disease

Editorial on the Research Topic

The Role of Exosomes in Metabolic and Endocrine Disease

The discovery of exosomes has a history of 36 years, but it has gradually become a research hotspot in the recent 10 years. The study of exosomes is multisystem; however, the role of exosomes in the frontier of metabolism and endocrinology has become more important since the crosstalk between cells and organs is an immensely important function of exosomes, and exosomes exert their effects in an endocrine or paracrine manner.

In the present Research Topic includes four research articles and five reviews, which focused on exosomes as messengers of intercellular or inter-organ communication in metabolic and endocrine diseases.

There are four papers that discussed the role of exosomes in bone and muscle metabolism.

The original research of Li et al., titled "*Exosomes Derived From M2 Macrophages Facilitate Osteogenesis and Reduce Adipogenesis of BMSCs*", investigated exosomes derived from M2 macrophages that facilitate osteogenesis and decrease the adipogenesis of bone marrow mesenchymal stem cells through the miR-690/IRS-1/TAZ axis, which suggested that exosomes derived from M2 macrophages might possibly provide a therapeutic tool for bone loss diseases.

In the field of bone metabolism, the review by Yang et al., "*Exosomes: A Friend or Foe for Osteoporotic Fracture?*", discussed the role of exosomes in the occurrence and healing of osteoporotic fractures. Bone-related cell-derived exosomes have dual function in osteoporosis. Exosomes secreted by osteoclasts, osteoblasts, and mesenchymal stem cells have bilateral effects of accelerating and slowing down osteoporosis. Exosomes derived from vascular endothelial cells mainly inhibit the process of osteoporosis. More research on the potential mechanism of myocyte- and macrophage-derived exosomes in osteoporosis is still needed to make up for the limited data available. The authors concluded the diagnostic and therapeutic applications of exosomes in osteoporotic fracture by regulating osteoblastogenesis, osteoclastogenesis, and angiogenesis.

In addition, Aoi et al., in the mini review "*Roles of Skeletal Muscle-Derived Exosomes in Organ Metabolic and Immunological Communication*", discussed that exosomes derived from skeletal muscles can be recognized as myokines, which play their role by regulating the physiological and pathological states of the target organ, including physical fitness, muscle disorders, and lifestyle-related diseases. In their opinion, exosomes have potential application as biomarkers reflecting the metabolic and immune status.

Moreover, Li et al., in another research titled "*BMSC-Derived Exosomes Inhibit Dexamethasone-Induced Muscle Atrophy via the miR-486-5p/FoxO1 Axis*", investigated the mechanism of bone marrow stromal cell-derived exosomes (BMSC-Exos) in suppressing dexamethasone-induced

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myotube atrophy and muscle wasting by delivery of miR-486-5p, which reduced the expression of forkhead box protein O1 (FoxO1) in the muscle nucleus. The authors suggested that it might be a new biotherapy for muscle atrophy based on exosomes.

Four papers focused on diabetes, metabolic syndrome, or obesity.

The original study "*Metabolic Syndrome Is Associated With Altered mRNA and miRNA Content in Human Circulating Extracellular Vesicles*" reported by Li et al. provided data on the changes in the significance of the modified cargo of circulating extracellular vesicles in human subjects with metabolic syndrome (MetS) or age-matched lean controls. The authors concluded that exosomes can potentially serve as important regulators, biomarkers, and targets in the progression and treatment of MetS.

The review "*Adipocyte–Endothelium Crosstalk in Obesity*" by Sabaratnam and Svenningsen illustrated how obesity changes the adipose tissue microenvironment through exosome-mediated communication and how this communication regulates systemic energy homeostasis in metabolic disorders. Exosomes mediate the information exchange between a variety of cell types in adipose tissue, especially between adipocytes and endothelial cells. The adiponectin secreted by adipocytes promotes endothelial cells in the whole body to secrete exosomes, which remove excess or unnecessary substances in endothelial cells and maintain cell homeostasis.

The review of Hu et al., "*Clinical Translational Potentials of Stem Cell-Derived Extracellular Vesicles in Type 1 Diabetes*", summarized the therapeutic potential of stem cell-derived extracellular vesicles for the treatment of type 1 diabetes (T1D) by modulating the immune response and overcoming the deficit of islet β cells. The authors illustrated that the diversity and low yield of production of native extracellular vesicles, as well as their short half-life and the off-target effects of their functions post-administration, have limited their application, while bioengineered extracellular vesicles can facilitate the clinical translation of extracellular vesicles for the treatment of T1D.

The review by Mattke et al., "*Role of Exosomes in Islet Transplantation*", discussed the potential of exosomes as biomarkers of islet stress and injury, inflammation or immune response, and/or as therapeutic methods for clinical islet transplantation. More and more miRNAs are identified in exosomes collected during islet isolation, which can be used as biomarkers for islet stress and injury, inflammation, or immune response. On the other hand, therapy of mesenchymal stem cell-derived exosomes has the potential to increase the vitality and function of isolated and transplanted islets.

Moreover, one paper revealed the diagnostic function of exosomes on 11β-HSD2-related hypertension.

As the relevance of exosomal cargo alterations has been confirmed in the pathology of numerous metabolic and endocrine diseases, the use of messenger RNA (mRNA) levels in exosomes as a diagnostic tool has gained interest. De Santis et al., in "*Detection of Urinary Exosomal HSD11B2 mRNA Expression: A Useful Novel Tool for the Diagnostic Approach of Dysfunctional 11β-HSD2-Related Hypertension*", investigated the relationship between urinary exosomal HSD11B2 and hypertension status, aiming to identify an accurate method to assess HSD11B2 mRNA from urinary exosomes in samples from family members affected by apparent mineralocorticoid excess and in patients with low renin essential hypertension. The authors provided evidence that the detection of mRNA in exosomes is of great significance for the diagnosis of hypertension with 11β-HSD2 imbalance.

Therefore, the special issue summarizes the current knowledge and focuses on new insights into the role of exosomes in metabolic and endocrine diseases.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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REVIEW
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Exosomes: A Friend or Foe for Osteoporotic Fracture?

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The clinical need for effective osteoporotic fracture therapy and prevention remains urgent. The occurrence and healing of osteoporotic fracture are closely associated with the continuous processes of bone modeling, remodeling, and regeneration. Accumulating evidence has indicated a prominent role of exosomes in mediating multiple pathophysiological processes, which are essential for information and materials exchange and exerting pleiotropic effects on neighboring or distant bone-related cells. Therefore, the exosomes are considered as important candidates both in the occurrence and healing of osteoporotic fracture by accelerating or suppressing related processes. In this review, we collectively focused on recent findings on the diagnostic and therapeutic applications of exosomes in osteoporotic fracture by regulating osteoblastogenesis, osteoclastogenesis, and angiogenesis, providing us with novel therapeutic strategies for osteoporotic fracture in clinical practice.

Keywords: exosomes, osteoporosis, fracture healing, bone physiology, osteoporotic fracture

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INTRODUCTION

Bone is an essential component of the musculoskeletal system, providing structural support for tendons and ligaments as well as protection to vital internal organs. Bone fracture is a worldwide public health problem and often leads to dire consequences, exerting a strong threat on life quality and even mortality (1). It results from injury, or a mild stress under certain pathological conditions that weakens the bones, like osteoporosis (OP), bone cancer, or osteogenesis imperfecta (2). In particular, OP is a major systemic musculoskeletal disorder characterized by deterioration of micro-architecture and bone loss, with an inclination to high risks of bone fragility and even fracture (3). As a matter of fact, osteoporotic fracture accounts for a large proportion of all fractures. Osteoporotic fracture-related impaired healing, especially non-union, is another critical problem, resulting in prolonged treatment and aggravated socio-economic burden.

Exosomes were first identified in 1981 as exfoliated membranes by Trams (4). At present, exosomes are defined as cell-derived spherical lipid bilayer vesicles (EVs) with a diameter around 40-160nm (5). Exosomes can be secreted by nearly all sorts of cells and are found in various biological fluids, such as plasma, serum, and cerebral spinal fluid (6). Previous studies have

demonstrated that the constituents of exosomes are macromolecules including DNAs, RNAs (mRNA, microRNA, and other non-coding RNA), proteins, lipids, and cytokines (7, 8). Size and content are two major manifestations of the heterogeneity for exosomes, which vary with cellular origin, metabolic status, and environment of cells (5). Size heterogeneity is beneficial to distinguish exosomes and other EV subtypes. This property is also highly relevant to exosome isolation. Nowadays, some researchers usually use differential centrifugation to purify exosomes, but these exosome pellet fractions contain more than just exosomes. Hence, we require modified novel techniques for exosome purification, which is directly associated with the results of many exosomes-related experiments. Content heterogeneity determines the functions of exosomes since exosomes can convey their contains into recipient cells *via* endocytosis, receptor- ligand interaction, or fusion of membranes (9), thus mediating lots of responses of these recipient cells. Moreover, numerous studies have demonstrated that exosomes were obviously related to immune responses (10), pregnancy (11), and the occurrence and development of many disease, such as cardiovascular disease (12), central nervous system-associated diseases (13), and cancer (9). For example, exosomes secreted by apoptotic vascular endothelial cells were rich in miR-126, which could suppress the apoptosis of endothelial cells by activating chemokine ligand 12 (CCL12). Moreover, miR-126 containing exosomes inhibited the penetration of macrophages into the blood vessel wall, thereby stabilizing the hardened plaque and exerting the anti-atherosclerosis effect (14). Exosomes derived from prostate cancer cells could induce neoplastic reprogramming and tumor formation of adipose stem cells *via* transferring their cargos, like miR-125b, miR-130, miR-155, HRas, and Kras mRNAs (15). Apoptotic glioblastoma cells could secret spliceosomal proteins such as RBM11 and small nuclear RNAs (snRNAs) containing exosomes to modify mRNA splicing (MDM4, CCND1) of recipient cells, resulting in tumor aggressiveness and drug resistance (16). Significantly, translation of more comprehensive understanding of exosomes in various diseases into diagnosis and therapeutic applications has already occurred. First, the intrinsic properties of exosomes have exhibited their great potential in diagnosis of multiple diseases. Lewis et al. presented a simple method which integrated and analyzed exosomes as well as other extracellular vesicles directly from whole blood, plasma, or serum onto an AC electrokinetic microarray chip. They detected the samples from pancreatic ductal adenocarcinoma (PDAC) patients as well as healthy objects, finding that glypican-1 and CD63 were useful biomarkers to predict the occurrence of PDAC. These researchers also developed a bivariate model to detect PDAC with 99% sensitivity and 82% specificity (17). In addition, exosomes have been implicated as therapeutic targets in many fields, with potential utility in delivering therapeutic payloads directly to the desired place (18). Exosomal microRNA (miRNA) could target mRNA and modify related gene expression in the recipient cells, and some laboratories have attempted to use exosomes for the delivery of miRNA or small interfering RNA (siRNA) to treat mammary carcinoma (19), glioma (20), and pancreatic cancer (21). Moreover, clinical-grade MSC-derived exosomes with $Kras^{G12D}$ siRNA payload (iExosomes) have been applied to the treatment of pancreatic cancer in various animal models (22). Taken together, exosomes play an important role in mediating a variety of physiological and pathological processes through the special intracellular communication, which provides us a promising avenue to conquer numerous disorders, including osteoporotic fractures (23).

In this review, we aimed to illustrate the relationship between the exosomes and osteoporotic fracture, while referring to the complicated occurrence and healing processes. Moreover, we summarized the applications of exosomes in preventing and treating osteoporotic fracture, which may be an invaluable tool for the intervention of osteoporotic fracture and other related musculoskeletal disorders.

BONE PHYSIOLOGY AND EXOSOMES

Bone consists of minerals and organic material. The minerals include crystals of hydroxyapatite $Ca_{10}(PO_4)_6(OH)_2$ and other ions, while the organic material comprises osteogenic cells and extracellular matrixes like collagen fibers (24). Osteogenic cells mainly include three types of cells: the osteoblasts, osteoclasts, and osteocytes (25). In fact, exosomes exerted a dominant effect on bone turnover and remodeling by mediating these important elements of bone. On the one hand, exosomes played a key role in the process of production of bone extracellular matrixes. For instance, the exosomes membrane rich in phosphatidylserine (PS) participated in the formation of hydroxyapatite crystal during osteogenesis, a process that was also accelerated by the calcium stored in exosomal annexins and the phosphate generated by exosomal ATPases, nucleotidases, phosphatases, pyrophosphatases, and membrane transporters (24). On the other hand, exosomes acted as messengers to mediate signals transmitting in the same cluster (autocrine) or different cluster (paracrine) of osteogenic cells, thus participating in their differentiations and activations. Wei et al. found let-7 was contained in both osteoblast precursors and differentiated osteoblasts-derived exosomes, which could promote osteogenesis through mediating high-mobility group AT-hook 2 (HMGA2) and Axin 2 (26). Another study by Li et al. showed osteoclasts-derived exosomal miR-214-3p suppressed osteoblastic bone formation (27). Further, bone modeling and remodeling were tightly correlated with the endocrine system. And parathyroid hormone (PTH), estrogen hormone, and glucocorticoid were important factors in mediating the bone microenvironment for osteoanabolism (28). However, the specific relationships between exosomes and steroid or protein hormones in bone physiology is still not fully understood and needs further exploration. Overall, exosomes exert an indispensable role in these processes and future research analyzing their properties and functions may help to build a multi-targeted system to maintain the balance of bone resorption and formation.

RELATIONSHIPS BETWEEN EXOSOMES AND THE OCCURRENCE OF OSTEOPOROTIC FRACTURE

Bone loss and a fall bias lead to great susceptibility to fractures for the aging and menopausal population (29). Clinically, osteoporotic fractures are common, have become a serious public health issue worldwide, and cause an ever-increasing burden to the healthcare system. OP has an obvious clinical and public health impact which is estimated to affect 75 million people worldwide (30). Moreover, the number of annual incident fragility fractures is about 9 million (31).

Mechanically, OP is a result of excessive bone resorption and inadequate formation of new bone upon aberrant activities of osteoclasts and osteoblasts (32). Under normal circumstances, bone metabolism follows the strict control of various factors. And among them, receptor activator of NF-κB (RANK)/receptor activator of NF-κB ligand (RANKL), Wnt/β-catenin, and Jagged1/Notch1 are the three best studied pathways which exert a strong influence on bone mass density (33). Strikingly, exosomes have been widely studied for their roles in OP, which make great contributions to the imbalance of osteoblasts and osteoclasts, hence promoting the prevalence of fragility fracture. Therefore, we comprehensively reviewed the role of exosomes in OP initiating bone fracture and presented their potential in retarding the occurrence and progress of osteoporotic fracture.

The Functions of Exosomes in OP

As mentioned above, exosomes functioned as "the carrier pigeons of the cell" in intercellular communication and materials exchange to mediate a series of responses of adjacent or distant recipient cells (34, 35). Multiple studies have shown that bone-related exosomes were tightly associated with bone modeling and remodeling (27, 36) by transferring biologically essential molecules to interfere with the activities of osteoblasts and osteoclasts (**Figure 1**). Besides, the circulatory exosomes possessed pathophysiological functions in the development of senile OP and thus would be helpful for its diagnosis and therapy.

Circulatory Exosomes in OP

A large number of reports detected the main differences of serum-derived exosomes (SDEs) between OP or osteopenia

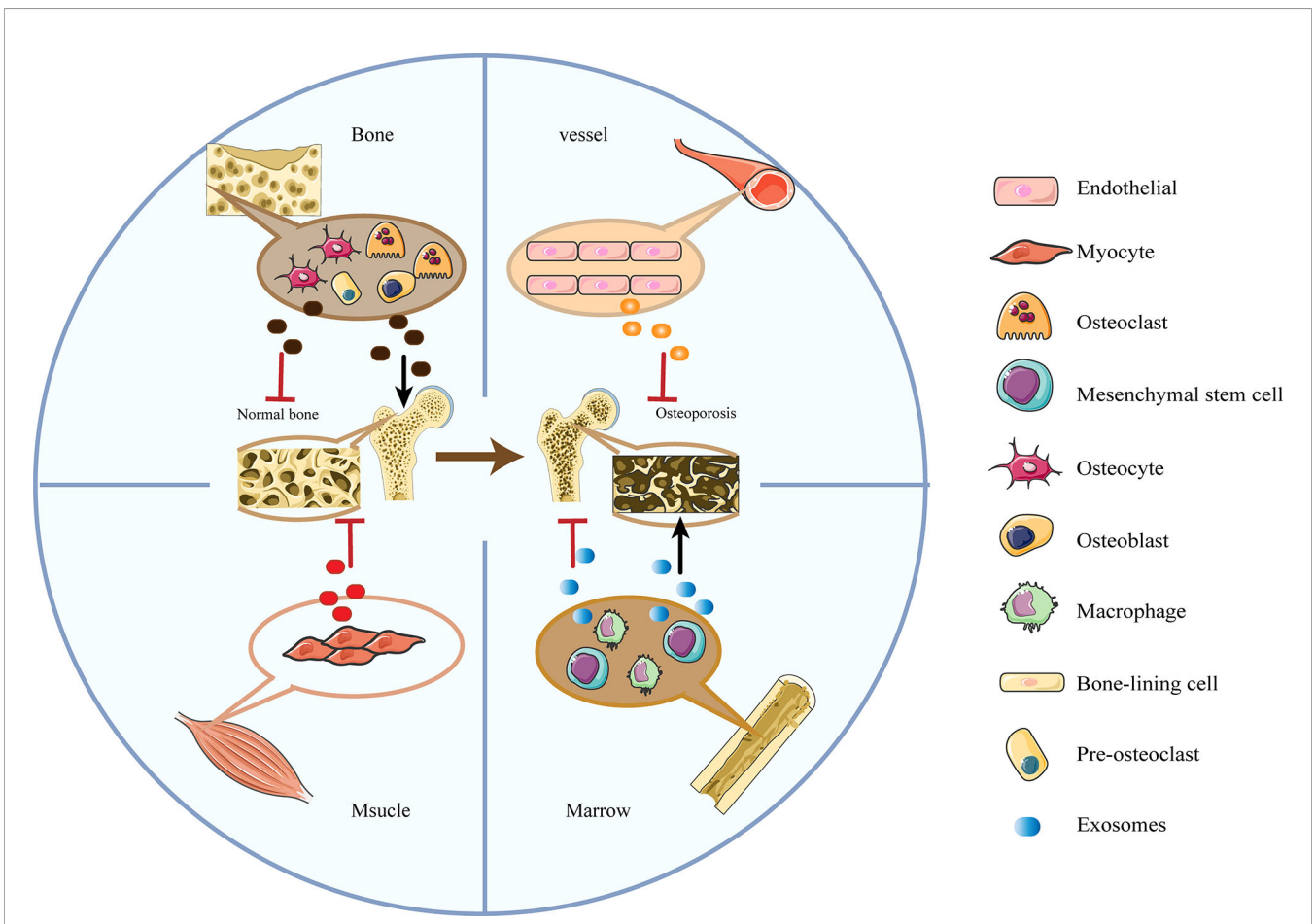

FIGURE 1 | The diplex functions of bone-related cells-derived exosomes in OP. Exosomes secreted by osteoblasts, osteoblasts and mesenchymal stem cells have bilateral effects in promoting and suppressing OP; Exosomes derived from myocytes and vascular endothelial cells mainly inhibit the process of OP.

and normal bone mass by related techniques to get a more comprehensive understanding of the functions of exosomes in OP (**Table 1**). Xie et al. identified 1371 proteins from SDEs, especially, 585 differentially expressed proteins (DEPs) of OP. These DEPs not only participated in inhibiting integrin-related activation and function of osteoblasts, but also strengthened the capacity of osteoclasts. Notably, the integrin β_1 (ITGβ_1), integrin β_3 (ITGβ_3), and hematopoietic progenitor cell antigen CD34 (CD34) were three representatives of downregulated proteins, which acted as hubs in suppressing bone mineralization of osteoblasts (37). Chen et al. utilized the PLAGH Hip Fracture Database, finding a total of 45 significant DEPs and verifying four exosomal proteins, namely PSMB9, PCBP2, VSIR, and AARS, with an AUC of 0.805 in the classification of OP (38). Zhang et al. investigated the functions of transfer RNA-derived fragments (tRFs), a novel type of small non-coding RNAs derived from tRNAs contained in plasma exosomes in OP. They found 29 differentially expressed tRFs, which played an active role in some important pathways related to OP, including Wnt, PI3K-Akt, MAPK, TGF-β, and calcium signaling pathway. More importantly, plasma exosomal tRF-25-R9ODMJ6B26 (tRF-25), tRF-38-QB1MK8YUBS68BFD2 (tRF-38), and tRF-18-BS68BFD2 (tRF-18) were at highly-expressed levels in OP patients compared to normal controls and were utilized to develop a more accurate model for OP diagnosis with an average AUC of 0.815 (39). Furthermore, a study by Teng et al. detected 393 differentially expressed lncRNAs and found co-located mRNAs were highly enriched in OP-associated processes, such as MAPK pathway, insulin secretion, cellular response to metal ions, fucosylation, and proteolysis (40). More importantly, Shao et al. isolated serum exosomes from menopausal females with or without OP and detected 191 aberrant miRNAs, which were related to Wnt, MAPK, and Hippo pathways (41). All of these findings offered forceful evidence to illustrate exosomes from the circulation could be potential biomarkers of evaluating bone status for OP diagnosis and analyzing the results of therapy.

Bone-Related Cell-Derived Exosomes in OP

Bone-related cells like osteoblasts, osteoclasts, and endothelial cells, could achieve mutual interaction through exosomes, the cell-cell communicators (**Table 2**). Over the past few years, many researchers have drawn attention to the relationship between bone-related cells-derived exosomes and OP. First, some laboratories have extensively investigated exosomes derived from bone-related cells by using techniques including Nanoparticle Tracking Analysis (NTA) (69), Transmission electron microscopy (TEM) (50), western blotting (WB), immuno-EM, or bead-based fluorescence-activated cell sorting (FACS) (63). More significantly, these scientists not only identified the classical bone-related cell-derived exosomal markers of OP, but also revealed the ability of exosomes to mediate osteogenic cells differentiation and activity as well as matrix formation in OP. Overall, this evidence indicated the essential role of bone-related cell-derived exosomes in OP and provided us serviceable tools in the treatment of OP.

MSCs-Derived Exosomes in OP

MSCs are pluripotent stem cells with the capability to proliferate extensively and maintain the potential to differentiate into various types of cells (70). In 2010, MSCs-derived exosome was first isolated from conditioned medium of human embryonic-derived MSCs (hESC-MSCs) (71). Nowadays, MSCs have been identified as the most ferocious producer of exosomes (72). Moreover, Chen et al. detected more than 850 gene products and 150 miRNAs in MSCs-derived exosomes (73, 74), indicating their potential clinical efficacy for a variety of diseases, including OP. In fact, a considerable amount of research has confirmed that BM-MSCs-derived exosomes could improve OP by triggering osteoblasts proliferation, differentiation, and activation as well as inhibiting cell apoptosis (42, 43) through miR-196a (44), miR-150-3p (45), miR-181a (46), miR-218 (47), lncRNA MALAT1 (48), and let-7 (26). In addition, miR-29a loaded in BM-MSCs-derived exosomes could not only promote angiogenesis and osteogenesis but also restrain osteoclastogenesis, which was down-regulated in the aged

TABLE 1 | Exosomes from circulation in OP.

Origin of exosomes	Detected technique	Exosomes contains	Main difference	Reference
SDEs of patients with 31 osteoporosis, 46 osteopenia, and 62 normal volunteers.	TMT-based quantitative MS	Proteins	1,371 proteins were identified with an overlap of 1,160 Gene IDs among the ExoCarta proteins. 585 osteoporosis differentially expressed proteins were detected (255 upregulated and 360 downregulated).	(37)
Plasma exosome of patients with 30 osteoporosis, 10 osteopenia, and 20 normal controls	MS	Proteins	2351 proteins were identified in all groups, and 45 differentially expressed proteins were identified in the discovery dataset	(38)
Plasma exosome of patients with 40 osteoporosis, and 40 healthy controls	Small RNA sequence	tRFs	Found 288 total tRFs and 29 differentially expressed tRFs (11 upregulated and 18 downregulated)	(39)
SDEs of 9 elderly patients with fracture and 9 age-matched patients without fracture at the age between 60 and 90 years old	RNA-Seq experiments	LncRNAs	Detected 393 differentially expressed lncRNAs (296 upregulated and 97 downregulated)	(40)
SDEs of 6 menopausal females without osteoporosis and 12 menopausal females with osteoporosis	miRNA high-throughput sequencing	MiRNA	191 aberrant miRNAs were found in the group of menopausal females with osteoporosis (72 upregulated and 121 downregulated)	(41)

lncRNAs, long non-coding RNA; MS, mass spectroscopy; SDEs, serum-derived exosomes; TMT, tandem mass tag; tRFs, transfer RNA-derived fragments.

TABLE 2 | Bone-related cells derived exosomes in OP.

Origin of exosomes	Exosomes contains	Recipient cell	Involved pathway	Function	Reference
BM- MSCs	Not referred	Osteoblasts	Not referred	Promoted osteoblasts proliferation and inhibited cell apoptosis	(42)
BM- MSCs	Not referred	Osteoblasts	MAPK pathway	Promoted osteoblasts differentiation	(43)
BM- MSCs	MiR-196a	Osteoblasts	ALP, OCN, OPN and Runx2	Promoted osteoblasts differentiation, activation and proliferation	(44)
BM- MSCs	MiR-150-3p	Osteoblasts	Not referred	Promoted osteoblasts proliferation and differentiation	(45)
BM- MSCs	MiR-181a	Osteoblasts	TGF- and Wnt signaling pathways	Promoted osteoblastic differentiation	(46)
BM- MSCs	MiR-218	Osteoblasts	Wnt signaling pathways	Accelerated osteoblasts differentiation and mineralization	(47)
BM- MSCs	LncRNA MALAT1	Osteoblasts	MiR-34c/SATB2 axis	Alleviated osteoporosis	(48)
BM- MSCs	Let-7	Osteoblasts	HMGA2	Increased osteogenesis and bone formation	(26)
BM- MSCs	MiR-29a	HUVECs, osteoblasts, osteoclasts	PCAF-mediated RANKL and CXCL12 or Frizzled 4	Promoted angiogenesis and osteogenesis and inhibited osteoclastogenesis.	(49)
BM- MSCs	Not referred	BM- MSCs	Not referred	Promoted the proliferation and osteogenic differentiation of BM-MSCs	(50)
hiPSC-MSCs	Not referred	BM- MSCs	Not referred	Enhanced angiogenesis and osteogenesis	(51)
BM- MSCs	MiR-186	BM- MSCs	Hippo signaling pathway	Promoted osteogenesis	(52)
BM- MSCs	Not referred	BM-MSCs, osteoblasts	Wnt/β-catenin signaling	Restored the function of BM-MSCs	(53)
BM- MSCs	MiR-31a-5p	Osteoblasts, osteoclasts	SATB2 and E2F2 pathways; RhoA pathway	Reduced osteoblastogenesis and promote osteoclastogenesis	(54)
BM- MSCs	MiR-148a	Osteoclasts	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B	Promoted osteoclasts differentiation	(55)
BM- MSCs	MiR-21	BM- MSCs	Targeted SMAD7	Inhibited osteogenesis	(56)
Osteoblasts	MiR-677-3p	BM- MSCs	Increase AXIN1	Enhanced BM-MSCs differentiation	(57)
Osteoblasts	MiR-378	BM- MSCs	PI3K/Akt signaling pathway	Activated the glucose-mediated osteogenic differentiation	(58)
Osteoblasts	RANKL	Osteoclasts	RANKL-RANK	Led to osteoclasts information	(59)
Osteoblasts	MiR-30d-5p	Osteoblasts	RUNX2	Suppressed osteoblasts differentiation	(57)
Osteoblasts	MiR-133-3p	Osteoblasts	RUNX2	Suppressed osteoblasts differentiation	(57)
Osteoblasts	MiR-140-5p	Osteoblasts	BMP-2	Diminished osteoblast activity	(60)
Osteoblasts	Not referred	BM- MSCs	Not referred	Inhibit BM-MSCs differentiation	(61)
Osteoclast precursors	Not referred	Osteoclasts	Vitamin D-dependent pathway	Promote osteoclasts formation	(62)
Osteoclasts	RANK	Osteoclasts	RANKL-RANK	Inhibited osteoclastogenesis	(63)
Osteoclasts	MiR-214	Osteoblasts, osteoclasts	EphrinA2/EphA2, ATF4; PI3K/Akt pathway	Inhibited osteoblastogenesis, promoted osteoclastogenesis	(27, 64)
Osteocytes	MiR-218	Osteoblasts	Not referred	Promoted osteoblastic differentiation	(65)
Muscle	MiR-34a-5p	BM- MSCs	Sirt1	Induced BM-MSCs senescence	(66)
Endothelial cells	MiR-155	Osteoclasts	Spi1, Mitf, Socs1	Suppressed osteoclasts differentiation and activation	(67)
Endothelial cells	Not referred	Osteoblasts	ferroptosis	Rescued the glucocorticoid-induced osteogenic inhibition of osteoblasts	(68)

AXIN1, axis inhibition protein 1; BMP-2, bone morphogenetic protein 2; hiPSCs, human induced pluripotent stem cells hiPSC-MSC-Exos; HMGA2, high-mobility group AT-hook 2; HUVECs, human umbilical vein endothelial cells ; Mitf, microphthalmia-associated transcription factor; RUNX2, runt-related transcription factor 2; Socs1, suppressor of cytokine signaling 1.

populations, providing us a novel way to improve therapeutic strategy for OP (49). BM-MSCs-derived exosomes also have been identified as inducers to motivate undifferentiated MSCs toward osteogenic differentiation both *in vivo* and *in vitro* (50–52), showing its potential as a bone regenerative drug for OP. Moreover, Zuo et al. expounded that BM-MSCs-derived exosomes could promote bone formation by banishing reactive oxygen species (ROS), assisting in DNA repair, rescuing cell (mainly osteoblasts) proliferation and differentiation ability, suppressing the senescence-related protein and adipogenic gene expression, and accelerating osteogenic expression, thus ameliorating OP (53).

However, some studies also showed that BM-MSCs-derived exosomes could be helpful to OP with the function of promoting maturation and activity of osteoclasts. Previous studies have proven that miR-31a-5p was over-expressed in aged BM-MSCs- derived exosomes, which could reduce osteoblastogenesis by the SATB2 and E2F2 pathways and promote osteoclastogenesis by the RhoA pathway (54). Hence miR-31a-5p contained in BM-MSCs exosomes would lead to osteoporotic bone loss in aging bone tissue. Cheng et al. also detected that miR-148a from BM-MSCs-derived exosomes could stimulate osteoclasts differentiation *via* targeting V-maf musculoaponeurotic fibrosarcoma oncogene

homolog B (55). Besides, a recent study by Jiang and colleagues demonstrated miR-21 was at a high level in BM-MSCs derived exosomes from OP patients, which inhibited osteogenesis by targeting SMAD7 (56).

Therefore, exosomes secreted by BM-MSCs are capable of mediating proliferation and differentiation of osteoblasts and osteoclasts, suggesting a novel method to improve therapeutic strategy for OP. For instance, researchers have successfully used exosomes from processed BM-MSCs, like miR-935-modified BM-MSCs and circ-Rtn4-modified BM-MSCs, to treat OP (75, 76).

Osteoblasts-Derived Exosomes in OP

Paracrine/autocrine in communication between osteoblasts and other bone-related cells in the field of OP has provided us with new knowledge and evoked further detection in this area. Ge et al. isolated osteoblasts-derived exosomes and analyzed their cargos, finding they were tightly combined with osteogenesis (57). Consistently, accumulating studies indicated that the contents of osteoblasts-derived exosomes, like miR-677-3p (59) or miR-378 (58), could accelerate osteogenesis, while RANKL (61), miR-30d-5p, miR-133-3p (59), and miR-140-5p (60) from osteoblast-derived exosomes possessed the ability to suppress this progress. Niedermair et al. recently revealed osteoblasts-derived exosomes from OP patients could also hamper osteogenic differentiation of BM-MSCs (77). Therefore, it remains a mystery for us as to what the specific functions of osteoblasts-derived exosomes in OP are.

Osteoclasts-Derived Exosomes in OP

Osteoclasts-derived exosomes have positive as well as negative feedback in the progress of osteogenesis, which played dual roles in OP. A recent study showed that exosomes secreted by osteoclast precursors facilitated vitamin D-dependent osteoclast formation in whole mouse marrow cultures, while exosomes from osteoclast-enriched cultures suppressed osteoclastogenesis in the same cultures which referred to an important factor-RANK (62). Furthermore, miR-214-containing exosomes from osteoclasts have been reported to have a significant effect on osteogenesis. On one hand, it could restrain osteoblasts *via* targeting EphrinA2/EphA2 (63) and activating transcription factor 4 (ATF4) (27). On the other hand, it could promote osteoclastogenesis through the PI3K/Akt pathway (64). Therefore, miR-214-3p may be a potential clinical target to reverse established OP. In fact, another study performed by Zhu et al. also found that the magnetic hydroxyapatite scaffold (MHA) facilitated osteoblasts' proliferation in a model of OP through altering the osteoclasts-derived exosomal cargos and suppressing exosomes intussuscept by osteoblasts (78), indicating exosomes could act as tools to modify the interactions of bone cells or direct drugs to treat OP.

Osteocytes-Derived Exosomes in OP

Osteocytes are vital components of bone tissue that originated from osteoblasts and play multifaceted roles in bone remodeling. Emerging studies found osteocytes-derived exosomes could enter into circulation and carry some miRNAs, such as miR-29, miR-484, and miR-221 (79). Besides, Qin et al. offered evidence that exosomes from osteocytes could co-localize with the nucleus of MC3T3-E1 cells and reduce osteoblastogenesis. More significantly, they expounded the specific mechanism that myostatin possessed the ability to suppress osteocytes-derived exosomal miR-218, which was an unprecedented method for muscle-bone communication (65).

Muscle-Derived Exosomes in OP

Muscle has a mechanical crosstalk with bone. As aforementioned, muscle is identified as a secretory endocrine organ that takes part in biochemical interplay and influences the functions mutually. Bone-derived factors compromise fibroblast growth factor (FGF)-2, prostaglandin E2, osteocalcin, and sclerostin, while the muscle secreted factors are described as "myokines" (80), such as myostatin, interleukin (IL)-6, irisin, and RANKL. Recently, Fulzele et al. found that miR-34a-5p was overexpressed in muscle and muscle-secreted exosomes with aging and with myoblast exposure to oxidation (66). Further research showed that muscle-derived exosomes containing miR-34a could suppress Sirt1 expression in BM-MSCs and accelerate BM-MSCs senescence (81). The evidence represented a potential pathway by which muscle could affect bone physiology and provide us a different perspective to understand OP.

Endothelial Cells-Derived Exosomes in OP

Blood vessels occupy vital positions in bone homeostasis. Endothelial cells are stationed at the inner layer of vascular vessels, which actively participate in the progress of internalizing and secreting substances like exosomes. Song et al. found that miR-155 loaded in endothelial cells-derived exosomes suppressed osteoclasts' differentiation and activation by several targets, like Spi1, microphthalmia-associated transcription factor (Mitf), and suppressor of cytokine signaling 1 (Socs1) (67). Furthermore, endothelial cells-derived exosomes could rescue the glucocorticoid-induced osteogenic suppression of osteoblasts by inhibiting ferritinophagy-dependent ferroptosis (68). Hence, endothelial cells-derived exosomes might be promising and biocompatible nanomedicine for OP.

Macrophages-Derived Exosomes in OP

Osteal macrophages are a subtype of bone-resident macrophages, which are close to the bone surface and adjacent to osteoblasts, with the function of mediating bone formation (82). Current knowledge about macrophage-derived exosomes is limited. A study by Wei et al. showed that BMP2/macrophage-derived exosomes could up-regulate the expression of osteogenesis-associated genes such as ALP, Runx2, BMP-2, BMP-7, and osteopontin, playing essential roles in OP (83). Collectively, the explicit mechanism of the macrophage-derived exosomes in OP remains unclear. However, with more in-depth study, it may show great potential acting as the therapeutic target for OP in the future.

RELATIONSHIPS BETWEEN EXOSOMES AND OSTEOPOROTIC FRACTURE HEALING

Bone is a unique form of tissue that can heal without a fibrous scar (84). Similar to the process of bone remodeling, bone regeneration is highly orchestrated and precisely controlled, and is influenced by

multiple extrinsic and intrinsic factors (85). Extrinsic factors include smoking, alcohol, and certain use of medicines While intrinsic factors cover injury to the periosteum or endosteum and poor vascularization at the injury site (86). Although we have achieved great improvement in surgical techniques nowadays, sometimes osteoporotic fractures-related impaired bone regeneration like non-union would happen, which greatly affects the life quality of a number of patients (87). Therefore, it is of great need to do intensive investigations to fill the clinical gaps and develop effective therapeutic approaches for these affected patients.

In general, fracture healing is subdivided into four biological phases followed in a chronological order: hematoma formation and inflammatory response, proliferation and differentiation, ossification, and remodeling (88). These phases are involved in many physiological processes, like inflammation, angiogenesis, stem cell differentiation, osteogenesis, and chondrogenesis with the involvement of various types of bone-related cells (89). Exosomes are closely connected with these processes through mediating cell-to-cell communications (**Table 3**). In fact, exosomes could promote osteoblasts' differentiation, suppress osteoclasts formation and differentiation, and accelerate angiogenesis. In addition, some important intrinsic factors mentioned above could modify the functions of exosomes to regulate the healing process. BM-MSCs could be induced to recruit, proliferate, migrate, and differentiate into osteoblasts and chondrocytes during intramembranous ossification and endochondral ossification, which played important roles in osteoporotic fracture healing (99). Reliable evidence has illustrated the capacities of osteogenic differentiation for BM-MSCs-derived exosomes. For example, Furuta et al. used the $CD9^{-/-}$ mouse, an established model with reduced levels of exosomes, and found there was an obvious delay of endochondral ossification and fracture healing compared with the wild-type mouse. More interestingly, the delay could be rescued by injecting BM-MSCs-exosomes (90). Besides, Narayanan et al. incubated exosomes isolated from osteogenic differentiated BM-MSCs with BM-MSCs, finding BM-MSCs internalized these exosomes and gave rise to an extensive upregulation of several important genes like bone morphogenetic protein 9 (BMP9), transforming growth factor β1 (TGFβ1), transcription factors, and ECM molecules (50). Xu et al. showed similar results and detected this impact was involved in the modulatory effect of miRNAs on target genes and pathways (100). Among these, miR-196a acted as one of the most significant molecules in the modulatory process and miR-21 and miR-25 have also proven to possess the ability to accelerate osteogenesis and angiogenesis (90, 91). More importantly, BM-MSCs-exosomes contained a variety of bone repair-related cytokines such as monocyte chemotactic protein 1 (MCP-1), monocyte chemotactic protein 3 (MCP-3), stromal cell-derived factor-1 (SDF-1), and angiogenic factors, which could promote fracture healing (101). Recently, some researchers transplanted BM-MSCs-derived exosomes into the fracture site in a rat model of femoral non-union, obtaining the results that these exosomes could significantly trigger osteogenesis and angiogenesis to promote bone healing process though activating the BMP-2/Smad1/RUNX2 and the HIF-1α/VEGF signaling pathways (92). Furthermore, some studies also revealed that exosomes secreted by human umbilical cord mesenchymal stem cells (uMSCs) could enhance angiogenesis to accelerate bone healing by overexpressing VEGF, HIF-1α (93), or Wnt signaling pathway (94), which provided a new view for us regarding uMSCs-related bone fracture. As we mentioned above, some intrinsic factors could change the functions of exosomes and thus affect the osteoporotic fracture healing. Xu et al. observed that aged exosomes had obvious attenuated effects on MSCs osteogenic differentiation *in vitro* and facture healing *in vivo*. Under further investigation, they found miR-128-3p was dramatically over-expressed in aged-BM-MSCs-derived exosomes, which functioned as a suppressor in the process of fracture healing by directly targeting the 3'-UTR of Smad5 (95). Hence, exosomal miR-128-3p antagomir could be an ideal treatment for bone fracture healing, especially for the elderly. Wang et al. observed that the healing time was longer in obese fracture patients than normal weight patients. Interestingly, using bioinformatics analysis and related assays, they further found that high-fat treatment could decrease the secretion of BM-MSCs-derived exosomes and reduce the carried lncRNA H19 *via* miR-467/HoxA10 axis, hence affecting osteogenic differentiation and fracture (96). Liu et al. performed a serious of *in vivo* and

TABLE 3 | Exosomes in fracture healing.

Origin of exosomes	Exosomes contains	Recipient cell	Involved pathway	Function	Reference
MSCs	MiR-196a, miR-21	Not referred	Not referred	Promoted bone healing	(90)
BM-MSCs	MiR-25	Osteoblasts	(SMURF1), Runx2	Accelerated osteogenic differentiation, proliferation, and migration of osteoblasts	(91)
BM-MSCs	Not referred	HUVECs and MC3T3-E1	BMP-2/Smad1/RUNX2 and the HIF-1α/VEGF signaling pathways	Enhanced osteogenesis, angiogenesis	(92)
uMSCs	Not referred	HUVECs	HIF-1α	Promoted angiogenesis	(93)
uMSCs	Not referred	Not referred	Wnt signaling pathway	Accelerated bone healing	(94)
Aged-BM-MSCs	MiR-128-3p	MSCs	Smad5	Inhibited bone healing	(95)
High-fat treatment BM-MSCs	LncRNA H19	Osteoblasts	MiR-467/HoxA10 axis	Inhibited osteogenesis	(96)
Hypoxia preconditioning BM-MSCs	MiR-126 and	MSCs	the SPRED1/Ras/Erk signaling pathway	Promoted bone healing	(97)
M2 macrophage	MiR-5106	Osteoblasts	SIK2 and SIK3	Promoted osteoblast differentiation	(98)

SMURF1, Smad ubiquitination regulatory factor 1; SIK2, Salt-inducible kinase 2; SIK3, Salt-inducible kinase 3.

in vitro experiments to verify that hypoxia preconditioning BM-MSCs-derived exosomes exerted a greater effect on promoting fracture healing. Hypoxic MSC-derived exosomes were enriched with miR-126, which possessed the abilities of being pro-angiogenesis, pro-proliferative, and pro-migratory by suppressing SPRED1/Ras/Erk signaling pathway. Therefore, hypoxia-induced BM-MSCs transplantation might have great potential as a therapy for fracture, but several challenges remain to be overcome to achieve clinical applications (97). Moreover, a study by Xiong et al. explored the function of M2 macrophages in bone formation and its underlying mechanisms. They co-cultured M2 macrophages and BM-MSCs and isolated the exosomes secreted by M2 macrophages, finding M2 macrophages-derived exosomes (M2D-Exos) were rich in miR-5106, which could be internalized by BM-MSCs and promote osteoblasts' differentiation to accelerate healing *via* targeting the osteogenic related genes, like Salt-inducible kinase 2 and 3 (SIK2 and SIK3). Therefore, local injection of M2D-Exos might be a significant therapeutic strategy to accelerate bone fracture healing (98).

