EXOSOMES IN CARDIOVASCULAR DISEASES: MECHANISM, DIAGNOSIS AND THERAPY

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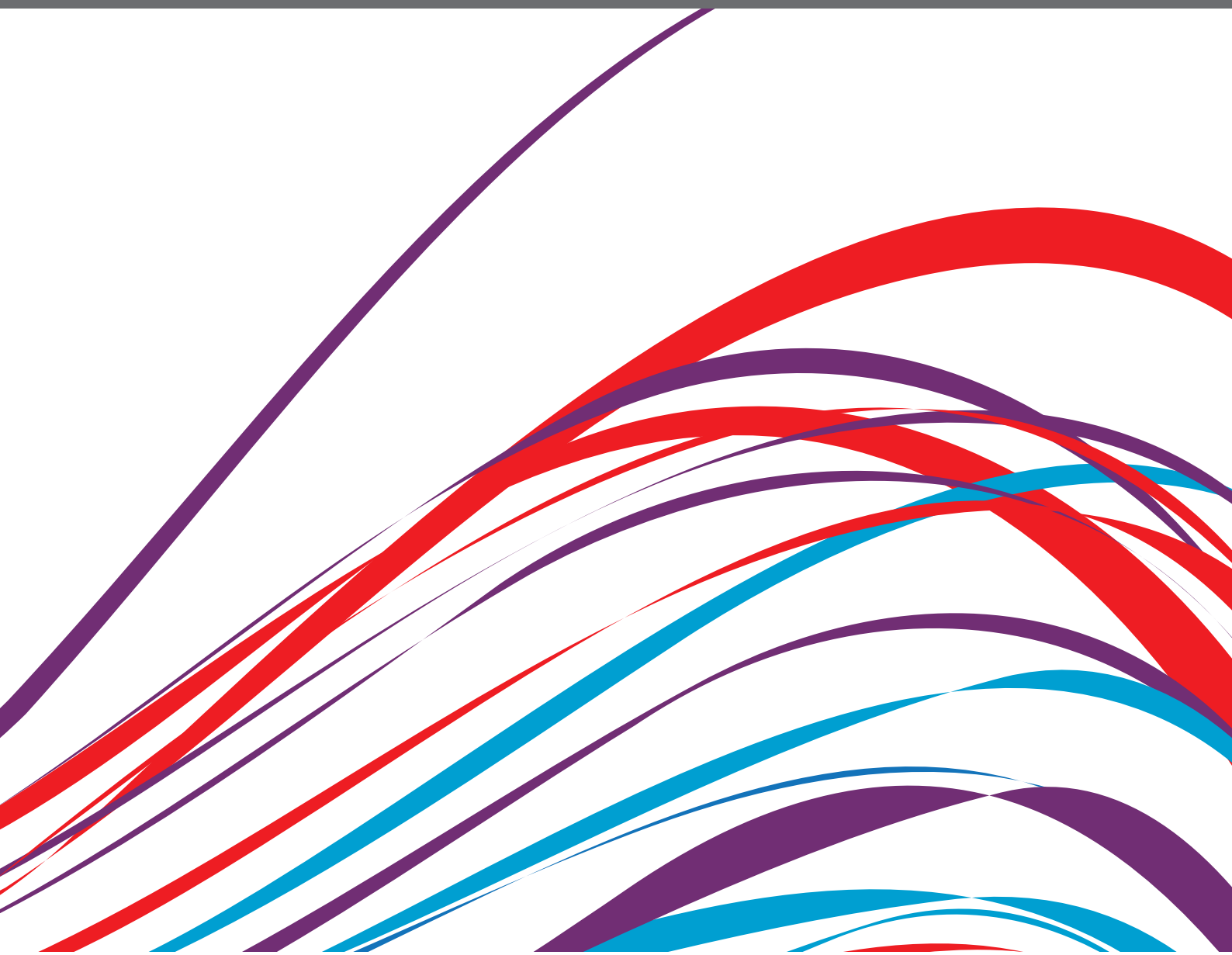