Current studies provided powerful evidence that exosomes appeared to show the ability to enhance osteogenesis and angiogenesis to promote fracture healing *via* multiple pathways,

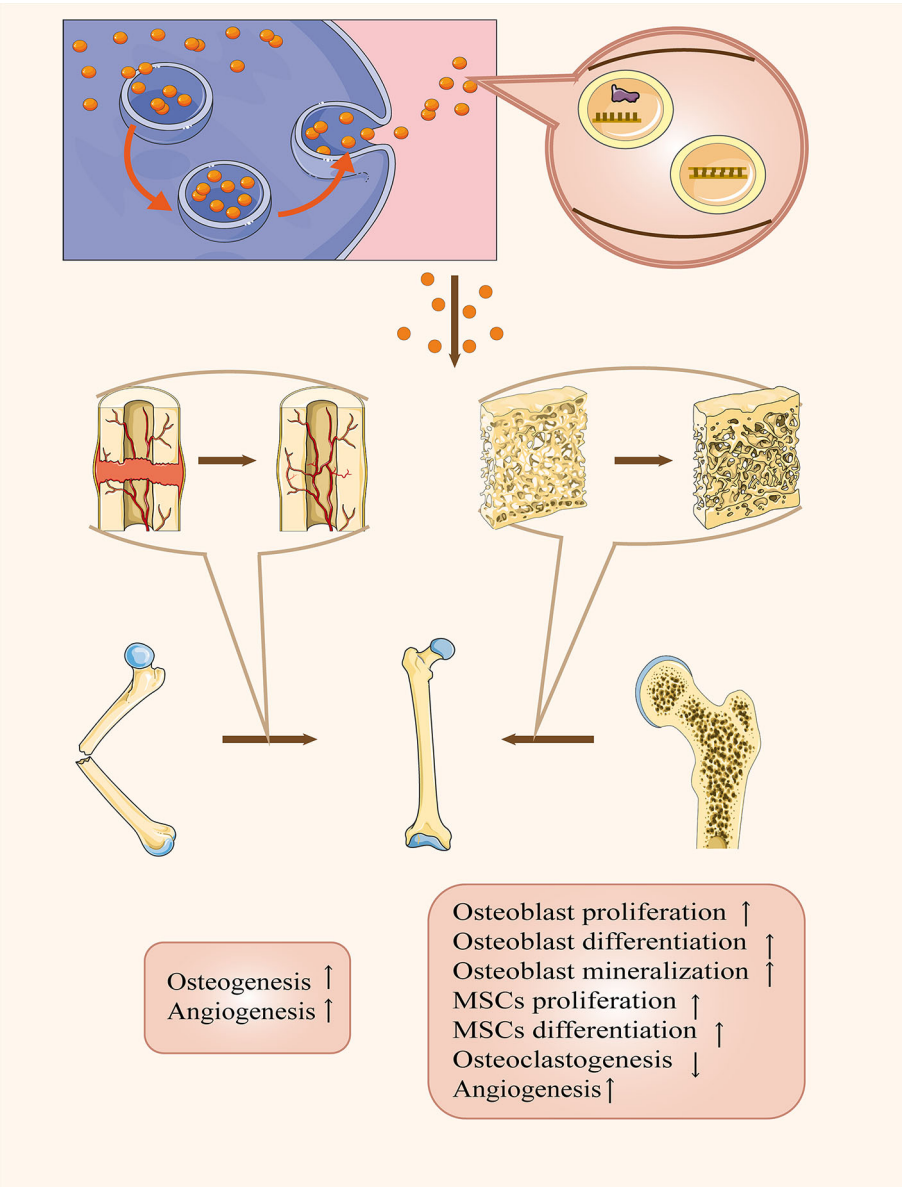

FIGURE 2 | The therapeutic applications of exosomes in OP and related fracture healing. Exosomes can act as therapeutic agents or drug carriers not only to remit OP by facilitating osteoblast proliferation, differentiation, mineralization, and MSCs proliferation, differentiation and angiogenesis as well as suppressing osteoclastogenesis but also accelerating fracture healing via promoting osteogenesis and angiogenesis.

implying a novel insight to comprehend the process of bone regeneration. Therefore, we need to spare no effort to do related research and detect an effective treatment for osteoporotic fracture.

SUMMARY AND PERSPECTIVES

The number of people with OP is increasing rapidly among generations, coupled with a growing number of osteoporotic fractures (102). Moreover, osteoporotic fractures increase the incidence of abnormal fracture healing, like delayed healing or non-union, which aggravates the heavy burden placed on healthcare systems as well as the economy. Hence, we should endeavor to look for more effective ways to prevent and treat osteoporotic fractures. Nowadays, the application of exosomes in osteoporotic fractures has received much attention, and lots of scientists have revealed multiple functions of exosomes in this field. As outlined above, exosomes played an essential role in mediating osteoporotic fractures. First, many researchers found obvious dysregulations of certain contents in circulatory exosomes from patients with OP or related impaired fracture healing, which could be promising biomarkers for the diagnosis of OP. Second, exosomes were a double-edged sword for OP by transporting their cargos to modify the activities of surrounding or distant bone-related cells. Third, exosomes have been applicated as a brilliant drug delivery system to treat osteoporotic fractures by accelerating osteogenesis and angiogenesis (**Figure 2**). However, the specific mechanisms of intercellular communications *via* exosomes is still not fully understood. A recent study by Ge et al. detected 1536 proteins contained in osteoblasts-derived exosomes and 172 among them overlapped with proteins in the bone database, such as ephrinB1 (EFNB1), transforming growth factor beta receptor 3 (TGFBR3), lipoprotein receptor-related protein (LRP6), bone morphogenetic protein receptor type-1 (BMPR1), and Smad ubiquitylation regulatory factor-1 (SMURF1) (103). Besides, increasing data unveiled there were nine overexpressed miRNAs (let-7a, miR-199b, miR-218, miR-148a, miR-135b, miR-203, miR-219, miR-299-5p, and miR-302b) and four down-regulated (miR-221, miR-155, miR-885-5p, miR-181a, and miR-320c) in MSCs-derived exosomes (100). However, these complicated mechanisms and the therapeutic potential for osteoporotic fractures remain a mystery and there is a clear need for us to perform further investigations. On the other hand, Xie et al. found that BM-MSCs-derived exosomes could promote bone formation when mixed with decalcified bone matrix scaffolds (104). But it is still a serious challenge to quantify and separate different exosome subpopulations as well as modify these exosomes for us. Overall, it should be noted that several significant developments are expected to occur, including: (*a*) clarifying the specific mechanisms of cargos within bone-related cells-derived exosomes in OP and osteoporotic fracture; (*b*) elucidating the functions of exosomes in the occurrence and healing of osteoporotic fractures, which possess both positive and negative effects; (*c*) discovering credible biomarkers contained in circulatory exosomes of clinical significance in the early diagnosis of OP; (*d*) improving the methodologies of extraction and separation of exosomes; and (*e*) propelling clinical application in the treatment of osteoporotic fracture through utilizing them as therapeutic agents or drug carriers. Advances in these areas will likely require new experimental techniques, superior creativity, and much work. But at the same time, such advances will help us to get a more comprehensive understanding of osteoporotic fractures and allow scientists to translate this knowledge into exosome-based therapies and diagnosis in clinic. Therefore, exosomes provide a promising method to improve the therapeutic effects of osteoporotic fractures, even though an increase in research is demanded.

AUTHOR CONTRIBUTIONS

ZY: Writing - Original Draft and Editing. WZ: Writing - Review and Editing, Visualization. XR: Writing - Original Draft. CT: Writing - Review and Editing, Supervision, Funding acquisition. ZL: Writing - Review and Editing, Supervision, Funding acquisition. All authors contributed to the article and approved the submitted version.

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Exosomes Derived From M2 Macrophages Facilitate Osteogenesis and Reduce Adipogenesis of BMSCs

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Bone regeneration is a complex process that requires the coordination of osteogenesis and osteoclastogenesis. The balance between osteogenesis and adipogenesis of bone marrow mesenchymal stem cells (BMSCs) plays a major role in the process of bone formation. Recently, intercellular communication between bone cells and surrounding cells has been gradually recognized, and macrophages on the surface of bone have been proven to regulate bone metabolism. However, the underlying mechanisms have not been fully elucidated. Recent studies have indicated that exosomes are vital messengers for cell-cell communication in various biological processes. In this experiment, we found that exosomes derived from M2 macrophages (M2D-Exos) could inhibit adipogenesis and promote osteogenesis of BMSCs. M2D-Exo intervention increased the expression of miR-690, IRS-1, and TAZ in BMSCs. Additionally, miR-690 knockdown in M2 macrophages with a miR-690 inhibitor partially counteracted the effect of M2D-Exos on BMSC differentiation and the upregulation of IRS-1 and TAZ expression. Taken together, the results of our study indicate that exosomes isolated from M2 macrophages could facilitate osteogenesis and reduce adipogenesis through the miR-690/IRS-1/TAZ axis and might be a therapeutic tool for bone loss diseases.

Keywords: macrophage, bone marrow mesenchymal stem cell, exosomes, osteogenesis, adipogenesis

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INTRODUCTION

The skeletal system undergoes constant remodeling through functional changes in osteocytes, osteoclasts, and osteoblasts throughout life (1). The orderly progress of bone reconstruction is a complex process that involves many factors, including enzymes, hormones, and cytokines (2). Once the balance of bone metabolism is disrupted, a variety of bone disorders will develop. Bone marrow mesenchymal stem cells (BMSCs) are stromal stem cells that are derived from bone marrow and possess multiplex differentiation potential (3). Osteoblasts mature from BMSCs to osteoprogenitor cells, to osteoblast precursors, and ultimately to osteoblasts. Therefore, BMSCs play a critical role in bone formation (4). In recent decades, the osteogenic and adipogenic properties of BMSCs have been found to maintain a dynamic balance (5–8). Thus, regulating the balance between osteogenesis and adipogenesis may be a potential treatment strategy for bone loss diseases.

Recently, our understanding of the bone system has moved beyond bone cells. The crosstalk between bone cells and surrounding cells as well as the relationship between the bone and bone microenvironment has been gradually recognized and regarded as a relatively important factor in bone metabolism (9). Among these factors, the effect of macrophages on bone has attracted increased attention. Osteal macrophages are located on the bone surface and are tightly related to the osteogenesis of BMSCs (10). As early as 2002, Champaign found that macrophages could promote the osteogenic differentiation of BMSCs (11). A recent study suggested that human bone marrow mesenchymal stem cells showed stronger proliferation and osteogenic differentiation when cocultured with inactive human monocytes (12). Polarized macrophages can be divided into the M1 type and M2 type. M1 macrophages, positive expression of CD86, can produce tumor necrosis factor-α (TNF-α), induced nitrogen monoxide synthase (iNOS), Interleukin-12 (IL-12), and other proinflammatory cytokines to induce immune response. M2 macrophages, positive expression of CD206, contribute to tissue repair and anti-inflammatory functions. Recently, M2 macrophages have been gradually recognized as a positive regulator of bone formation during fracture healing (13). However, the underlying mechanism of this beneficial effect remains elusive. In addition, whether M2 macrophages can regulate the balance of osteogenic and adipogenic differentiation of BMSCs needs to be further explored.

Exosomes are extracellular vesicles formed by invagination or endocytosis that contain various types of biological information (14). Recent studies have found that exosomes play a vital role in bone diseases (15, 16). In a rat calvarial defect model, macrophage exosomes could mediate bone regeneration (17). However, it has also been reported that exosomes from M0, M1, and M2 macrophages might have different effects on the osteogenic differentiation of BMSCs (18, 19). This may be due to the different information substances contained in different exosomes. A recent study has shown that M2 exosomes are rich in miR-690 and could regulate insulin sensitivity (20). Insulin receptor substrate 1 (IRS-1) is the major signaling adapters in insulin/IGF-1 pathways and has been proved to play an important role in bone metabolism (21, 22). Our previous study found that IRS-1 regulates the osteogenic and adipogenic differentiation of BMSCs through Transcriptional co-activator with PDZ-binding motif (TAZ) (23, 24). TAZ could be combined with peroxisome proliferator-activated receptor γ (PPARγ) and Runt-related transcription factor 2 (Runx2) to regulate the balance between osteogenesis and adipogenesis (25). In this study, we explored whether exosomes derived from M2 macrophages could regulate the balance between osteogenesis and adipogenesis through miR-690-IRS-1-TAZ.

MATERIALS AND METHODS

Cell Culture

All the experiments in the present research were approved by the Third Hospital of Hebei Medical University. The macrophage cell line RAW264.7 was incubated in α-MEM culture medium containing 10% fetal cattle serum (FBS) and 1% penicillin-streptomycin. For induction of M2 macrophage differentiation, RAW264.7 cells were cultured with 20 ng/ml Interleukin-4 (IL-4) for 24 h. Ten BALB/c male mice were obtained from the animal experimental center of Hebei Medical University and were used to separate BMSCs. Briefly, primary BMSCs were isolated from bone marrow and subsequently cultured in DMEM complete medium. For induction of osteogenesis, BMSCs were grown in osteogenic medium containing 10 mM β-glycerophosphoric acid, 10 nmol/L dexamethasone, and 50 μg/ml ascorbic acid. Adipogenesis was induced by 10 mg/ml insulin, 500 mmol/L methyl-isobutylxanthine, and 1 μmol/L dexamethasone. All cells were cultured at 37°C with 5% CO_2.

Identification of M2 Polarization Macrophages and Exosome

After RAW264.7 cells were cultured with 20 ng/ml Interleukin-4 (IL-4) for 24 h, the positive surface markers CD206 (BioLegend, San Diego, CA, USA) was analyzed by flow cytometry analysis.

After M2 differentiation of macrophages was induced, the MinuteTM efficient exosome precipitation reagent purchased from Inent Biotechnologies Company was used to obtain the exosomes according to the instructions. In short, the culture medium of M2 macrophages was harvested after culture in serum-free medium for 15 h. Then, cells were removed from the samples by low-speed centrifugation (5 min, 1,000 g), and the culture medium was obtained for further separation. The collected media was subsequently incubated with exosome precipitant overnight at 4°C. Next, the samples were centrifuged for 1 h at 10,000 g, and the supernatant was removed. The isolated exosomes were harvested and stored at −80°C for future use.

For further identification, the morphology and diameter of exosomes were characterized by transmission electron microscopy (Hitachi HT7700 TEM, Tokyo, Japan). Twenty microliter exosome samples were aspirated with a pipette gun and placed in carbon membrane copper mesh for 5 min. Then, the excess liquid was aspirated with filter paper. The sample was subsequently stained with 2% phosphotungstic acid for 2 min. The excess liquid was absorbed with filter paper. Finally, images of exosomes were collected by TEM. The number and size of exosomes were characterized by nanoparticle tracking analysis (NTA) using NanoSight NS-300. In addition, CD63 and CD81, exosomal specific surface proteins, were detected by western blotting.

Exosome Uptake Assay

According to the instructions, exosomes were labeled with PKH26 (Sigma-Aldrich, Germany). First, 30 μl of exosomes was diluted in 1 ml of diluent C and 6 μl of PKH26 dye for 5 min. Then, 10% bovine serum albumin (BSA) was added to neutralize the excess dye, followed by washing in PBS for 60 min. Finally, BMSCs were treated with the labeled exosomes and analyzed by a fluorescence microscope.

miRNA Inhibitor and Si-IRS-1 Plasmids Transfection

Cells were transfected with 30 nM miR-690 inhibitor or 30 nM inhibitor negative control (inhibitor NC) using Lipofectamine 3000 (Thermo Fisher Scientific, USA) based on the manufacturer's protocol.

Briefly, miR-690 inhibitor or inhibitor NC was mixed with Lipofectamine 3000 for 20 min separately before being cultured with cells. Cells were collected for further miRNA analysis at 48 h post-transfection. The miR-690 inhibitor and inhibitor negative control were all obtained from Zhongshi Tongtru (Tianjin, China). The sequences were as follows: miR-690 inhibitor 5'-UUUGGUUGUGAGCCUAGCCUUU-3' and miR-690 inhibitor NC 5'-CAGUACUUUUGUGUAGUACAA-3'.

BMSCs were transfected with Si-IRS-1 or control plasmid (Genechem, Shanghai, China) using Lipofectamine 3000 after reaching 80% confluence, in accordance with manufacturer's instructions.

Alizarin Red Staining (A-R Staining)

A-R staining was performed after 2 weeks of osteoblast induction. In short, BMSCs were fixed with 4% paraformaldehyde (Solarbio, China) for 10 min after being washed twice with PBS. Then, 1% A-R staining solution was incubated for half an hour. Finally, red mineralized nodules were observed. In addition, the absorbance at 570 nm was subsequently measured *via* a microplate spectrophotometer (BioTek Instruments, San Jose, CA, USA).

Oil Red O staining

Lipid droplet formation was detected by oil red O staining after culture in adipogenic medium for 2 weeks. Briefly, BMSCs were washed twice with PBS followed by fixation with 4% paraformaldehyde for 10 min. Then, oil red O staining solution was added to stain the cells for half an hour. Finally, the formation of lipid droplets was captured by a microscope. Moreover, the absorbance at 520 nm was subsequently analyzed.

Real-Time Reverse Transcription Polymerase Chain Reaction (Real-Time RT-PCR)

For the analysis of mRNA, total RNA was extracted using TRIzol® reagent (Tiangen, Beijing, China) before reverse transcription into cDNA *via* a RevertAid™ First Strand cDNA synthesis kit (Thermo, Waltham, USA) based on the instructions. Then, RT-PCR analysis was performed according to the protocols for Tiangen SuperReal PreMix Plus.

For the analysis of miRNA, microRNA assay kits were purchased from Zhongshi Tongtru (Tianjin, China) and used following the manufacturer's instructions.

Table 1 shows the PCR primer sequence, which is designed by primer software.

Western Blotting

RIPA lysis buffer containing 1% protease inhibitor (Zhongshi Tongtru, Tianjin, China) was used to obtain total protein from the samples. Then, 20 μg protein was separated *via* SDS-PAGE and transferred to PVDF membranes before being blocked with 5% milk for 1 h. The PVDF membranes were stained at 4°C overnight with antibodies specific for IRS-1 (1:1,000, #2382, Cell Signaling, Beverly MA,USA), TAZ (1:1,000, #72804, Cell Signaling, Beverly MA,USA), OCN (1:1,000, ab274873, Abcam, Cambridge, England), RUNX2 (1:1,000, ab236639, Abcam, Cambridge, England), CEBPβ (1:1,000, ab32358, Abcam, Cambridge, England), PPARγ (1:1,000, ab272718, Abcam, Cambridge, England), and GAPDH (1:1,000, ab8248, Abcam, Cambridge, England). Finally, blots were stained with fluorescence secondary antibodies and detected with the Odyssey Infrared Imaging System (Li-COR Biosciences).

TABLE 1 | Primers used for RT-PCR.

Gene	Forward Sequence (5′—>3′)	Reverse Sequence (5′—>3′)
GAPDH	AGTTCAACGGCACAGTCAAGG	AGCACCAGCATCACCCCAT
IRS-1	CCTGACATTGGAGGTGGGTC	TTACCACCACCGCTCTCAAC
TAZ	GTCACCAACAGTAGCTCAGATC	AGTGATTACAGCCAGGTTAGAAAG
RUNX2	GGACTGGGTATGGTTTGTAT	GCTGAAGAGGCTG TTTGA
OCN	ACCACATCGGCTTTCAGG	CATAGGGCTGGGAG GTCA
C/EBPβ	GCGGGGTTGTTGATGTTT	CTTTAATGCTC GAAACGG
PPARγ	CCTTGCTGTGGGGATGTCTCA	CTCCTTCTCGGCCTGTGGCAT
miR-690	5'-CTCAACTGGTGTCGTGGAGTCGGCAA TTCAGTTGAGTTTGGTT- 3'	
U6	5'-AACGCTTCACGAATTTGCGT-3′	

Statistical Analysis

Experimental data were obtained from at least three replicates and are shown as the mean ± standard deviation. Student's t-tests and one-way ANOVA with Tukey's *post hoc* test were used to compare the data between different groups as appropriate. $p < 0.05$ was considered statistically significant.

RESULTS

Characterization of M2D-Exos

Compared to PBS-treated macrophages, IL-4-treated macrophages showed significant upregulation of CD206 (**Figure 1A**), suggesting that the RAW264.7 cells were successfully induced to M2 polarization after stimulation with IL-4.

Exosomes were first harvested from the supernatants of M2 macrophages *via* exosome separation reagent. TEM, NTA, and western blotting analyses were performed to identify the collected M2D exosomes. Typical round or cup-shaped exosomal structures were revealed by TEM (**Figure 1B**). Western blotting analysis further provided evidence that the isolated particles were positive for exosomal surface markers, including CD63 and CD81 (**Figure 1C**). NTA analysis revealed that the diameters of these exosomes ranged from 50 to 150 nm (**Figure 1D**).Therefore, these above analyses confirmed the successful collection of M2D-Exos.

Subsequently, to verify whether the M2D-Exos could be endocytosed by BMSCs, we labeled the M2D-Exos with PKH26 and further cocultured them with BMSCs. As shown in **Figure 1E**, PKH26-labeled exosomes were localized in the BMSC region, which exhibited efficient internalization of the M2D-Exos by BMSCs.

M2D-Exos Facilitated BMSCs Osteogenesis *In Vitro*

To further analyze the effect of M2D-Exo function on the osteogenesis of BMSCs, we performed western blotting, RT-PCR, and Alizarin red staining. The expression of the osteogenic differentiation-related proteins RUNX2 and osteocalcin (OCN) was upregulated after culture with M2D-

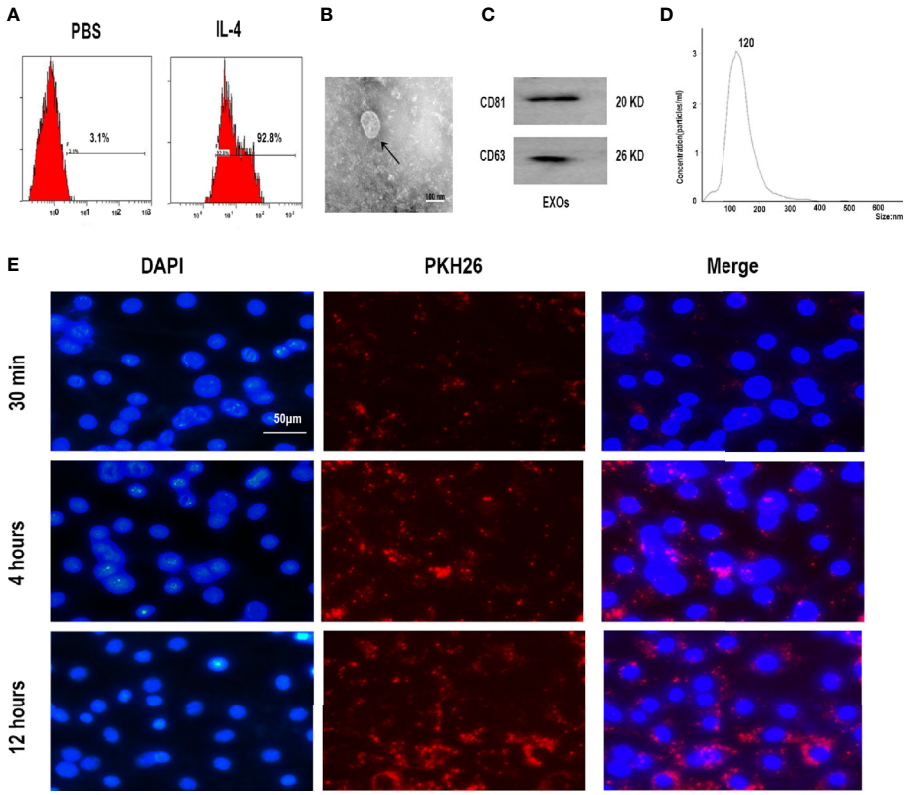

FIGURE 1 | Characterization of M2 macrophages and exosomes derived from M2 macrophages. **(A)** Expression of M2 marker CD206 in polarized RAW 264.7 cells analyzed by flow cytometry. **(B)** Morphology identified by TEM, scale bar:100 nm. **(C)** The surface biomarkers CD63, CD81 were analyzed by Western blotting assay. **(D)** Size distribution profiles of M2D-Exos was detected by nanoparticle tracking analysis. **(E)** The exosomes derived from M2 macrophages was marked with red fluorescence dye PKH26 and co-cultured with BMSCs, scar bar = 50 μm.

Exos (**Figures 2A, B**). Consistent with this finding, significantly higher mRNA levels of RUNX2 and OCN were detected in the M2D-Exo group than in the control group (treated with PBS) (**Figure 2C**). In addition, the Alizarin red staining results revealed that the mineral deposition staining in the M2D-Exo-treated BMSCs was significantly larger than that in the control group (**Figures 2D, E**). The above findings indicate that M2D-Exos could increase the osteogenic differentiation of BMSCs.

M2D-Exos Inhibited BMSCs Adipogenesis *In Vitro*

For further elucidation of the function of M2D-Exos in the adipogenesis of BMSCs, adipogenic differentiation-related genes, and proteins as well as the formation of lipid droplets were analyzed. The expression of CCAAT/enhancer binding protein β (C/EBPβ) and PPARγ was downregulated after treatment with M2D-Exos (**Figures 3A, B**). Similarly, the RT-PCR results showed lower mRNA levels of C/EBPβ and PPARγ in the M2D-Exo group compare to PBS-treated BMSCs (**Figure 3C**). In addition, oil red O staining revealed reduced formation of lipid droplets in the M2D-Exo-treated cells (**Figures 3D, E**), indicating an inhibitory effect of M2D-Exos on adipogenesis.

M2D-Exos Delivered miR-690 Into BMSCs and Increased the Expression of IRS-1 and TAZ

To explore the mechanism involved in the regulation of the differentiation of BMSCs by M2D-Exos, we then detected the expression of miR-690, IRS-1, and TAZ. Western blotting analysis showed that TAZ and IRS-1 expression during osteogenesis was significantly increased in the M2D-Exo-induced BMSCs (**Figures 4A, B**). Moreover, M2D-Exo intervention led to an increased level of miR-690 during osteogenic differentiation (**Figure 4C**). During adipogenic differentiation, we also detected the expression of miR-690 and the IRS-1 and TAZ proteins. The results were consistent with those for osteogenic differentiation. M2D-Exo administration resulted in an increase in the levels of miR-690, IRS-1, and TAZ (**Figures 4D–F**). We then knocked down IRS-1 expression using si-IRS-1 plasmids (**Figures 4G, H**). Western blot analyses suggested that Si-IRS-1 significantly blocked the upregulation of TAZ expression after the intervention of M2D-Exos (**Figures 4I, J**). The above results indicated that M2D-Exos might up-regulate miR-690 of BMSCs and increase the expression of IRS-1 and TAZ to regulate the balance between adipogenesis and osteogenesis.

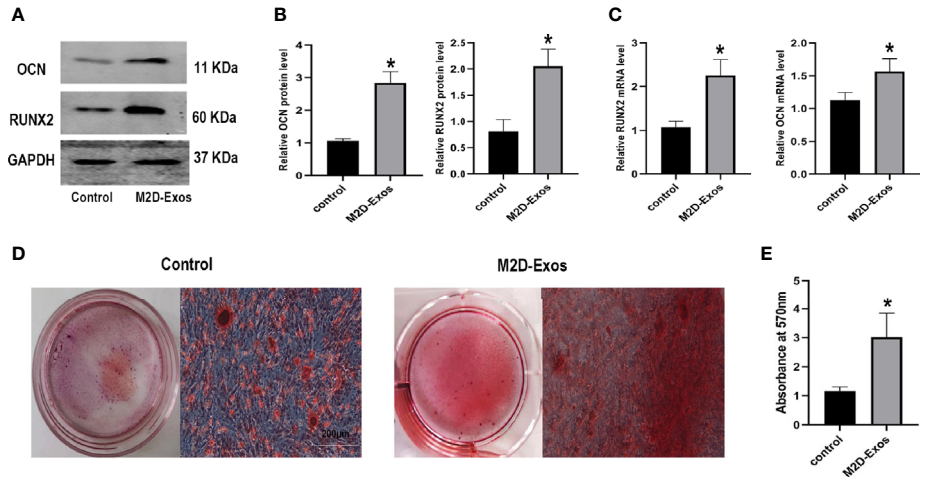

FIGURE 2 | Exosomes derived from M2 macrophages enhanced osteogenic differentiation of BMSCs. **(A, B)** Osteogenic proteins, OCN and RUNX2, were measured by western blotting analysis and quantified, Control group was BMSCs treated in osteogenic inducer. **(C)** Osteogenic genes, OCN and RUNX2, were measured by RT-PCR analysis, Control group was BMSCs treated in osteogenic inducer. **(D)** Alizarin red staining was used to measure the formation of bone nodules in BMSCs after treated by PBS (control group) or M2D-Exos for 14 days. Scale bar = 200 μm. **(E)** The statistical data of Alizarin red-mediated calcium staining. *p < 0.05 compared to the control group. Control group was BMSCs treated in osteogenic inducer.

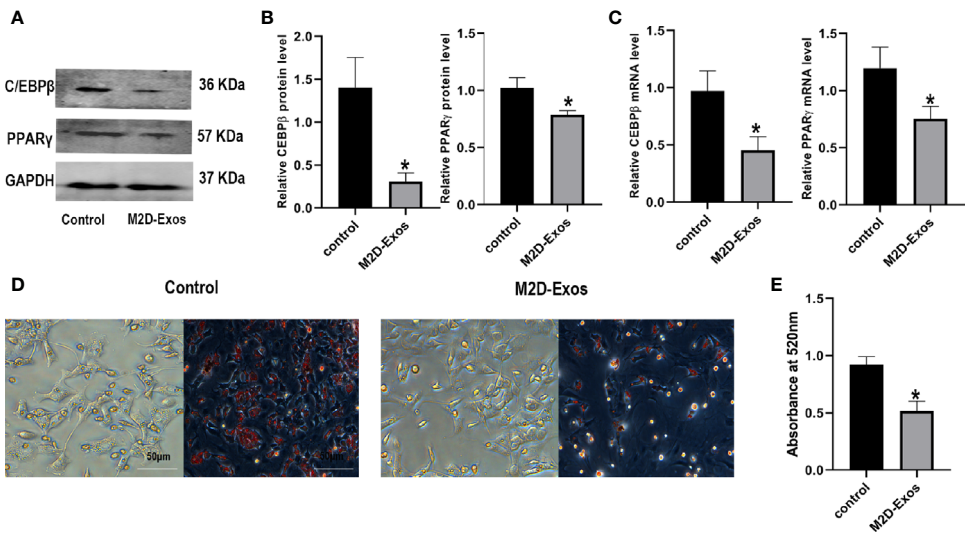

FIGURE 3 | Exosomes derived from M2 macrophages inhibited adipogenic differentiation of BMSCs. **(A, B)** Adipogenic proteins, C/EBPβ and PPARγ, were measured by western blotting analysis and quantified. Control group was BMSCs treated in adipogenic inducer. **(C)** Osteogenic genes, C/EBPβ and PPARγ, were measured by RT-PCR analysis. Control group was BMSCs treated in adipogenic inducer. **(D)** Oil red O staining was used to measure the formation of lipid droplets in BMSCs after treated by PBS (control group) or M2D-Exos for 14 days. Scale bar = 50 μm. **(E)** The statistical data of Oil red O staining. *p < 0.05 compared to the control group. Control group was BMSCs treated in adipogenic inducer.

miR-690 Inhibitor Abolished the Effect of M2D-Exos on Osteogenesis and Adipogenesis of BMSCs

To investigate whether miR-690 mediates the M2D-Exo-derived regulation of adipogenesis and osteogenesis, we first detected the expression of miR-690 in BMSCs after the intervene of M2D-Exos. The level of miR-690 was significantly increased after treated with M2D-Exos (**Figure 5A**), showing that M2D-Exos might transmit miR-690 to BMSCs. We then transfected a miR-690 inhibitor and inhibitor control into M2 macrophages.

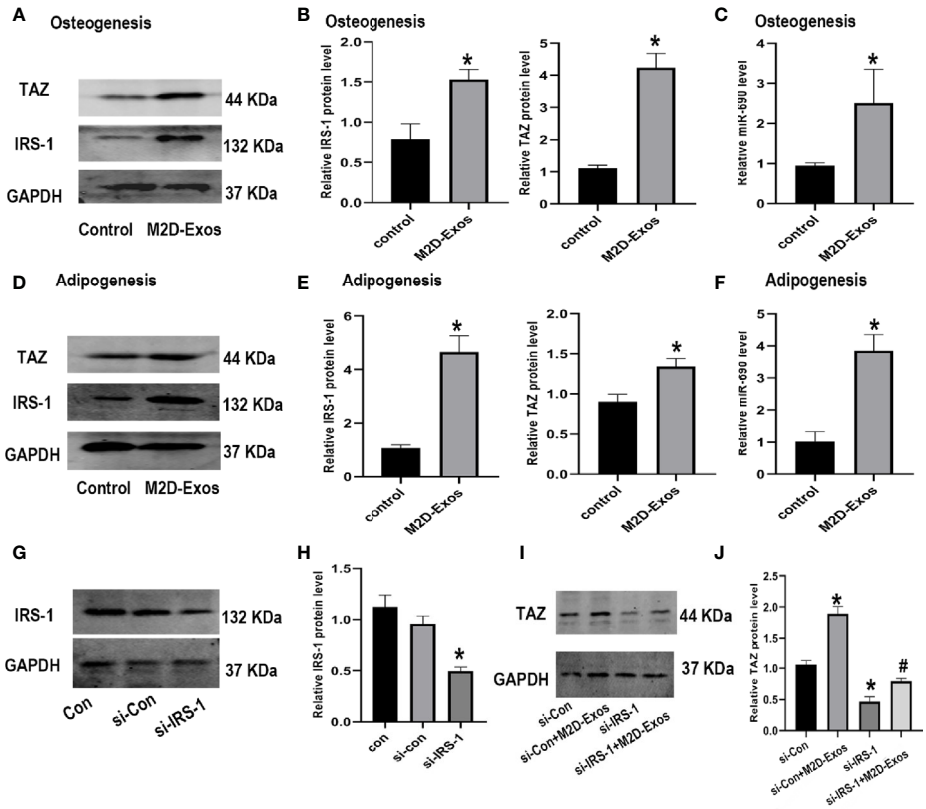

FIGURE 4 | Exosomes derived from M2 macrophages increased the level of IRS-1 and TAZ protein of BMSCs. **(A, B)** IRS-1 and TAZ protein of BMSCs after treated with M2D-Exos or PBS during osteogenesis were measured by western blotting analysis and quantified. Control group was BMSCs treated in osteogenic inducer. **(C)** The level of miR-690 of BMSCs after treated with M2D-Exos during osteogenesis were measured by qRT-PCR analysis. Control group was BMSCs treated in osteogenic inducer. **(D, E)** IRS-1 and TAZ protein of BMSCs after treated with M2D-Exos or PBS during adipogenesis were measured by western blotting analysis and quantified. Control group was BMSCs treated in adipogenic inducer. **(F)** The level of miR-690 of BMSCs after treated with M2D-Exos during adipogenesis were measured by qRT-PCR analysis. Control group was BMSCs treated in adipogenic inducer. **(G, H)** IRS-1 expression was analyzed using western blotting after transfected with different plasmids. **(I, J)** TAZ expression was analyzed using western blotting after different treatments. *$p < 0.05$ compared to the control group.

Compared with the inhibitor control, the miR-690 inhibitor significantly inhibited the level of miR-690 in M2D-Exos (**Figure 5B**). In addition, BMSCs were cocultured with miR-690 inhibitor-treated exosomes or miR-690 inhibitor control-treated exosomes, and the miR-690 inhibitor-treated exosome group showed a lower level of miR-690 than the inhibitor control group (**Figures 5C, D**). Furthermore, the miR-690 inhibitor attenuated the M2D-Exo-induced increases in IRS-1 and TAZ levels and the subsequent upregulation of osteogenic marker gene (RUNX2 and OCN) expression in osteogenic medium (**Figure 5E**). Similarly, the miR-690 inhibitor-treated exosomes inhibited the mRNA expression of IRS-1 and TAZ and increased the mRNA expression of C/EBPβ and PPARγ during adipogenic differentiation (**Figure 5F**). Furthermore, the results of Alizarin Red staining and oil red O staining were consistent with the real-time RT-PCR analyses and verified the mechanism by which M2D-Exos facilitated osteogenic differentiation and inhibited adipogenic differentiation by miR-690 (**Figures 5G–J**).

DISCUSSION

The close coupling of bone resorption and bone formation is vital to maintain bone remodeling. BMSCs have been confirmed to have an indispensable role in this continuous process (4). Recently, impaired bone formation with aging and diseases has been shown to be accompanied by decreased osteogenesis and increased adipogenesis (26, 27). Therefore, exploring the mechanism regulating the balance between osteogenesis and adipogenesis of BMSCs might be beneficial to further treatment for bone loss. Macrophages on the surface of bone may play an important role in the regulation of bone formation (18). In the present study, M2D-Exos promoted osteogenic differentiation and inhibited adipogenic differentiation of BMSCs through up-regulate miR-690, IRS-1, and TAZ.

The balance between osteogenesis and adipogenesis of BMSCs plays a vital role in bone remodeling (28). However, the differentiation of BMSCs is a complex process that is regulated by various factors. Recently, the bone microenvironment has been

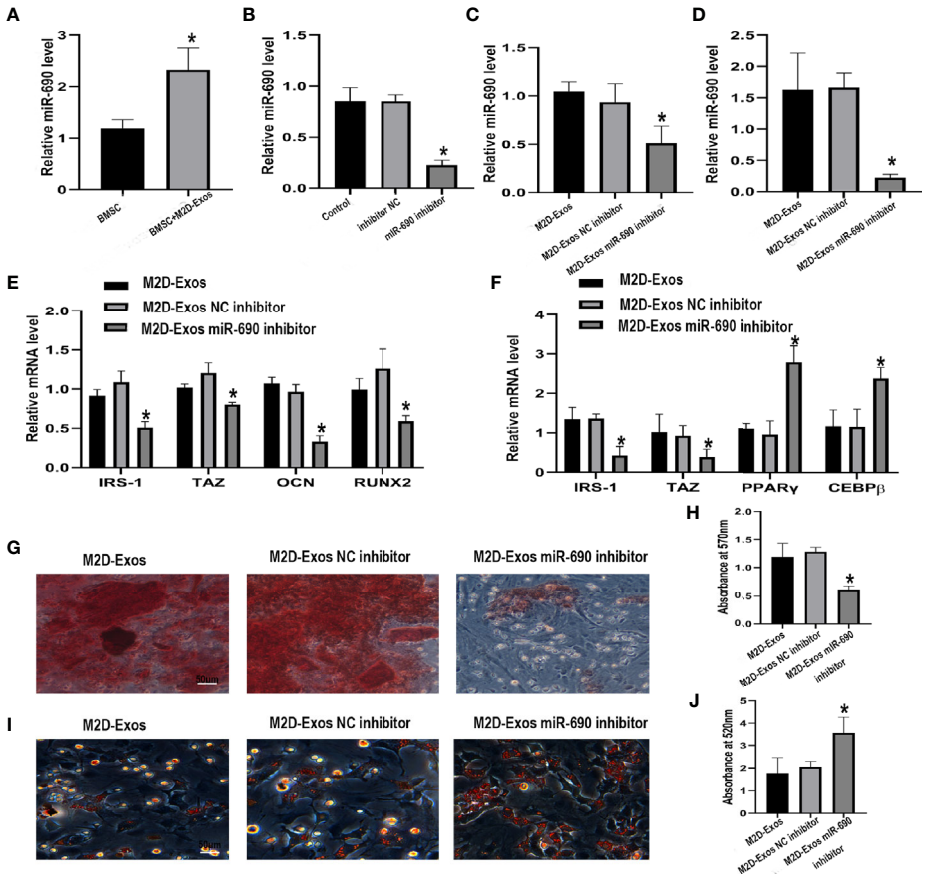

FIGURE 5 | The inhibitor of miR-690 partially reversed the up-regulation osteogenesis and down-regulation adipogenesis induced by M2D-Exos. **(A)** The level of miR-690 in BMSCs after treated with M2D-Exos or PBS. *p < 0.05 compared to the BMSC group. **(B)** The level of miR-690 in M2 macrophages after treated with lipo3000, miR-690 inhibitor, or inhibitor control measured by qRT-PCR analysis. Control group was M2 macrophages treated with lipo3000. *p < 0.05 compared to the inhibitor control group. **(C)** The level of miR-690 in BMSCs after treated with M2D-Exos, miR-690 inhibited M2D-Exos, or inhibitor control M2D-Exos before cultured in osteogenic inducer were measured by qRT-PCR analysis. *p < 0.05 compared to the M2D-Exos NC inhibitor group. **(D)** The level of miR-690 in M2 macrophages after treated with lipo3000, miR-690 inhibitor, or inhibitor control before cultured in adipogenic inducer were measured by RT-PCR analysis. *p < 0.05 compared to the M2D-Exos NC inhibitor group. **(E)** IRS-1, TAZ, OCN, RUNX2 mRNA level of BMSCs after treated with M2D-Exos, miR-690 inhibited M2D-Exos, or inhibitor control M2D-Exos during osteogenesis were measured by qRT-PCR analysis. *p < 0.05 compared to the M2D-Exos NC inhibitor group. **(F)** IRS-1, TAZ, C/EBPβ, and PPARγ mRNA level of BMSCs after treated with M2D-Exos, miR-690 inhibited M2D-Exos, or inhibitor control M2D-Exos during adipogenesis were measured by qRT-PCR analysis. *p < 0.05 compared to the M2D-Exos NC inhibitor group. **(G)** A-R staining was used to measure the formation of bone nodules in BMSCs following treated by M2D-Exos, miR-690 inhibited M2D-Exos, or inhibitor control M2D-Exos for 14 days. Scale bar = 50 μm, **(H)** The statistical data of Alizarin red-mediated calcium staining. **(I)** Oil red O staining was used to measure the formation of lipid droplets in BMSCs following treated by M2D-Exos, miR-690 inhibited M2D-Exos, or inhibitor control M2D-Exos for 14 days. Scale bar = 50μm. **(J)** The statistical data of Oil red O staining. *p < 0.05 compared to the M2D-Exos NC inhibitor group.

confirmed to have an indispensable function in osteogenesis (29). Macrophages are adjacent to bone. Macrophage-derived TNF-α can increase the chemotactic ability of osteoblasts (30). BMP-2 released by macrophages plays a vital role in the process of ossification through the Wnt and Wnt/LRP5 signaling cascades (31, 32). Macrophages can readily differentiate into different subtypes to regulate tissue homeostasis according to local cellular and secreted signals. In short, there are two polarized (M1 and M2) states and one unpolarized state (M0) of macrophages (32). M1 macrophages exert proinflammatory functions (33). In contrast, M2 macrophages contribute to the repair of tissue and exert anti-inflammatory functions (34). Different subtypes of macrophages exert different effects on bone formation. Recently, the positive role of M2 macrophages in bone remodeling during bone fracture has been gradually recognized. However, the exact mechanism is still unclear. Cell-conditioned medium generated by M2 macrophages influences the cellular behaviors of BMSCs (35), suggesting a paracrine effect of macrophages on BMSCs.

Exosomes have been demonstrated to be ideal transport devices for delivering regulatory substances to target cells. Cells receive messages within exosomes (including proteins and nucleic acids) through internalization to achieve communication between different types of cells. Recently, the regulatory effect of macrophage-derived exosomes on intestinal stem cells, endothelial cells, and tumors has

been confirmed (36). Moreover, the role of M2D-Exos in bone metabolism has been gradually recognized (37). In the present study, PKH26 staining indicated that M2D-Exos can be internalized by BMSCs. M2D-Exo-treated BMSCs had a stronger osteogenic differentiation ability. This result is consistent with previous studies and suggests that exosomes are important mediators during M2 macrophage-induced osteogenesis of BMSCs (13). In addition, we further verified the effect of M2D-Exos on the adipogenic differentiation of BMSCs. The results revealed that M2D-Exos could inhibit adipogenic differentiation and the formation of lipid droplets in BMSCs. The above results suggest that M2D-Exos could regulate the balance of osteogenesis and adipogenesis, providing a potential therapeutic strategy for the treatment of bone loss diseases.

Small RNA molecules and miRNAs are involved in many diseases and processes, such as cancer and endocrine diseases (38). Evidence has also shown that exosomes can transport miRNAs to cells. A recent study showed that M2D-Exos are rich in miR-690 (20). In the present study, a higher level of miR-690 was also found in the M2D-Exo-treated BMSCs. Previous study has reported that miR-690 might up-regulate osteogenic differentiation by targeting NF-kappaB p65 (39). In addition, miR-690 could repress transcription factor CCAAT/enhancer-binding protein α (C/EBPα), which is an important regulation factor during adipogenesis (40). Therefore, miR-690 might mediate the regulation of M2D-Exos in the process of osteogenesis and adipogenesis. In the present study, we found that M2D-exos enhanced osteogenesis, decreased adipogenesis, and increased miR-690 level of BMSCs. At the same time, the levels of IRS-1 and TAZ were increased by M2D-exos intervention. A previous study showed that miR-690 can improve insulin sensitivity (20). As a major signaling adapter of the insulin/IGF-1 signaling pathway, IRS-1 stimulates a variety of downstream pathways to participate in the regulation of insulin resistance and cell differentiation (41). Our previous studies have shown that IRS-1 targets TAZ to facilitate osteogenesis and reduce adipogenesis in rat BMSCs (23, 24). TAZ is a transcription modulator that can influence stem cell fate determination through transcription factors (42). This molecule regulates signaling cascade genes to regulate the balance of osteogenesis and adipogenesis (43). In the present study, the level of TAZ and IRS-1 expression was increased after the administration of M2D-Exo. Knockdown of IRS-1 partially abolished the M2D-Exos induced elevation of TAZ in BMSCs. Thus, we hypothesized that M2D-Exos might regulate the differentiation of BMSCs *via* IRS-1/TAZ. To further verify the role of miR-690, we used a miR-690 inhibitor to reduce the level of miR-690 in M2D-Exos. The results showed that the expression of IRS-1 and TAZ was lower in the miR-690 inhibited-M2D-Exo-treated BMSCs than the inhibitor control-treated cells. Moreover, miR-690 inhibition in M2D-Exos partly counteracted the effect of M2D-Exos on promoting osteogenesis and inhibiting adipogenesis. Taken together, the results of our study indicate that exosomes isolated from M2 macrophages could facilitate osteogenesis and reduce adipogenesis through miR-690/IRS-1/TAZ.

The balance of osteogenesis and adipogenesis of BMSCs is vital for maintaining bone mass, which is regulated by various factors. Recently, macrophages had been found to play an important role in bone metabolism. However, the specific mechanism is still unclear. In the present study, we observed that exosomes derived from M2 macrophages could facilitate osteogenesis and reduce adipogenesis of BMSCs *in vitro*. This positive effect is at least partially mediated by miR-690, which is enriched in M2D-Exos and can upregulate the levels of IRS-1 and TAZ. Therefore, our findings suggested a potential role of M2D-Exos as a therapeutic tool for bone loss.

However, there are still some limitations in this study. First, we used RAW264.7 cells instead of primary macrophages. Second, we have not explored the direct target of miR-690 in BMSC. Third, this study is carried out *in vitro*. Next, we will further explore the direct target of miR-690 regulating IRS-1/TAZ in primary cells, and verify the positive role of M2D-exos in osteoporosis animal models.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Ethics Association of the Third Hospital of Hebei Medical University.

AUTHOR CONTRIBUTIONS

YL and ZL designed the study. ZL, SL, and YW collected the data, carried out the data analysis, and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Role of Exosomes in Islet Transplantation

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Exosomes are known for their ability to transport nucleic acid, lipid, and protein molecules, which allows for communication between cells and tissues. The cargo of the exosomes can have a variety of effects on a wide range of targets to mediate biological function. Pancreatic islet transplantation is a minimally invasive cell replacement therapy to prevent or reverse diabetes mellitus and is currently performed in patients with uncontrolled type 1 diabetes or chronic pancreatitis. Exosomes have become a focus in the field of islet transplantation for the study of diagnostic markers of islet cell viability and function. A growing list of miRNAs identified from exosomes collected during the process of isolating islets can be used as diagnostic biomarkers of islet stress and damage, leading to a better understanding of critical steps of the isolation procedure that can be improved to increase islet yield and quality. Exosomes have also been implicated as a possible contributor to islet graft rejection following transplantation, as they carry donor major histocompatibility complex molecules, which are then processed by recipient antigen-presenting cells and sensed by the recipient immune cells. Exosomes may find their way into the therapeutic realm of islet transplantation, as exosomes isolated from mesenchymal stem cells have shown promising results in early studies that have seen increased viability and functionality of isolated and grafted islets *in vitro* as well as *in vivo*. With the study of exosomes still in its infancy, continued research on the role of exosomes in islet transplantation will be paramount to understanding beta cell regeneration and improving long-term graft function.

Keywords: exosome, biomarker, diabetes mellitus, miRNA, islet transplantation, islet stress, cytokines, nucleic acids

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INTRODUCTION

Diabetes mellitus is characterized by chronic hyperglycemia due to partial or absolute insulin deficiency (type 1 diabetes, T1D) or pancreatic beta cell dysfunction and/or insulin inaction (type 2 diabetes, T2D) (1–3). In T1D, autoimmune cells infiltrate pancreatic islets (insulitis) and target pancreatic beta cells for destruction, leading to loss of the beta cell mass necessary for maintenance of euglycemia. T2D is a heterogeneous disease, with impairments or abnormalities in synthesis/secretion of insulin from islet beta cells, tissue sensitivity to insulin, or insulin action. Type 3c

diabetes mellitus (T3cD), the least known or recognized form of diabetes mellitus (4, 5), is caused by pathologies of the exocrine pancreas that cause islet inflammation and damage (6). Chronic pancreatitis (CP), the most common cause of T3cD, is treated based on the etiology on a case-by-case basis. For refractory CP, partial or total pancreatectomy is performed to alleviate severe pain associated with chronic inflammation and necrosis. Current therapeutic options vary based on the type of diabetes. T2D is typically managed through lifestyle changes and medications targeting insulin resistance and/or beta cell function (7). For T1D and some cases of T3cD, exogenous insulin therapy is the standard of care. Even with aggressive therapy, a subset of diabetic patients present with glycemic lability, hypoglycemia unawareness, severe hypoglycemic episodes, diabetic ketoacidosis, and other complications of diabetes, including cardiovascular and kidney disease (8).

Pancreatic islet transplantation is an effective, minimally invasive treatment option for T1D or T3cD. In the 1980s, the first allo- (for T1D) and auto- (after pancreatectomy) islet transplants in humans were performed (9, 10), with poor long-term outcomes due to peri-transplant inflammation and ineffective immunosuppression (11, 12). In 2000, the Edmonton protocol with improved induction immunotherapy, a corticosteroid-free immunosuppressive regimen, and optimal islet dose, was established (13). Despite initial success in achieving insulin independence 1 year after transplantation, these patients exhibited poor long-term islet graft function. Several reports including our own data suggest that 50% to 70% of transplanted islet cells are lost during the islet isolation process and in the peri-transplant period due to an innate immune response called instant blood-mediated inflammatory reaction. This acute response involves the complement and coagulation systems and activation of inflammatory mediators (14, 15). Post-transplant inflammatory events lead to the recognition of the islet graft (either auto or allo) by the host immune system (innate or adaptive) and to the eventual rejection of transplanted islet cells. In allogeneic islet transplantation, alloantigen presentation to the host immune system triggers immune cell infiltration of the graft tissue, resulting in graft rejection. Observations in independent CP and T1D cohorts suggest that an optimal islet yield (>5000 islet equivalents/kg), islet survival in the peri-transplant period and engraftment, and optimal immunosuppressive regimen are important for achieving post-transplant insulin independence (16, 17). Despite the advancements made, short- and long-term graft survival and function remain suboptimal and a challenge to be overcome (18, 19).

Islet graft function and survival after transplantation are commonly assessed by glucose tolerance tests, C-peptide and hemoglobin A1c levels, exogenous insulin usage, and noninvasive imaging techniques (20, 21). However, these assessments tend to reflect the loss of islet function, offering little insight into islet stress, islet engraftment, and the ongoing loss of islet function. Establishing robust noninvasive methods to monitor islet survival and function after transplantation will help in the development of novel strategies to alleviate islet damage and improve transplantation outcomes. These challenges warrant further research on 1) delineating mechanisms of acute and chronic graft rejection, 2) monitoring islet stress and damage during and after transplantation, and 3) tailoring existing protocols to achieve improved short- and long-term outcomes.

In this regard, extracellular vesicles called 'exosomes' have emerged as prominent players in the assessment of islet function. Exosomes play an important role in donor-to-host cell communication, especially in presenting donor antigens to host immune cells and in horizontal transfer/dissemination of their content. Recent research also suggests a role for exosomes in carrying islet stress or damage molecular markers in circulation. In this review, we provide an overview of the roles of exosomes in allogeneic and autologous antigen presentation to the recipient immune system, exosome cargo, and the utility of exosomes for diagnosis and therapy.

EXOSOME FORMATION AND RELEASE

Extracellular vesicles are released by almost all types of cells under physiological or pathophysiological conditions. These include microvesicles (100–1000 nm), apoptotic bodies (1–5 μm), oncosomes (1–10 μm), and exosomes (30–100 nm) (22, 23). Exosomes consist of a lipid bilayer membrane and an inner lumen containing diverse bioactive molecules including lipid, protein, and nucleic acid species (24, 25). After endocytosis, intraluminal vesicles (ILVs) are formed by inward budding of the endosomal membrane and are ultimately released as exosomes by exocytosis (26). These late endosomes containing ILVs are referred to as multivesicular bodies (27). During formation of ILVs, endosomal sorting complex required for transport (ESCRT) aids in the packaging of bioactive species, including nucleic acid species, proteins, and lipids in the ILVs. The first step in this process involves the ubiquitination of the cytoplasmic domain, which is recognized by ESCRT-0 (28) and then sequentially interacts with ESCRT-I, -II, -III to regulate cargo loading into the ILVs (29, 30). Another process for loading cargo into ILVs occurs independent of ESCRT, utilizing lipids such as lysobisphosphatidic acid and ceramides, exploiting the hydrophobic nature of lipids to bend the endosomal membrane inward to form vesicles (31). Following their formation, multivesicular bodies with ILVs may then be transported to lysosomes for degradation or fuse with the plasma membrane, resulting in exocytosis of the ILVs as exosomes and their contents (26). Circulating exosomes transfer their cargo between their parental and target cells, enabling cell-to-cell communication.

EXOSOMAL PROTEIN CARGO IN ISLET TRANSPLANTATION

Exosomes contain distinct nucleic acid and protein profiles reflecting the phenotypes of their cell source and cellular state.

Exosomes are enriched in endosome-associated proteins including flotillins and annexins owing to their origin from endosomes. Exosomes also contain tetraspanins (CD9, CD81, CD82, CD37, and CD63), ESCRT proteins, heat shock proteins (HSC70 and HSC90), Alix, and TSG101 and are commonly used as exosome markers for research purposes (23).

Exosomes share surface major histocompatibility complex (MHC) antigens with their lineage. For example, dendritic cell–derived exosomes express CD80, CD86, and MHC class II molecules (32, 33). In a human-to-mouse xenogeneic islet transplant model and allogeneic human islet transplantation, transplanted human islets released donor human leukocyte antigen (HLA, MHC class I)–specific exosomes into the recipient circulation. Acute and long-term follow up of these recipients revealed gradual reduction in circulating donor HLA-specific exosomes, and elevated recipient T cell–derived CD3-specific exosomes, reflecting graft rejection (34). In both a mouse model and human allogeneic islet transplant recipients, donor HLA-specific exosomes contained islet endocrine hormones including insulin, glucagon, and somatostatin (protein and mRNA), which decreased after immune rejection of islet grafts (34). Exosomes derived from mouse insulinoma clonal cells (MIN6) expressed insulin and glutamic acid decarboxylase (GAD65) (T1D-associated autoantigen) in addition to exosome markers (35, 36). Ex vivo, human and rat islets released exosomes containing GAD65 and IA-2, autoantigens in T1D (37). In an allogeneic human islet transplant recipient, GAD65 antigen was detectable in donor HLA-specific exosomes at 455 days post-transplantation, with development of GAD65 autoantibodies at 1001 days post-transplantation (38). Our recently published observations revealed that human islets exposed to proinflammatory conditions released exosomes containing cytokines including IL-6, IL-8, MCP-1, and IP-10 (39). Proteomic profiling of MIN6-derived exosomes demonstrated enrichment of proteins involved in glycolysis, gluconeogenesis, citrate cycle, fructose and mannose metabolism, pyruvate metabolism, purine metabolism, and other metabolism-related pathways (35).

EXOSOMAL NUCLEIC ACID CARGO IN ISLET TRANSPLANTATION AND ITS UTILITY AS A BIOMARKER

Apart from proteins, intra-exosomal cargo contains nucleic acids, including DNA, mRNA, and miRNA. Loading of miRNA into exosomes is not a random process, as the types and diversity of miRNAs vary by cell type and cellular state at the time of sampling (40–43). Following intraportal transplantation, islets are exposed to hypoxic and inflammatory conditions, leading to loss of islet mass (**Figure 1**). *Ex vivo* mRNA and small RNA profiling of human islet–derived exosomes reveals distinct profiles depending on the islet culture conditions. Exosomes derived from human islets exposed to proinflammatory cytokines and/or hypoxia contain significantly higher levels of specific miRNAs. The Venn diagram in **Figure 2A** depicts the numbers of islet-derived exosomal miRNAs with differential expression under a) hypoxic, b) cytokine stress, and c) hypoxic and cytokine-induced cellular stress. Time course analysis revealed that miR-29b-3p and miR-216a-5p were released in exosomes starting 6 hours after hypoxia and cytokine exposure, relating to islet stress. miR-375 and miR-148a-3p were released in exosomes after 24 hours of hypoxic and cytokine-induced stress and damage, coinciding with onset of apoptosis (**Figure 2**). These early cellular stress and damage-induced exosomal miRNAs were also detected in plasma following islet transplantation in mice with streptozotocin-induced diabetes (43). During islet infusion and after transplantation in patients undergoing total pancreatectomy with islet autotransplantation, these miRNA markers were elevated in circulation, signaling islet stress and damage during transplantation and in the peri-transplant period (**Figure 1**) (43). In an independent study, human islets exposed to cytokines released exosomes containing 19 differentially expressed miRNAs, of which miR-155-5p, a well-known miRNA involved in inflammation, was the most upregulated miRNA (44). Apart from miRNAs, other small RNAs including piRNAs, lncRNAs, snoRNAs, and tRNAs were also identified in exosomes in this study (44). Among the small RNAs studied, exosomal miRNAs have potential as biomarkers of islet stress and damage in islet transplantation. Of particular interest are miR-375, miR-29b-3p, miR-148a-3p, miR-216a-5p, miR-200c-3p, miR-122-5p, miR-155-5p, and miR-221-3p, as these miRNAs have been identified consistently in an islet transplantation setting (43, 45). KEGG analysis revealed that miR-29b-3p, miR-216a-5p, miR-148a-3p and miR-375 target key molecules in PI3K/Akt, FOXO, mTOR signaling pathways and platelet activation (43). Further investigations are necessary to understand whether these islet stress and damage specific exosomal miRNAs alter key signaling pathways in immune cells including antigen presenting cells and lymphocytes in the context of islet transplantation.

In the context of diabetes, several studies have shown elevated levels of circulating exosomal miRNAs including miR-25-3p in T1D (46) and miR-125a-3p, miR-99b-5p, miR-197-3p, miR-22-3p, miR-27b-3p, miR-200a-3p, and miR-141-3p in gestational diabetes (47). Although a number of circulating miRNAs have been reported as elevated or reduced in circulation in T1D, T2D, obesity, and gestational diabetes, these studies were performed using plasma or serum fractions (45) and hence do not necessarily represent the exosomal miRNA content. Other types of extracellular vesicles including microvesicles may also contribute to the miRNAs in circulation.

Islet-derived exosomal mRNA cargo differs based on cellular state similar to exosomal miRNAs. After exposure to cytokines, 133 mRNAs were differentially expressed in human islet-derived exosomes. Although not differentially expressed, these human islet-derived exosomes also contained mRNAs coding for *IAPP, INS, MAFA, NEUROD1, NKX6.1, FOXO1, NEUROG3, PAX4*, and *SOX9* (44). In four allogeneic human islet transplant recipients, donor islet-specific exosomes contained insulin and glucagon mRNAs up to 1197 days after transplantation. One of these patients undergoing graft rejection also demonstrated circulating exosomes containing GAD65 mRNA at 455 and

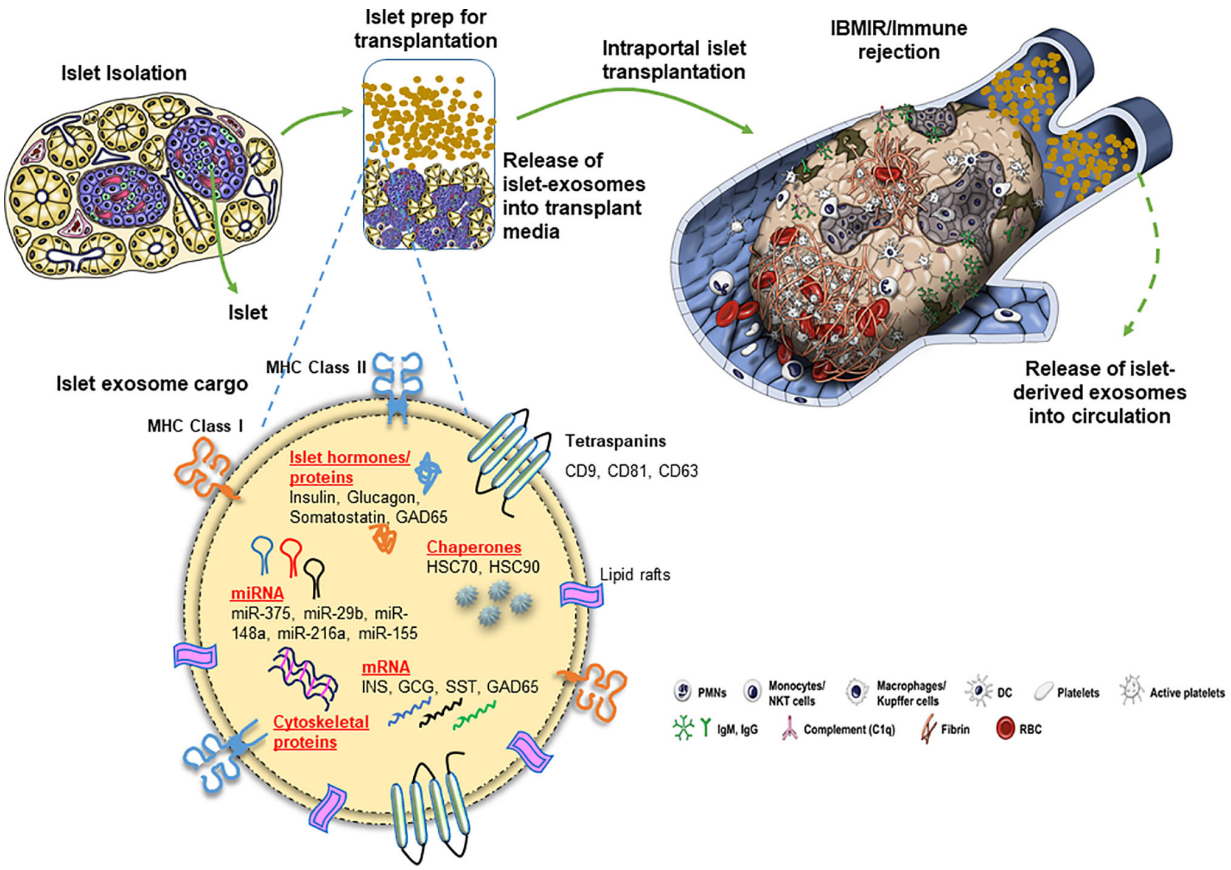

FIGURE 1 | Release of exosomes and their cargo from human pancreatic islets during clinical islet transplantation.

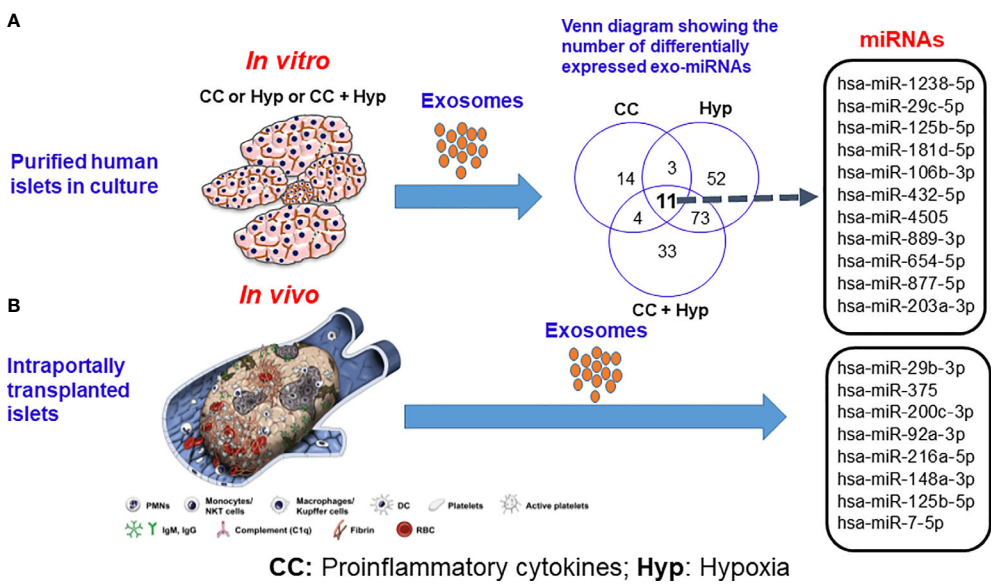

FIGURE 2 | Release of exosomal microRNAs from human islets subjected to proinflammatory cytokines and hypoxic conditions **(A)** *in vitro* and **(B)** following intraportal transplantation of autologous islets *in vivo*. Source: Saravanan et al., *Diabetologia*, 2019 (36). CC indicates proinflammatory cytokines; Hyp, hypoxia.

1197 days after transplantation (38). In a human-to-mouse xenogeneic islet transplant model, donor HLA-specific exosomes contained mRNAs coding for insulin, glucagon, somatostatin, and FXYD2 (34). Thus, long-term graft function and survival can be monitored using circulating exosomal mRNA and endocrine hormones. Thus, non-invasive monitoring of islet graft function and survival by exosomal cargo using simple, non-invasive method supersedes existing diagnostic practices.

Although it is still unclear, exosomal cargo containing metabolism-, inflammation-, and cellular stress-related molecular species (**Table 1**) may exert distinct biological actions in target cells. In the context of islet transplantation, apart from their utility as biomarkers of islet stress and damage, transplanted islet-derived exosomes may serve as auto- or alloantigens triggering an immune response. Future anterograde tracing studies focusing on the actions of islet-derived exosomes on target cells in physiological and pathophysiological conditions are necessary.

EXOSOME RECOGNITION BY IMMUNE CELLS

Transplant rejection by the host immune system is a complex process, initiated by a series of events starting from recognition of the allograft or autoantigens, stimulation of the host's immune system, and T cell–dependent rejection of the graft. Recent research has emphasized the roles of donor tissue–derived exosomes in recognition of the transplanted tissue by the host immune system. Purified allogeneic donor exosomes stimulate alloimmune responses by T cells *in vitro* and *in vivo*, emphasizing the significance of exosome recognition, uptake, and immune responses in transplantation (48). Exosomes weakly stimulate or fail to elicit immune responses in T cell lines and naïve T cells, respectively, and processing of exosomes by antigen-presenting cells (APCs) is required for T-cell activation (49–51).

After transplantation, recipient T cells can be stimulated through three pathways. The first is a *direct pathway*, where recipient T cells are stimulated by donor passenger APCs presenting an alloantigen on allogeneic MHC proteins (52). If the recipient T cells are naïve, donor passenger APCs must migrate to secondary lymphoid organs to induce T-cell responses. Structural differences in the donor MHC:alloantigen complex (multiple binary complexes hypothesis) (53) or each allo-MHC molecule on donor APCs (high-determinant density hypothesis) (54) may be recognized by T cells. The direct pathway is the driving mechanism behind acute graft rejection (55). The second pathway is the *indirect pathway*, where T cells recognize donor MHC/alloantigen-derived peptides processed and presented on self-MHC molecules by recipient APCs. T cells primed to recognize alloantigens are able to respond to peptides derived from allogeneic α and β chains of class II MHC molecules (56). Skin grafts from MHC II knockout mice were detected and rejected by MHC I knockout mice even though the recipient MHC I knockout mice lacked $CD8^+$ T cells (57). The indirect pathway is important for alloantibody production and chronic rejection leading to graft vasculopathy and fibrosis (55). The third pathway is the *semidirect pathway:* Immediately after transplantation, donor passenger leukocytes do not travel to recipient regional lymph nodes for antigen presentation. Instead, recipient APCs take up donor exosomes containing donor MHC and antigens and process and present them on self-MHC molecules (cross-dressing), triggering T cell activation (32, 33, 55, 58). Recipient dendritic cells were able to acquire significant amounts of MHC molecules from both donor dendritic cells and endothelial cells (59). Graft rejection was evident in recipients who lacked the indirect allorecognition pathway (60). Following heart and islet transplantation in allogeneic mouse transplant

TABLE 1 | Exosomal cargo in human islet transplantation.

Exosome content	Exosome source	Comments	References
hsa-miR-375	Culture supernatant	"Damage-induced exo-miRNA" increased in response to hypoxia, streptozotocin, and cytokine stress after 24 h	(36, 43)
	Xenotransplant mouse serum	Elevated 24 h following transplant	
	TPIAT supernatant/human Serum	Elevated throughout islet isolation	
hsa-miR-216a-5p	Culture supernatant	"Stress-induced exo-miRNA" increased after 6 h ctyokine and hypoxic stress	
	Xenotransplant mouse serum	Elevated 24 h following transplant	
	TPIAT supernatant/human serum	Elevated throughout islet isolation	
hsa-miR-148a-3p	Culture supernatant	"Damage-induced exo-miRNA" increased after 24 h cytokine and hypoxic stress	
	Xenotransplant mouse serum	Elevated 24 h following transplant	
	TPIAT supernatant/human serum	Elevated throughout islet isolation	
hsa-miR-29b-3p	Culture supernatant	"Stress-induced exo-miRNA" increased after 6 h ctyokine and hypoxic stress	
	Xenotransplant mouse serum	Elevated 24 h following transplant	
	TPIAT supernatant/human serum	Elevated throughout islet isolation	
hsa-miR-200c-3p	TPIAT supernatant/human serum	Elevated throughout islet isolation	
hsa-miR-3613-5p	Xenotransplant mouse serum	Increased in normoglycemic xenotransplant mice	(34)
Angiopoietin-1	Xenotransplant mouse serum	Increase associated with normoglycemia following xenotransplant	
HSC70	Xenotransplant mouse serum	Increase associated with normoglycemia following xenotransplant	
Complement C3	Xenotransplant mouse serum	Increase associated with rejection following xenotransplant	
Hemopexin	Xenotransplant mouse serum	Increase associated with rejection following xenotransplant	

TPIAT indicates total pancreatectomy with islet autotransplantation.

models, recipient cells were cross-dressed with donor MHC antigens in draining and non-draining lymph nodes and spleen, with only a few passenger leukocytes at these sites (48). In a human-to-mouse xenogeneic islet transplant model, donor passenger leukocyte-derived exosomes were negligible in the donor HLA-specific exosome fraction, suggesting transplanted human islets as their source (34). Exosomes alone derived from rat mast cells only weakly stimulated specific T cells, and T-cell activation increased by fixation of exosomes to latex beads *in vitro* (49). MIN6-derived exosomes induced splenocytes isolated from NOD mice to produce proinflammatory cytokines including IL-6, IFN-γ, and TNF-α *via* the TLR-MyD88 signaling pathway. MIN6-derived exosomes increased CD86 expression on class II MHC-positive splenocytes and induced splenic T cell proliferation (36). After pro-inflammatory cytokine exposure, human islet-derived exosomes induced mRNA expression of NOS2 and COX2 in THP-1 cells, a macrophage cell line (39). The semidirect pathway is particularly important when recognizing donor peptides and MHC molecules by recipient immune cells. The involvement of the semidirect pathway in alloantigen presentation and stimulation of T cell responses is reviewed in detail elsewhere (55).

Thus, exosomes containing donor antigens, MHC molecules, and miRNAs can elicit immune responses directly (mostly negligible due to suboptimal levels of exosomal cargo to activate T cells) or indirectly through recipient APCs. In the context of islet autotransplantation, exosomes carrying islet stress and damage markers, sequestered self-antigens, and MHC molecules may be important in inducing autoimmune responses.

THERAPEUTIC POTENTIAL OF EXOSOMES IN ISLET TRANSPLANTATION

Of particular interest is induction of allogeneic tolerance or tolerance to sequestered self-antigens in autotransplantation. The immunogenicity of allogeneic or self-antigens depends on the dose, presentation site, other signals, and nature/state of APC activation. The ability of exosomes to induce immune tolerance is still unexplored, but exosomes as therapeutic delivery vehicles have been studied recently. Human bone marrow mesenchymal stem cells transfected with si-Fas and anti-miRNA-375 were co-cultured with peripheral blood mononuclear cells. Exosomes were collected from co-culture supernatant and transplanted into NOD-SCID gamma mice, resulting in decreased immune response and rejection by enhancing regulatory T cell function (61). In living donor liver transplant recipients, infusion of donor regulatory dendritic cells resulted in cross-dressing of recipient immune cells with surface expression of PD-L1, which was most likely transferred through donor exosomes containing PD-L1, CD73, and CD39. There was an increase in recipient regulatory $CD4^+$ T cells as well as decreased activated $CD8^+$ T cells after the infusion of donor regulatory dendritic cells (62).

More importantly, exosomes exhibit low toxicity, do not present a risk for tumor formation, and easily diffuse across biological barriers owing to their small size, which enables them to be used as injectable therapeutics (63). *In vitro*, mesenchymal stem cell–derived exosomes alleviated the detrimental effects of hypoxia-induced DNA damage, resulting in increased viability of porcine islet cell clusters in hypoxic conditions (64). Mouse islets cultured with mesenchymal stem cell–derived exosomes showed reduced expression of the pro-apoptotic genes *BAD* and *BAX* and increased expression of prosurvival genes *PI3K* and *BCL-2*, as well as increased production of vascular endothelial growth factor in mouse islets, resulting in increased production of insulin mRNA (65). Mesenchymal stem cell derived exosomes protected beta cells from apoptosis *via* the actions of miR-21 on ER stress and inhibition of phosphorylation of p38 (66). In streptozotocin diabetic mice, infusion of exosomes derived from bone marrow–derived mesenchymal stem cells induced regeneration of pancreatic islets (67). Administration of adipose tissue–derived exosomes into streptozotocin diabetic mice increased the regulatory T cell ratio in splenic mononuclear cells and improved insulitis (68). Exosomes isolated from lean adipose tissue explants also increased viability and functionality of isolated pancreatic β cells (69). These observations are proof of concept that stem-cell-derived exosomes may improve islet engraftment and functional outcomes of islet transplantation. Transplantation of MIN6-derived exosomes improved median survival time, glucose tolerance, insulin content, and islet architecture and reduced macrophage infiltration in streptozotocin diabetic mice (70). However, in diabetes-resistant NOR mice, immunization using MIN6-derived exosomes accelerated insulitis (36), highlighting that exosomes may either induce immune tolerance or induce a response to allogeneic or autologous antigens.

CONCLUSIONS AND FUTURE RESEARCH DIRECTIONS

Exosomes have emerged as important players in transplant rejection, as they carry donor antigens, which can weakly activate alloimmune responses through the direct or indirect allorecognition pathways. However, their major contribution to allorecognition comes in the form of cross-dressing recipient immune cells, leading to rejection of allogeneic transplanted tissue. Exosomal protein and nucleic acid cargoes also have potential for use as biomarkers for monitoring graft function and survival. Recent research highlights the utility of mesenchymal stem cell–derived exosome therapy during transplantation to improve islet survival and function, induce immune regulatory responses, and improve transplantation outcomes. Although exosome biology has been studied extensively, exosome research has its limitations. Exosome yield and quality vary between different isolation methods including ultracentrifugation, ultrafiltration and precipitation. Although exosomal miRNA, mRNA isolation and qPCR methods are robust, lack of exosomal housekeeping controls for normalization may influence experimental observations across laboratories. Additionally, there is no commercially

available kit to measure very small amounts of exosomal miRNA/mRNA content accurately. Thus, there is a need for optimization and standardization of exosome research methods. Future studies should also be carried out using larger sample cohorts to validate and append the growing list of biomarkers for use in diagnostics. Exosomes in inducing transplant tolerance is an interesting area of research and may open up exciting and novel avenues in post-transplant immunosuppressive regimen. Given the contribution of islet-derived exosomes to graft rejection (34), future studies should focus on the fine line between induction of rejection and tolerance. With several studies demonstrating exosomes as safe therapeutic agents, continued studies into engineering and administration of exosomes in order to attenuate immune responses and prolonging graft survival will be of vital importance to the field. As exosome research is still in its infancy, its utility as biomarkers of islet stress and damage, inflammation or immune response and/or as therapeutics in the context of clinical islet transplantation should be validated and well-established independently across institutions.

AUTHOR CONTRIBUTIONS

JM, SV wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Metabolic Syndrome Is Associated With Altered mRNA and miRNA Content in Human Circulating Extracellular Vesicles

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As mediators of intercellular communication, circulating extracellular vehicles (EVs) can modulate tissue and cellular pathways by altering transcription profiles in recipient cells, and their content may reflect the status of their parent cells. However, whether their cargo is altered in the metabolic syndrome (Mets) remains unclear. We hypothesized that MetS altered mRNAs and miRNAs packed within circulating-EVs. EVs were collected from plasma of patients with MetS or age-matched Lean controls (n=4 each). RNA sequencing was performed to identify dysregulated mRNAs and miRNAs, and analyze genes targeted by miRNAs, top pathways, and diseases associated with MetS-EVs. MetS patients showed elevated body weight, blood pressure, glucose, insulin, and liver injury markers levels. 1,446 mRNAs were downregulated and 32 upregulated in MetS- compared to Lean-EVs, whereas 40 miRNAs were selectively enriched and 10 downregulated in MetS-EVs. MetS upregulated in EVs genes involved in apoptosis, mitochondrial regulation, transport, and lipoproteins, but downregulated vessel and heart development, protein complex biogenesis, and angiogenesis. MetS also upregulated miRNAs targeting genes implicated in cellular processes, including oxidation–reduction, and downregulated miRNAs capable of modulating catalytic activity, as well as heart, blood vessel, and skeletal development, transcriptional regulation, apoptosis, and cell cycle. Our study, thus, indicates that human subjects with MetS show modified cargo of circulating EVs, which in turn may modulate several critical cellular functions and fate. These EVs may reflect the anomalous status of their parent cells, and potentially serve as important regulators, biomarkers, and targets in the progression and treatment of MetS.

Keywords: metabolic syndrome, RNA sequencing, circulation, extracellular vesicles, extracellular vehicles

BACKGROUND

The metabolic syndrome (MetS) is a collective cluster of disease risk factors, including dyslipidemia, obesity, inflammation, insulin resistance, and hypertension, affecting numerous people worldwide (1). The presence of MetS significantly increases disease risk for type 2 diabetes, atherosclerosis, cardiovascular, and chronic kidney disease, and ultimately leads to organ injury and dysfunction (2). The pathogenesis of MetS involves activation of a plethora of signaling pathways and potential damage to cell types, tissues, and target organs (3).

Extracellular vesicles (EVs), membrane-bound nanoparticles, can be released from almost every cell type and play an essential role in a wide range of physiological and pathological process *in vivo* (4). Besides apoptotic bodies, the two main types of EVs are exosomes and microvesicles, which are the most studied EVs populations. Known as carriers for circulating miRNAs, plasma EVs also contain other nucleotides, lipids, and proteins (5). EVs function as mediators of intracellular communication by shuttling bioactive cargo between the parent cells in which they originate and neighboring or distant recipient cells (6). By altering transcription profiles in recipient cells, EVs can modulate tissue metabolism and cellular pathways (7). Growing evidence indicates that EVs are prevalent in biofluids, including serum/plasma, saliva, bronchoalveolar lavage fluid, and urine, and that their composition changes in disease states (8), positioning EVs as novel circulating biomarkers (9) that reflect the status of their parental cells.

Our group recently demonstrated that EVs released by pig adipose tissue-derived mesenchymal stem cells (MSCs) are selectively packed with micro-RNAs (miRNAs), mRNAs, and proteins, which possess the capacity to modify selective pathways in recipient cells (10, 11). miRNAs are small non-coding RNAs that regulate gene expression post-transcriptionally and may play a crucial role in the pathophysiology of MetS (12), suggesting them as a new class of endocrine factors (13). Importantly, recent studies have revealed that miRNAs are detectable in the peripheral circulation packed in different types of EVs (14).

Furthermore, we have also shown that MetS can modify the cargo and size of porcine adipose tissue-derived MSC, including mRNAs, miRNAs, and proteins, which might in turn control some important cellular functions (15, 16). In addition, the transcriptome and proteome of particularly genes in MSCs involved in mitochondria, inflammation, and transcription regulation were found to be altered in MetS (17).

Identifying miRNA and mRNA cargo of circulating EVs, and elucidating alternations imposed by disease status, could illuminate their potential as biomarkers of tissue and organ injury. However, how the presence of MetS affects the overall transcriptome of circulating EVs in human subjects and which pathways are primarily altered remains unknown. Therefore, we hypothesized that MetS is associated with altered mRNA and miRNA content in EVs isolated from systemic human plasma. To test this, we took advantage of high-throughput mRNA and miRNA sequencing to compare the gene and miRNAs profile between Lean- and MetS-human subjects.

Abbreviations: MetS, metabolic syndrome; EVs, extracellular vesicles; miRNA, micro-RNA; eGFR, estimated glomerular filtration rate; ALT, alanine aminotransferase; AST, aspartate aminotransferase; ALP, alkaline phosphatase; PRA, plasma renin activity. MSCs: mesenchymal stem cells.

MATERIALS AND METHODS

Patient Population

MetS patients and healthy subjects (n=4 each group) were recruited in the First Hospital Affiliated to Jinan University (Guangdong, China). Our study followed the Declaration of Helsinki, was approved by the Institutional Research Ethics Committee, and written informed consent obtained from all subjects. All study participants were reviewed for medical history.