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EXOSOMES IN CARDIOVASCULAR DISEASES: MECHANISM, DIAGNOSIS AND THERAPY

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Editorial: Exosomes in cardiovascular diseases: Mechanism, diagnosis, and therapy

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Editorial on the Research Topic
Exosomes in cardiovascular diseases: Mechanism, diagnosis, and therapy

Among non-communicable diseases, cardiovascular disease (CVD) is the leading cause of mortality worldwide, which is associated with increased morbidity and hospitalization (1). To date, few effective strategies have been developed to cure CVD including heart failure and ischemic-reperfusion injury, etc. Therefore, exploring effective therapeutical strategies is essential to CVD treatment. In recent years, extracellular vesicles (EV) have attracted growing attention in the diagnosis and treatment of CVD (2, 3) due to their specific characteristics, including excellent biocompatibility and low immunogenicity (4). This Research Topic aims to provide more constructive scientific findings and new horizons in CVD diagnosis and treatment, which may help promote future clinical trials of EV in treating CVD.

In this issue, Ma et al. creatively conduct an overview of EV research in CVD *via* a bibliometric analysis. The authors systematically collect the last 20 years of research on Web of Science Core Collection and perform a bibliometric analysis with visual tools (Citespace and Vosviewer). The results demonstrate that increasing attention was significantly paid to the capacity of EV in CVD from 2017 onwards, indicating the increasing popularity of the subject in this field. Furthermore, the authors conclude most of these studies focused on EV as biomarkers for CVD diagnosis, delivery vehicles, and a potential strategy for treating myocardial infarction.

The study of Yao et al. prospectively performs atrial fibrillation (AF) modeling in Canines and investigates the function of EV in AF development. The authors reveal that blocking the release of small EV by GW4869 could alleviate AF by reducing atrial

fibrosis. Mechanistically, EV-enclosed miR-21-5p targets the downstream TIMP3/TGF-β1 pathway and induced fibrosis. Inhibiting the release of EV may be a potential strategy for AF treatment, which may help accelerate new clinical trials of AF treatment. Chen et al. systematically review the role of EV and EV-enclosed non-coding RNAs (NcRNAs) in the diagnosis and treatment of AF in more detail. Notably, EV-enclosed NcRNAs may also play a crucial role in the progression of AF.

The EV-mediated interaction between adipose tissue and blood vessels may play an important role in CVD. In this Research Topic, Liu et al. and Yang et al. explore the role of adipose-derived EV in lipid metabolism, which is closely associated with vascular homeostasis. Epicardial adipose tissue (EAT)-derived EV-enclosed miR-3064-5p is identified as a key molecule in regulating lipogenic differentiation.

Interestingly, EV is proven to be involved in air-pollution-associated cardiac injury. Hu et al. investigate the crosstalk between macrophages and cardiomyocytes. The authors reveal that ambient particulate matter could promote the release of EV and subsequently activate macrophages. EV-enclosed TGF-β derived from macrophages promotes the fibrotic alteration of cardiomyocytes, ultimately leading to cardiac dysfunction. This study sheds light on the underlying mechanism and potential therapeutical strategy for air pollution-associated cardiovascular disorders.

Notably, increasing evidence shows that platelet-derived EV are a crucial component of circulating nanoparticles in blood, indicating that platelet-derived EV play important roles in multiple pathological processes. In this Research Topic, Wei et al. summarize the role of platelet-derived EV in mediating intercellular communication, which contributes to arterial thrombosis. This review provides a new horizon as platelet-derived EV could provide promising biomarkers for the diagnosis of CVD.

Overall, this Research Topic provides a relatively comprehensive understanding of the role and potential application of EV in CVD, including a bibliometric analysis, overviews, and some surprising experimental articles. These studies identify a novel cargo and signaling axis (EV-miR21-TIMP3/TGF-β1, miR-3064-5) in the development of CVD and provide a novel way of using different sources of EV and EV-enclosed cargo in diagnosing CVD. Nevertheless, EV-associated basic, translational, and clinical studies are still on the way. Here we sincerely hope that this Research Topic can provide readers with different viewpoints and new horizons in EV & CVD diagnosis and treatment, inspiring future studies on the therapeutical application of EV. In addition, we also hope the work can help to stimulate novel ideas in associated fields and improve the research progression of CVD.