Inclusion criteria for MetS patients included older than 18 years and diagnosis of MetS, based on criteria presented by the International Diabetes Federation (18). The diagnosis of the MetS centered on obesity (body mass index[BMI] >30 kg/m2) and two or more of the following: abnormal lipids metabolism (HDL-cholesterol <50 mg/dl in females and <40 mg/dl in males, triglycerides ≥150 mg/dl), systolic blood pressure ≥130 mm Hg or diastolic blood pressure ≥85 mm Hg, previously diagnosed hypertension or type 2 diabetes or fasting glucose concentration ≥100 mg/dl. Exclusion criteria included cancer, heavy smoking, drug abuse, severe cardiac valvular diseases, any severe systemic diseases, or alcohol consumption in the past 3 months.

Inclusion criteria for healthy controls included overall health status, older than 18 years. Exclusion criteria for healthy controls included any significant disease, drug abuse, heavy smoking, or alcohol consumption in the past 3 months. None of these Lean or MetS subjects have been taking non-steroidal anti-inflammatory drugs (NSAIDs) or changed their exercise regimen within 3 months of enrolment.

Blood samples were collected under fasting condition for assessment of metabolic, renal, and liver functions following routine procedures in the clinical laboratories of the First Hospital Affiliated to Jinan University. Estimated glomerular filtration rate (eGFR) was calculated by the Modification of Diet in Renal Disease eGFR Equation (19).

Blood EV Harvesting

EVs were isolated using the exoRNeasy Serum/Plasma (Qiagen cat# 77044) assay, followed by RNA isolation using the miRNeasy serum/plasma advanced kit (Qiagen cat# 217204), as per manufacturer's directions. Briefly, thrombin was added to the plasma, incubated for 5 minutes at room temperature, and centrifuged at 2,500*g* for 15 min. The supernatant was then mixed with precipitation buffer and incubated for 60 min at 4°C. Following centrifugation at 13,000*g* for 5 min, the pellet was resuspended and served for RNA isolation. The resuspended pellet was lysed, protein was precipitated and removed, isopropanol was added to the supernatant, and the sample loaded onto the column. Following three washes, RNA was eluted and stored at −80°C.

EVs were characterized post-isolation based on transmission electron microscopy and expression of EV markers (CD9, CD63, CD81) by Western blot, as previously published (10), and their concentrations determined using nanoparticle tracking analysis. In addition, their cargo was compared to typical EV markers listed in ExoCarta (http://www.exocarta.org), a web-based resource of exosomal cargo.

mRNA Sequencing Analysis

RNA sequencing was performed and analyzed as described previously (10). RNA libraries were prepared according to the manufacturer's protocol (TruSeq RNA Sample Prep Kit v2, Illumina, San Diego, USA) and loaded onto flow cells (8–10 pM) to generate cluster densities of 700,000/mm^2 following the standard protocol. Then cells were sequenced on an Illumina HiSeq 2000 using TruSeq SBS kit version-3 and HCS v2.0.12 data collection software, with data analyzed using the MAPRSeq v.1.2.1 system and the Bioinformatics Core standard tool. The mRNA-Seq data were analyzed using CAP-miRSeq v1.1, normalized, and differential expression analyzed using edgeR 2.6.2. Gene expression was normalized to 1 million reads and corrected for gene length (reads per kilobasepair per million mapped reads). DEseq analysis was performed and mRNAs showing fold-change >1.4 between the groups, and $p<0.05$ were considered upregulated, whereas those with fold-change <0.7 and $p<0.05$ were considered downregulated. Functional annotation clustering analysis was performed using DAVID 6.7 database (http://david.abcc.ncifcrf.gov/) to obtain a ranking of primary gene ontology categories for upregulated and downregulated mRNAs.

miRNA Sequencing and Data Analysis

EVs total RNA libraries were prepared using QIAseq Stranded Total RNA Kit. MiRNA sequencing libraries prepared with QIAseq miRNA Library Kit were sequenced with an Illumina NGS system (MiSeq Personal Sequencer, NextSequence500, HiSeq-1000, HiSeq-1500, HiSeq- 2000, HiSeq-2500, and GaIIx). The data were analyzed with CLC (Biomedical) Genomics Workbench. Starting with unaligned FASTQs, the workflow generates aligned BAMs and then both raw and normalized known mature miRNA expression counts. The R-based tool from Bioconductor, edgeR2.6.2 was used to perform DEseq analysis to identify miRNAs enriched in MetS-EVs compared to Lean-EVs (fold-change >2.0 or fold-change <2 and $p<0.05$). TargetScan 7.1 and miRWalk 2.0 were used to predict target genes of significantly upregulated and downregulated miRNAs. Subsequent functional annotation-clustering analysis utilized the PANTHER (http://www.pantherdb.org/) and DAVID 6.7 database. Gene targets of miRNAs enriched in Lean- and MetS-EVs were compared on different categories, including cellular component, molecular function, biological process, and biological pathway.

Validation of miRNA and mRNA Expressions

To validate expression of representative mRNAs and miRNAs in circulating human EVs, the expression of several candidate RNAs was confirmed by quantitative polymerase chain reaction (qPCR). Total RNA was isolated from plasma-derived EVs, and probed with primers (Ribobio, Guangzhou, Guangdong Province, China; GZP2020081200495, GZP2020081200501, GZP2020081200497, GZP2020081200484, GZP2020081200483, and GZP20200 81200482). All results were adjusted by GAPDH.

Integrated mRNA/miRNA Analysis

Target prediction analysis of dysregulated miRNAs was performed using miRWalk 3.0 (http://mirwalk.umm.uni-heidelberg.de/) and target genes that overlapped with those mRNAs dysregulated in MetS-EVs analyzed using Venny 2.1 (https://bioinfogp.cnb.csic.es/tools/venny/).

Statistical Analysis

Statistical analysis was performed using JMP 14.0 (SAS Institute, Cary, NC). Data are expressed as mean ± standard deviation. The Shapiro–Wilk test was used to test for deviation from normality. Normally distributed data were compared using unpaired Student's t-test and ANOVA. Nonparametric tests (Wilcoxon and Kruskal Wallis) were used when data did not follow a Gaussian distribution. Statistical significance was accepted if $p \leq 0.05$.

RESULTS

Systemic Characteristics of Lean and MetS Patients

Table 1 shows the demographic, clinical, and laboratory characteristics of the study patients. BMI, systolic, and diastolic blood pressures were all markedly higher in the MetS compared to

TABLE 1 | Clinical, laboratory, and demographic data of Lean and Mets patients (n = 4 each).

Parameters	Lean	Mets
Age (years)	24.5 (21–29)	29.3 (24–32)
Sex (female/male)	2/2	2/2
Duration of MetS (years)	–	5.25 ± 1.71
Body mass index (Kg/mm2)	19.1 ± 0.9	62.2 ± 14.2*
Systolic blood pressure (mmHg)	113 ± 11.9	150.3 ± 9.7*
Diastolic blood pressure (mmHg)	63.8 ± 5.9	95 ± 4.5*
Hemoglobin A1C (%)	5.4 ± 0.2	7.2 ± 0.8*
Total cholesterol (mmol/l)	4.5 ± 0.4	5.1 ± 0.5
Triglycerides (mmol/l)	1.75 ± 0.2	2.42 ± 0.8
High-density lipoprotein (mmol/l)	0.96 ± 0.1	0.97 ± 0.2
Low-density lipoprotein (mmol/l)	1.6 ± 0.3	3.1 ± 0.6*
Blood urea nitrogen (mmol/l)	3.7 ± 0.5	5.4 ± 1.6*
eGFR (ml/min/1.73m2)	129.3 ± 39.1	193.4 ± 7.4*
Fasting blood sugar (mmol/l)	4.9 ± 0.4	8.2 ± 1.6*
Insulin (mIU/L)	14.0 ± 4.4	42.4 ± 16.1*
C-peptide (ng/ml)	2.5 ± 0.6	6.0 ± 0.8*
Alanine aminotransferase (U/L)	29 ± 7.0	100 ± 23.5*
Aspartate aminotransferase (U/L)	25.5 ± 7.4	86.5 ± 29.4*
Alkaline phosphatase (U/L)	63.5 ± 5.2	85.3 ± 15.4*
White blood cells *10^9/L	4.15 ± 1.1	8.28 ± 2.3*
Plasma renin activity (ng/ml/h)	0.6 ± 0.1	3.5 ± 0.5*

*$P < 0.05$ vs Lean.
eGFR, estimated glomerular filtration rate.

Lean subjects, whereas age and sex were similar between the groups. Low-density lipoprotein, fasting blood sugar, C-peptide, insulin, and hemoglobin A1C levels were also higher in MetS compared to Lean, underscoring development of MetS. Elevated blood urea nitrogen (BUN) and eGFR were consistent with development of hyperfiltration that characterizes obese individuals, and plasma renin activity (PRA) was increased. Elevated alanine aminotransferase (ALT), aspartate aminotransferase (AST), and alkaline phosphatase (ALP) indicated early liver injury in the MetS group, whereas a higher white blood cell count was consistent with systemic inflammation.

Characterization of Lean- and MetS Circulating EVs

Transmission and scanning electron microscopy demonstrated that the plasma contained EVs with the traditional "cup-like" morphology (**Figure 1A**). Circulating EVs expressed typical EV (CD9, CD81, and CD63) markers, and NTA demonstrated a typical size distribution of microparticles and exosomes (**Figures 1B, C**). Furthermore, of the top 100 conventional EV markers listed in ExoCarta, 95 mRNAs were identified in isolated EVs (**Figure 1D**), confirming their nature.

EV mRNAs Cargo

Of all annotated genes (n=32,392), mapping of RNA reads revealed 32 genes (0.1%) upregulated in circulating MetS-compared with Lean-EVs (**Figures 2A** and **S1**). The genes encode for metabolite interconversion enzymes with protein binding and catalytic activity (**Figures 2B, C**). Functional analysis revealed that these proteins are primarily implicated in regulation of apoptosis (RHOB, PPP2R2D, DYNLT3), and mitochondrial function (MRPL18, MTIF3, UCP2), followed by transport and lipoproteins (**Figure 2D**). Contrarily, 1,446 (4.5%) mRNAs were downregulated in MetS compared to Lean-EVs. Functional annotation clustering analysis showed that those top 100 genes (**Figure 3A**) encode for nucleic acid binding and scaffold-adaptor proteins with binding and catalytic activity (**Figures 3B, C**), primarily involved in tube, vascular or heart development (NEBL, AKAP13, ERBB4), protein complex biogenesis (TRIOBP, TBCD, SMARCA4), and angiogenesis (APP, PRKX), followed by hemopoiesis, mitochondria, cytoskeleton, and regulation of apoptosis (**Figure 3D**).

EV MicroRNAs Cargo

Of 1,515 annotated miRNAs, 40 (2.6%) distinct miRNAs were selectively enriched in MetS EVs (**Figures 4A** and **S2**). Functional annotation clustering analysis showed that those upregulated miRNAs targeted genes encoding for cell junction proteins with binding activity (**Figures 4B, C**), primarily involved in redox regulation and oxidation-reduction (OXA1L, PNPT1, SDHC), cell structure (DCTN1, EFHC2, RHOA), and Ras protein signal transduction (BAD, RAF1, RELA), followed

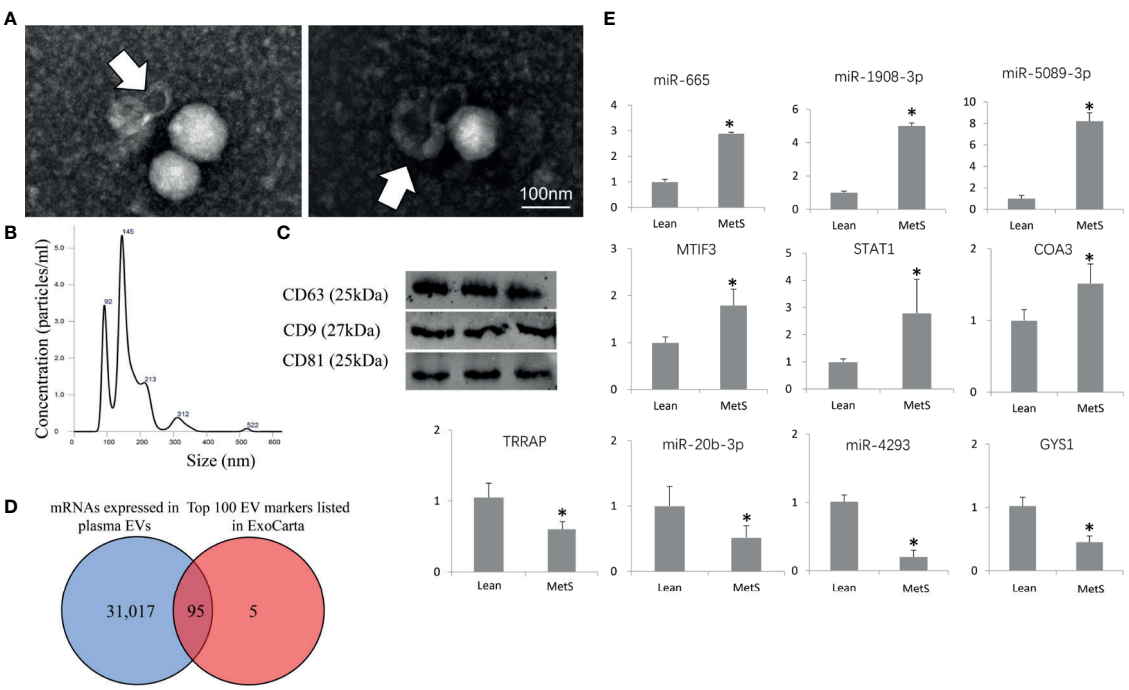

FIGURE 1 | Characterization of circulating extracellular vesicles (EVs) and validation of dysregulated miRNAs. **(A)** Transmission electron microscopy (negative staining) showing EV clusters (arrows) with the classic "cup-like" morphology. **(B)** Size distribution of isolated EVs revealed a composition of about 2/3 small microvesicles (~145 nm in size) and 1/3 exosomes (~92 nm). **(C)** Western blotting analysis showing that isolated EVs expressed common EV markers (CD9, CD63, and CD81). **(D)** Venn diagram showing that 95 mRNAs of the top 100 EV markers listed in ExoCarta were found in the isolated EVs. **(E)** Expression of candidate mRNAs and miRNAs (qPCR) was concordant with miRNA-seq and mRNA-seq results. *p ≤ 0.05 vs. Lean.

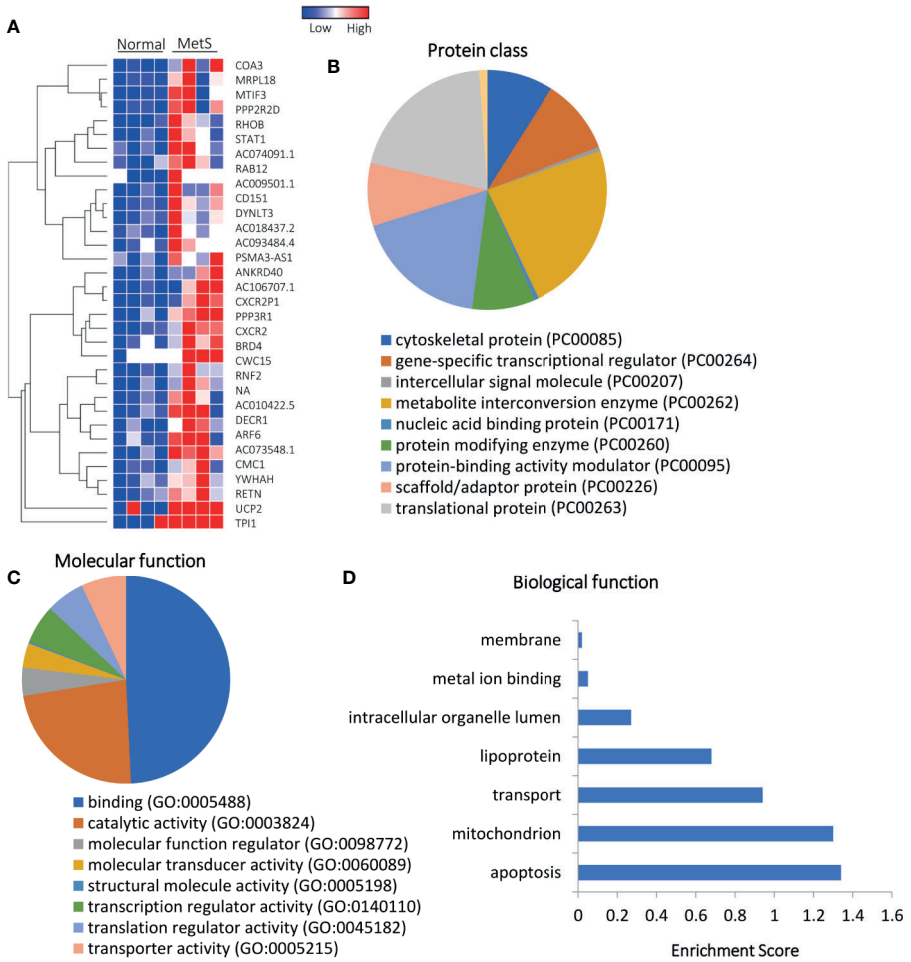

FIGURE 2 | Upregulated mRNAs in Lean and MetS plasma-EVs. **(A)** Heat-map showing 32 upregulated mRNAs in MetS compared with Lean plasma-EVs. Panther analysis showed protein class **(B)** and molecular function **(C)**. **(D)** Enrichment of functional pathways of the 32 upregulated genes using DAVID 6.7.

by regulation of apoptosis and transcription (**Figure 4D**). Contrarily, 10 miRNAs (0.7%) were downregulated in MetS-EVs compared with Lean-EVs (**Figure 5A**). The downregulated miRNAs targeted genes encode for gene-specific transcriptional regulators with binding and catalytic activity (**Figures 5B, C**), mostly implicated in heart and skeletal development (HEY2, SRF, NOTCH1), phosphoprotein (PPM1D, RPL26), and transcription regulation (RLF, TRIP4, ZNF45), followed by blood vessel development, apoptosis, and cell cycle (**Figure 5D**). qPCR confirmed that the expression pattern of several candidate mRNAs and miRNAs was similar to RNAseq results (**Figure 1E**).

Integrated mRNA/miRNA Analysis

Integrated miRNA/mRNA analysis evaluated the interactions among dysregulated mRNAs and miRNAs. It revealed that 76.2% of mRNAs dysregulated in MetS-EVs were targets of miRNAs dysregulated in the same MetS-EVs (**Figure 6**).

DISCUSSION

Our study used high-throughput RNA-sequencing to interrogate mRNA and miRNA content of circulating EVs and explore the putative function of enriched or excluded genes and miRNAs within those EVs obtained from patients with MetS compared to lean controls. We demonstrated that MetS is associated with altered content of genes and miRNAs in human circulating EVs. Specifically, we found in MetS circulating EV upregulated genes involved in regulation of apoptosis, mitochondria, transport, and lipoproteins, but downregulated genes responsible for cardiovascular development and angiogenesis. MetS-EVs also showed upregulated miRNAs that target genes and proteins implicated in multiple cellular processes, including redox regulation and cell structure. Contrarily, miRNAs downregulated in MetS-EVs are capable of modulating proteins implicated in several cellular processes including heart, blood vessel, and skeletal development, transcription regulation, metal ion binding, apoptosis, and cell cycle. These observations suggest impaired

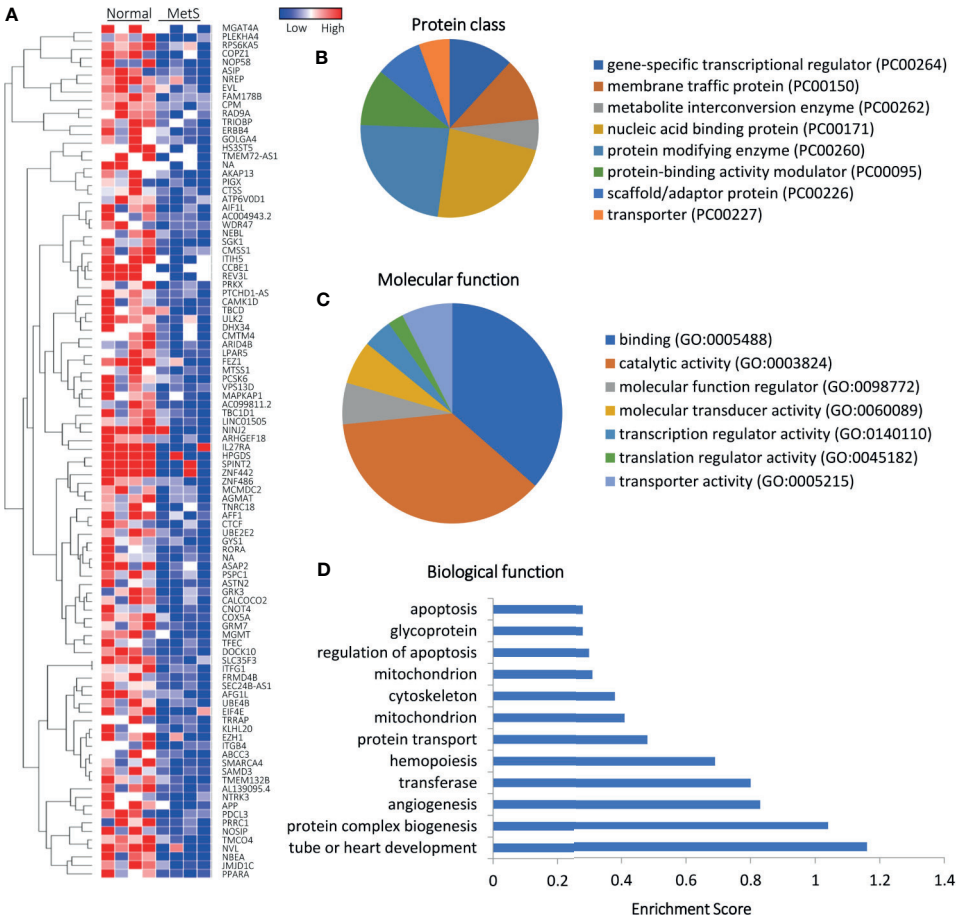

FIGURE 3 | Downregulated mRNAs in Lean and MetS plasma-EVs. **(A)** Heat-map showing top 100 downregulated mRNAs in MetS compared with Lean plasma-EVs. Panther analysis showed protein class **(B)** and molecular function **(C)**. **(D)** Enrichment of functional pathways of the top 100 downregulated genes using DAVID 6.7.

cellular structure, function, and fate in MetS, which may blunt development or repair of blood vessels.

About one third of US adults and over a billion people globally have MetS (20). MetS fosters development of type 2 diabetes, lipid disorders, cardiovascular disease, hepatic steatosis, and other circulatory disorders (1, 2). Fundamental manifestations of MetS include insulin resistance and adipocyte dysfunction, which promote oxidative stress and chronic inflammation, and in turn damage in target tissues such as the kidney, liver, brain, and vasculature. In the kidney, MetS induces hyperfiltration and contributes to microvascular remodeling, podocyte injury, and mitochondrial dysfunction (21), whereas in the liver, non-alcoholic fatty liver disease is a manifestation of MetS (22). Congruently, we found that our patients with MetS showed elevated BUN, eGFR, ALT, AST, and ALP, indicating early renal and liver injury.

Circulating EVs are released from various tissues and organs, reflect the status of their parental cells, and may also mediate important processes in target cells (5). We found that several biological protein functions, including protein complex biogenesis and transport are targeted by dysregulated miRNAs within those EVs. In addition, MetS is a proinflammatory state associated with oxidative stress and apoptosis (19, 23), as also reflected in circulating EVs in our study. Biological functions targeted by upregulated miRNAs included redox regulation, which might be altered in parent cells that released those EVs to the systemic circulation. Interestingly, apoptosis and cell cycle are targeted by dysregulated miRNAs, consistent with our recent findings showing that MetS dysregulates in MSC-derived EVs miRNAs that regulate cellular senescence (24). In addition, MetS may alter angiogenesis and blood vessel development (25), which we found to be targeted by dysregulated miRNAs, suggesting that MetS might impair vascular reparative processes in response to ischemia or wound healing.

Mitochondrial dysfunction plays a role in MetS and advances as the disease progresses from insulin resistance to type 2 diabetes (26). Our previous studies have shown that obesity, as observed in our patients, impairs MSC mitochondrial structure and function, possibly mediated partly through miRNA-induced mitochondrial gene regulation, leading to increased oxidative stress (15, 27, 28). The current study extends our previous observations, demonstrating that MetS-EVs contain mitochondria-related mRNAs, which may reflect mitochondrial damage in the parent

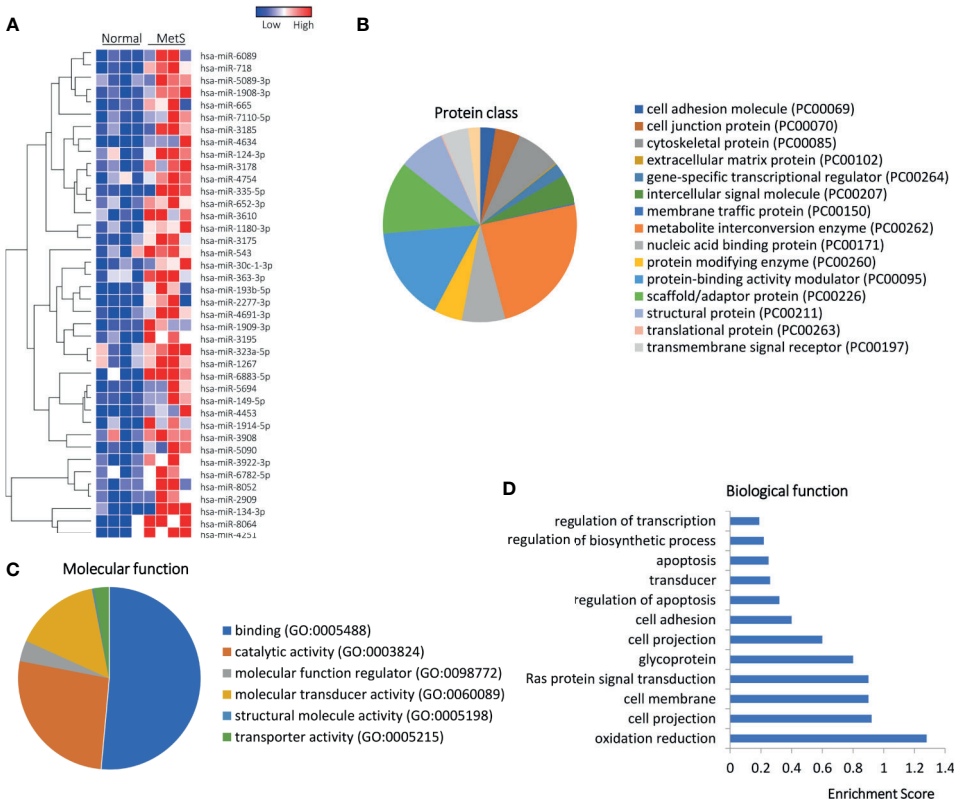

FIGURE 4 | Upregulated miRNAs in Lean and MetS plasma-EVs. **(A)** Heat-map showed 40 upregulated miRNAs in MetS compared with Lean plasma-EVs. Panther analysis illustrated protein class **(B)** and molecular function **(C)**. **(D)** Enrichment of functional pathway of the 40 upregulated miRNAs target genes using DAVID 6.7.

cells that released those EVs to the systemic circulation. Several mitochondrial genes upregulated in MetS-EVs included cytochrome oxidase assembly factor-3, a novel regulator of mitochondrial COX1 translation and cytochrome oxidase assembly (29), mitochondrial translational initiation factor-3, and signal transducer and activator of transcription. The product of this gene is required for recognition and regulation of translation initiation of mitochondrial mRNAs and for coordinated assembly of oxidative phosphorylation complexes *in vivo* (30). Likewise, the transcription factor signal transducer and activator of transcription regulates mitochondria-mediated oxidative stress response, PKCδ activation, and autophagy (31). Hence, our observations suggest mitochondrial dysfunction in MetS detectable in circulating EVs.

We found upregulation of genes linked to lipoprotein metabolism, consistent with the marked dyslipidemia in our MetS patients. Similarly, genes upregulated in MetS-EVs are implicated in apoptosis, including RHOB, PPP2R2D, dynein light chain Tctex-type 3, and C-X-C motif chemokine receptor-2 genes, which modulate specific apoptosis pathways (32). Apoptosis is a vital component of fundamental processes including normal cell turnover, proper development and functioning of the immune system, hormone-dependent atrophy, and others (33). RhoB, a Rho family GTPase, regulates cell cycle progression and is required for the apoptotic response of transformed fibroblasts to DNA damage (34). RhoB also contributes to cancer progression by regulating cell cycle progression, apoptosis, DNA damage responses, invasion, and migration. Recently, dynein light chain Tctex-type 3 has been reported to foster ovarian cancer through promoting cell proliferation, migration, and invasion (35). C-X-C motif chemokine receptor-2 has been found to promote anti-apoptosis, anti-senescence, and epithelial-to-mesenchymal transition of breast cancer cells, leading to enhanced metastasis and chemoresistance (36). Therefore, our findings suggest that MetS might impact cellular fate.

miRNAs play an essential role in regulating gene expression in several different physiological and pathophysiological conditions (15, 37), including metabolic diseases. In this study, many miRNAs were dysregulated in circulating EVs from MetS patients compared with healthy individuals. miRNAs upregulated in MetS plasma could be delivered to recipient cells and modulate cellular pathways. For example, miR-124, which participates in inflammation, autophagy, mitochondrial function, and neurotransmission (38), was selectively enriched in MetS EVs. Similarly, mir-149 that mediates inhibition of cell proliferation, migration, and invasion, and induces apoptosis, was also enriched in MetS-EVs (39). Taken together, these observations suggest that dysregulated miRNAs in circulating MetS-EVs might reflect imbalanced homeostasis and modulate cellular pathways.

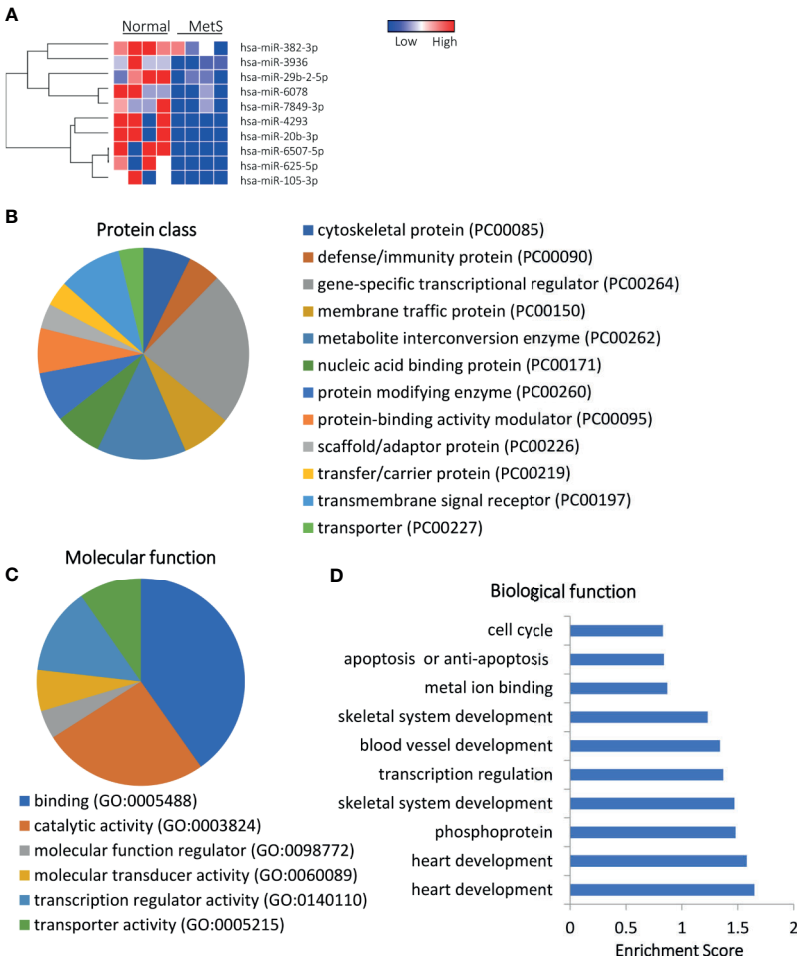

FIGURE 5 | Downregulated miRNA profile in Lean and MetS plasma-EVs. **(A)** Heat-map showed 10 downregulated miRNAs in MetS compared with Lean plasma-EVs. Panther analysis depicted protein class **(B)** and molecular function **(C)**. **(D)** Enrichment of functional pathway of the 10 downregulated miRNAs target genes using DAVID 6.7.

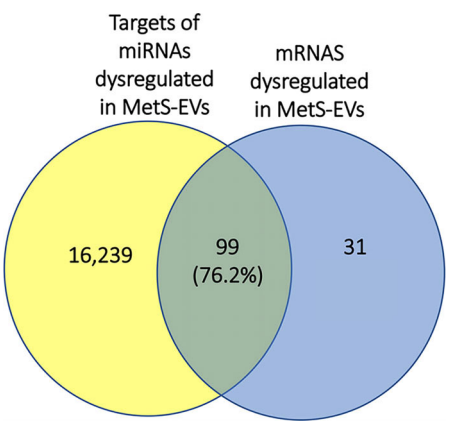

FIGURE 6 | Representative Venn diagram showing that 76.2% of mRNAs dysregulated in MetS-EVs could be targeted by miRNAs dysregulated in MetS-EVs.

Our study combined and comprehensively analyzed the mRNAs and miRNAs content in human circulating-EVs, and has a number of strengths. Using next-generation sequencing analysis, we identified differential mRNAs and miRNAs expression signatures in circulating EVs in MetS compared with Lean human subjects. These observations suggest that the mRNA and miRNA cargo of circulating EVs might represent novel biomarkers in MetS. However, further studies are needed to analyze different body fluids in chronic metabolic diseases to identify additional EV biomarkers (mRNAs and miRNAs) that can be used for diagnosis, prognosis, and therapeutics. In addition, further studies are needed to determine the relative contributions of the individual components of MetS (e.g., obesity, hypertension, hyperlipidemia, etc.), as well as the causal relationship between EV content, MetS, and the cellular damage that it is known to induce. Our study is limited by the small sample size, as is often used in mRNA- and miRNA-seq studies that produce large datasets (15, 24). Our patients were relatively young, arguing against a major role of aging in our changes in the cargo of circulating EVs. Lastly, MetS

patients were extremely obese, so whether milder forms of obesity and MetS would lead to comparable alterations remains to be defined. Further studies are also needed to explore in detail genes and molecules that regulate pathogenic pathways in MetS, as well as techniques to blunt them.

CONCLUSION

In summary, we found in human MetS modifies cargo of circulating EVs, which may in turn modulate several important cellular functions and fate, and potentially serve as key regulators, biomarkers, and targets in the progression and treatment of MetS. Genetic message related to mitochondrial function, apoptosis, angiogenesis, oxidative stress, and inflammatory pathways were dysregulated. Further studies are needed to determine whether these changes in circulating EVs could be delivered to recipient cells and modulate cellular pathways.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: www.ncbi.nlm.nih.gov/bioproject/672664, RPJNA672664.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the First Hospital Affiliated to Jinan University Institutional Research Ethics Committee (Guangdong, China). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

YL participated in the experimental design, data collection and assembly, data analysis, and interpretation, manuscript writing. YM participated in the collection and assembly of data, financial support, data analysis, and interpretation. AW participated in the data analysis and interpretation, manuscript editing. AE participated in the conception and design, data collection and analysis, manuscript editing. LL participated in the conception and design, financial support, manuscript editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.687586/full#supplementary-material

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Conflict of Interest: LL is an advisor to AstraZeneca, Butterfly Biosciences, and Janssen Pharmaceuticals.

The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Adipocyte-Endothelium Crosstalk in Obesity

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Obesity is characterized by pathological adipose tissue (AT) expansion. While healthy AT expansion enhances systemic insulin sensitivity, unhealthy AT expansion through increased adipocyte size is associated with insulin resistance, fibrosis, hypoxia, and reduced adipose-derived adiponectin secretion. The mechanisms causing the unhealthy AT expansion are not fully elucidated; yet, dysregulated crosstalk between cells within the AT is an important contributor. Evidence from animal and human studies suggests a crucial role of the crosstalk between vascular endothelium (the innermost cell type in blood vessels) and adipocytes for metabolic homeostasis. Arterial endothelial cells are directly involved in maintaining normal organ functions through local blood flow regulation. The endothelial-dependent regulation of blood flow in AT is hampered in obesity, which negatively affects the adipocyte. Moreover, endothelial cells secrete extracellular vesicles (EVs) that target adipocytes *in vivo*. The endothelial EVs secretion is hampered in obesity and may be affected by the adipocyte-derived adipokine adiponectin. Adiponectin targets the vascular endothelium, eliciting organ-protective functions through binding to T-cadherin. The reduced obesity-induced adiponectin binding of T-cadherin reduces endothelial EV secretion. This affects endothelial health and cell-cell communication between AT cells and distant organs, influencing systemic energy homeostasis. This review focuses on the current understanding of endothelial and adipocyte crosstalk. We will discuss how obesity changes the AT environment and how these changes contribute to obesity-associated metabolic disease in humans. Particularly, we will describe and discuss the EV-dependent communication and regulation between adipocytes, adiponectin, and the endothelial cells regulating systemic energy homeostasis in health and metabolic disease in humans.

Keywords: adipose tissue, endothelial cells, hypoxia, extracellular vesicles, endocrine, nitric oxide

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INTRODUCTION

Obesity, defined as excessive fat accumulation, is a worldwide epidemic accompanied by an increased risk of developing cardiovascular diseases (CVDs), certain types of cancers, Alzheimer's disease, non-alcoholic fatty liver disease, and type 2 diabetes mellitus (T2D) (1). The link between the increased disease risks and excessive fat accumulation is not completely

understood but appears to rely on impaired white adipose tissue (WAT) function. WAT functions as an energy buffer that stores and releases energy (2); however, WAT is also an essential endocrine organ and plays a key role in regulating systemic glucose and energy metabolism by secreting an array of adipokines, including leptin and adiponectin (3). WAT is recognized as a highly dynamic and heterogeneous organ, and the adaptation to expanding WAT requires coordinated actions of multiple cell types to ensure a healthy adipocyte environment.

The WAT adapts to the excessive energy intake through two mechanisms 1) an increase in adipocyte number (hyperplasia) and/or 2) size (hypertrophy) (4). The hyperplastic WAT expansion is characterized by the formation of new adipocytes from adipose progenitor cells, which is associated with enhanced systemic insulin sensitivity. Hypertrophic WAT expansion is, on the other hand, characterized by insulin resistance, dysfunctional prolipolytic action, increased inflammation, fibrosis, and altered adipokine secretion profile, including decreased adiponectin levels (4, 5). Unhealthy WAT expansion is the *sine qua non* of metabolic unhealthy obesity, causing ectopic lipid accumulation in peripheral tissues such as the liver and skeletal muscle (4). The molecular mechanisms underlying the transition from a healthy WAT to an unhealthy pathological expansion are yet to be elucidated. Here, we will review and summarize our current understanding of the crosstalk between the adipocytes and the arterial endothelial cells within the WAT and how this communication potentially regulates systemic energy homeostasis in metabolic disorders.

THE ADIPOSE TISSUE MICROENVIRONMENT

Adipose tissue is a heterogenous cell population and contains, besides adipocytes, fibroblasts, stem cells, immune cells, and endothelial cells, and their intercellular crosstalk is crucial for the microenvironment (6). There is substantial evidence, mainly from animal studies, that hypertrophic obesity is associated with low oxygen tension in AT and increased expression of hypoxia-response genes (7), including the master regulator of hypoxia, hypoxia-inducible factor 1 (HIF1) (8). In primary adipocytes and macrophages from lean mice, hypoxia increases expression of inflammatory markers such as TNF-α, IL-1, IL-6, and TGF-β, chemokine (MIF), extracellular enzyme (MMP9), and macrophage markers (CD11 and F4/80) (7). The effect of hypoxia on the expression of adipokines in human adipocytes revealed increased gene expression levels of FIAF/angiopoietin-like protein 4, IL-6, leptin, MIF, PAI-1 and vascular endothelial growth factor (VEGF) (9). In 3T3-L1 cells, the promoter activity of NFkappaB and TNF-α was activated by hypoxia, causing reduced adiponectin promoter activity (7). Adipocyte-specific knockout of Hif1β – the obligate partner of hypoxia-inducible factors (Hif1a, Hif2a, and Hif3a) - reduced weight gain relative to wild-type controls and showed decreased high-fat diet (HFD)-induced obesity and glucose intolerance (10), indicating that the adipocyte-response to hypoxia is negative.

Oxygen tension in human WAT is challenging to measure, and the measured levels between lean and obese are dependent on the techniques used (11). Goossens et al. found that oxygen tension in WAT was higher in obese, and the obese subjects had lower WAT oxygen consumption (12). On the other hand, Pasarica et al. found that obesity was associated with lower WAT partial O_2 pressure, and obese subjects had a lower capillary density and decreased gene expression of WAT VEGF (13). Despite these differences in these two studies, the obese subjects had insulin resistance, high expression of inflammatory cell markers, and lower WAT capillarization (12, 13). Cifarelli et al. reported that the human AT expansion in obesity is associated with reduced AT pO2, which contributes to increased AT HIF-1α expression (14). These alterations decrease the branched-chain amino acid (BCCA) catabolism and increase the AT inflammation and fibrosis. Ultimately, this leads to an increase in circulating BCAAs and PAI-1 causing systemic insulin resistance (14). Very recently, Todorčević et al. demonstrated that markers of subcutaneous AT hypoxia are elevated in severely obese patients with obesity hypoventilation syndrome but not in moderately obese individuals, suggesting that in moderate obesity, AT dysfunction may not be driven by hypoxia (15).

Capillarization is endothelial-dependent, and blockage of vascularization in WAT causes unhealthy tissue expansion, enhanced inflammation and fibrosis, leading to systemic insulin-resistance (16–21). On the other hand, stimulation of angiogenesis results in healthy WAT expansion even during HFD-feeding and is associated with maintained insulin-sensitivity (16–21). Collectively, it appears that the interplay between AT cells is crucial for metabolic homeostasis and hampered endothelial-dependent regulation of WAT blood flow affects AT plasticity.

Using single-cell RNA-sequencing (scRNA-seq), it has become possible to perform large-scale transcript profiling of heterogeneous cell populations obtained from WAT from mice (22, 23) and humans (24). scRNA-seq of the stromal vascular fractions from visceral and subcutaneous WAT samples from obese patients undergoing bariatric surgery classified the cells into three subpopulations: 1) progenitors or stem cells (55%), 2) immune cells (37%), and 3) endothelial cells (8%) (24). Interestingly, endothelial cells could furthermore be divided into three types of endothelial cells (EC1-3). EC1 cells express genes (*FABP4*, *LGALS1*, *RBP7*, *GPX3*, and *CD36*) involved in lipid handling machinery, while EC2 cells had pronounced expression of canonical endothelial markers (*ACKR1*, *SELE*, *TM4SF1*, *VCAM1*, *TMEM173*, *PLVAP*, *ICAM1*, *PECAM1*, *VWF*, *ADAMTS9*, and *TFPI*). EC3 cells were highly enriched in LYVE1 expression, which is a marker of lymphatic endothelial cells (24). The lymphatic endothelial cells were predominantly present in visceral WAT samples (24). Although it is beyond the present review's scope, it is worth mentioning that lymphatic vessels (25) and immune cells (26) have been shown to contribute to unhealthy obesity. For example, lymphatic vasculature dysfunction was associated with an adult-onset obesity phenotype (27), and ablation of macrophages, through

transgenic expression of diphtheria-toxin receptor under control of CD11c promoter, in mice fed an HFD normalized insulin sensitivity and reduction in local and systemic inflammation markers (28). Nonetheless, the vascular endothelial cells, representing only a minor fraction of the total cell population, have prominent physiological and biological roles in health and metabolic disease. Importantly, endothelial dysfunction is an early vascular abnormality in metabolic disorders, and emerging evidence supports a critical role of endothelial cells in the development of metabolic disorders.

VASCULAR ENDOTHELIAL CELLS ARE CRUCIAL FOR WHOLE-BODY METABOLISM

The vascular endothelial cells are the inner most cell type of arteries, veins and capillaries. The vascular endothelial cell function sustains organ homeostasis by regulating vascular tone, recruitment of blood cells, exchanging tissue factors, forming new blood vessels, and providing organ-specific barrier function (29). Unlike other healthy cell types, endothelial cells generate most of their ATP from glycolysis (30). Endothelial cells have an insulin-independent glucose uptake through glucose transporter 1 (GLUT1) (31), and hyperglycemia, an associated consequence of obesity, is likely to increase the endothelial cells glucose concentration, which in itself is enough to cause oxidative stress and endothelial dysfunction (32). Although the endothelial glucose uptake is insulin-independent, impaired insulin signaling in endothelial cells can affect systemic insulin sensitivity. HFD fed mice have a reduction in insulin receptor substrate (Irs) 1 and 2 in the endothelial cells, and endothelial-specific knockout of Irs2 impaired insulin-induced glucose uptake in skeletal muscle (33). A vital function of the endothelium is to induce relaxation of the underlying vascular smooth muscle cells through the release of nitric oxide (NO) and thereby increase blood flow. The endothelium NO is generated primarily by the endothelial NO synthase (eNOS), and in endothelial-specific Irs2 knockout mice, the insulin-induced eNOS activation by phosphorylation was blocked, likely through reduced phospho-Akt (33). eNOS-phosphorylation was restored by the stable prostaglandin I2 analog beraprost sodium, which reestablished glucose uptake by the skeletal muscle in the endothelial Irs2 knockouts on a normal and HFD (33). Moreover, the antidiabetic drug metformin, which enhances whole-body insulin sensitivity, improves endothelial-dependent relaxation (34), indicating a critical role for endothelial blood flow regulation for the AT.

ENDOTHELIAL-DEPENDENT BLOOD FLOW

The endothelial-dependent vasodilatation is compromised in metabolic diseases, including obesity, and aging and is linked to reduced release of NO (35), increased oxidative stress (36), decreased endothelium-dependent hyperpolarization (37), and upregulation of released endothelium-derived contracting factors (38), which in combination results in reduced relaxation to vasodilatory substances such as acetylcholine in *ex vivo* arterial preparations. In humans and mice, increased body weight is associated with decreased endothelial-dependent vasodilatory in response to vasodilatory substances (39–43). Ingestion of food increases adipose tissue blood flow (ATBF) (44), and an oral glucose load increased ATBF in lean but not in obese subjects (12). The mediators responsible for this fast adjustment of ATBF are unknown, but intervention studies have provided evidence for the factors involved. Although increased glucose levels enhance plasma insulin levels, insulin micro infusion did not directly affect ATBF in humans (45). However, the ATBF increase correlated with changes in plasma norepinephrine (45). Plasma norepinephrine is likely to derive from spill over of sympathetic activity in muscle and AT (46). Norepinephrine is an endothelium-dependent arterial vasodilator (47) and high ATBF responding subjects had greater changes in plasma norepinephrine (44). Moreover, pharmacological intervention studies in humans have demonstrated that ATBF relies on the endothelial NO system (11, 48, 49), indicating that sympathetic activity is involved in ATBF regulation through an endothelial-dependent mechanism in arteries. HFD treatment of mice causes a reduced eNOS expression in WAT and transgene eNOS overexpression in whole body endothelial cells protected against high-fat diet-induced obesity (50). In agreement, eNOS deficient mice exhibit systemic insulin resistance (51, 52). Although the genetic interventions are not specific to WAT endothelium but affects all endothelial cells, the data suggest an essential role of eNOS regulation and endothelial health.

We have recently shown that mice fed an HFD developed endothelial dysfunction, which was abrogated in mice with global knockout of the T-type Ca^{2+} channel Cav3.1 (53). Consistent with this, in hypertensive patients treated with T-type/L-type channel blocker Efonidipine improved endothelial function (54), and pharmacological and genetic inhibition of Cav3.1 likewise protects against HFD-induced obesity in mice (53, 55). Cav3.1 is, among others, expressed in AT and endothelial cells (53), but the pharmacological and genetic inhibition studies of Cav3.1 do not pinpoint which cell types are involved in the phenotype. In endothelial cells, Cav3.1 interacts with eNOS (56). Nonetheless, global Cav3.1 knockout mice fed a regular diet display eNOS activity both *in vivo* and *in vitro* (56), and how Cav3.1 deficiency affects eNOS activity in HFD mice is still not known.

eNOS activity is also affected by caveolin-1 (Cav-1) – an integral membrane protein critically involved in the invagination of caveolae from the plasma membrane. Global Cav-1 knockout mice have endothelial dysfunction (57), and transgenic Cav-1 re-expression in the endothelium of Cav-1 knockout mice rescues the endothelial function (58). Cav-1 inhibits eNOS (59) and HFD-induced obesity increases vascular Cav-1 expression and accompanies impaired NO-mediated vasodilatation (60). In summary, the data suggest

that adequate eNOS regulation in AT endothelial cells is important for ATBF and whole-body energy homeostasis, and that Cav-1 appears to play a significant role.

Cav-1 is not only expressed in vascular endothelial cells but also highly expressed in adipocytes. Recently knockout of the Cav1 gene in mice uncovered a significant extracellular vesicle (EV)-mediated signaling between endothelial cells and adipocytes (61).

EXTRACELLULAR VESICLES ARE INVOLVED IN ENDOTHELIAL-ADIPOCYTES CROSSTALK

The term EV encompasses several distinct vesicle types but can broadly be divided into microvesicles and exosomes (62). Microvesicles originate from the plasma membrane through outward budding, while exosomes are created through invagination of the plasma membrane that ultimately causes the formation of multivesicular bodies (MVB), which through fusion with the plasma membrane release exosomes to the extracellular medium. The EVs all contain constituents of a cell, including nucleic acid, lipids, and nuclear, cytosolic, and membrane proteins (63, 64). Although the function(s) of EVs are still unknown, the fact that all cells, pro- and eukaryotes (65), release EVs points to their contribution to normal physiology, and EV appears to be involved in cell-cell communication and cellular waste management.

Extracellular Vesicles and Cell-Cell Communication

EVs have been suggested as entities for horizontal transfer of genetic material and proteins between cells. RNA is the dominant form of nucleic acid in EVs, and EVs appear to be enriched for several specific RNA species, including a number of microRNAs (miRNAs). In agreement, it was detected early that the correlation between cellular and EV RNA concentrations was poor (66), hinting at an active transport of RNA molecule into vesicles. Indeed, a short RNA motif has been identified that guides RNA into EVs (67). Nonetheless, using the golden standard for EV isolation – differential ultracentrifugation – has revealed that the average number of miRNAs per EV is low – approximate 1 miRNA per 100 EV (68). This low RNA/EV ratio suggests that EVs and their cargo may be heterogeneous and that some EVs carry a lot of RNA while others are non-RNA-carriers. Consistent with this, new EV separation and isolation techniques such as asymmetric-flow field-flow fractionation indicate that there exists a much wider variety of EVs than has previously been recognized (69). It should be noted that the analysis of EVs is complicated, and current EVs isolation techniques carry a significant risk of analysis of contaminations such as co-precipitated RNAs and proteins (62). This is even true for EVs isolated from serum-free medium where supplements may carry a significant amount of miRNA (70). Interestingly, though, in humans and mice, adipocyte-derived EV contain a significant fraction of circulating miRNAs (71). This was determined using mice with adipocyte-specific knockout of Dicer, a critical enzyme required for the conversion of pre-miRNA molecules into a mature miRNA (71). Disruption of the adipocyte processing of miRNAs caused significantly reduced plasma EVs level of miRNAs (71). The circulating adipocyte EV reduced hepatic FGF21 expression causing a decreased plasma FGF21 level and improved glucose tolerance, indicating that the EV miRNAs were functional (71). Collectively, it appears that adipocyte-derived EVs mediated cell-to-cell communication that affects distant organ function and surrounding cells.

Hypoxia is a potent stimulator of EV secretion. EVs released from adipocytes cultured at 1% O_2, compared to normal air with 5% CO_2, and EVs from obese subjects impaired insulin-stimulated glucose uptake in adipocytes (72). Moreover, in the initial stages of HFD-induced AT expansion, the increased AT oxygen consumption limits O_2 availability imposing a state of relative AT hypoxia that stimulated VEGF expression, and increased angiogenesis and HIF1α expression (73). In 3T3-L1 adipocytes, proteomic analysis of the EVs from the normoxic (20% O_2) and low oxygen (1% O_2) cultured 3T3-L1 cells showed that 75 and 67 proteins were up- and down-regulated, respectively, by the low oxygen conditions and that the EVs were enriched in proteins involved in *de novo* lipogenesis (74). Importantly, the low oxygen-derived EVs promoted the accumulation of lipids in recipient cells (74). EVs from liver cells are also important for lipid accumulation. Mice fed an HFD display rapid lipid accumulation in the liver (within hours), and the liver has been shown to respond to this by increased EV secretion (likely exosomes), which target adipocytes (75). Inhibition of EV secretion from liver cells by knockdown of Geranylgeranyl diphosphate synthase (Ggpps) improved glucose tolerance in HFD-fed mice but did not improve insulin resistance. The liver-derived EVs enhance adipocyte lipid deposition by increasing lipogenesis and inhibiting lipid oxidation through Pgc1α. Thus, liver cells may be an early metabolic sensor of lipid overload and respond by increased EV signaling to adipocytes (75).

In the WAT, the adipocytes are also targeted by EV derived from endothelial cells (61). Endothelial-derived EVs transferred Cav-1 protein to adipocytes, and, importantly, the EV-mediated transfer was regulated by fasting and feeding (61). Fasting increased endothelial Cav1 transfer, and this effect was blunted in HFD treated and *ob*/*ob* mice (61). Although the glucagon receptor expression did not differ between WAT- and lung-derived endothelial cells (61), glucagon only increased endothelial-derived EV secretion from WAT-derived endothelial cells (61). An essential function of the endothelial cells is the transcytosis of plasma components to the underlying parenchyma (29). Crewe et al. found that cultured endothelial cell-derived EVs were enriched in FBS-derived protein components (61). Glucagon and insulin increased BSA (fatty acid-free, low endotoxin) uptake in cultured endothelial cells, and Cav-1 participated in the process but was not essential for the glucagon and insulin-stimulated BSA uptake in endothelial cells (61). The BSA was secreted in EVs and indicates that endothelial cells contribute significantly to transcytosis by uptake of plasma components and secretion in EVs (61). Thus,

the intercellular and interorgan EV communication to and from the WAT appears to be important for metabolic regulation.

Extracellular Vesicles as Cellular Waste Management

Accumulating evidence suggests that EVs are part of the cellular waste management system and shares many features with secretory autophagy (76). For example, blockage of EV secretion causes accumulation of harmful DNAs and activation of cellular damage response (77). The important role of autophagy for adipocytes and endothelial cells has been demonstrated in experimental models. Mice with the adipocyte-specific knockout of autophagy genes Atg3 and Atg16L had normal weight and body composition; however, the gene disruptions caused a massive influx of inflammatory cells in AT even in the regular diet-fed mice (78). This occurred without an increase in cytokines such as TNF-α, IL-6, or MCP1 (78). The knockouts developed insulin resistance and impaired glucose tolerance, and together, suggests an essential role for adipocyte autophagy in the development of insulin resistance independent of obesity (78). Autophagy is also crucial for endothelial cells. Obesity-induced endothelial dysfunction is associated with the upregulation of endothelial autophagy machinery (79) and vascular ceramide content (80). Exosome production and release are modified by ceramide synthesis (81), and, interestingly, adiponectin signaling in endothelial cells increased exosome secretion and reduced cellular ceramide levels (74). Thus, exosome secretion may be a critical mechanism to reduce the intracellular accumulation of toxic material and endothelial dysfunction through adipocyte secretion of adiponectin.

ADIPONECTIN - T-CADHERIN-AXIS AND CELLULAR CROSSTALK

Adiponectin is an adipokine, which acts in an autocrine/paracrine and endocrine fashion (82) and is highly expressed in human and mouse AT (82, 83). Various adipokines may play a key role in AT biology, on systemic metabolism or tissue crosstalk such as leptin, however, in this review we are only focusing on adiponectin. Typically, plasma concentration of adiponectin is high and in the micromolar range. Low adiponectin levels are reported in humans with metabolic diseases such as obesity and T2D (84) and are inversely correlated with insulin resistance (85) and fat mass in humans (86). Moreover, decreased levels of adiponectin are also reported in coronary artery disease (87) and myocardial infarction (88). Thus, reduced circulating adiponectin levels can reflect metabolic perturbations and can potentially serve as a critical marker of WAT fitness.

Adiponectin belongs to the C1q-like superfamily of protein, and its structure consists of a 22 collagen repeats and a C-terminal C1q-like globular domain (82). Endogenous adiponectin forms homo-oligomeric structures consisting of trimers, dimers of trimers, and 4- and 5-mers of trimers that is referred to as low molecular weight (LMW), medium molecular weight (MMW), and high molecular weight (HMW), respectively, complexes (89, 90) with different biological functions through binding of surface receptors (91–93).

Activation of the adiponectin receptors AdipoRs (94–96) and calreticulin (91) have important metabolic and immunological roles. Skeletal muscle is an important site of insulin-mediated glucose uptake; thus, considerable emphasis was placed on studying the possible metabolic effects of adiponectin on muscle. In cultured muscle cell lines, adiponectin improves insulin sensitivity (97), increases glucose uptake (63, 98) and increases fatty acid oxidation (63, 64). In mouse models of obesity and T2D, physiological doses of adiponectin enhanced insulin sensitivity (99). In muscle, adiponectin acts through AdipoR1 to activate AMPK (100). The anti-inflammatory effects of adiponectin have been demonstrated in different cell studies. Treatment of human macrophages with adiponectin revealed that adiponectin inhibits mature macrophages' phagocytic activity, and adiponectin also inhibited the lipopolysaccharide (LPS)-induced TNF-α production and TNF-α mRNA expression (101). In line with this, another study showed that treatment of peritoneal macrophages with recombinant adiponectin enhanced transcript levels of a marker of the M2 phenotype such as IL-10 (102), indicating that adiponectin promotes macrophage polarization toward an anti-inflammatory M2 phenotype. In the liver, adiponectin binds to AdipoR1 and AdipoR2 to suppress hepatic glucose production and glycogenolysis (103), leading to reduced plasma glucose levels. Decreased hepatic glucose production can possibly be explained by studies showing that adiponectin suppresses the key regulators involved in gluconeogenesis, including phosphoenolpyruvate carboxykinase and glucose-6-phosphatase (104, 105).

In addition to adiponectin's beneficial effects on muscle and liver, it is also protective through its effects on the vascular endothelium (106). In the vascular endothelium, adiponectin acts through AdipoR1 and AdipoR2 to increase NO production through AMPK, which activates eNOS, leading to vasodilation (106). In addition to AdipoR1 and AdipoR2, endothelial cells also bind adiponectin through T-cadherin expression.

As described above, adiponectin increases endothelial exosome secretion, and this effect is mediated through the binding of adiponectin to T-cadherin (107). T-cadherin is a member of the cadherin family, but in contrast to the other family members, it lacks a C-terminal intracellular domain and is attached to the extracellular side of the plasma membrane through a glycosylphosphatidylinositol (GPI)-anchor (108). There is strong *in vivo* support for the adiponectin/T-cadherin interaction from human and murine studies. Thus, genome wide-association studies (GWAS) for plasma adiponectin levels suggest the CDH13 (the gene encoding T-cadherin and outside of the *ADIPOQ* locus) is strongly linked to adiponectin levels (109), and CDH13 single nucleotide polymorphisms (SNPs) are linked to increased adiponectin plasma levels in humans (110, 111). In mice, T-cadherin deficiency causes 3-fold increased plasma adiponectin levels (112). T-cadherin is expressed in the heart, skeletal muscle, aorta, and vascular endothelium (112–114). Adiponectin in its hexameric and HMW – but not trimeric

and globular-forms bind T-cadherin (92, 115), and the T-cadherin expression tissues are also the site for the accumulation of HMW and hexameric adiponectin in mice (112–114, 116, 117), suggesting critical biological functions of the adiponectin binding to T-cadherin. Adiponectin and T-cadherin knockout mice have lower plasma exosome levels, and viral overexpression of adiponectin caused increased plasma exosome levels (107). The mechanisms and cellular signaling pathways that are involved are still unknown. It has, however, been shown that oligomerization of membrane-anchored stimulates their sorting of cargo into exosome (118, 119); thus, the binding of the cellular attached T-cadherin to adiponectin might cause T-cadherin oligomerization, internalization, and sorting into the multivesicular bodies (MVBs) before being released as exosomes. Thus, the crosstalk between adipocytes and endothelial cells involves EVs; however, this crosstalk's biological significance is still largely unknown but could be an important mechanism mediating tissue-crosstalk and endothelial health. Further research should be undertaken to investigate if other adipokines also play a key role in crosstalk mediated by EVs.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The WAT microenvironment is critical for a whole-body metabolism; thus, gaining a mechanistic understanding of the crosstalk between the different cell populations in WAT is crucial. The endothelial cells are important for regulating WAT blood flow, and inadequate blood flow may cause hypoxia in the WAT and reduced adiponectin secretion (**Figure 1**). The reduced adiponectin secretion may cause reduced EV secretion from endothelial cells and thereby accelerate the development of endothelial dysfunction and decreasing adipocyte function further (**Figure 1**). The reduced adipocyte function affects multiple organs, e.g., through decreased adiponectin signaling in skeletal muscle, liver and the heart, and targeted treatment that restores the adipocyte/endothelial crosstalk in WAT may thus provide therapeutic opportunities that improve whole-body metabolism.

Since the crosstalk involves physiological adaptations, such as changes in blood flow, and molecular changes, the investigations are heavily dependent on integrated models. In line with this, the exact role of EVs for WAT biology regulation also requires integrated models and needs the development of new experimental tools. The effect of EVs is often inferred from correlation studies, and tools that block exosome signaling in a cell-specific manner are still not available. The use of fluorescent-tagged proteins that are transferred from endothelial cells to adipocytes has convincingly shown that transport does indeed occur between different cell types *in vivo* (61); however, to obtain information on EV function directly, cell-specific manipulation of EVs needs to be established. Possible solutions could be to use single-cell assays such as CD63-pHluorin (120, 121) and *in vivo* models for tracking intercellular EV communication (122). The

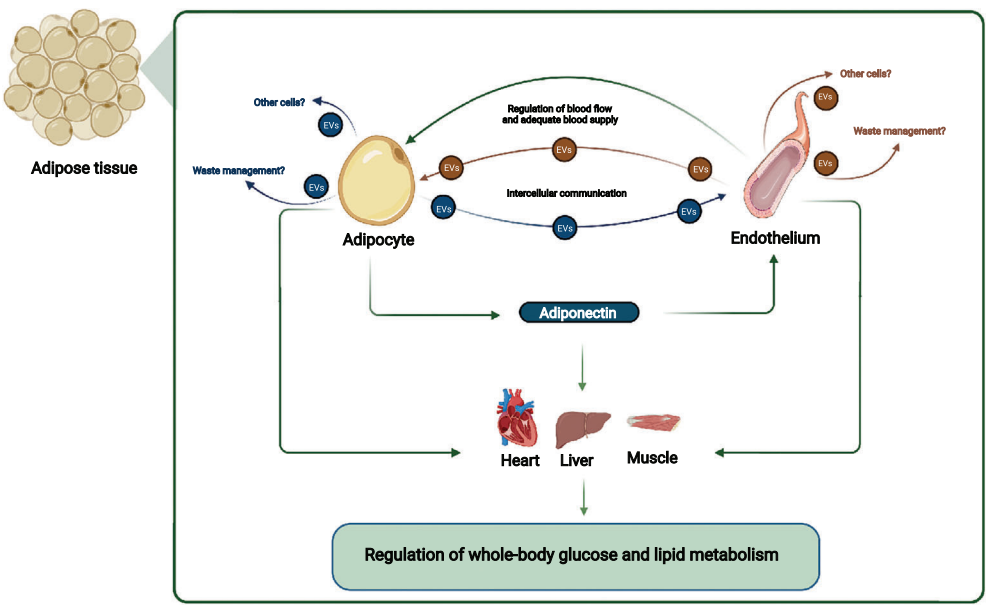

FIGURE 1 | Adipocyte and endothelial crosstalk in adipose tissue contribute to regulation of whole-body glucose and lipid metabolism: In this working hypothesis, endothelial cells regulate blood flow adequately to adipocytes, which secrete adiponectin that communicate locally with endothelial cells and with distant organs such as muscle, liver, and heart. Adiponectin stimulates EV secretion from endothelial cells, ensuring their health e.g. through cellular waste management, and stimulates muscle, liver, and heart metabolism, improving systemic metabolism. EVs from adipocytes and endothelial cells may also target other cell types. Other adipocyte-derived factors, and overall endothelial health also contributes to regulation of whole-body glucose and lipid metabolism. Figure created with BioRender.com.

tagged EVs will allow for cell-specific quantification of EV release rate and enable the identification of genetic and pharmacological interventions that interferes with EVs.

From a translational perspective, cells – including adipocytes and endothelial cells – release EVs to the circulation and provide non-invasive access to organs within the body. We have shown, using paired samples of human kidney and urine samples, that the EVs protein abundance is not a reliable marker of its tissue abundance (123). Nonetheless, the AT-derived EVs may be used to monitor clinical intervention studies and for early detection and differentiating of individual subjects based on whether or not they have healthy or unhealthy obesity. The identification of adipocyte- and endothelial-specific EV markers will provide an approach that enables the non-invasive interrogation of crosstalk between the cell-types in humans, enabling the translation of the findings from animal models to humans, and thereby provide new treatment options for alleviation of the negative health impacts of obesity.

AUTHOR CONTRIBUTIONS

RS prepared the figure. RS and PS drafted the manuscript. RS, PS revised the manuscript. Both authors contributed to the article and approved the submitted version.

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Detection of Urinary Exosomal HSD11B2 mRNA Expression: A Useful Novel Tool for the Diagnostic Approach of Dysfunctional 11β-HSD2-Related Hypertension

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Objective: Apparent mineralocorticoid excess (AME) is an autosomal recessive disorder caused by the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme deficiency, traditionally assessed by measuring either the urinary cortisol metabolites ratio (tetrahydrocortisol+allotetrahydrocortisol/tetrahydrocortisone, THF+5αTHF/THE) or the urinary cortisol/cortisone (F/E) ratio. Exosomal mRNA is an emerging diagnostic tool due to its stability in body fluids and its biological regulatory function. It is unknown whether urinary exosomal HSD11B2 mRNA is related to steroid ratio or the HSD11B2 662 C>G genotype (corresponding to a 221 A>G substitution) in patients with AME and essential hypertension (EH).

Aim of the Study: To detect and quantify HSD11B2 mRNA from urinary exosomes in samples from family members affected by AME and EH, and to evaluate the relationship between exosomal HSD11B2 mRNA, steroid ratio, 662C>G genotype, and hypertension.

Methods: In this observational case–control study, urinary steroid ratios and biochemical parameters were measured. Urinary exosomes were extracted from urine and exosomal HSD11B2 mRNA was quantified by Droplet Digital PCR (ddPCR). B2M (β-2 microglobulin) gene was selected as the reference housekeeping gene.

Results: Among family members affected by AME, exosomal urinary HSD11B2 mRNA expression was strictly related to genotypes. The two homozygous mutant probands showed the highest HSD11B2 mRNA levels (median 169, range 118–220 copies/μl) that progressively decreased in 221 AG heterozygous with hypertension (108, range 92–124 copies/μl), 221 AG heterozygous normotensives (23.35, range 8–38.7 copies/μl), and wild-type 221 AA subjects (5.5, range 4.5–14 copies/μl). Heterozygous hypertensive subjects had more HSD11B2 mRNA than heterozygous normotensive subjects. The F/E urinary ratio correlated with HSD11B2 mRNA copy number ($p < 0.05$); HSD11B2 mRNA strongly decreased while THF+5αTHF/THE increased in the two probands after therapy.

In the AME family, HSD11B2 copy number correlated with both F/E and THF+5αTHF/THE ratios, whereas in EH patients, a high F/E ratio reflected a reduced HSD11B2 mRNA expression.

Conclusions: HSD11B2 mRNA is detectable and quantifiable in urinary exosomes; its expression varies according to the 662 C>G genotype with the highest levels in homozygous mutant subjects. The HSD11B2 mRNA overexpression in AME could be due to a compensatory mechanism of the enzyme impairment. Exosomal mRNA is a useful tool to investigate HSD11B2 dysregulation in hypertension.

Keywords: apparent mineralocorticoid excess, 11β-hydroxysteroid dehydrogenase type 2, urinary cortisol metabolites ratio, urinary exosomal mRNA, Droplet Digital PCR, HSD11B2, essential hypertension

INTRODUCTION

Apparent mineralocorticoid excess (AME) is a rare autosomal recessive disorder caused by the 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzyme deficiency that consequently leads to an activation of the mineralocorticoid receptor (MR) not mediated by aldosterone (1). The MR is characterized by similar affinity for both aldosterone and cortisol, but in spite of the higher plasma concentration of cortisol as compared to that of aldosterone (about 100-fold), specific MR activation by aldosterone is physiologically guaranteed because of the specific 11β-HSD2 enzymatic activity. The enzyme, in fact, converts the active steroids cortisol and corticosterone respectively into their inactive metabolites, i.e., cortisone and 11-dehydrocorticosterone, thus allowing a functional selectivity of aldosterone to activate MR and regulate the epithelial sodium transport (2, 3). An impaired 11β-HSD2 function leads to an accumulation of active steroid forms in the renal distal tubular cells, with subsequent MR activation and sodium retention with the development of a clinical syndrome characterized by sodium retention, hypokalemia, salt-dependent hypertension, low renin, and suppressed aldosterone concentrations (1, 4–7). The activity of the 11β-HSD2 enzyme can be estimated either by measuring the urinary cortisol metabolites ratio (tetrahydrocortisol+allotetrahydrocortisol/tetrahydrocortisone, THF+5αTHF/THE) or by measurement of serum or urinary free cortisol/cortisone ratio (F/E). An increase in urinary (THF +5αTHF)/THE ratio or urinary F/E indicates a decreased 11β-HSD2 activity.

AME is a rare disease *per se* although it was hypothesized that a mild reduction of 11β-HSD2 activity can have a role also in low renin essential hypertension (EH). Milder forms of AME, recently named as "non-classic" AME, are at higher prevalence than classic AME, have a different phenotype and genotype, and are mainly related only to a partial enzymatic deficiency (8–10).

Urinary extracellular vesicles and especially urinary exosomes are secreted by renal tubular epithelial cells and carry nucleic acids, proteins, and lipids; they can be easily detected in urine and provide diagnostic and pathophysiological information without an invasive tissue biopsy (11–13).

Exosomes have a size of 40–100 nm, are secreted by all cell types, and are composed of a lipid bilayer with membrane receptors and nucleic acids inside. They are involved in extracellular trafficking, differentiation, and survival, and could provide information about transcription in cells of urogenital tissue. HSD11B2 mRNA is expected to be present in urinary exosomes, considering the tubular localization of the enzyme; however, its presence in exosomes has not been documented so far. Some authors hypothesized the usefulness of detecting HSD11B2 or even other exosomal mRNAs for the study of the pathophysiological mechanisms of hypertensive diseases and in particular for the diagnosis of mineralocorticoid hypertension (14). Urinary exosomes are considered extremely valuable for diagnostic purposes as they are considered as kidney liquid biopsies (15) because urinary EVs (uEVs) are mainly derived from renal cells while circulating serum EVs, under physiological conditions, cannot cross the nephron barrier (12, 16). The investigation of the urinary exosomal expression of HSD11B2 can therefore open up novel perspectives in the complex diagnostic and prognostic processes in non-classic forms of EH and especially in AME. In this context, our study was aimed to identify an accurate method to detect and quantify HSD11B2 mRNA from urinary exosomes in samples from family members affected by AME (17) and in EH patients to investigate a possible relationship between urinary exosomal HSD11B2 mRNA, steroid ratio, HSD11B2 662 C>G genotype (corresponding to a 221 Ala>Gly substitution in the amino acid sequence), and hypertension status.

MATERIALS AND METHODS

In this observational case–control study, we compared HSD11B2 exosomal mRNA copy numbers and urinary steroid ratios measured by two different methods, in AME family members and hypertensive subjects.

Subjects Selection

To study HSD11B2 urinary exosomal mRNA, two groups of subjects were enrolled: (1) hypertensive patients and (2) members of a family affected by AME as previously described (17).

Blood sample for biochemical routine parameters, 24-h urine, and second morning urine samples were collected for each subject. Second morning urine was obtained the same day of

the collection of the 24-h urine vessel. A sterile container was given to patients who provided the second morning void directly at the outpatient clinic.

Measurement of biochemical, hormonal, and routine laboratory tests were performed at the laboratory of the Clinical Chemistry Institute of the Verona University Hospital. Plasma renin and aldosterone levels were measured by commercially available methods (Dia Sorin Diagnostics, Vercelli, Italy), as previously described (18).

The study was approved by the Ethics Committee of our Institution (Azienda Ospedaliera Universitaria Integrata, Verona, Italy) and patients gave their informed written consent after full explanation of the study.

Hypertensive Subjects

Patients were enrolled among those referring to the Hypertension Unit of the Verona University Hospital for resistant hypertension or for possible secondary causes of hypertension. After the exclusion of secondary forms of hypertension (such as nephro-parenchymal disease, primary aldosteronism, renovascular hypertension, cathecolamine excess, and cortisol excess) only patients for whom a diagnosis of EH was made were included in the study. Similarly, patients currently treated with glucocorticoids, or with a clinical history of previous glucocorticoid treatment, were excluded. None was taking licorice-containing sweets, or 11β-HSD2 inhibitors, such as cancer treatment dithiocarbamates (DTCs) or fungicides such as itraconazole, hydroxyitraconazole (OHI), and posaconazole (19, 20).

According to the study protocol, all patients had not taken any hypotensive drugs other than verapamil and/or alpha-blockers over the previous 4 weeks. Plasma samples for aldosterone and renin were obtained after at least 2 h in the upright position and a subsequent period of 10 min in the seated position. Blood samples for hormonal and routine parameters were collected after overnight fasting between 8:00 and 9:00 a.m. Biochemical parameters were determined and 24-h urine cortisol/cortisone metabolite assay was performed on 24-h collected urine.

Fourteen subjects with a definite diagnosis of EH (equal to the number of the AME family members included in the study) were selected based on the availability of biological samples for the evaluation of HSD211B exosomal mRNA.

AME Family Members

Fourteen members of a family previously studied for a story of AME syndrome (14) were enrolled for this study. The subjects were subdivided according to their HSD11B2 662 C>G genotype (homozygous, 662 GG; heterozygous, 662 AG; wild type, 662 AA) and phenotype [normotensive (N); hypertensive (H)]. Clinical and biochemical follow-up of the two probands was also available at approximately 3 years after the diagnosis.

The two homozygous probands were not taking antihypertensive drugs at the time of enrolment whereas the other family members with a previous diagnosis of hypertension were already on treatment. The two probands were followed over time, with clinical and biochemical evaluation.

F/E and THF+Allo-THF/THE Ratio Measurement

Urinary free cortisol (F) and cortisone (E) quantification was performed using a validated liquid chromatography tandem mass spectrometry (LC-MS/MS) method, as extensively described elsewhere (21). Briefly, separation and quantification of both steroids was performed by using the MS urinary free cortisol/cortisone kit (ISBN-BSN, Castelleone, Italy) on a Nexera X2 series UHPLC (Shimadzu, Kyoto, Japan) coupled with a 4500 MD triple quadrupole MS (Sciex, Milan, Italy) detector. The mean intra- and inter-assay imprecision were between 1.7% and 11.3%

Total tetrahydro-cortisol (THF), 5α-tetrahydrocortisol (5α-THF), and tetrahydro-cortisone (THE) were quantified by gas chromatography–mass spectrometry (GC-MS) method, as previously described (17, 22). Instrumentation used comprised an Agilent Technologies 7890A gas chromatograph and an Agilent Technologies 5975C inert MSD detector (Agilent Technologies Inc., Santa Clara, CA, USA). The mean intra- and inter-assay imprecision were between 3.9% and 13% for both methods.

Either F/E or THF+allo-THF/THE was measured on 24-h collected urine.

Urinary Extracellular Vesicles (Exosome) Isolation

Second morning urine samples from AME family members and patients affected by EH were collected, processed as previously described with minor modifications (23) and stored at −80°C. Second morning void was chosen as good option for UV isolation based on previous reports (24, 25). Samples were thawed at room temperature and extensively vortexed to increase exosome yield. Aliquots of 5 ml of urine were mixed with the same volume of a commercially available precipitating reagent (Total Exosome Isolation Reagent from urine; ThermoFisher Scientific, Waltham, Massachusetts, USA) and incubated for 1 h at RT according to the manufacturer's instructions. Urine mixed with reagent were centrifuged at 10,000 *g* and 4°C for 1 h. The obtained pellet was used for analysis after careful removal of the supernatant. Vesicle size was checked by electron microscop.

RNA Extraction

Total exosomal RNA was extracted by a commercial kit (PureLink RNA Micro kit; Invitrogen, Carlsbad, California, USA). According to the manufacturer's instructions, RNA was extracted, purified, and eluted in a final volume of 15 μl and stored at −80°C.

Synthesis of cDNAs was carried out using a commercial kit (iScript Advanced cDNA synthesis kit for RT-PCR; Biorad, Hercules, California, USA) and a preamplification step was included (obtained by the use of SsoAdvanced PreAmp Supermix; Biorad, Hercules, California, USA) to improve mRNA detection and measurement. Preamplification primer mix included the genes HSD11B2 and β-2 microglobulin (B2M).

Reverse Transcription-Droplet Digital Polymerase Chain Reaction (ddPCR)

Droplet Digital PCR was performed at the Genomics and Transcriptomics Platform of "Centro Piattaforme tecnologiche"

of the University of Verona by the use of Bio-Rad QX200 Droplet digital PCR system (Bio-Rad). Reactions and processing of the samples were obtained following the manufacturer's instructions. In brief, the mix completed with cDNA samples were loaded with a multichannel pipette into a droplet generator cartridge and 70 µl of oils was added to the lower wells and the cartridge or reaction plates containing the samples was placed into a QX200 Automatic Droplet Generator (Bio-Rad) to produce the individual droplets. Each reaction was partitioned into ~20,000 nanoliter-sized droplets. Procedures were standardized and performed with high care. After the PCR was completed, the sealed plate containing the droplets was loaded in a QX200 droplet reader for the detection of completed PCR reaction individual droplets. Data were analyzed with Quanta Soft Software version 1.7.4.0917 (Bio-Rad) with the thresholds for detection set manually based on results from negative control wells containing water instead of RNA. During all the preparation, and in particular for the steps of droplet generation and droplet handling, high accuracy was required to preserve droplet integrity. The detection of HSD11B2 RNA was assumed valid only if the housekeeping gene B2M was present. Optimization was evaluated by the separation between positives and negatives. qPCR results were calculated according to the standard curves. Mean values and standard deviations of ddPCR and qPCR were assessed using Student's *t*-test. Reproducibility was tested with three independent samples and intra-assay coefficients of variation (CV) calculated for HSD11B2 and B2M mRNA were <5%. Statistical significance was achieved with p-value < 0.05. More details on RNA quantification are available in the Supplementary Material.

Statistical Analysis

Data analysis for comparison of clinical/biochemical data was performed using the SPSS 24.0 for Windows (SPSS, Chicago, Illinois, USA). Quantitative data are expressed as median values with minimum–maximum range, while categorical variables were expressed as percentage. Continuous variables showing a skewed distribution were analyzed on log-transformed values. Correlations between continuous variables were assessed by Pearson's test. Taking into account the very limited sample size of the different study subgroups (e.g., AME family members with different genotypes or EH patients analyzed according to exosomal HSD11B2 RNA copy number), all the comparisons are shown only as descriptive and not quantitative statistics.

RESULTS

Subjects enrolled in the study were characterized in terms of hormonal and biochemical parameters. In particular, the members of the AME family presented the features shown in **Supplementary Table S1** detailed for each family member and in **Table 1** according to genotype. Family members affected by AME displayed the typical phenotype of the disease, with aldosterone, renin, and K lower in 221 GG subjects than in heterozygous 221 AG and wild-type 221 AA subjects as shown in Supplementary Figure S1. Characteristics of EH patients are reported in **Table 2**. THF+aTHF/THE and F/E ratios showed a good correlation in the whole population. As illustrated in **Figure 1**, there was a positive significant correlation between the two parameters ($r = 0.9134$, $p < 0.0001$).

Values of THF+αTHF/THE ratio measured in the AME family were higher in 221 GG subjects ($n = 2$, 7.66, range 6.41–8.91) than those in the other groups, 221 AG_H ($n = 4$, 2.14, range 1.74–2.54), 221 AG_N ($n = 3$, 1.91, range 1.6–2.43), and 221 AA ($n = 4$, 0.99, range 0.29-1.8) (**Figure 2A**). In parallel, the highest F/E ratio values were found in 221 GG subjects ($n = 2$, 3.06, range 2.75–3.37) and the lowest values were found in 221 AA subjects ($n = 5$, 0.16, range 0.09-0.39) (**Figure 2B**). The F/E ratio values also showed a decreasing trend according to hypertensive status, 221 AG_H ($n = 4$, 0.70, range 0.42–0.97), 221 AG_N ($n = 2$, 0.47, range 0.25–0.49) (**Figure 2** and **Table 1**).

In EH patients, mean values for THF+allo-THF/THE and F/E were 1.59 ± 1.09 and 0.52 ± 0.35, respectively (**Table 2**).

Urine samples were processed for exosome extraction and HSD11B2 exosomal mRNA was measured by ddPCR. Method optimization and standardization was performed in order to obtain reliable results. Samples positive for the presence of both the housekeeping gene and the target gene were used for further analysis. HSD11B2 mRNA was expressed as copies/µl. The distribution of the enzyme in the different experimental groups investigated is shown in **Figure 2**. In the AME family, HSD11B2 exosomal mRNA was higher in mutated subject 221 GG (169, range 118–220 copies/µl) and progressively lower in 221

TABLE 1 | Characteristics of the AME family members according to genotype*.

	221 GG	221 AG	221 AA
Age	10 (7–13)	51 (25–81)	49 (20–79)
Hypertension (%)	100	57.1	20
THF+aTHF/THE ratio	7.66 (6.41–8.91)	1.91 (1.60–2.54)	0.99 (0.29–1.80)
F/E ratio	3.06 (2.75–3.37)	0.49 (0.25–0.97)	0.16 (0.09–0.39)
K (mmol/L)	2.94 (2.60–3.28)	3.97 (3.12–4.33)	3.9 (3.64–4.80)
P-Renin (pg/ml)	2.85 (2.46–3.24)	8.46 (0.84–15.96)	8.23 (6.06–40.40)
P-Aldosterone (pg/ml)	<15	106 (15–134)	128 (70–185)
P-creatinine (mg/dl)	0.56 (0.44–0.68)	0.76 (0.68–0.94)	0.75 (0.71–0.99)
P-cortisol (µg/dl)	16.25 (14.80–17.70)	11.1 (10.20–18.30)	12.7 (9–22)
ACTH (pg/ml)	53.4	19 (10.70–25.50)	17.3 (0.76–51)

**Data are expressed as median with minimum–maximum range.*

TABLE 2 | Biochemical and clinical features of EH patients*.

Age	49 (22–68)
Gender (M/F)%	36/64
THF+aTHF/THE ratio	1.15 (0.6–4.67)
F/E ratio	0.41 (0.18–1.63)
K (mmol/L)	3.86 (3.19–4.28)
P-Renin (pg/ml)	9.71 (1.2–33.16)
P-aldosterone (pg/ml)	185 (103–456)
P-creatinine (mg/dl)	0.82 (0.44–1.11)
P-cortisol (µg/dl)	13.95 (7–25.6)
ACTH (pg/ml)	15.96 (4.82–26.7)

Data are expressed as median with minimum-maximum range.