Author contributions

HW, YH, and JX are the topic editors of this issue and they have contributed to the writing and revising of the article. XZ, MZ, and YL contributed to drafting the Editorial. All authors approved it for publication.

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Conflict of interest

The 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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ORIGINAL RESEARCH
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MiR-3064 in Epicardial Adipose-Derived Exosomes Targets Neuronatin to Regulate Adipogenic Differentiation of Epicardial Adipose Stem Cells

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Backgroud: The metabolism of epicardial adipose tissue (EAT) is closely related to coronary atherosclerotic heart disease (CAHD), but the specific mechanism is not fully understood. In this study, we investigated the effects of EAT microenvironment on adipose metabolism from the viewpoint of EAT-derived exosomes and epicardial adipose stem cells (EASCs).

Methods: EAT samples from CAHD patients and non-CAHD patients were collected to obtain exosomes via tissue culture. MiRNA sequencing was performed to analyze differences in miRNA expression in exosomes between groups. Luciferase reporter assay was then performed to verify the miRNA target gene. EAT was digested by collagenase to obtain EASCs, which were induced to mature adipocytes *in vitro*. Immunochemical staining and western blotting were performed to detect protein expression levels.

Results: The results showed that CAHD patients had higher levels of EASCs in EAT, and no significant difference in the adipogenic differentiation ability of EASCs was observed between CAHD and non-CAHD patients *in vitro*. This indicates that the EAT microenvironment is a key factor affecting the adipogenic differentiation of EASCs. The EAT-derived exosomes from CAHD patients inhibited adipogenic differentiation of EASCs *in vitro*. Sequencing analysis showed that miR-3064-5p was highly expressed in EAT-derived exosomes in CAHD patients, and its inhibitor could improve the adipogenic differentiation of EASCs. Luciferase reporter assay results showed that the target gene of miR-3064-5p is neuronatin (Nnat). Nnat remained silent in EASCs and was less expressed in EAT of CAHD patients.

Conclusion: Abovementioned results suggest that Nnat is the key to regulating the adipogenic differentiation of EASCs, and miR-3064-5p in EAT-derived exosomes can inhibit the expression of Nnat by targeting its mRNA, thereby affecting the adipogenic differentiation of EASCs.

Keywords: coronary atherosclerotic heart disease, epicardial adipose stem cells, exosomes, miR-3064-5p, neuronatin

INTRODUCTION

Atherosclerosis (AS) causes ∼20 million deaths worldwide each year. AS is a slowly progressive disease with complex pathogenesis, and its exact etiology is still not fully understood. It is now generally believed that AS is a chronic inflammatory disease, closely related to hyperlipidemia, hypertension, diabetes, and genetic factors (1). Therefore, elucidating the pathogenesis of AS and finding new targets and treatments are the key issues in the prevention and treatment of AS.

Epicardial adipose tissue (EAT) is the adipose tissue located between the myocardium and the pericardium, which surrounds and directly contacts the cardiac blood vessels. Owing to its elasticity and compressibility, EAT can protect coronary arteries from excessive distortion caused by arterial pulsation and myocardial contraction (2). EAT, as a local storage site of excess free fatty acids, maintains myocardial energy supply and prevents the toxic effects of high circulating free fatty acids on the myocardium and coronary arteries (3). There is no connective tissue or aponeurotic tissue between EAT and myocardium, indicating a close and strong interaction between them. Unlike pericardial fat, EAT angiogenesis depends on the branches of the coronary arteries, further indicating a close relationship between EAT and myocardial tissue (2). Although not fully elucidated, a growing body of evidence supports that EAT with metabolic disorders promotes the progression of coronary atherosclerotic heart disease (CAHD) (4). Thus, correcting the disordered EAT metabolism may be a potential method for the prevention and treatment of CAHD.