AG_Hypertensive (108, range 92–124), 221 AG_Normotensive (23.35, range 8–38.7), and wild-type 221 AA subjects (5.5, range 4.5–14). EH patients had HSD11B2 exosomal mRNA (20.9, range 5.6–79.2) lower than homozygous and heterozygous AG H (**Figure 3**). HSD11B2 mRNA data were available only for nine AME and nine EH subjects; as for the other subjects, the housekeeping gene was undetectable.

Furthermore, the two probands of the AME family, homozygous for the GG allele, were assessed also at 3 years follow up. The analysis revealed that HSD11B2 exosomal mRNA levels were greatly reduced, −86% with a decrease from 118 copies/µl to 16.6 copies/µl, and −98%, from 220 copies/µl to 5.1 copies/µl. By contrast, levels of THF+allo-THF/THE ratios were higher than those at the time of the enrolment, from 8.91 to 12.8 and from 6.41 to 9, respectively (**Figures 4A, B**). Other clinical–biochemical parameters improved from baseline, with a better blood pressure control (in relation to antihypertensive therapy including eplerenone, a MR receptor antagonist) and

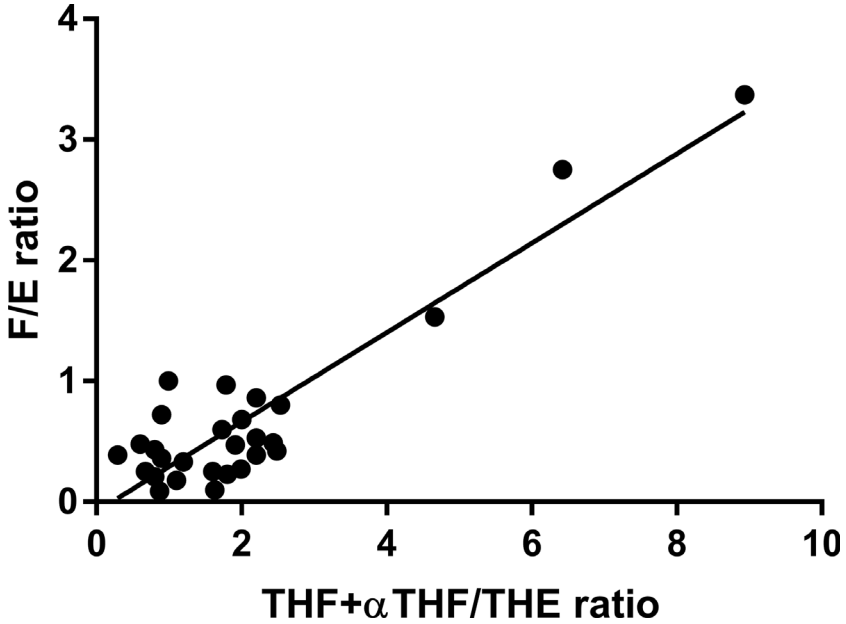

FIGURE 1 | THF+allo-THF/THE and F/E ratio correlation in the complete study population (n = 28). r = 0.9134, p < 0.0001.

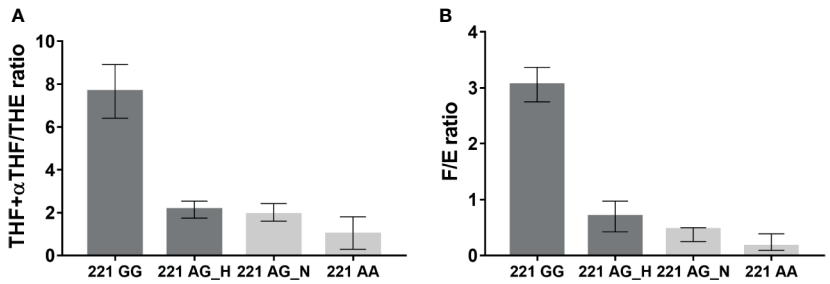

FIGURE 2 | THF+allo-THF/THE **(A)** and F/E **(B)** ratios in family affected by AME according to genotype (GG homozygous, AG heterozygous, AA wild type) and hypertensive status (H, Hypertensive; N, Normotensive).

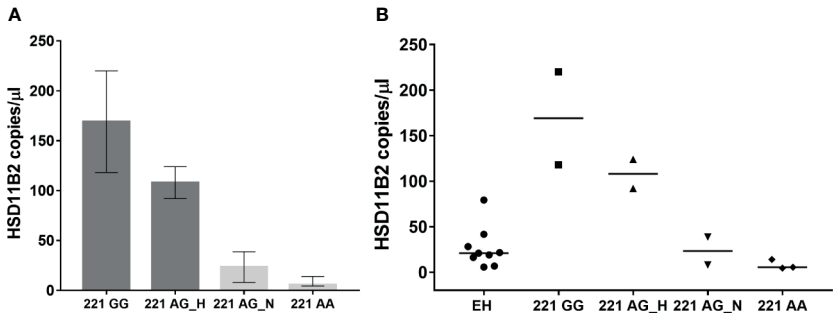

FIGURE 3 | HSD11B2 exosomal mRNA (copies/µl) in AME subject according to genotype (GG homozygous, AG heterozygous, AA wild type) and hypertensive status (H, Hypertensive; N, Normotensive) **(A)** and in all subjects including EH **(B)**.

normalization of kalemia. We then analyzed the correlation between HSD11B2 mRNA copies and other biochemical parameters, in particular THF+αTHF/THE and F/E ratio.

The EH population was divided into two subgroups according to HSD11B2 mRNA copy number: the first group within the first tertile and the second group above the first tertile value (17.12 copies/µl) (**Figure 5A**). Both ratios were higher in the EH group with a lower HSD11B2 mRNA copy number (2.20, range 0.9–4.67 *vs.* 1.6, range 0.68–2.2 for THF+αTHF/THE and 0.86, range 0.72–1.53 *vs.* 0.34, range 0.25–0.68 for F/E, respectively) (**Figure 5B**). The two EH subgroups differed also in K concentrations and age, as reported in **Table 3**. The main correlations studied between HSD11B2 mRNA and biochemical parameters are illustrated in **Figure 6**, where AME subjects are represented on the left and EH patients are represented on the right side of the figure. In the AME family, both urinary steroid ratios correlated positively with HSD11B2 mRNA (**Figures 6A, C**); by contrast, the correlation was negative in the EH population (**Figures 6B, D**), even if statistical significance was reached only for F/E in the AME subjects (**Figure 6C**, $r = 0.7825$, p-value = 0.0127). In AME, the mRNA exosomal copies of HSD11B2 significantly inversely correlated with aldosterone and renin ($r = -0.6646$, p-value = 0.0184 and $r = -0.7649$, p-value = 0.0164), respectively (**Figures 6E–G**). In EH patients, aldosterone levels did not show any correlation with HSD11B2 mRNA levels (**Figure 6F**), while renin values displayed a positive correlation with HSD11B2 mRNA levels ($r = 0.692$, $p = 0.039$ **Figure 6H**). In addition, when data were analyzed for a possible influence of age and BMI on both urinary steroid ratios in the whole population, no significant correlation was observed by Pearson's test. Furthermore, steroid ratios were also evaluated in relation to aldosterone and renin values either in AME or in EH subjects (**Supplementary Figure S2**). THF+αTHF/THE ratio showed an inverse correlation with aldosterone ($r = -0.555$, p-value = 0.0394) and an inverse but non-significant correlation with renin ($r = -0.4491$, p-value = 0.1237) in the AME family (**Supplementary Figure 2A**) despite the fact that, in EH, no correlation was found (**Supplementary Figures 2A, B**). Similar trends were observed for F/E ratio with renin and aldosterone levels; a significant negative correlation was in fact found in AME ($r = -0.6225$, p-value = 0.0231; $r = -0.666$, p-value = 0.0093, respectively) while there was no significant correlation in EH patients (**Supplementary Figures 2C, D**).

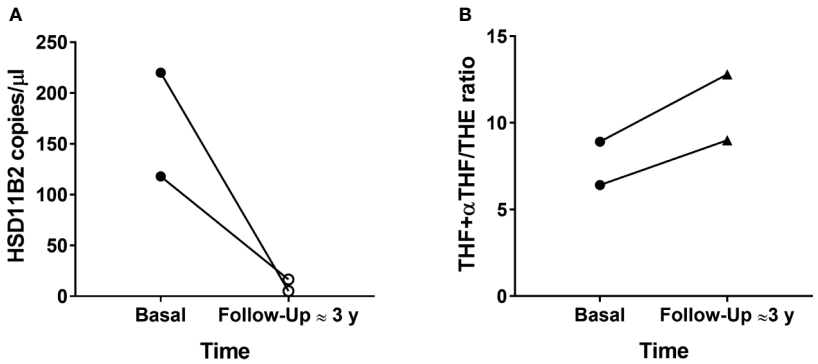

FIGURE 4 | HSD11B2 exosomal mRNA (copies/µl) **(A)** and THF+allo-THF/THE ratio **(B)** in the two probands 221 GG mutated at enrolment and follow-up (≈3 years).

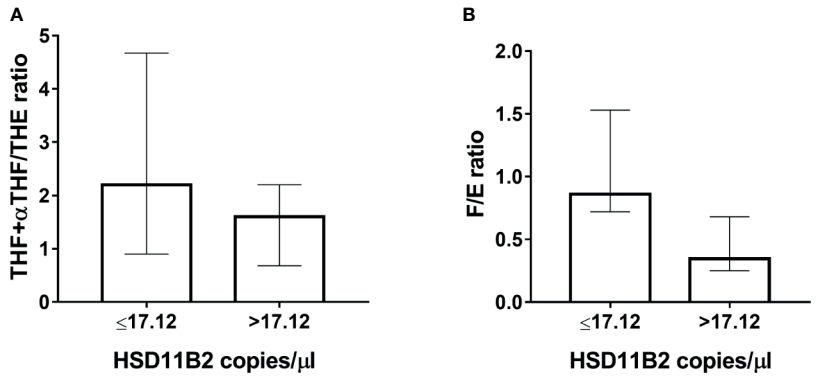

FIGURE 5 | Classification of EH patients in low and high HSD11B2 mRNA when below or higher than the first tertile (17.12 copies/μl), respectively. **(A)** THF+allo-THF/THE ratio. **(B)** F/E ratio.

DISCUSSION

To the best of our knowledge, this is the first report showing that HSD11B2 mRNA is detectable in urinary exosomes and correlates with the 11β-HSD2 activity estimated by urinary steroid ratios. The results highlight, moreover, that the urinary exosomal HSD11B2 mRNA expression varies according to the HSD11B2 662C>G genotype in an AME family, suggesting a potential use of it as a molecular marker of hypertensive disease, due to an impaired 11β-HSD2 enzyme function.

The-11β-HSD2 is a crucial enzyme for the modulation of MR activation, a key receptor at the kidney cellular level for the regulation of sodium and water balance in arterial hypertension. Cortisol inactivation by 11β-HSD2 mediates the ligand selectivity of aldosterone for MR, and an impaired enzymatic activity led to the cortisol activation of MR in AME disease.

The study of 11β-HSD2 enzyme is of interest because, even if AME is a rare disease, with about 100 cases and overall 50 mutations described in the literature, a partial deficiency of this enzyme was proposed also in other conditions, i.e., arterial hypertension and renal failure. A moderate impairment of 11β-HSD2 was reported in some EH patients, and a number of polymorphisms or genetic mutations with a mild influence on 11β-HSD2 activity were described in EH (7, 26, 27). Moreover, in relatives of AME patients, a mild form of hypertension, indistinguishable from EH, was often described (28, 29). We previously demonstrated that a number of EH patients and glucocorticoid-treated patients shared a similar phenotype, characterized by both arterial hypertension and elevated urinary THF+aTHF/THE ratio (22). Recent research suggests that milder forms of AME could be present in the general population, and hypertensive patients were mislabeled as idiopathic. This condition was previously described in literature as AME type II (1, 7, 30), and more recently described as "non-classic" AME, with a much higher prevalence (7.1%) than classic AME (8, 9, 31).

In humans, the 11β-HSD iso-enzyme 11β-HSD1 (converting the inactive cortisone into the active cortisol) is widely distributed but it is mostly abundant in liver and adipose tissue, whereas the 11β-HSD2 iso-enzyme (inactivating cortisol to cortisone) is primarily expressed in MR target tissues, i.e., kidney, colon, and salivary gland. To study the functional role of 11β-HSD2 in the regulation of water balance, the most critical issue is that the kidney cannot be easily available unless a renal biopsy, i.e., an invasive procedure is made. The 11β-HSD2 enzyme activity is, therefore, usually estimated by a surrogate marker, i.e., the urinary cortisol-to-cortisone metabolite ratios. As a proof of this assumption in a cohort of patients undergoing

TABLE 3 | Characteristics of EH patients divided into low and high HSD11B2 mRNA copy number*.

	<17.12	≥17.12
Age	59 (41–78)	44 (33–53)
Gender (M/F) %	33/67	50/50
THF+aTHF/THE ratio	2.2 (0.90–4.67)	1.60 (0.68–2.20)
F/E ratio	0.86 (0.72–1.53)	0.34 (0.25–0.68)
K (mmol/L)	3.40 (3.19–3.68)	4.01 (3.35–4.26)
P-Renin (pg/ml)	7.04 (1.20–7.60)	10.3 (5.28–33.16)
P-Aldosterone (pg/ml)	190 (171–283)	261 (103–456)
P-creatinine (mg/dl)	0.86 (0.59–1.11)	0.81 (0.44–0.99)
P-cortisol (μg/dl)	13.25 (11.7–15.60)	14 (7–25.6)
ACTH (pg/ml)	19.86 (14.7–26.7)	13.65 (4.82–24)

**Data are expressed as median with minimum-maximum range.*

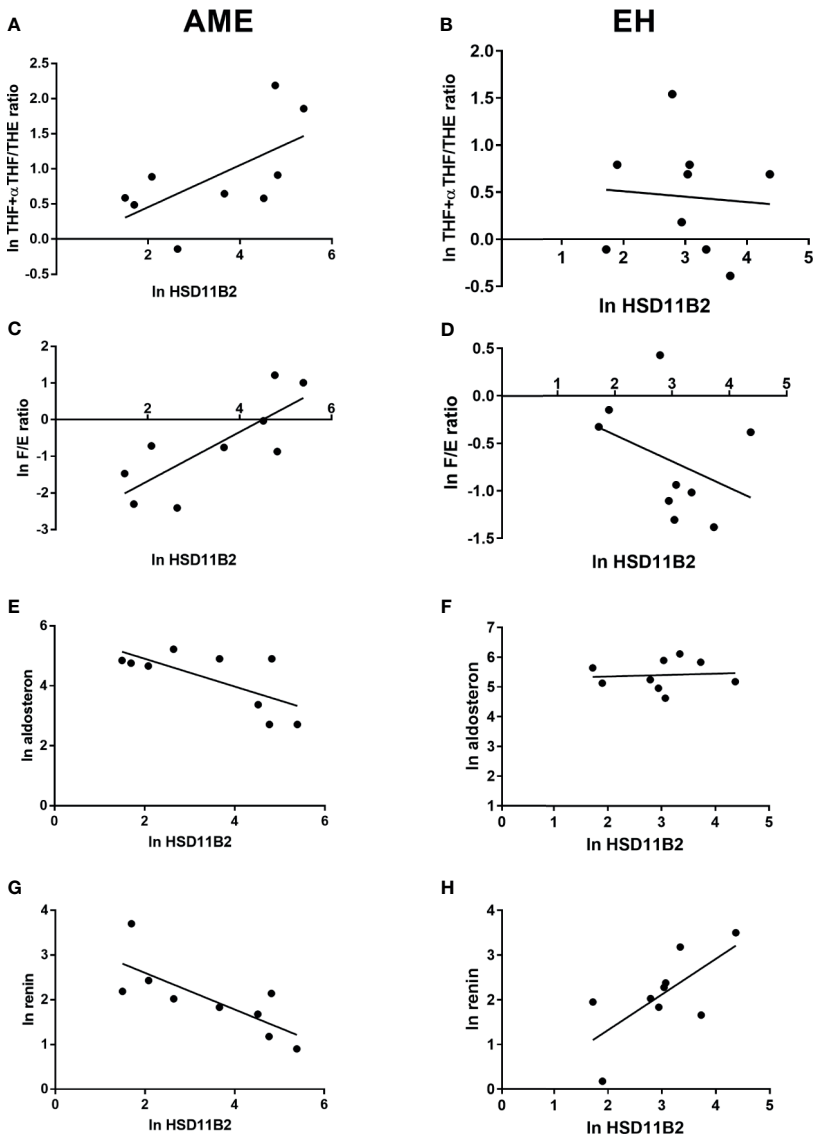

FIGURE 6 | HSD11B2 exosomal mRNA levels and steroid ratio and aldosterone and renin correlations in AME subjects (on the left) and in EH patients (on the right). 11β-HSD2 exosomal mRNA showed a positive correlation with THF+allo-THF/THE ratio in AME subjects, $r = 0.6266$, p-value = 0.0710 **(A)** and an inverse correlation in EH patients, $r = -0.07793$, p-value = 0.8420 **(B)**. Similar trend was found between 11β-HSD2 exosomal mRNA and F/E ratio in AME, $r = 0.7825$, p-value = 0.0127 **(C)** and inverse in EH, $r = -0.3762$, p-value = 0.3184 **(D)**. On the contrary, an inverse and significant correlation was found between 11β-HSD2 exosomal mRNA and aldosterone and renin in AME and a positive significance for renin in EH, $r = -0.6921$, p-value = 0.0388 **(E)** and $r = -0.7649$, p-value = 0.00164 **(G)**, $r = 0.692$, p-value = 0.039 **(H)**, respectively. No correlation was found in EH for 11β-HSD2 exosomal mRNA and aldosterone, $r = 0.081$, p-value = 0.8359 **(F)**.

renal biopsy because of suspected underlying renal disease, it was demonstrated that renal 11β-HSD2 mRNA expression relates to both urinary cortisol metabolite ratios (THF+aTHF/THE and F/E). Moreover, a decreased HSD11B2 expression was observed among the patients with worse renal function (32). In different patients, such as AME and EH, similar results have never been obtained so that the same relationships between cortisol metabolites ratios and 11β-HSD2 activity is far from being firmly proved. In this context, we hypothesized that urinary extracellular vesicles and exosomes might represent a useful tool for investigation and a possible source of information. Since the 11β-HSD2 enzyme is expressed in the kidney, we hypothesized that HSD11B2 mRNA is part of the urinary exosome cargo and therefore a possible liquid biomarker of disease related to 11β-HSD2 enzyme dysregulation. The results here presented confirm our hypothesis.

We demonstrated that HSD11B2 mRNA is detectable in urinary exosomes both in patients with AME syndrome and in patients with EH. The relationship with clinical–biochemical parameters are different in the two conditions. In the AME family, the expression of HSD11B2 mRNA varied according to

the 662 C>G genotype: the homozygous probands showed the highest levels, the heterozygous subjects showed intermediate levels, and the wild-type subjects showed the lowest levels. Among heterozygous carriers, hypertensive subjects had higher HSD11B2 exosomal mRNA than normotensives. These data suggest the following: (i) higher expression of the gene when the enzymatic activity is mostly reduced, at least as estimated by urinary steroid ratios (urinary THF/THE and THF+5αTHF/THE ratios); (ii) 11β-HSD2 mRNA is inversely related to the renin and aldosterone concentrations, mirroring a pronounced inhibition of the renin–aldosterone axis. The first point may be explained by an increased rate of transcription of the mutated gene as a feedback mechanism to compensate for the reduced enzymatic activity. However, this effect could be specific for the 662 C>G variant, while other mutations at different points of the gene sequence could have inhibitory consequences on the transcription process with reduced or absent mRNA.

The mechanisms previously investigated of the modulation of 11β-HSD2 gene expression were at pre- and post-transcriptional levels: the presence of polymorphisms, variations in microsatellite regions (33, 34), and epigenetic modifications, i.e., differentially expressed miRNAs (35, 36) and CpG methylation at the HSD11B2 promoter region (17, 37–39).

DNA methylation is the main epigenetic feature in mammalian cells, leading to a gene transcriptional regulation by transcriptional repression when the gene promoter site is hypermethylated (40). In a cohort of patients treated with glucocorticoid with high urinary THF+aTHF/THE, HSD11B2 promoter methylation in peripheral leukocytes was associated with the development of hypertension, suggesting a possible role of a modulation of HSD11B2 gene expression in the pathogenesis of steroid-induced hypertension (39). In the same AME family investigated in this study, we previously demonstrated a lower HSD11B2 promoter methylation in the two affected probands, compared to the wild-type individuals (17), and this result is consistent with the present finding of a great amount of HSD11B2 exosomal mRNA measured in the two probands.

A further indirect demonstration of the role of a gene expression modulation of HSD11B2 related to the entity of MR activation derives from the data of the two probands at follow-up. At baseline, when the two patients were free from specific therapy, with a florid disease characterized by low-renin, low-aldosterone hypokalemic hypertension, we observed a high exosomal HSD11B2 mRNA expression. After a long period of antihypertensive therapy including MR antagonist (eplerenone), along with a good blood pressure control and normalization of renin and kalemia, we observed a clear reduction in exosomal HSD11B2 mRNA. If HSD11B2 gene expression at baseline was increased in order to compensate for the excessive MR activation, a therapy-mediated MR inhibition also reduced HSD11B2 mRNA. Interestingly, the higher THF+aTHF/THE ratio as compared to baseline observed after eplerenone treatment in the two boys is consistent with the finding of a lower exosomal HSD11B2 gene expression.

The enzymatic activity of 11β-HSD2 is estimated by either urinary THF+aTHF/THE or serum or urinary F/E ratio. It was hypothesized that F/E may be a better marker of the activity of 11β-HSD2 isoenzyme, while THF+5αTHF/THE may represent a proxy of global HSD11B2 activity (i.e., both 11β-HSD1 and 11β-HSD2 isoforms) (7, 41–46). Which parameter is better to assess 11β–HSD2 activity in humans remains unclear. In the population of this study, F/E has a better correlation with exosomal HSD11B2 gene expression than the THF+aTHF/THE ratio: in the AME family, the positive correlation between HSD11B2 exosomal mRNA was positive for both urinary ratios, but reached the statistical significance only for the F/E, and the negative correlation between HSD11B2 exosomal mRNA and renin was significant only for the F/E ratio. In the EH population, U/F better discriminated patients according to median values of HSD11B2 mRNA than the THF+aTHF/THE ratio. However, because of the limited sample size, we cannot definitively state that urinary F/E is better than the THF+aTHF/THE ratio in estimating enzymatic 11β-HSD2 activity. Further studies are needed to confirm our data.

A further strength of the study is the accuracy of the method to quantify the urinary HSD11B2 mRNA expression since we utilized an advanced and sensitive novel technology, namely, the ddPCR (47) that is particularly suited for extremely low-target quantitation from variably contaminated samples as it is based on the partitioning of the PCR reaction into thousands of individual reaction vessels prior to amplification and on the acquisition of data at reaction end point. DdPCR is thus able to give more precise and reproducible data than qPCR (48).

The present study has some limitations that need to be acknowledged. The study sample is very limited and, therefore, mostly descriptive, which implies that they should be interpreted with caution. We also recognize the substantial overlap in exosomal HSD11B2 RNA copy number between different subgroups, including 221 GG homozygotes and hypertensive 221 AG heterozygotes, which limits the diagnostic value of such assay. Moreover, the comparison between AME family members and EH subjects is limited by the lack of age and sex matching. Digital PCR has proven to be the right tool for investigating HSD11B2 mRNA, as other approaches were not successful in allowing the detection of such a minimal amount of material such as the mRNA present in UVs, a limitation to get enough mRNA to analyze all samples, even after the necessary step of preamplification. Further studies are indeed required to confirm these findings in a larger data set.

Despite the study limitations, our results appear biologically plausible and could be seen as a potential proof of concept about the role of 11βHSD2 activity in mineralocorticoid hypertension.

HSD11B2 mRNA is detectable and quantifiable in urinary exosomes by ddPCR technology. In AME family members (662 C>G genotype), we observed an increased HSD11B2 expression in homozygous as compared to heterozygous and wild types and a positive correlation between exosomal mRNA and the 11βHSD2 enzyme activity as estimated by the urinary steroid ratios, i.e., opposite to the trend observed in EH patients. The overexpression of HSD11B2 mRNA is a possible mechanism of compensation of the enzyme deficiency. The study of exosomal expression is a useful tool to investigate 11β-HSD2 functional activity in kidney through the analysis of exosomal mRNA cargo in mineralocorticoid hypertension.

The present study, moreover, adds to the current knowledge and understanding that it is possible to perform a kidney tissue gene expression analysis simply by evaluating a urine sample. It is also possible to speculate that also other genes of interest related to diseases with a renal involvement could be investigated and provide novel insights for novel perspectives in clinical approaches.

DATA AVAILABILITY STATEMENT

The datasets generated for this study will not be made publicly available due to privacy policy. Requests to access the datasets should be directed to FP, francesca.pizzolo@univr.it.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of our Institution (Azienda Ospedaliera Universitaria Integrata, Verona, Italy). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

Conceived and designed research: SF, FP, and OO. Performed experiments: LB, FM DD, ED, SU, and MV. Analyzed data: LB, DD, AC, FM, and SU. Interpreted results of experiments: OO, FP, SF, NM, and ED. Prepared figures: DD and NM. Drafted the manuscript: FP, AC, and DD. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2021.681974/full#supplementary-material

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Roles of Skeletal Muscle-Derived Exosomes in Organ Metabolic and Immunological Communication

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Skeletal muscles secrete various factors, such as proteins/peptides, nucleotides, and metabolites, which are referred to as myokines. Many of these factors are transported into extracellular bodily fluids in a free or protein-bound form. Furthermore, several secretory factors have been shown to be wrapped up by small vesicles, particularly exosomes, secreted into circulation, and subsequently regulate recipient cells. Thus, exosome contents can be recognized as myokines. In recipient cells, proteins, microRNAs, and metabolites in exosomes can regulate the expression and activity of target proteins associated with nutrient metabolism and immune function. The levels of circulating exosomes and their contents are altered in muscle disorders and metabolic-related states, such as metabolic dysfunction, sarcopenia, and physical fitness. Therefore, such circulating factors could mediate various interactions between skeletal muscle and other organs and may be useful as biomarkers reflecting physiological and pathological states associated with muscular function. Here, this review summarizes secretory regulation of muscle-derived exosomes. Their metabolic and immunological roles and the significance of their circulating levels are also discussed.

Keywords: skeletal muscle, myokine, exosome, metabolism, microRNA

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INTRODUCTION

In the last 20 years, skeletal muscle has emerged as a secretory organ. Early studies showed that interleukin-6 (IL-6) is a typical secretory protein produced by muscle cells (1). Thereafter, various proteins and peptides were identified as muscle-secreted factors. Classically, muscles are known to generate and secrete a metabolite, lactate through glycolysis during muscle contraction. The majority of lactate is consumed as an energy substrate in metabolic organs, a process known as the "lactate shuttle" (2). Furthermore, novel metabolites have been reported using developed metabolomic analysis techniques. Many secretory factors are transported into the circulation and mediate the communication between skeletal muscle and other organs, referred to as the myokine theory (1). In recipient cells, myokines can regulate the expression and activity of target proteins in the muscle itself in autocrine and paracrine manners or other organs *via* an endocrine route, thereby contributing to physiological and pathological phenotypes, such as metabolic capacity, muscle mass, bone density, hormone secretion, cognitive function, and tumorigenesis.

Many secretory factors exist in free form or in a protein-bound form in circulation. Growing evidence shows that several secreted factors also contain in extracellular vesicles (EVs). Generally, two major subtypes of EVs have been characterized with differences of their biogenesis. In contrast to plasma membrane-derived "microvesicles", "exosomes" are defined as the EVs derived from endosome (3). Although it is difficult to distinguish each subtype completely, EVs differ based their components and functions. A noncoding RNA, microRNA (miRNA) is a typical component. Over 60% of protein-coding genes may be regulated by miRNAs (4), and many of these miRNAs are thought to play important roles in a range of biological processes. Some intracellular miRNAs are wrapped up by small vesicles, particularly exosomes, secreted into circulation, and then regulate recipient cells. Indeed, over 1000 RNAs have been detected in exosomes (5), in addition to proteins/peptides, lipids, and metabolites. Secreted exosomes dock to the plasma membrane of target cells, where they can bind or fuse with the plasma membrane or be endocytosed and then deliver their cargo (6). Through this process, exosomes mediate cell-cell and organ-organ communication (**Figure 1**). Skeletal muscle cells secrete exosomes; thus, their contents can be recognized as myokines and have potential functions over free-form secretory factors.

Here, we discuss the significance of circulating exosomes and their contents in the context of various physiological, and pathological states, such as muscle disorders, lifestyle-related diseases, and physical fitness.

EXOSOME COMPONENTS AND FUNCTIONS SECRETED FROM SKELETAL MUSCLE

The production of EVs, which includes exosomes, by skeletal muscle cells was first demonstrated by Guescini et al. (7). In a proteomic assay, muscle-derived exosomes were found to contain exosome-associated proteins and signal transduction proteins. Interestingly, mitochondrial DNA was also shown to be taken up into exosomes and released into the extracellular medium in cultures of C2C12 cells (7). In parallel, miRNAs have been reported to be present in blood. Several miRNAs are highly enriched in muscle tissues and are often referred to as myomiRs. Four myomiRs, namely, *miR-1*, *miR-133a*, *miR-133b*, and *miR-206*, together account for nearly 25% of miRNA expression in skeletal muscles in both humans and mice (8, 9) and contribute to the development of animal and human skeletal muscle cells (10, 11). Skeletal muscle cells were shown to secrete exosomes containing these four myomiRs (12, 13), which were found to be involved in myoblast differentiation into myotubes *via* an autocrine route. In addition, several animal and human studies have suggested the existence of some circulating

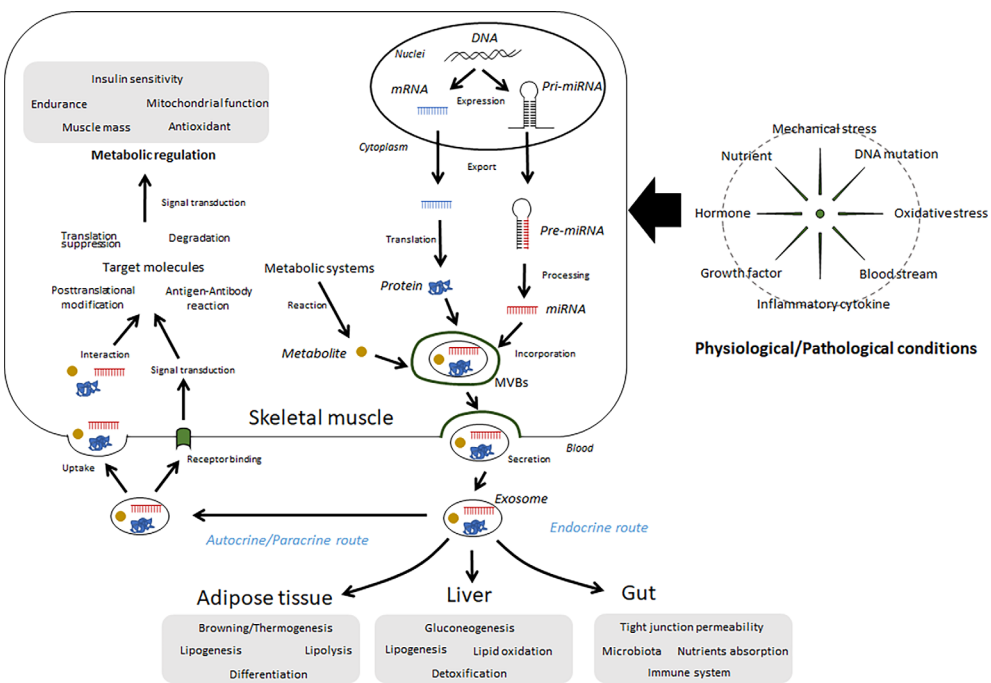

FIGURE 1 | Summary of the regulation and functions of exosomes in skeletal muscle. Several miRNAs, proteins, and metabolites are wrapped up by intracellular vesicles, such as exosomes, in skeletal muscle cells and secreted into extracellular fluids. These components can be affected by various conditions, including physical activity, diet, diseases, and aging. Circulating exosomes migrate to the skeletal muscle as well as other organs and regulate metabolic and immunological functions.

myomiRs in the living body (14–16). Recently, intact skeletal muscle tissues secrete exosomes containing *miR-1*, *miR-133a*, *miR-133b*, and *miR-206*, which depends on their expression levels in the tissues (13). In addition to the four miRNAs, several muscle-enriched miRNAs, including *miR-208*, *miR-486*, and *miR-499*, have also been identified in circulation (14, 17). Additionally, in human satellite cells, extracellular guanosine 5'-triphosphate was shown to increase the level of myomiRs and also induce the release of exosomes containing guanosine (18). These studies suggest that circulating muscle-specific miRNAs likely can act as myogenetic factors in autocrine and paracrine manners, and some are even released into circulation (13). However, their involvement in sarcopenia and training-induced muscle hypertrophy is unclear.

Although few proteins have been identified in exosomes secreted from skeletal muscle, several proteins related to the growth and metabolism of muscle have been reported. Proteomic findings using two-dimensional gel electrophoresis and liquid chromatography tandem mass spectrometry have shown that exosomes secreted from C2C12 cells show different in compositions after differentiation into myoblasts and myotubes. Several protein components, including integrin subunit beta 1, CD9, CD81, neural cell adhesion molecule, CD44, and myoferlin, likely play key roles in the differentiation of these cells (19). Additionally, growth factors such as insulin-like growth factors and fibroblast growth factor-2 are also present in exosomes from differentiating human skeletal muscle cells (20). Such exosomes likely contribute to skeletal myogenesis. A previous study showed that mouse and human myoblasts cultured in exosome-depleted serum form fewer myotubes than myoblasts grown in normal serum (21). This effect was not reversed, even when the medium was replaced with normal medium. Moreover, the expression levels of myomiRs, such as *miR-1*, *miR-206*, and *miR-133a*, are decreased during myoblast proliferation. As mentioned above, exosomes containing guanosine may also contribute to regulated contents of myomiRs (18). In addition, differentiating C2C12 exosomes increase motor neuron survival in NSC-34 cells (22). Therefore, skeletal muscle-secreted exosomes can mediate the maintenance of physiological homeostasis in muscle tissue in autocrine and endocrine manners.

In general, there are two pathways for exosome formation: endosomal sorting complex required for transport (ESCRT)-dependent and ESCRT-independent pathways (23). ESCRT-dependent pathways involve four types of ESCRT complexes and the proteins that bind to them which regulate protein recruitment, vesicle budding and separation. On the other hand, the ESCRT-independent pathway is mediated by sphingolipid ceramide *via* neutral sphingomyelinase. Although the regulatory mechanism of their biogenesis and secretion in muscle have not been fully clarified, several studies have provided some information. Using proteomic and bioinformatic analysis, Forterre et al. (12) suggested the existence of ESCRT proteins in exosomes from C2C12 differentiation. The release of EVs from C2C12 myoblasts was found to be modulated by intracellular ceramide levels in a Ca^{2+}-dependent manner (15). In *mdx* mice, the inhibition of ceramide synthesis suppressed EV secretion and improved muscle dystrophy (15).

Since the report by Valadi et al. (5), evidences regarding not only the secretory mechanism but also body dynamism of exosomes have been developed. Particularly, Aswad et al. (24) showed that information. fluorescently labeled muscle-derived exosomes injected into mice were found to be taken up into various tissues, including the lung, liver, spleen, brain, heart, pancreas, and gastrointestinal tract. In addition, a high-fat diet was shown to accelerate the release of exosomes from muscle tissues in mice (24). Muscle-derived exosomes from mice fed a high-fat diet induced myoblast proliferation and altered the expression of cell cycle and differentiation factors. These findings suggest that muscle-derived EVs have paracrine signaling functions *via* alteration of muscle homeostasis in response to a high-fat diet and may have endocrine functions *via* targeting other tissues *in vivo*. The same group showed that EVs from the muscles of mice on a high-fat diet increased the sizes of isolated islets *in vitro* and induced the proliferation of murine insulin-secreting MIN6B1 cells (25).

EXOSOMES IN PHYSICAL ACTIVITY/ MUSCLE CONTRACTION

Skeletal muscles function as a supporting organ during physical activity. The muscles consume lipids and glucose as substrates and generate energy for muscle contraction; thus, exercise training adaptively leads to muscle building and endurance. In addition, daily physical activity reduces the risk of various noncommunicable diseases, such as cardiovascular disease, type 2 diabetes, and cancer. Habitual exercise also counteracts the development of age-related muscle atrophy by regulating myogenesis and protein metabolism. Muscle-secreted factors have been known to contribute to physical fitness, preventive diseases, and anti-aging effects.

Initial studies have shown that circulating miRNAs are altered in response to a single bout of exercise and training (14, 26–30). However, the miRNAs are not limited to the cargo in exosomes and their secretory sources cannot be identified. In a last decade, EVs have been isolated in circulation and their secretory characteristics examined in physiological and pathological states (**Table 1**). Differences in exosomal or vesicle-free contents may affect the results. For example, Guescini et al. (31) reported that muscle-enriched *miR-133b* and *miR-181a-5p* in EVs were elevated after acute exercise, and a positive correlation was found between aerobic fitness and circulating muscle-enriched miRNAs. Additionally, Yin et al. (17) examined the secretory dynamics of cell-free and exosomal muscle-enriched miRNAs with exercise patterns in rats. In cell-free miRNAs, only *miR-1* and *miR-133a* were increased immediately after uphill exercise, whereas more miRNAs (*miR-1*, *miR-133a*, *miR-133b*, *miR-206*, *miR-208a*, and *miR-499*) increased after downhill running. In exosomes, only *miR-133a* increased following uphill running, whereas *miR-1*, *miR-133a*, and *miR-499* increased after downhill running (17). Mild to moderate muscle-damaging exercise affects *miR-31* in circulating EVs after exercise but does not alter myomiRs in EVs (32). Although the

TABLE 1 | Muscle-derived exosome components in physiological and pathological states.

Status			Subject	Change	Components	Reference number
Exercise	Acute	• Cycling	Healthy young (serum)	↓	miR-486	(14)
		• Running	Healthy young (EVs)	↑	miR133b, miR-181a-5p	(31)
		• Uphill running	Rat (cell-free)	↑	miR-1, miR-133a	(17)
		• Uphill running	Rat (exosome)	↑	miR-133a	(17)
		• Downhill running	Rat (cell-free)	↑	miR-1, miR-133a, miR-133b, miR-206, miR-208a, miR-499	(17)
		• Downhill running	Rat (exosome)	↑	miR-1, miR-133a, miR-499	(17)
		• Jump and downhill running	Healthy young (plasma)	↓ →	miR-31 myomiRs	(32)
	Chronic	• Cycling	Healthy young (serum)	↓	miR-486	(14)
		• Running, cycling	Healthy elderly (exosome)	↑ ↓	miR-486-5p, miR-215-5p, miR-941 miR-151b	(33)
Disease	Neuromuscular disorder	• Duchenne muscular dystrophy	Mouse (serum)	↑	miR-1, miR-133a, miR-206	(34)
		• Duchenne muscular dystrophy	Patient (serum)	↑	myomiRs	(35)
		• Denervation	Mouse (exosome)	↑ ↓	miR-206 miR-1, miR133a, miR 133b	(16)
	Diabetes	• Streptozotocin treatment	Rat (skeletal muscle)	↓	miR-23a	(36)
		• Dexametazon treatment	Cultured myotube (exosome)	↑	miR-23a	
		• Type 2 diabetes	Rat (serum) Patient (serum)	↑ ↑	miR-144 miR-144	(37)
		• Precursor miR-23a/27a injection	Mouse (skeletal muscle)	↑	miR-23a, miR27a	(38)
	Chronic kidney disease	• Exo/miR-26a injection	Mouse (skeletal muscle)	↑	miR-26	(39)
	Chronic obstructive lmonary disease		Sarcopenic condition (serum)	↑	miR-1, miR-206, miR-499	(40)
	Rhabdomyosarcoma tumors		Patient (serum)	↑	miR-1, miR-133a, miR-133b, miR-206	(41)
Aging		• Aging	Mouse (serum)	↑	miR-29b, miR-34a	(42)
Diet	High fat diet	• Obesity	Mouse (sketetal muscle)	↑	exosome	(24)
Ischemia reperfusion			Mouse (sketetal muscle)	↑	complement component C3 prepropeptide, PK-120 precursor, alpha-amylase 1 precursor, beta-enolase isoform 1, adenylosuccinate synthetase isozyme 1	(43)

↑, *increase;* ↓, *decrease;* →, *no change.*

levels of exosomal miRNAs do not change as easily in response to exercise, training status can influence the response. For example, Nair et al. (33) showed that regular exercise adaptively increased baseline levels of *miR-486-5p*, *miR-215-5p*, and *miR-941* and decreased the levels of *miR-151b*. These miRNAs were associated with IGF-1 signaling. In particular, circulating *miR-486*, a muscle-enriched miRNA, is altered in response to exercise and correlated with endurance capacity (14), suggesting that it may act on certain recipient cells. A major putative target of *miR-486* is phosphatase and tensin homolog (PTEN), which is a negative regulator of insulin signal transduction. Therefore, exosomal *miR-486* has a potential for protein synthesis through the IGF-1/Akt/mTOR pathway. Release of EVs into circulation in response to exercise is also affected by the physical characteristics of the individuals, including sex, body mass index, and fitness level (44). Furthermore, as reported by Whitham et al. (45), many proteins are contained in exosomes and small vesicles, and their profiles can be changed by exercise. Therefore, future investigations should focus on proteins and peptides as cargo in exosomes.