Histological analysis of EAT showed that it is a mixed cell structure, mainly comprising adipose stromal cells, and contains a large number of inflammatory cells including lymphocytes, macrophages, and mast cells (5). Studies have confirmed that EAT is rich in adipose stem cells (ASCs), which express stem cell markers (6). Epicardial adipose stem cells (EASCs) have higher myocardial and angiogenic potential compared with stem cells derived from pericardial and omental adipose tissues (5, 6). However, few studies have analyzed the existence and the functions of EASCs, in both animal and human studies, and the relationship between EASCs and CAHD is also rarely reported. In the preliminary experiments, we found that the abundance of EASCs in EAT of CAHD patients was higher than that in the EAT of non-CAHD patients. We speculate that the abnormal adipogenic differentiation of EASCs may be the cause of the metabolic disorder of EAT in CAHD patients, and have explored EAT-derived exosomes form that perspective in this study.

MATERIALS AND METHODS

Reagents

The antibodies for CD9, CD44, CD81, calnexin, neuronatin (Nnat), and GAPDH were all purchased from Proteintech (Rosemont, IL, USA). The Oil red O stain was commercially obtained from Solarbio (Beijing, CHN). Lipofectamine 2000 was purchased from ThermoFisher (Waltham, MA, USA).

TABLE 1 | Characteristics of patients.

	non-AS group ($n = 15$)	AS group ($n = 24$)	*P*-value
Age (year)	62.47 ± 2.13	67.13 ± 1.39	0.0636
Male/Female	5/10	16/8	0.0549
Body mass index (kg/m^2)	24.41 ± 0.49	25.81 ± 0.48	0.0605
Clinical diagnosis			
Rheumatic heart disease	15	–	
Coronary atherosclerotic heart disease	–	24	
Reason for surgery			
Valvular lesion	15	–	
Multivessel coronary stenosis	–	24	

Sample Acquisition

The patients signed an informed consent form, which was approved by the hospital ethics committee, and the study complied with the Declaration of Helsinki. The patient samples were collected in accordance with the relevant Chinese laws and regulations, and the samples obtained were registered with the relevant government departments. From January 2020 to December 2020, there were 39 planned cardiac surgery patients in total (**Table 1**). According to clinical diagnosis, the patients were divided into the AS group ($n = 24$) and non-AS group ($n = 15$). During the operation, an EAT biopsy sample (0.5–1.0 g) was collected from the aortic root near the right coronary artery and divided into two parts. One part was put in the sample preservation solution and transported to the laboratory and stored at a low temperature, and the other part was fixed with formaldehyde.

EASCs Culture

In the biosafety cabinet, fresh EAT samples were washed with PBS, and then digested with 0.075% type I collagenase by shaking at 37°C for 1 h. Following this, the same volume of cell culture medium was added, with repeated blow and mix. After centrifugation, the supernatant and residual fat were discarded to obtain cell sediment. The primary cell culture medium (Procell, Wuhan, CHN) was added to resuspend the cells and inoculated into T25 culture bottle. The cells were cultured in a humidified atmosphere with 5% CO_2. After 24 h, the medium was discarded to remove the non-adherent cells. The cells were then fed every 3 days, and sub-cultured at 70–80% confluency. CD44 and CD45 were detected in EASCs via immunofluorescence and flow cytometry.

Exosomes Extraction

In the biosafety cabinet, fresh EAT tissue sample was divided into 2 × 2 × 2 mm tissue blocks, and transferred into the culture bottle. Turning over the culture bottle gently, an appropriate amount of culture medium prepared with exosome-free fetal bovine serum was added to the bottle. After 2 h, the culture bottle was turned back gently and the culture medium slowly covered the tissue blocks which would be cultured in the incubator conventionally. The culture medium was collected regularly to

separate the exosomes by high-speed centrifugation, and the morphology of exosomes was observed by transmission electron microscopy (HITACHI, Tokyo, Honshu, JPN).

Oil Red O Staining

EASCs from CAHD patients or non-CAHD patients were induced differentiation into mature adipocytes using an adipogenic differentiation medium (Procell, Wuhan, HB, CHN) according to the instruction of manufacturer. EAT-derived exosomes were categorized into the non-AS group and AS group, and inoculated with EASCs from CAHD patients at a concentration of 20 μg/mL, which was determined based on preliminary experiments. After 21 days of adipogenic induction, the culture medium was discarded and the cells were stained with oil red O dye as reported previously (7).

Immunohistochemical Analysis

Briefly, paraffin sections of EAT samples and climbing slices of EASCs were incubated with target antibodies. All sections were photographed at 20× magnification (Leica Microsystems Inc., IL, USA), and analyzed using the Image Pro Plus 6.0 software (Media Cybernetics, Rockville, MA, USA) accordingly.

MiRNA Sequencing

The total RNA in exosomes was extracted using the miRNA isolation kit (mirVana, Austin, TX, US), qualified by electrophoresis, and sequenced accordingly. The sequencing reagent was prepared according to user guide of Illumina (San Diego, CA, USA), and the sample was analyzed by an Illumina sequencer. The single-read program was used for single-end sequencing. The sequencing process was controlled by the data collection software provided by Illumina, and real-time data analysis was carried out.

Quantitative Polymerase Chain Reaction (qPCR) Assay

in vitro delivery of miR-3064-5p inhibitor-loaded exosomes to EASCs by incubating in culture medium. After 24 h, the expression of miR-3064-5p in EASCs was detected using qPCR. Briefly, total RNA was reverse transcribed using the