MUSCLE-DERIVED EXOSOMES AND NONCOMMUNICABLE DISEASES

miRNAs have been shown to function as modulators of myogenesis, muscle mass, and metabolic capacity in skeletal muscle (46–48). In addition, several muscle-enriched miRNAs are released into the circulation, and these miRNAs can be altered by muscle disorders (**Table 1**). Therefore, several researchers have investigated the functions of these miRNAs in the muscle itself and in other organs through paracrine and endocrine routes. In animal models and human patients of Duchenne muscular dystrophy, circulating levels of *miR-1*, *miR-133a*, and *miR-206* were suggested to be increased and

were correlated with the progression of muscle pathology (34, 35). Another neuromuscular disorder, denervation, resulted in higher levels of *miR-206* and lower levels of *miR-1*, *miR-133a*, and *miR-133b* in muscle-derived exosomes (16). These secretory characteristics of miRNAs may be closely involved in the development of pathological muscle conditions. Indeed, exposure of C2C12 muscle cells to EVs from the serum of *mdx* mice resulted in a decrease in cell death (15). Moreover, inhibition of ceramide synthesis and injection with GW4869, an inhibitor of exosome secretion, ameliorate muscular dystrophy in *mdx* mice, suggesting that EVs and myomiRs are closely associated with skeletal muscle degeneration.

Several exosomal contents have been shown to change with associated metabolic dysfunction. For example, *miR-23a* levels were found to be decreased in atrophied gastrocnemius muscles from streptozotocin-induced diabetic rats. Additionally, *miR-23a* was present in exosomes isolated from the medium of C2C12 myotubes, and dexamethasone treatment increased its abundance in exosomes (36). Thus, selective packaging of *miR-23a* into exosomes may reduce its content in muscles. Jalabert et al. (25) found that skeletal muscle-derived exosomes injected into mice could specifically target *miR* pancreatic islet cells and affect gene expression and proliferation of βcells. Although direct evidence has not yet shown whether muscle-derived exosomes can influence insulin secretion, regular exercise is known to promote the secretory capacity of insulin (49, 50). Karolina et al. (37) found that circulating *miR-144* levels are increased in animals and humans with type 2 diabetes. This elevation is negatively correlated with insulin receptor substrate 1 in insulin-responsive tissues, including skeletal muscle, suggesting that the elevation of *miR-144* levels in circulation may be associated with the development of insulin resistance. Furthermore, overexpression of *miR-23a/-27a* in muscle prevents diabetes-induced muscle cachexia and attenuates renal fibrosis lesions *via* muscle-kidney crosstalk (38). In addition, overexpression of *miR-26* in muscle prevents chronic kidney disease (CKD)-induced muscle wasting *via* exosomal *miR-26a* (39). This result supports the potential therapeutic applications of exosome delivery of specific miRNAs to treat and prevent CKD and sarcopenia.

Levels of muscle-specific miRNAs, i.e., *miR-1*, *miR-206*, and *miR-499*, were also higher in the plasma of patients with chronic obstructive pulmonary disease (COPD), who often have a sarcopenic condition (40). In plasma samples from 103 patients with COPD and healthy controls, plasma *miR-499* and *miR-206* levels were associated with inflammatory responses in skeletal muscle and circulation (40). This may cause sarcopenic conditions in patients with COPD. In addition, serum levels of myomiRs (i.e., *miR-1*, *miR-133a*, *miR-133b*, and *miR-206*) were significantly higher in patients with rhabdomyosarcoma tumors than in those with non-rhabdomyosarcoma tumors (41). Ischemia-reperfusion stimulation of skeletal muscle induces different responses of circulating exosomal proteins between nuclear factor-κB-knockout and wild-type mice (43). The results showed that various proteins, including complement component C3 prepropeptide, PK-120 precursor, alpha-amylase one precursor, beta-enolase isoform 1, adenylosuccinate synthetase isozyme 1, and glyceraldehyde-3-phosphate dehydrogenase, were altered. Interestingly, exosome-like vesicles released from inflamed myotubes induce myoblast inflammation and inhibit myogenic mechanisms while stimulating atrophic signals (51), supporting the roles of muscle-derived EVs in the development of inflammatory diseases *via* organ-organ crosstalk.

Several studies have shown that aging and dietary habits can also affect the quality of muscle-derived exosomes. An aging-dependent miRNA, *miR-29b-3p*, was observed in exosomes isolated from differentiated atrophic myotubes. The *miR-29b-3p*-containing exosomes released from myotubes were taken up by neuronal SH-SY5Y cells, leading to downregulation of neuronal-related genes and inhibition of neuronal differentiation. Muscle-derived *miR-34a* also increases with age in circulating EVs and induces the senescence of bone marrow stem cells (42). Thus, such age-associated exosomes in circulation may promote aging through modulation of crosstalk between muscle cells and neurons. In addition, a high-fat diet can promote the secretion of exosomes from skeletal muscles, thereby affecting proliferation and differentiation of myoblast (24). Thus, exosomes contribute to lipid transfer through mediating communication between cells in muscle tissues. Regarding the cardiovascular system, the muscle-derived exosomes with angiogenic miRNAs such as *miR-130a*, induces angiogenesis *via* redox signaling in endothelial cells (52). This prevents age-related loss of capillarization, found in type 2 diabetes and chronic heart failure. Furthermore, the influence on the function and survival of β-cells under normal and diabetic conditions has been discussed (25). *miR-133a*, *miR-206*, and *miR-16* in muscle-derived exosomes likely result in the abnormal regulation of insulin secretion in pancreatic islets (53). In contrast, *miR-133a*, *miR-133b*, and *miR-206* may have a tumor suppressive function. Under several cancer conditions, the levels of these myomiRs levels in circulation are lower than in the normal conditions (54). Although the relationship between exosomes and disease is still unclear, these observations support the significant role of muscle-derived exosomes in physiological homeostasis and pathological changes.

CONCLUSION

In this review, we discussed the roles of exosomes in metabolic and immunological communication between muscles and other organs. Many components are contained in muscle-derived exosomes and are likely contributed to maintaining or impairing the homeostasis of muscles and organs throughout the body. Abnormalities in glucose tolerance and lipid metabolism occur as a result of impaired metabolic function of skeletal muscle. Furthermore, it is now known that age-related muscle atrophy, or sarcopenia, is associated with central diseases including respiratory and cardiovascular diseases, chronic kidney disease, dementia, and depression. There is also a negative correlation between physical activity and the risk of developing cancer, as well as an association between intestinal bacteria and leaky gut syndrome. Exosomes may be

involved in these associations between skeletal muscle and multiple organs. Thus far, the search for bioactive factors in circulation has targeted free proteins, peptides, and metabolites, but in the future it is necessary to include the components encapsulated in exosomes. In addition, exosomes have potential applications as biomarkers reflecting metabolic and immunological conditions. However, it is unclear whether muscle-derived exosomes can be useful as a biomarker to reflect physical fitness, diseases, or aging. Many factors lack specificity because common factors are secreted by multiple organs and different stimuli. Therefore, special factors with highly expressed in skeletal muscle should be found in a future. In contrast to the identification of many miRNAs in exosomes, few proteins and metabolites have been identified, although it is assumed that many exist. Accordingly, further studies are needed to elucidate the detailed characteristics of exosomes under physiological and pathological conditions.

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WA and YT wrote the manuscript. All authors contributed to the article and approved the submitted version.

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BMSC-Derived Exosomes Inhibit Dexamethasone-Induced Muscle Atrophy *via* the miR-486-5p/FoxO1 Axis

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Sarcopenia, characterized by reduced muscle function as well as muscle mass, has been a public health problem with increasing prevalence. It might result from aging, injury, hormone imbalance and other catabolic conditions. Recently, exosomes were considered to regulate muscle regeneration and protein synthesis. In order to confirm the effect of BMSC-derived exosomes (BMSC-Exos) on muscle, dexamethasone-induced muscle atrophy was built both *in vitro* and *in vivo*. In the present research, BMSC-Exos attenuated the decrease of myotube diameter induced by dexamethasone, indicating that BMSC-Exos played a protective role in skeletal muscle atrophy. Further mechanism analysis exhibited that the content of miR-486-5p in C2C12 myotubes was up-regulated after treated with BMSC-Exos. Meanwhile, BMSC-Exos markedly downregulated the nuclear translocation of FoxO1, which plays an important role in muscle differentiation and atrophy. Importantly, the miR-486-5p inhibitor reversed the decreased expression of FoxO1 induced by BMSC-Exos. In animal experiments, BMSC-Exos inhibited dexamethasone-induced muscle atrophy, and miR-486-5p inhibitor reversed the protective effect of BMSC-Exos. These results indicating that BMSC-derived exosomes inhibit dexamethasone-induced muscle atrophy *via* miR486-5p/Foxo1 Axis.

Keywords: muscle atrophy, bone marrow mesenchymal stem cell, exosomes, miR-486-5p, FoxO1

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INTRODUCTION

Sarcopenia, with the character of loss of physical performance and muscle mass, leads to increased risk of disability and mortality (1). Muscle fiber loss accelerates after 40 years old, and nearly 30% of muscle mass will be lost by 80 years of age (2). Sarcopenia is expected to affect more than 200 million people worldwide in the next 40 years according to the European Working Group on Sarcopenia in Older People (2). Recent studies have found that sarcopenia often occurs simultaneously with osteoporosis, indicating that there should be a close relationship between bone and muscle (3). Importantly, the crosstalk between skeletal muscle and bone plays an important role during the process of aging (4).

Bone marrow mesenchymal stem cells (BMSCs) are considered as ideal seed cells for tissue engineering. BMSCs transplantation was demonstrated to contribute to skeletal muscle regeneration in rat. However, the protective role might not be produced by the myogenic differentiation of BMSCs, but by a paracrine effect of BMSCs on muscle regeneration (5). Recently, exosomes, the small phospholipid bilayer structures that containing proteins, RNA and other active substances, are considered to mediate the crosstalk between cells (6). Exosomes can exert the effects on target cells by delivering signal factors and activating downstream signaling cascades. Exosomes, which were released by different type of cells, undertake a vital role in promoting muscle health (7–10). It has been certified that exosomes derived from human skeletal myoblasts could up-regulate myogenesis of human adipose stem cells (11). MSC-derived exosomes had also been indicated to promote the myogenesis of C2C12 cells *in vitro* (12). However, the underlying mechanism of this beneficial effect remains elusive.

In this study, BMSC-Exos attenuated the decrease of myotube diameter induced by dexamethasone. Further mechanism analysis showed that the miR-486-5p/FoxO1 axis mediates this protective effect. Moreover, we demonstrated that BMSC-Exos alleviated dexamethasone-induced muscle atrophy *in vivo*.

MATERIALS AND METHODS

Cell Culture

All animal experiments in this research were approved by the ethics committee of the Third Hospital of Hebei Medical University. BMSCs were separated from bone marrow of 4 Balb/c male mice which were obtained from the animal experimental center of Hebei Medical University. In short, mice were killed off and soaked in 75% alcohol for disinfection. Then, the femur was obtained by dissecting both lower limbs, and the bone marrow was flushed out with PBS. The primary BMSCs were blown into a single cell suspension and subsequently cultured in DMEM media containing 10% heat-inactivated fetal bovine serum. The skeletal muscle cell line, C2C12 myotubes, were obtained from Procell (Wuhan, China) and incubated in DMEM high glucose medium. C2C12 myotubes were cultured under myogenic induction (2% horse serum) for 6 days, followed by treatment with PBS (con group), BMSC-Exos (Exos group), 50 μM DEX (DEX group) or 50 μM DEX with BMSC-Exos (DEX+ Exos group) for a further 24 h. All cells were cultured in a stable environment of 37°C and 5% CO2.

Isolation and Identification of Exosome

The MinuteTM efficient exosome precipitation reagent (Inent Biotechnologies Company, Beijing, China) was used to separate exosomes form the conditioned medium of BMSCs according to the instruction. Briefly, the BMSCs were cultured in serum-free medium for more than 15 hours after washed by PBS. Then, the culture supernatant was collected. Removing cells from the collected supernatant low-speed centrifugation (5min, 1000g) before incubating with exosome precipitant. Finally, centrifuging the samples for 1 h at 10,000g and removing the supernatant. The exosomes were collected and stored for further use. The concentration of exosomes was determined using a BCA protein assay kit (Servicebio, Wuhan, China). Ten μg/ml of BMSC-Exos was used to treat C2C12 cells.

The character of exosomes derived from BMSCs were further identified. TEM (Hitachi HT7700 TEM, Tokyo, Japan) were used to observe the morphology and diameter of exosomes. Briefly, exosomes samples were aspirated with pipette gun and placed in carbon membrane copper mesh for 5 minutes, then the excess liquid was aspirated with filter paper. The samples were subsequently stained with 2% phosphotungstic acid for 2 mins. Finally, the images of exosomes were collected by TEM. The specific surface proteins of exosomes, CD63 and CD81, were detected by western blotting assay.

Exosome Uptake Assay

Firstly, exosomes were stained with PKH26 diluent for 5 minutes. Secondly, the excess dye was neutralized with 10% bovine serum albumin before washing the labeled exosomes in PBS for 60 minutes. Finally, C2C12 cells were treated with the labeled exosomes for 24h, stained with 4',6-diamindino-2-phenylindole and phalloidin.

MiRNA Inhibitor and Mimics Transfection

miR-486-5p inhibitor and mimics was transfected into BMSCs by Lipofectamine 3000 (TermoFisher Scientific, USA) according to the protocol. miR-486-5p inhibitor and miR-486-5p mimics were separately mixed with Lipofectamine 3000 for 20 min and then cultured with BMSCs for 6 hours. Then, the transfected medium was renewed by complete culture medium. Cells were harvested for further analysis after 48 h post-transfection. The miR-486-5p inhibitor and miR-486-5p mimics were all purchased from ZHONGSHI TONGTRU (Tianjin, China).

Real-Time RT-PCR Analysis

For the analysis of mRNA, total RNA was extracted through TRIzol® reagent (TIANGEN, Beijing, China) before reversed-transcribed into cDNA *via* RevertAid™First Strand cDNA synthesis Kit (Thermo, Waltham, USA) based on the instructions. Then, RT-PCR analysis was performed according to TIANGEN SuperReal PreMix Plus' protocols.

For the analysis of miRNA, microRNA assay kit was purchased from ZHONGSHI TONGTRU (Tianjin, China) and performed following the manufacturer's introductions.

Primers used in the present research are compiled as follows: GAPDH: 5'-GCAAGTTCAACGGCACA-3', 5'-CGCCAG TAGACTCCACGAC-3'; Atrogin-1: 5'- AAGGCTGTTGGAGC TGATAGCA -3', 5'- CACCCACATGTTAATGTTGCCC -3'; MuRF1: 5'-TGTCTCACGTGTGAGGTGCCTA-3'; 5'- CACCA GCATGGAGATGCAGTTAC -3'; MyoG: 5'- GTAGTAGGC GGTGTCGTAGC -3', 5'-CCACGATGGACGTAAGGGAG-3'.

Western Blotting

The nuclear protein extraction kit (Beyotime, China) was used to get total protein of samples. Then, 20μg protein was separated *via* 12% SDS-PAGE and transferred to PVDF membranes before being blocked with 5% milk for 1 hour at room temperature. The PVDF membranes were stained at 4°C overnight with antibodies

specific for FoxO1 (1:1000, Abcam, England), MuRF1(1:1000, Abcam, England), MyoG (1:1000, Abcam, England) and Lamin B1 (1:1000, Abcam, England). Finally, Blots were then stained with fluorescence secondary antibodies (1:20,000, Rockland, USA) and analyzed by the Odyssey Infrared Imaging System (Li-COR Biosciences).

Animals and Treatment

A total of 20 male mice were randomly divided into four groups: control (CON), dexamethasone (DEX), dexamethasone + BMSC-Exosomes (DEX +inhibitor control-Exos), dexamethasone +miR-486-5p-inhibitor-Exos (DEX +miR-486-5p-inhibitor-Exos) after adaptive feeding. Dexamethasone (5mg/kg) dissolved in normal saline and was intraperitoneal injected once a day for 2 weeks until the end of experiment. BMSCs-Exo and miR-486-5p-inhibitor-Exos (100 μg exosomes resuspended in PBS) were injected into the muscle of limbs at multi-points every two days. Mice were maintained under suitable growth environment with free access to food and water.

Measurements the Mass and Strength of Muscle

After 2 weeks of treatment, grip strength and running test were performed to evaluate the muscle force. For grip strength, mice were allowed to grip the T-bar using forelimbs. Then, the animals' tails were pulled directly toward the tester. The pull force for each mouse should keep the same. Grip strength was calculated as force divided by the final body weight (N/g). For running test, total running time and distance were recorded. Body weight and muscle composition of each mouse was measured at the end of muscle function test.

Immunostaining Analysis and the Measurement of Fiber Size

The mice were sacrificed after grip strength analysis. Muscle tissue was cut into thick serial sections for further staining. For muscle histology, the samples were stained with HE staining. The images were captured using an optical microscope.

Immunostaining of laminin for muscle tissue sections was performed after fixation and permeation. The images were captured and processed with a fluorescence microscope and analyzed using Image J software.

Statistical Analysis

For the experiments *in vitro*, the data were obtained from at least 3 replicated tests, and for the experiments *in vivo*, there were 5 mice in each group. The data were described by mean ± standard deviation. Students t-tests and one-way ANOVAs with Tukey's *post hoc* test were performed to compare the data between groups as appropriate. Statistically significant was measured by $p < 0.05$.

RESULTS

Characterization of Exosomes Derived From BMSCs

Typical spherical exosomes were captured by TEM (**Figure 1A**). The exosome-specific markers (CD63 and CD81) proteins were further detected by Western blotting analysis (**Figure 1B**). Subsequently, to further verify the endocytosis of BMSC-Exos in C2C12 myotubes, the fluorescent dye PKH26 was used to stain the BMSC-Exos. As shown in **Figure 1C**, PKH26-labeled BMSC-Exos were localized in the C2C12 region, which exhibiting the efficient internalization of the BMSC-Exos by C2C12 myotubes.

BMSC-Exos Inhibited DEX-Induced Decreases in Myotube Diameter

Cell diameter were measured to confirm the positive role of BMSC-Exos on DEX-induced atrophy of C2C12 myotubes. C2C12 myotubes cultured under myogenic induction for 6 days, followed by treatment with PBS (con group), BMSC-Exos (Exos group), 50 μM DEX (DEX group) or 50 μM DEX with BMSC-Exos (DEX+ Exos group) for a further 24 h. The results showed that the cell diameter decreased due to the treatment of DEX, whereas the BMSC-Exos treatment attenuated the reduction of cell diameter by DEX (**Figures 2A, C**). Meanwhile, the results of MHC immunofluorescence staining indicated that BMSC-Exos significantly improve the quantity of myotubes (**Figures 2B, D**).

BMSC-Exos Attenuated DEX-Induced FoxO1 Transmission Involved in Muscle Atrophy and Myogenesis

qRT-PCR analysis and western blot were performed to evaluate the effect of BMSC-Exos on muscle atrophy and myogenesis. The results demonstrated that DEX increased the level of atrogin-1

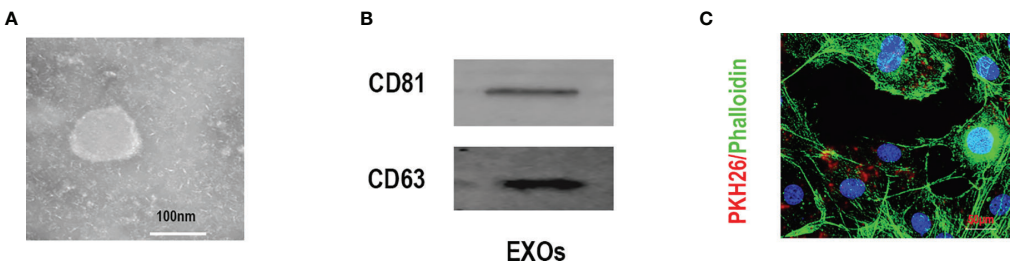

FIGURE 1 | Characterization of exosomes derived from BMSCs. **(A)** Morphology identified by TEM, scale bar:100 nm. **(B)** The specific surface biomarkers of exosomes were analyzed by Western blotting assay. **(C)** The exosomes derived from BMSCs were marked with PKH26 and co-cultured with C2C12 myotubes, mbar = 50μm.

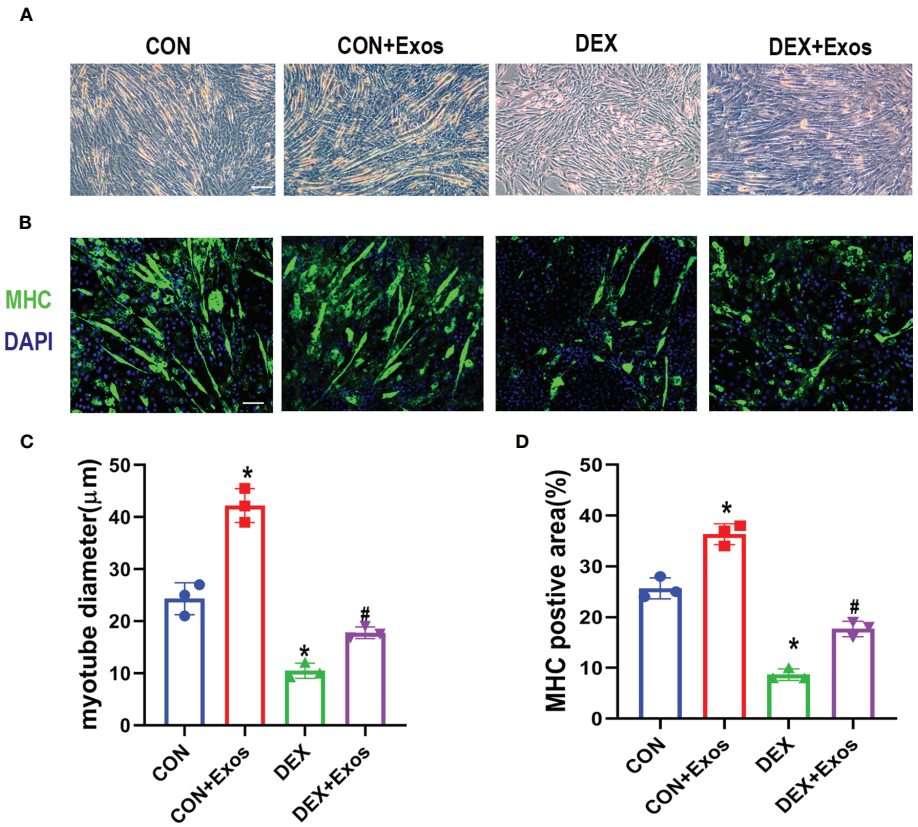

FIGURE 2 | Exosomes derived from BMSCs inhibited DEX-induced myotube atrophy. **(A)** Representative images of C2C12 myotubes under the treatment of DEX and BMSC-Exos. Scale bar = 50μm **(B)** Immunofluorescence staining of Myosin high chain (MHC) in C2C12 cells with the treatment of DEX and BMSC-Exos. Scale bar = 50μm. **(C)** The myotube diameters among different groups were quantified (n = 3). **(D)** The MHC positive area among different groups were quantified (n = 3). *$p < 0.05$ compared to the control group, #$p < 0.05$ compared to the DEX group.

and MuRF1. Meanwhile, DEX administration induced down-regulation of myogenin (MyoG) (**Figures 3A–C**). However, the treatment of BMSC-Exos significantly reduced the up-regulation of mRNA and protein expression of atrogin-1 and MuRF1 induced by DEX. The decreased expression of DEX-evoked MyoG was also inhibited by BMSC-Exos (**Figures 3A–C, F–I**).

Western blotting analysis was performed after C2C12 myotubes were treated with BMSC-Exos to further verify the potential mechanisms. DEX treatment significantly increased the level of FoxO1 in nuclear compared to the control group. However, the increased expression of FoxO1 in nucleus was reversed by BMSC-Exos (**Figures 3D, E**). The above results indicated that BMSC-Exos could inhibit muscle atrophy induced by DEX *via* the regulation of FoxO1.

MiR-486-5P Inhibitor Abolished the Effect of Exosomes Derived From BMSCs on Muscle Atrophy *In Vitro*

In order to explore the mechanism that involved in the regulation of the BMSC-Exos on muscle atrophy, we then detected the expression of miR-486-5p. BMSC-Exos intervention led to an increased level of miR-486-5p in C2C12 myotubes (**Figure 4A**). To further investigate the role of miR-486-5p in BMSC-Exos-derived inhibition of muscle atrophy, inhibitor and mimics of miR-486-5p was transfected into BMSCs, respectively. miR-486-5p inhibitor Exos significantly inhibited the level of miR-486-5p in C2C12 myotubes compared to the inhibitor control Exos, while miR-486-5p mimics Exos significantly increased the level of miR-486-5p in C2C12 myotubes (**Figure 4B**). Furthermore, miR-486-5p inhibitor attenuated the BMSC-Exos induced decreases of FoxO1 expression and the subsequent changes of the expression of muscle atrophy markers (including Atrogin-1 and MuRF-1) as well as MyoG (**Figures 4C–F**). miR-486-5p mimics further up-regulated the protective effect of BMSC-Exos in muscle atrophy(**Figures 4C–F**). These results verified the protective role of BMSC-Exos on Dex-induced muscle atrophy was at least partly mediated by miR-486-5p.

BMSC-Exos Increased Muscle Strength and Up-Regulated Muscle Weight *In Vivo*

To further verify the effect of BMSC-Exos on DEX-evoked muscle atrophy, the mass as well as the function of muscle was measured. DEX treatment significantly decreased the animals' body weight (**Figure 5A**), which could be reversed by inhibitor

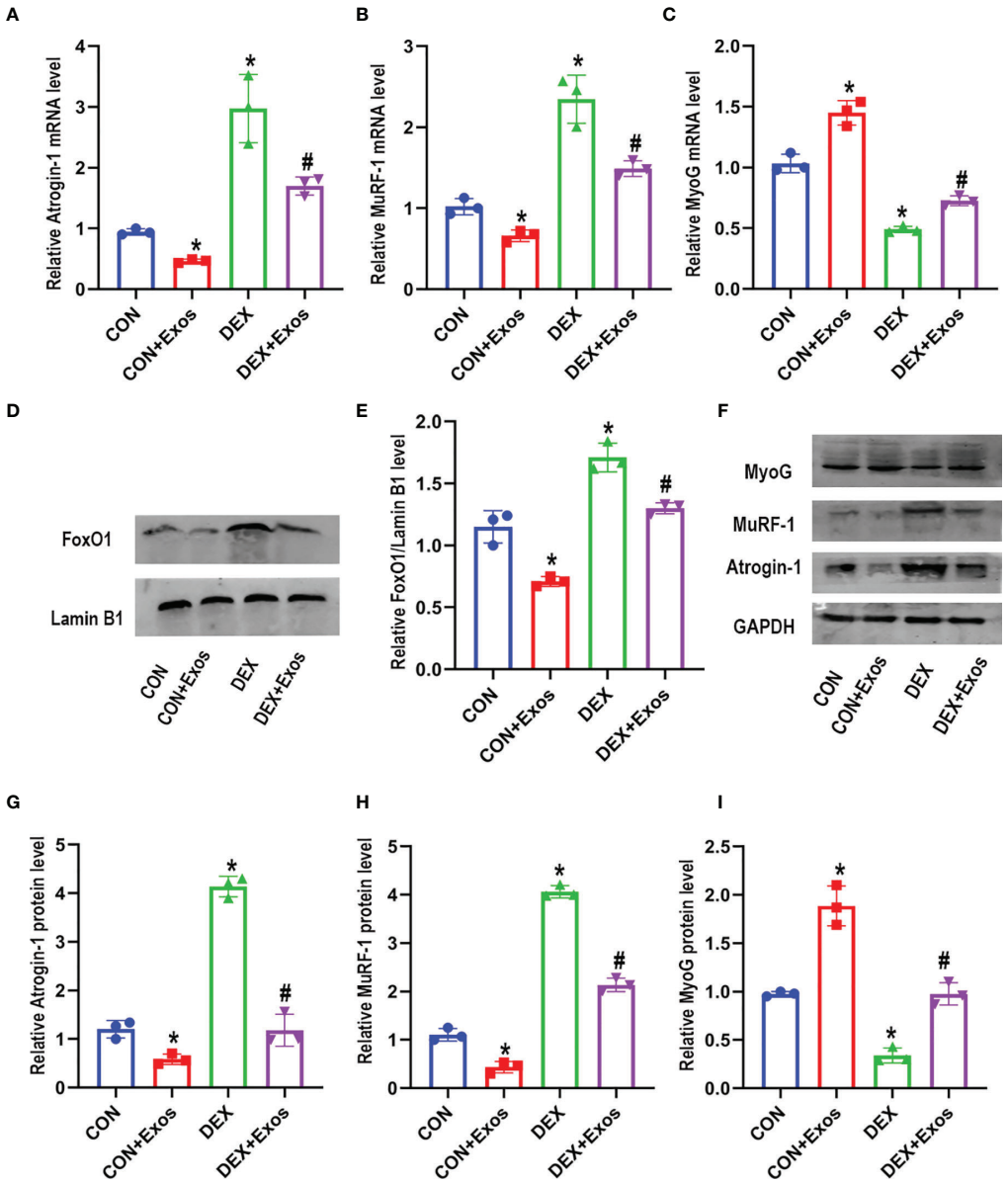

FIGURE 3 | Exosomes derived from BMSCs regulated mRNA and protein expression involved in muscle atrophy and myogenesis. **(A)** Measurements of Atrogin-1 mRNA expression in different groups (n = 3). **(B)** Measurements of MuRF-1 mRNA expression in different groups (n = 3). **(C)** Measurements of MyoG mRNA expression in different groups (n = 3). **(D, E)** The protein level of FoxO1 in nuclear was measured by Western blot analysis (n = 3). Lamin B1 was used as the internal reference. **(F–I)** Measurements of Atrogin-1, MuRF1 and MyoG protein level in in different groups (n = 3). *$p < 0.05$ compared to the control group. $^{\#}p < 0.05$ compared to the DEX group.

NC BMSC-Exos treatment. Meanwhile, the analysis of muscle weights indicated that BMSC-Exos significantly suppressed DEX-induced muscle atrophy (**Figure 5B**). The decreased ratio of lean body mass to total mass induced by DEX was partly reduced by the supplementation of inhibitor NC BMSC-Exos (**Figure 5C**). However, inhibiting miR486-5P in BMSC-Exos attenuated the protective role against DEX-induced muscle atrophy.

Muscle strength of the mice was measured by running test and grip strength (**Figures 5D–F**). DEX treatment decreased the activity time and muscle strength of mice, which was reversed by BMSC-Exos intervein. However, supplementation of miR-486-5p inhibitor-treated exosomes could not protect the DEX-induced muscle atrophy, and also could not increase the activity of DEX-treated mice.

BMSC-Exos Prevents DEX-Evoked Muscle Atrophy *via* miR-486-5p *In Vivo*

The histomorphological analysis indicated that DEX treatment decreased muscle fiber cross sectional area (CSA). BMSC-Exos treatment effectively increased CSA of muscle fiber. Meanwhile,

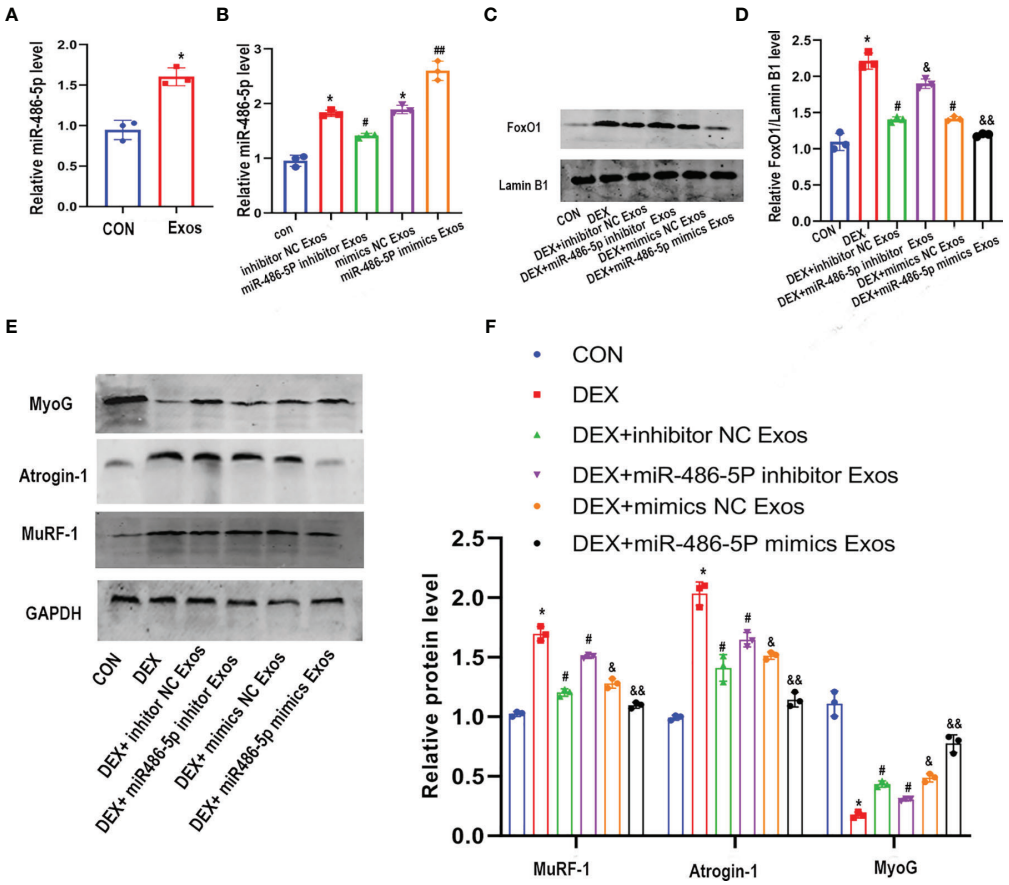

FIGURE 4 | The inhibitor of miR-486-5P partially reversed the protective role of BMSC-Exos on DEX -induced myotube atrophy. **(A)** The level of miR-486-5p in C2C12 myotube after treated with con or con+BMSC-Exos were measured by qRT-PCR analysis (n=3). *p < 0.05 compared to the control group. **(B)** The expression of miR-486-5P in C2C12 myotubes after treated with different exos was measured by qRT-PCR analysis (n = 3). *p < 0.05 compared to the con group, $^{\#}p$< 0.05 compared to the inhibitor NC Exos group, $^{\#\#}p$< 0.05 compared to the mimics NC Exos group. **(C, D)** FoxO1 protein level in nuclear of C2C12 cells was measured and quantified (n=3). *p<0.05 compared to the con group, $^{\#}$p < 0.05 compared to the DEX group, $^{\&}$p < 0.05 compared to the DEX + inhibitor NC group, $^{\&\&}$p < 0.05 compared to the DEX+mimics NC group. **(E, F)** Measurements of Atrogin-1, MuRF1, and MyoG protein in DEX and Exos co-treated C2C12 myotube (n = 3). *p < 0.05 compared to the con group, $^{\#}$p < 0.05 compared to the DEX group, $^{\&}$p < 0.05 compared to the DEX + inhibitor NC group, $^{\&\&}$p < 0.05 compared to the DEX+mimics NC group.

supplementation of miR-486-5p inhibitor-treated exosomes decreased muscle fiber CSA compared with inhibitor control exosomes treatment (**Figures 6A**–**D**). Simultaneously, western blot analysis results revealed that inhibitor NC-treated BMSC-Exos other than miR-486-5p inhibitor-treated BMSC-Exos inhibited the DEX-evoked up-regulation of the nuclear FoxO1 (**Figures 6E, F**). Meanwhile, the regulation of BMSC-Exos in Atrogin-1,MuRF-1and MyoG protein was also eliminated by miR-486-5p inhibitor (**Figures 6G–J**).

DISCUSSION

Skeletal muscle which accounts for more than 40% weight of the body, undertakes important tasks of physical strength, energy consumption and metabolism during life (13). However, muscle atrophy can be caused by drugs, diseases, injury as well as aging. Muscle loss results in decreased activity ability, increased fall risk and ultimately affect the quality of life. The reduction of muscle mass and function caused by muscle atrophy will increase morbidity and mortality. Moreover, as an important endocrine organ, muscle dysfunction can cause liver and fat metabolism disorder. Therefore, it is very important to prevent or delay the occurrence of muscle atrophy. However, there is no effective treatment for muscle atrophy (14). In the present research, exosomes secreted by BMSCs effectively ameliorates DEX-evoked muscle atrophy both *in vivo* and *vitro*. BMSC-Exos significantly downregulated the nuclear translocation of FoxO1, which plays a vital part in the regulation of skeletal muscle mass and myogenic differentiation. Furthermore, the protective effect of BMSC-Exos is due to the transfer of miR-486-5p from BMSC-Exos to muscle cells.

Dexamethasone, a common glucocorticoid drug, was used to treat various of inflammatory diseases (15). The effect of glucocorticoids in skeletal muscle is a double-edged sword

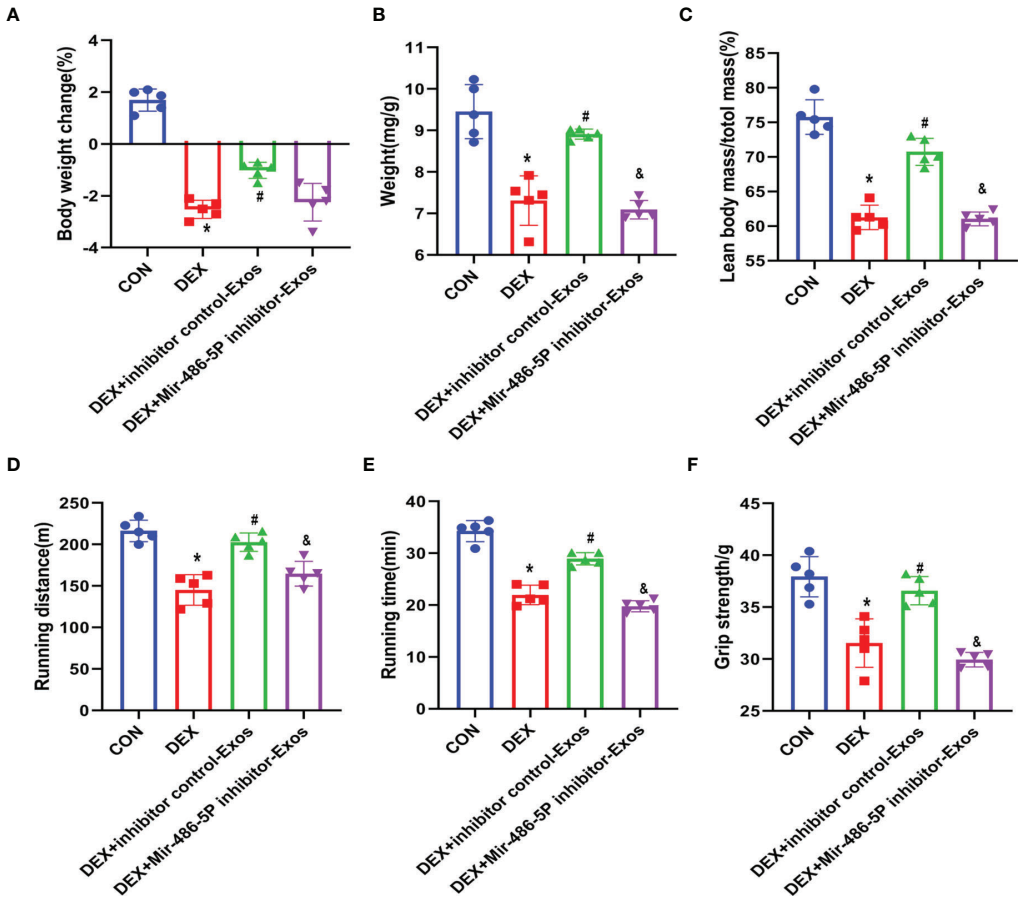

FIGURE 5 | BMSC-Exos inhibited DEX-evoked decline in activity tolerance. **(A)** Body weight was measured after 14 days of treatment (n = 5). **(B)** Muscle mass comparison of different groups (n = 5). **(C)** The ratio of lean body mass to total mass (n = 5). **(D, E)** Running distance and active time in different groups (n = 5). **(F)** Grip strength was measured in different groups (n = 5). *$p < 0.05$ compared to the control group. $^{\#}p < 0.05$ compared to the DEX group. $^{\&}p < 0.05$ compared to the inhibitor NC group.

(16). On the one side, glucocorticoids maintain the metabolic balance by coordinating the metabolism of glucose, lipid and protein. On the other hand, long-term as well as high doses usage of glucocorticoids might led to many passive effects, including muscular atrophy. The incidence rate of non-inflammatory myopathy is over 50% in patients who use steroid drugs for more than 4 weeks (17). Due to the multifactorial changes that contribute to the etiology of muscular atrophy, the underline pathogenesis of muscle atrophy is not completely understood. Current studies have found that DEX induced up-regulation of ROS, activation of the ubiquitin-proteasome and lysosomal pathways *via* the glucocorticoid receptor contribute to muscular atrophy (18, 19). In the present study, the administration of DEX led to a decrease of muscle mass, which verified the Dex-induced muscle atrophy model.

Stem cells with multi-differentiation potential are considered as an effective treatment for many kinds of diseases. Although MSCs transplantation increased the regeneration of skeletal muscle, the cells did not differentiate into skeletal myofibers indicating a paracrine role of MSCs in muscle regeneration (5). Accumulating evidence confirm that exosomes exert an essential role for cell-to-cell and organ-to-organ communication *via* endocrine signaling. It has been reported that stem cell-derived exosomes inhibited doxorubicin-induced muscle toxicity through decreasing pro-inflammatory cytokines. In addition, stem cell-derived exosomes showed a significant reduction in muscular atrophy and fibrosis in doxorubicin treated mice (20). MSC-derived exosomes have been reported to increase the expression of MyoG and MyoD1 of C2C12 cells, indicating that MSC-derived exosomes had the effect to promote myogenesis (12). In the present research, the diameter of C2C12 myotubes decreased after DEX treatment, which was partly rescued by BMSC-Exos administration. Furthermore, BMSC-Exos inhibited DEX-induced skeletal muscle atrophy in mice. BMSCs-Exos administration significantly ameliorated the up-regulation of atrogin-1 and MuRF1 gene level and the down-regulation of MyoG and MyoD gene level. Atrogin-1 and MuRF1 are atrophy-related genes. The level of atrogin-1 and MuRF1 were up-regulated at the early stage of muscle wasting and keeping high level throughout the period of muscle atrophy.

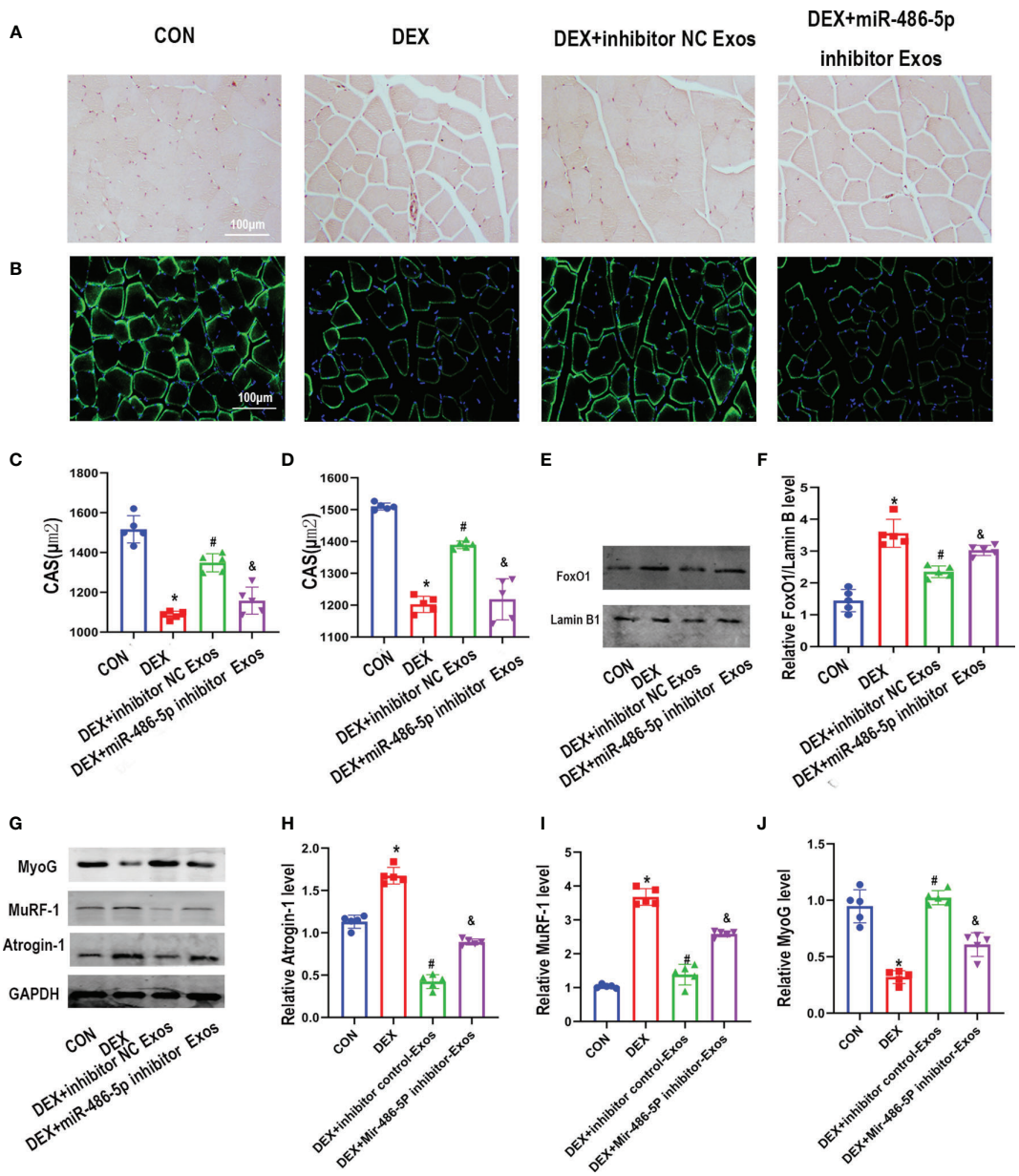

FIGURE 6 | BMSC-Exos inhibited DEX-evoked muscle loss *in vivo*. **(A)** Cross-sectional area (CSA) of muscle were captured by H&E staining and quantified **(C)** (n = 5). scar bar = 100μm. **(B)** CSA of muscle were captured by immunostaining with laminin antibody and quantified **(D)** (n = 5). scar bar = 100μm. **(E, F)** The protein level of FoxO1 in nuclear was measured by Western blot analysis (n = 5). **(G–J)** The protein level of Atrogin-1,MuRF-1and MyoG in muscle was measured by Western blot analysis (n = 5). *p < 0.05 compared to the control group. #p < 0.05 compared to the DEX group. &p < 0.05 compared to the inhibitor NC group.

Atrogin-1 and MuRF1 knock-out showed a protective role of denervation-induced muscle atrophy in mice (21, 22). Atrogin-1 and MuRF1 are regulated by Forkhead box O 1(FoxO1) during muscle atrophy (23, 24). FoxO1 is a member of Forkhead box O (FoxO) proteins, which are transcriptional factors that play critical roles in the regulation of skeletal muscle mass and myogenic differentiation (25–27). In our study, the level of Foxo-1 was down regulated by BMSC-Exos indicating the protective effect of BMSC-Exos was mediated by FoxO1.

Recently, miRNAs in exosomes have been considered as a major transport mechanism during the process of intercellular communication (28). MicroRNAs are short, non-coding RNAs that are involved in cellular processes due to their ability to inhibit the function of target mRNAs at the post-transcriptional level instead of translating into proteins. In recent decades, miRNAs have been confirmed to play vital roles in the development of sarcopenia. Clinical evidence showed that the expression of 13 miRNAs was changed in the muscles or blood of

sarcopenia patients (1). Meanwhile, 10 miRNAs were changed in muscles of rodent models of sarcopenia (1). These miRNAs were associated with the expression levels of many signaling molecules, including FoxO family, sirtuin 1 and insulin-like growth factor-1 (29). MiR-486 is a muscle-enriched miRNA and over-expression of miR-486 improves the physiological function of the muscle and its strength (30). A previous study found that miR-486-5p was significantly decreased in both aged senescence accelerated mouse-prone 8 (SAMP8) mice and late passage C2C12 cells (31). Previous studies proved that miR-486-5p is highly expressed in exosomes derived from BMSCs (32). Moreover, overexpression of miR-486 inhibit PTEN, accounting for increased AKT and diminished FOXO1, inhibited proteolysis and resulted in muscle growth (33). In the present study, BMSC-Exos treatment increased the expression of miR-486-5p in C2C12 cells. In order to further verify the role of BMSC-Exos-associated miR-486-5P in Dex-induced muscle atrophy, miR-486-5p inhibitor was further used to reduce the level of miR-486-5p in BMSC-Exos. The results showed that the expression of FoxO1 was higher in the miR-486 inhibited-BMSC-Exos treated C2C12 cells when compared with inhibitor control Exos group. At the same time, the results showed that the protective role of BMSC-Exos against Dex-induced muscle atrophy was partially eliminated by miR-486-5p inhibitor. In animal experiments, the muscle cross-sectional area of miR-486 inhibited-BMSC-Exos treated was less than that of inhibitor control group. Meanwhile, BMSC-Exos increases myotube diameter compared with control group. This may relate to the increased differentiation and fusion of myoblasts after the treatment of BMSC-Exos. FOXO1 also plays an important role in myogenesis through regulating MyoG and MyoD expression. Taken together, the results of our study indicating that exosomes isolated from the BMSCs could attenuates dexamethasone-induced muscle atrophy through miR-486-5p/FoxO1.

In summary, we found that BMSC-Exos effectively inhibited DEX-induced myotube atrophy and muscle wasting both *in vitro* and *in vivo*. Further exploration of underline mechanism proved that the BMSC-Exos delivered miR-486-5p to muscle cells, which reduced the expression of FoxO1 in nucleus, and reduced the expression of atrophy related genes. Our exploration on the functional mechanism of BMSC-Exos is of much significance to developing a new biotherapy of muscle atrophy based on exosomes. This research preliminarily explored a new approach to ameliorate muscle atrophy which need further exploration.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the ethics committee of the Third Hospital of Hebei Medical University.

AUTHOR CONTRIBUTIONS

PX and SJL designed the study. ZL, SLL, and CL did the experiments and collected the data. TL, YL, NW, and XB carried out the data analysis. ZL drafted the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Clinical Translational Potentials of Stem Cell-Derived Extracellular Vesicles in Type 1 Diabetes

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Type 1 diabetes (T1D) is an organ-specific disease characterized by the deficiency of insulin caused by the autoimmune destruction of pancreatic islet β cells. Stem cell-based therapies play essential roles in immunomodulation and tissue regeneration, both of which hold great promise for treating many autoimmune dysfunctions. However, their clinical translational potential has been limited by ethical issues and cell transplant rejections. Exosomes are small extracellular vesicles (EVs) released by almost all types of cells, performing a variety of cell functions through the delivery of their molecular contents such as proteins, DNAs, and RNAs. Increasing evidence suggests that stem cell-derived EVs exhibit similar functions as their parent cells, which may represent novel therapeutic agents for the treatment of autoimmune diseases including T1D. In this review, we summarize the current research progresses of stem cell-derived EVs for the treatment of T1D.

Keywords: extracellular vesicle, stem cell, type 1 diabetes, exosome, autoimmunity, immunomodulation, β-cell regeneration

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INTRODUCTION

Type 1 diabetes (T1D) is an autoimmune disorder characterized by impaired blood sugar control and insulin deficiency due to an autoimmune destruction of insulin-producing cells in the pancreas (1). Long-term hyperglycemia increases the risks of developing a number of diabetes-associated complications such as cardiovascular disease, kidney diseases, stroke, and blindness. These complications lead to a significant reduction in the quality of life for those affected (2). While the exact pathogenesis of T1D remains unknown, it is associated with a combination of environmental factors and genetic predisposition (3). The administration of exogenous insulin only alleviates symptoms and cannot fully mimic the physiological actions of endogenous insulin released from healthy pancreata. Pancreatic islet transplantation has become a potential treatment for T1D; however, drawbacks impeding its widespread application include high costs, a shortage of islet donations, and lifelong utilizations of immune-suppression drugs post-transplantation (4, 5). Recently, functional insulin-producing cells have been generated from embryonic stem cells (ESC) and induced pluripotent stem cells (iPSCs) (6–8). This has led to clinical trials for the treatment of T1D subjects including ViaCyte studies with VC-01 and VC-02 products (NCT04678557 and NCT03163511, respectively) and a Vertex study with VX-880 (NCT04786262).

Clinical applications of stem cell-derived insulin-producing cells may have ethical and safety concerns including potential tumor formations and immunological reactions (9). Therefore, understanding how to correct autoimmunity and overcome the deficiency of islet β cells are two critical concerns for the treatment of T1D.

Extracellular vesicles (EVs) are groups of small membranous particles that are released by various cells and play an essential role in the transfer of information between adjacent and distant cells (10), as well as the mediation of numerous physiological and pathophysiological processes (11). Since EVs have been found in all mammal biofluids, EVs containing biomolecules (RNAs and proteins) have been widely applied as biomarkers for diagnosis of diseases (12). Notably, increasing evidence has demonstrated the therapeutic potentials of EVs in various diseases including cancer, autoimmune diseases, and infection diseases (13, 14). To date, increasing evidence has demonstrated that stem cell-derived EVs with similar functions as their parent cells not only contribute to the promotion of tissue regenerations (15) but also the modulation of various functions of immune cells (16) and amelioration of autoimmunity in islets (17, 18). This review will focus on current advancements of stem cell-derived EVs for the treatment of T1D with a special focus on their immune modulations and therapeutic potentials to overcome the deficit of islet β cells.

EXTRACELLULAR VESICLES

EV Biogenesis and Isolation

EVs are divided into three categories according to their subcellular origin and secretion mechanisms: exosomes (30–150 nm), microvesicles (MVs, 100–1,000 nm), and apoptotic bodies (1,000–5,000 nm) (19). MVs, also termed "microparticles", are generated through direct budding at the plasma membrane. Apoptotic bodies are relatively large particles, with sizes ranging from 500 to 2,000 nm in diameter and derived from the late stage of apoptotic cells (19, 20). Exosomes, the smallest vesicles, are derived from endosomal budding and released into the lumen through exocytosis (21, 22). To explore the physiological and therapeutic functions of exosomes, the purification and quantification of exosomes are necessary to meet the requests of basic science and clinical practice. Several methods have been utilized to facilitate the isolation of EVs including precipitations, immune-affinity capture, ultracentrifugation (UC), sucrose density gradient ultracentrifugation, and size exclusion chromatography (SEC) (23). Each method is based on one particular feature of EVs, such as density, size, and surface-specific proteins. These methods have certain limitations in the purity and low yield of exosomes (24). Among these techniques, ultracentrifugation is the "gold" standard and is widely accepted for EVs experimental research (25). Recently, the microfluidics-based method has advanced exosome isolation with the high purity and high yield of exosomes (26). In the field of EVs, most studies have focus on the exosomes. Due to the overlapping range of size and density, as well as lack of specific protein markers for these three subtype EVs, the purification of separated exosomes, MVs, and apoptotic bodies is technically complicated. In this review, we use the term "EVs" on behalf of exosomes and MVs, and apoptotic bodies.

Composition of EVs

The membrane of EVs consisting of lipid bilayer is similar to that of cell plasma membrane and is in contrast with the single-layered high-density lipoprotein (HDL) and low-density lipoprotein (LDL) found in body fluids (27). Molecular characteristics revealed that there are a variety of biomolecules such as RNAs, DNA, proteins, and lipids inside EVs. Exosomes from different sources contain certain source-specific molecules, as well as common molecules found across all types of exosomes. Exosomes manufactured from the endocytic pathway inherit endosomal components such as Alix and Tsg101 molecules. Other molecules, including tetraspanin (CD9, CD63, and CD81), membrane molecules (integrins), and intercellular adhesion molecule 1 (ICAM-1), and cytoskeletal components (tubulin, actin, and annexins) are universally presented in almost all types of exosomes derived from different sources of cellular (28, 29). Microvesicle cargo was dependent on the cellular source since the formation of MVs was directly generated from outward budding of cell plasma membrane, along with cytosolic and plasma membrane-associated proteins (30). Several proteins commonly identified in MVs include tetraspanins, cytoskeletal proteins, heat shock proteins, and integrins (31). However, there were no EV-specific markers to distinguish MVs from exosomes. The compositions of apoptotic bodies were different among exosomes and MVs, in that they contain the degraded protein, DNA fragments, or even intact organelles (32). Therefore, recent studies showed that native EVs carry biological cargo from different cells acting as novel mediators. They may contribute to intercellular communications and modulate the recipient cells' function and demonstrate how EVs serve as biomarkers and therapeutic agents for both diagnosis and treatment among various diseases (33, 34).

THERAPEUTIC POTENTIALS OF STEM CELL-DERIVED EXOSOMES FOR THE CORRECTION OF MULTIPLE IMMUNE DYSFUNCTIONS IN T1D

Stem cell-derived EVs display great potential in immune modulation, which may be translated into clinical treatment of T1D. The released EVs enter into circulation and target different cells *via* direct fusion with plasma membrane, endocytosis by phagocytosis, or receptor–ligand interaction (35). Immediately, after EV's molecular content (miRNAs and proteins) is released into these targeted cells, contributing to the immunomodulation through different signaling pathways (36, 37). In the following section, we review current advancements for the modulation of stem cell-derived EVs on different immune cell compartments as alternative approach to correct the immune dysfunctions in T1D (**Figure 1**).

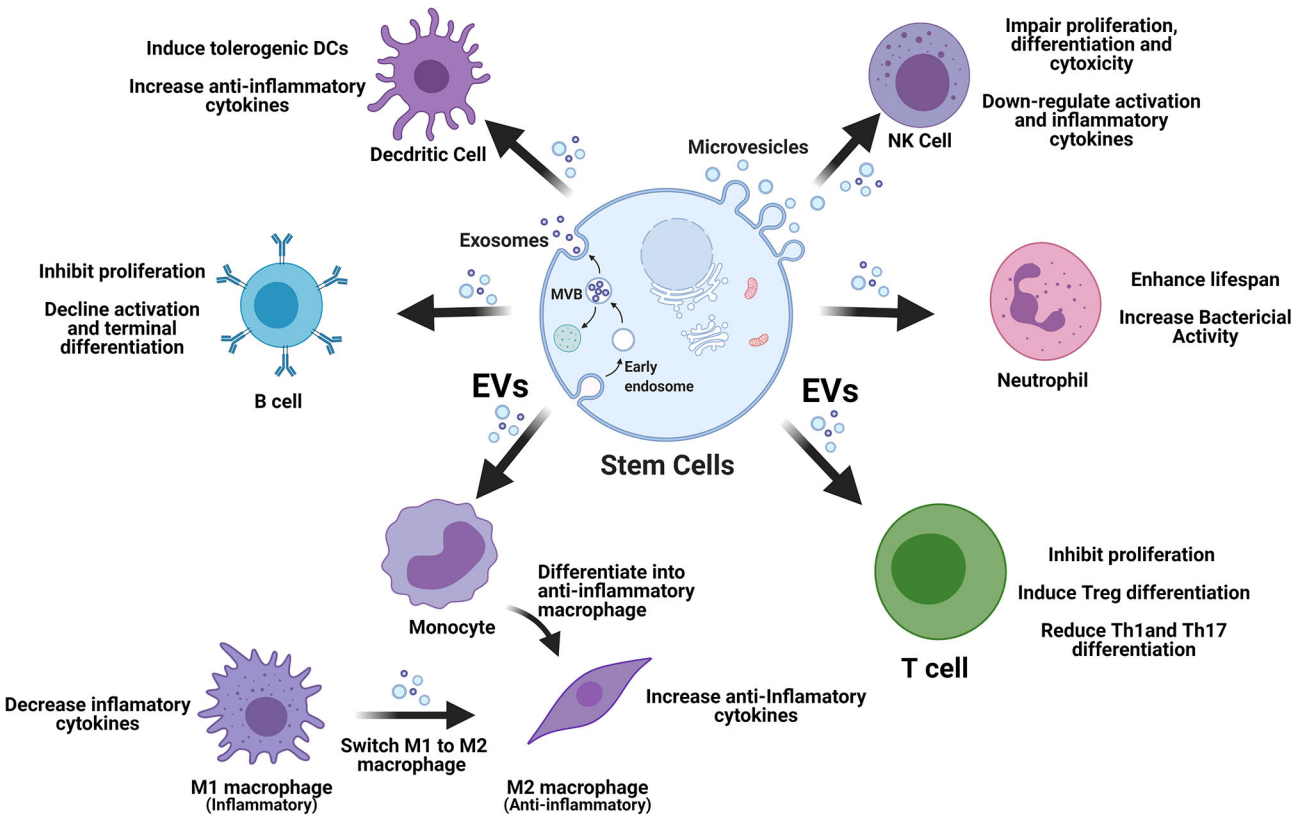

FIGURE 1 | Stem cell-derived EVs display multiple immune regulations on different types of immune cells. EVs, extracellular vesicles; M1, type 1 macrophages; M2, type 2 macrophages; NK, natural killer cells; Tregs, regulatory T cells.

Immune Modulation of Stem Cell-Derived EVs on T Cells

T cells as pathogenic effector cells in type 1 diabetes are well established. Both CD4+ and CD8+ cells play distinct and highly pathogenic roles driving diabetogenesis. CD8+ T cells are the predominant and inflammatory T-cell population contributing to the destruction of pancreatic islet β cells. Wiedeman et al. found that the rapid loss of C-peptides in T1D subjects is associated with prevalent islet-specific CD8+ memory T cells (38). CD8+ T cells predominantly infiltrate the islets, yet their activations are primed by CD4+ T cells (39). For example, diabetogenic CD8+ T-cell functions are maintained by CD4+ T cells in diabetic pancreata (40). CD4+ T cells can give rise to different functional subsets in response to different signals, offering to "help" effector immune cells in their immune response (41). Increasing evidence has demonstrated that multiple CD4+ T cells are involved in the development of insulitis, including T-helpers type 1, 2, and 17 (Th1, Th2, and Th17), regulatory T cells (Treg), and follicular B helper T cells (Tfh) (42–44). Studies have revealed an altered balance between Th1/Th17 and Th2 immune responses leading to T1D (45, 46). Tregs are critical regulators of peripheral tolerance, with defects in Treg phenotypes and suppressive capacities being reported in T1D patients (47–49). Tfh cells regulate germinal center (GC) formation and humoral response (50). Recently, studies have found that long-lived plasma cells secrete T1D-associated autoantibodies generated from GCs with the help of Tfh cells (51).

Stem cells with strong immune modulations have been applied to correct the dysfunction of T cells in T1D diabetes. Researchers found that both CD4+ and CD8+ regulatory T cells increase after coculturing with MSC. Fiorina et al. claim that allogeneic MSC administration can shift the Th1/Th2 balance among T1D mice models (52). Furthermore, several studies have reported that MSC could induce Treg differentiation and restore the balance between Th1/Th17s and Tregs through *ex vivo* and animal tests (53, 54). Moreover, cord blood-derived stem cells (CB-SC), with the effective immune modulation function, have been applied in the Stem Cell Educator® therapy for the treatment of T1D and autoimmune diseases. The attached CB-SC coculture with patient's apheresis mononuclear cells (MNC) for a short period (8–17 h). Consequently, the CB-S-educated MNC cells are returned back into the patient's blood circulation through infusion (55). Our previous clinical studies have demonstrated that Stem Cell Educator therapy utilizing cord blood-derived stem cells (CB-SC) can increase Th2-related cytokines (IL-4 and IL-12), decrease the level of Th17-associated cytokine (IL-17) (55), and reduce the percentages of CD4+ and CD8+ effector memory T

cells (T_{EM}) in T1D patients (56). Additionally, mechanistic studies have demonstrated the direct downregulation of the anti-CD3/CD28 bead-activated CD4+HLA-DR+ and CD8+HLA-DR+ T cells after the treatment with CB-SC-derived exosomes (57).

Stem cell-derived EVs display similar characterization as their parent cells and play an essential role in modulations of the T-cell proliferation, differentiation, and apoptosis (58, 59). It has been reported that stem cell-derived EVs inhibit the proliferation of T cells through the delivery of their specific protein or miRNAs (60). Stem cell-derived EVs could increase the percentage of Tregs and decrease Th1/Th17 subpopulations (61), highlighting the therapeutic potential of stem cell-derived EVs for the treatment of T1D. Recently, Shigemoto-Kuroda et al. found that human MSC-derived EVs can effectively prevent the onset of T1D in animal models. Using the mixed lymphocyte reaction (MLR) assays, these MSC-EVs are able to suppress the proliferation of Th1 and Th17 cells, which play a key role in the prevention of T1D onset (62). Similarly, Favaro et al. found that MSC-derived EVs can decrease the number of Th17 cells and cytokine IL-17, as well as increase the percentage of Tregs in peripheral blood mononuclear cells (PBMC) from recent-onset T1D patients (63). Additionally, this group found that MSC-EVs could decrease the level of IFN-γ in GAD65-stimulated PBMC and increase the level of anti-inflammation molecules, including the transforming growth factor-β (TGF-β), IL-10, and prostaglandin E2 (PGE-2) (64). Stem cell-derived EVs therefore have significant importance for T1D therapy. Nojedehi et al. applied EVs from adipose tissue-derived MSC (50 μg/ml in PBS, i.p., twice/week/mouse) for the treatment of chemical streptozotocin (STZ)-induced diabetic mice. Their study found that MSC-EVs are helpful in maintaining blood glucose and body weight. What is more, a mechanistic study revealed that there were significant upregulations in the levels of IL-4, IL-10, and TGF-β, as well as markedly decreased levels of IFN-γ and IL-17 among T1D mice after the treatment with MSC-derived EVs (65). Collectively, these data indicate the therapeutic potentials of stem cell-derived EVs as a novel approach to treating T1D through their immune modulations on T cells.

Immune Modulation of Stem Cell-derived EVs on the Antigen-Presenting Cells

Macrophages, one of the major antigen-presenting cells, have vital roles in the innate immune responses, glucose and lipid metabolism, and pathogenesis of diabetes (66, 67). Macrophages can be simplified into two subsets—pro-inflammatory (M1) and anti-inflammatory (M2) profiles (68). M1 macrophages are induced by Th1-related cytokines (IFN-γ and TNF-α) or microbial products [Toll-like receptors (TLRs), ligands, and lipopolysaccharide (LPS)] to kill pathogens and present their antigens to T cells for adaptive responses. M1 macrophages exhibit high levels of phagocytic activity and enhanced antigen-presenting capability through the expression of M1-associated markers including CD80, CD86, and nitric oxide synthase (iNOS). Additionally, M1 macrophages produce high levels of proinflammatory cytokines such as interleukin-12 (IL-12), IL-1β, and IL-23. In contrast, M2 macrophages are induced by Th2-response cytokines (IL-4 and IL-13). M2 macrophages are characterized by an anti-inflammatory profile that plays a crucial role in permitting the resolution of tissue repair (69, 70) and secrete a variety of anti-inflammatory mediators (e.g., IL-10 and TGF-β1) and reduce the level of proinflammatory cytokines secreted by M1 macrophages (71). In addition, M2-associated markers include CD163, mannose receptor (CD206), STAT6, and arginase 1 (72, 73). M2 macrophages with high expressions of arginase result in the production of polyamines and collagen, both of which favor tissue remodeling (74). Moreover, M2 macrophages with increased activity of arginase-1 can lower the level of NO secretion by competing for L-arginine, the substrate of iNOS (75). Both macrophages are essential players in the pathogenesis of T1D. While M1 macrophages trigger the immune response and initiate insulitis, M2 macrophages act as negative regulators by decreasing inflammation and insulitis in the T1D pancreas (70).

Macrophages may directly provoke or enhance insulin secretion through the production of factors such as retinoic acid (76). The depletion of islet-resident macrophages limits the islet leukocytic infiltration during early phases of diabetogenesis (77, 78). Both macrophage populations are central players in diabetes. M1 macrophages are responsible for triggering the inflammatory response, initiating insulitis and pancreatic cell death during the onset of T1D. M2 macrophages decrease hyperglycemia, insulitis, and inflammation in the pancreas, thereby negatively regulating T1D development (70). Fernando et al. found that LPS re-stimulation in diabetic bone marrow-derived macrophages (BMDM) resulted in higher secretions of TNF-α compared to non-diabetic BMDM (79). What is more, their study found that long-term high glucose-treated macrophages increased the levels of inflammatory cytokines (e.g., IL-1β and TNF-α) in macrophages (80). Similarly, Ferris et al. reported that an increased inflammatory signature in islet macrophages of non-obese diabetic (NOD) mice was correlated with the elevated expressions of chemokines and oxidative responses (81).

Increasing evidence has demonstrated that stem cell-derived EVs can reduce the inflammation through targeting macrophages (82, 83). Stem cell-derived EVs can both promote M2 macrophages and suppress M1 macrophage polarization by upregulating anti-inflammation cytokines and downregulating inflammation-related cytokines (84, 85). Interestingly, researchers have reported that MSC exosome-treated macrophages can reduce the inflammation and T-cell proliferation (86, 87). Recently, our studies demonstrated that CB-SC-derived exosomes can favorably target monocytes in the presence of PBMC and polarize these monocytes into M2 macrophages (57, 88). These functions may contribute to the clinical therapeutic potentials of Stem Cell Educator therapy to treat T1D (55) and other inflammatory-associated diseases (89).

Dendritic cells (DC) are another major population of antigen-presenting cells. Recent studies suggest that diabetic subjects have impaired functions of DC that may contribute to the pathogenesis of T1D (90, 91). Their findings revealed that adenosine deaminase (ADA) is upregulated in NOD dendritic

cells, which induce their spontaneous activation. Therefore, transferring the ADA-deficient DC to NOD mice can protect them from the development of diabetes (92). MSCs have been shown to inhibit the maturation and function of bone marrow-derived DC (BMDC) and induce the differentiation of DC into tolerogenic dendritic cells (93, 94). Similarly, MSC-derived exosomes suppress the maturation of BMDC with decreased secretion of pro-inflammatory cytokines (IL-12) and increase the production of anti-inflammatory cytokines (TGF-β), contributing to the DC-induced immune responses (95, 96). Mechanistic studies have demonstrated that MSC-derived exosomal miRNA-146a play an essential role in the immunomodulatory function of DC (97). Furthermore, Favaro et al. reported that the treatment of DC with MSC-derived EVs can increase the percentage of Tregs and decrease Th17, thus potentially leading to the inhibition of inflammatory T-cell response to islet antigens (63).

Immune Modulation of Stem Cell-Derived EVs in Other Immune Cells

In addition to T cells and monocytes/macrophages involved in the initiation of T1D, other immune cells contribute to the development of T1D (98, 99). Natural killer (NK) cells may be involved in the pathophysiology of diabetes since they partner with antigen-presenting cells or T cells for killing the targeted islet β cells (100). Literature has demonstrated that NK cells also release cytokines that transmit adaptive immunity. *In vitro* studies have confirmed that NK cells can lyse islet cells (101). Animal studies have demonstrated that the depletion of NK cells can significantly decrease T1D development (102). Stem cells can interact with NK cells for modulating these NK functions (103). For example, MSCs can inhibit IL-2-inuduced NK cell proliferation and downregulate expressions of activating NK receptors (104). Recently, MSC-derived exosomes were shown to reduce the release of interferon gamma (IFN-γ) and tumor necrosis factor alpha (TNF-α) by activated NK cells, alleviating the inflammatory response (105). Moreover, Fan and colleagues reported that human fetal liver MSC-derived exosomes impair NK proliferation, differentiation, and cytotoxicity through exosome-associated TGF-β (106).

B cells have an important role in the adaptive immune response including antibody production, antigen presenting, and multiple cytokine production (107, 108). Although there is much evidence that T cells play a major pathogenic role in T1D, B cells are also required for the development of diabetes, which has been demonstrated by the depletion of B cells using anti-CD20 or anti-CD22 monoclonal antibodies (mAb) (109, 110). Additionally, depleting B cells with anti-CD20 mAb in patients with newly diagnosed T1D can preserve islet β-cell function and delay the requirement for insulin administration among 1-year follow-ups (111, 112). Stem cells have proven immunomodulatory properties for both the activation and proliferation of B cells as well as the induction of regulatory B-cell generation (113, 114). MSC-derived EVs can affect the mRNA expression of B cells and impair their proliferation (115). Adamo et al. reported that MSC-derived exosomes can inhibit the proliferation and activation of B cells by downregulating their PI3K-AKT signaling pathway through the delivery of exosomal miR-155-5p (116).

Neutrophils are the primary innate cells to be recruited to the sites of inflammation, as they provide the front line of defense against the infection. Neutrophil functions have been reported to be closely related to β-cell autoimmunity, as a significant decrease in neutrophil numbers and chemotactic activity can be detected in T1D patients, but not among the healthy controls (117, 118). Additionally, the impaired phagocytosis and bactericidal activity of neutrophils were shown in marked correlation with the elevated blood glucose levels (119). There is more evidence demonstrating that treatment with stem cells can enhance the lifespan and bactericidal activity of neutrophils (120–122). Interestingly, since EVs have similar functioning as their parent cells, stem cell-derived EVs could significantly prolong the survival and function of neutrophils (123, 124).

THERAPEUTIC POTENTIALS OF STEM CELL-DERIVED EVS FOR OVERCOMING THE SHORTAGE OF ISLET BETA CELLS IN T1D

Pancreatic β cells are the only specialized cells in mammals that can secrete insulin. Cytoarchitectural studies in rodents have shown that they are located in the core of the rodent pancreatic islets and are surrounded by α cells, which can secrete glucagon, δ cells, which are few in number and secrete somatostatin, and PP cells, which secrete pancreatic polypeptide (125). Conversely, human islet β cells, α cells, and δ cells were found scattered through the human islets (126). The destruction of pancreatic β cells results in absolute insulin secretion deficiency—the hallmark characteristic of T1D (127). Understanding how to restore the islet β-cell population is one of the most challenging fields in the treatment of T1D. Nevertheless, a number of hurdles must be overcome. Currently, several known approaches have been applied to β-cell regeneration including endogenous regeneration of β cells, *in vitro* β-cell regeneration using stem cells, and the promotion of the remaining β cells that have survived (128, 129). Stem cells have great differentiation potential, being able to differentiate into endogenous β cells or *in vitro* insulin-producing cells. In addition, stem cells have powerful modulation functions for promoting the survival of remaining β cells (130, 131). Recently, more evidence has suggested that stem cell-derived EVs have positive effects for promoting the survival of β cells and generation of insulin-producing cells (132, 133).

Effects of Stem Cell-Derived EVs on Endogenous β-Cell Proliferation and Transdifferentiation

Endogenous regeneration of β cells, which occurs through the stimulation of existing β-cell proliferation or differentiation of other pancreatic progenitor cells or stem cells into functional insulin-secreting cells (designated neogenesis), is a potential

strategy for residual β-cell replication and neogenesis to treat diabetes (134). Pancreatic β-cell replication occurs readily during the fetal and neonatal stages and then declines after these stages. Increasing evidence has demonstrated that several mitogenic signaling pathways mediate the replication of β cells including IRS-PI3K-AKT, GSK3,cMYC, RAS/RAF/ERK, and mTOR (135). Interestingly, insulin signaling can regulate the mitotic FoxM1/PLK1/CENP-A pathway for promoting adaptive β-cell proliferation (136). In addition, multiple soluble factors, including GLP-1, lepin, and IL-6, have been implicated to control the proliferation of β cells. For example, studies have demonstrated that the transforming growth factor (TGF)-beta family and WNT/beta-Catenin signaling serve as the potential keys to controlling β-cell proliferation and differentiation (137–139). Moreover, increasing evidence has demonstrated that stem cell infusions can enhance the cell proliferation during the process of tissue repair (140, 141). Recently, scientists have applied human MSC with overexpressions of telomerase reverse transcriptase (TERT) to enhance the proliferation of autochthonous pancreatic β cells in half-pancreatectomized mice (142). Also, stem cell-derived EVs cause positive effects for the treatment of T1D by promoting existing β-cell proliferation. For instance, Mahdipour et al. found that applying EVs from menstrual blood-derived MSCs (10 μg/rat, i.v., on day 0, 2, and 10 after the injection of streptozotocin, respectively) can restore the β-cell mass and insulin production in diabetic rats. Mechanistic studies have revealed that EVs can induce β-cell regeneration through the activation of the pancreatic and duodenal homeobox 1 (PDX-1) pathways (143). Additionally, the reprogramming of cells into β-like cells has drawn increasing attention among the research community as an alternative means for endogenous β-cell regeneration. According to the development of embryonic pancreatic β cells, transcription factors play an essential role in pancreatic β-cell determination including PDX1, NGN 3, SOX9, NKX6.1, MAFA, and MNX1 (144). For example, Zhou et al. reported that the re-expression of transcription factor NGN 3, combined with PDX1 and MAFA, can efficiently reprogram pancreatic exocrine cells into insulin-producing cells (145). Additionally, their study found that the overexpression of Pref-1-activated MAPK and AKT signaling can help to increase insulin synthesis *via* the differentiation of human pancreatic ductal cells into β-like cells (146). Recently, research has demonstrated that by disrupting α cell-specific TFs such as DNMT1 and ARX, the reprogramming of α cells to β cells can be achieved (147). Until now, there has been a lack of direct evidence demonstrating that stem cells and their derived EVs can modify the process of reprogramming non-pancreatic cell into insulin-expression cells. However, Ribeiro et al. found that human pancreatic islet-derived EVs improve the differentiation of iPSC cluster culture in 3-D collagen hydrogel with increased pancreatic marker expression (148). Recently, Oh and colleagues showed that β-cell-derived EVs can directly trigger the differentiation of bone marrow mononucleated cells into insulin-producing cells (149). These findings suggest that stem cell-derived EVs may serve as possible mediators for the development of insulin-producing cells from non-beta pancreatic cells. Prospectively, investigating the function of stem cell-derived EVs on endogenous regeneration of β cells will provide deep insights into the process of cell reprogramming.

Protective Effects of Stem Cell-Derived EVs on Islet β Cells and Pancreatic Islets

The promotion of β-cell survival and functioning can be achieved *via* apoptosis protection. The apoptotic pathway mainly consists of two pathways—the extrinsic and intrinsic pathways. The extrinsic pathway is ignited by the cell surface death receptor (Fas and tumor necrosis factor receptor) bound with their ligand. The intrinsic pathway, also termed the "mitochondrial-mediated pathway", is triggered by the pre-apoptotic Bcl-2 family, leading to permeabilization of the mitochondrial outer membrane. Both pathways culminate in the activation of the caspase protease family, ultimately resulting in the dismantling of cells (150, 151). Autophagy is a cell survival mechanism that delays the cell death. There are increasing evidence supporting that coculture stem cells with β cells can delay the apoptosis of β cells (152, 153). Mechanistic studies have found that the secretome from stem cells can enhance autophagy and exert the protective effects on β cells (154, 155). Recently, researchers found that EVs among MSC secretome play a powerful role in the anti-apoptosis of β cells. Keshtkar et al. demonstrated that MSC-derived EVs can improve islet survival and function by upregulating insulin and vascular endothelial growth factor (VEGF) expressions (156). Furthermore, mechanistic studies have revealed that MSC-derived EVs preserve β-cell function, depending on their contained miRNA-21 for alleviating ER stress, and downregulate p38 MAPK phosphorylation to reduce hypoxia-induced apoptosis of β cells (157). Furthermore, *in vivo* studies have affirmed these findings, demonstrating that the administration of EVs from MSC can restore insulin secretions by inhibiting STZ-induced β-cell apoptosis in T1D mouse models (158).

Additionally, stem cell-derived EVs have a strong ability to promote angiogenesis during the tissue repair. An *in vitro* study found that human bone marrow stem cells enhance islet vascularization and preserve islet function with significantly increased expressions of insulin (159). Moreover, administration of MSC-derived EVs can preserve the architecture of islets with longer survival time and increased insulin content in STZ-induced diabetic mice. Histologic analysis has demonstrated that treatment with EVs improves the level of CD31 expression in pancreatic islets (which are markers of endothelial cells), indicating the enhanced islet vascularization (160). Interestingly, Cantaluppi et al. utilized EVs from endothelial progenitor cells with the islet transplantation mice model. Their study demonstrated that these EVs carry proangiogenic miR-126 and miR-196 enhanced islet vascularization, leading to sustained β-cell function (161). Nie et al. showed that human mesenchymal stem cell (MSC)-derived exosomes can improve the survival ratio, viability, and function of neonatal porcine islet cell clusters under hypoxic conditions (162), which are key factors causing islet graft dysfunction (163).

Recently, Gesmundo et al. reported that adipocyte-derived EVs regulated the survival and function of human pancreatic β cells and pancreatic islets (164). For the role of EVs in islet transplantation, readers are encouraged to refer to the prior review (165). These findings support the idea that stem cell-derived EVs are suitable candidates to improve the functioning and survival of β cells for the treatment of T1D.

Effects of Stem Cell-Derived EVs on Stem Cell Differentiation Toward β Cells

β-cell regeneration using stem cells means utilizing pluripotent stem cells with differentiation protocol to generate insulin-producing cells *in vitro*—a strategy for β-cell replacement therapy. Human pluripotent stem cells [either human embryonic stem cells (ESC) or induced pluripotent stem cells (iPS)] are attractive sources for β-cell differentiation since they can give rise to every cell type of the human body (166). To date, scientists have employed the multiple differentiation protocols, exposing cells to various growth factors and numerous signaling molecules in a particular sequence for differentiation of the cells into pancreatic endocrine cells (167, 168). Recently, other multipotent stem cells applied for the β-cell regeneration including MSCs and CB-SCs (169, 170). Interestingly, 3-dimensional cultures promote the differentiation of stem cells into insulin-producing cells with increased insulin and c-peptide secretion (171–173). Currently, there is no evidence suggesting that stem cell-derived EVs can definitively affect the differentiation of stem cells into functional islet β cells.

CONCLUSIONS

Currently, most clinical trials on EVs or exosomes are considering their function as valuable biomarkers for diagnosis and prognosis in a range of diseases. Based on their capabilities of immune modulations and anti-inflammation, some of the studies are actively exploring EVs as therapeutic carriers by using MSC-derived EVs in cases of chronic kidney disease (174), lung injury (175), and severe COVID-19 (176, 177). To date, a number of preclinical studies have implied the translational capability of stem cell-derived EVs to treat T1D through their multiple immunomodulations of different immune cells and their potential to improve β-cell regeneration. That being said, only one clinical trial posted in ClinicalTrials.gov in 2014 (NCT02138331) did not report results. In comparison with their parent cells, stem cell-derived EVs may have good safety profiles and can be easily stored and transported as cell-free products without losing their functions. However, parent cells at different *ex vivo* culture conditions (e.g., culture medium with or without serum) may markedly affect their exosomes' biochemical and biophysical features including the quantity and quality of bioactive molecules. Thus, it will be essential to develop a scalable and reproducible Standard Operating Procedure (SOP) for the EV production. Stem cell-derived EVs carry cargos of enriched biomolecules (RNAs, proteins) that need to be further characterized, clarifying their unique and synergistic effects for the treatment of T1D.

Overcoming the autoimmunity and shortage of islet β cells are two major issues for the treatment of T1D patients. Due to the limitations of native EVs such as the diversity, the low yield of EV production, as well as a short half-life and off-target effects of their actions post administration, it will be critical to direct a sufficient amount of EVs towards the specific targeting of autoimmune cells. Additionally, future attention should be placed on promoting the replication of residual β cells in pancreatic islets. To this respect, using the bioengineered EVs may facilitate the clinical translation of EVs for T1D treatment. However, the toxicity, purity, potency, and stability of these bioengineered EVs are mandatory for the FDA approval in clinical trials. These practical challenges must be overcome before stem cell-derived or bioengineered EVs can achieve their full therapeutic potentials for T1D and other autoimmune diseases.

AUTHOR CONTRIBUTIONS

YZ contributed to concepts, article revising, and final approval of article. WH and XS drafted the article. HY, JS, HW, and YZ edited the review article. All authors contributed to the article and approved the submitted version.

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Conflict of Interest: YZ is an inventor for Stem Cell Educator therapy and has a fiduciary role at Throne Biotechnologies.

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