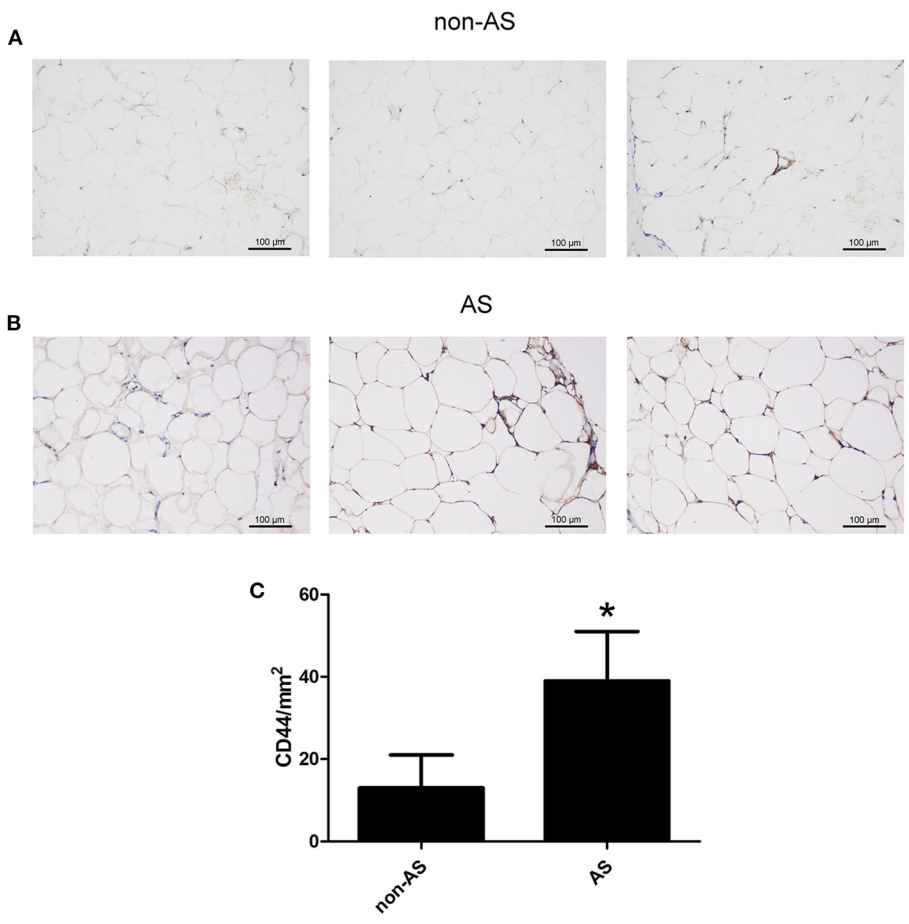

FIGURE 1 | The expression of CD44 in EAT of CAHD or non-CAHD patients. **(A)** The expression level of CD44 in EAT of non-CAHD patients. **(B)** The expression level of CD44 in EAT of CAHD patients. **(C)** The comparison of the number of CD44 positive cells in EAT of CAHD or non-CAHD patients. non-AS: the EAT of non-CAHD patients. AS: the EAT of CAHD patients. *$P < 0.05$ vs. non-AS.

MicroRNA Reverse Transcription Kit (Haoqinbio Inc., Shanghai, China) with specialized primers according to the manufacturer's instruction. RNU6 was used as a housekeeping reference. The synthesized first-strand cDNA samples were subjected to qPCR using hsa-miR-3064-5p specific TaqMan primer (Applied Biosystems, Foster City, USA) and TaqMan Universal PCR Master Mix in an ABI Prism 7700 Sequence Detector (ThermoFisher, Waltham, MA, USA). The oligonucleotide primer sequence of Nnat was designed using Primer 5.0 software and GAPDH was used as an internal control. The synthesized first-strand cDNA samples were subjected to qPCR using a SYBR Green PCR Master Mix (Toyobo Bio-Technology, Shanghai, CHN) and the qPCR reaction was also performed on the ABI Prism 7700 Sequence Detector (ThermoFisher).

Luciferase Reporter Assay

The EASCs were cultured *in vitro* to induce differentiation into mature adipocytes. The reporter plasmid and miRNA mimics were co-transfected into adipocytes using the Lipofectamine 2000 (ThermoFisher, Waltham, MA, USA) transfection reagent, and the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) was used to observe luciferase activity. Furthermore, RT-PCR and western blotting were sequentially performed to further verify mRNA and protein levels.

Western Blotting

Proteins were extracted from EAT or EASCs using radioimmunoprecipitation assay (RIPA) lysis buffer and size fractionated by SDS polyacrylamide gel electrophoresis. Membranes were incubated with target antibodies at 4°C overnight. Then, membranes were incubated with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature and washed with tris-buffered saline and Tween 20. The immune complexes were visualized by enhanced chemiluminescence after washing again, and the band intensity was measured quantitatively and analyzed with the Image J v2.1.4.7 software (National Institutes of Health, Bethesda, MD, USA).

Statistical Analysis

All data are presented as means ± standard error of the mean and analyzed using SPSS version 20.0 (IBM Corp., Armonk, NY, USA). Two-tailed Student's *t*-tests were performed to compare means between two groups. A P-value < 0.05 was considered to be statistically significant.

RESULTS

EASCs in EAT of CAHD Patients Are More Abundant Than Those in the EAT of Non-CAHD Patients

The EAT from CAHD patients (AS group) and non-CAHD patients (non-AS group) were collected to perform immunohistochemical staining for CD44, one of biomarkers of EASCs. As shown in **Figure 1**, the number of CD44-positive cells in the EAT of CAHD patients was significantly higher than

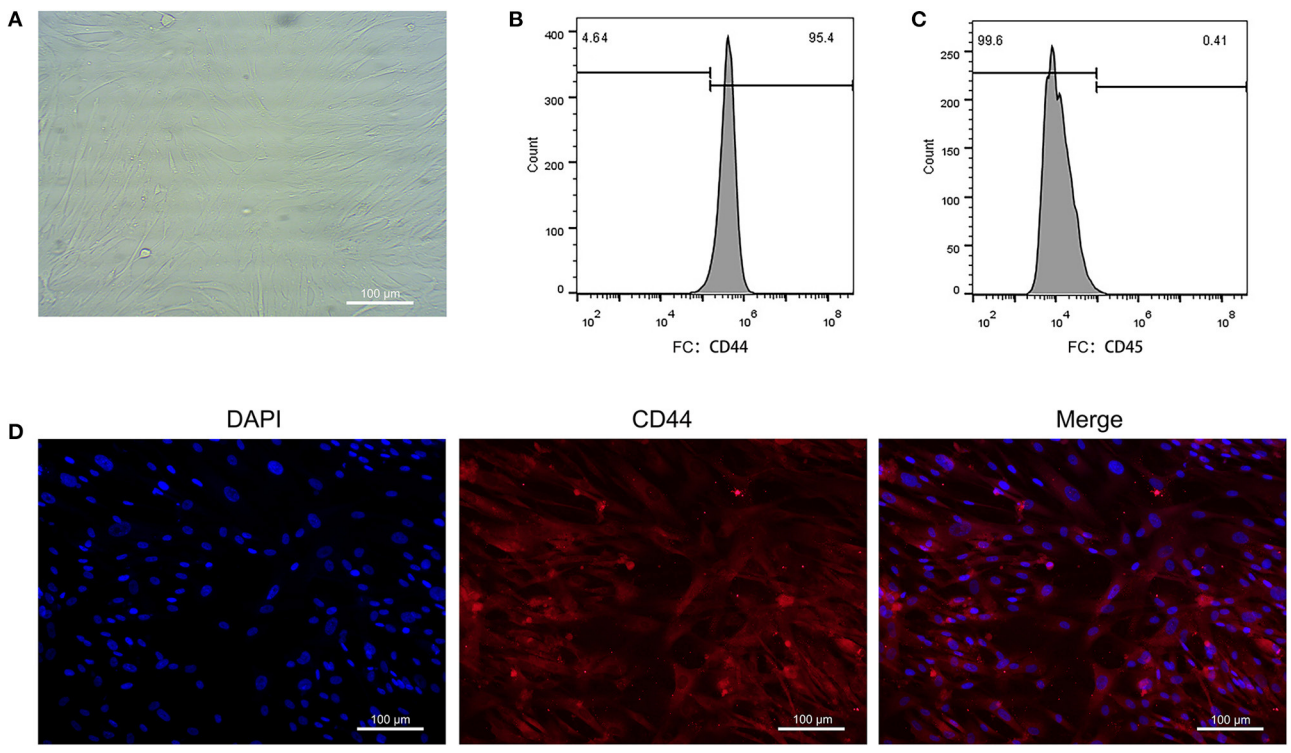

FIGURE 2 | Primary culture of EASCs and its identification. **(A)** Morphology of EASCs under microscope. **(B)** Flow cytometry of CD44 positive cells in EASCs. **(C)** Flow cytometry of CD45 positive cells in EASCs. **(D)** Immunofluorescence staining of CD44 in EASCs climbing slices.

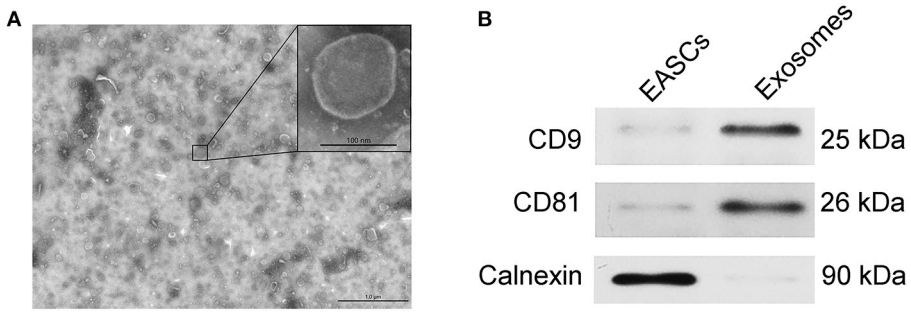

FIGURE 3 | Identification of exosomes from EAT. **(A)** Electron micrographs of exosomes. **(B)** The protein expression levels of CD9, CD81 and calnexin in EAT-derived exosomes.

that in the EAT of non-CAHD patients ($P < 0.05$), indicating that CAHD patients show a higher abundance of EASCs than non-CAHD patients. Human EAT was digested with collagenase to screen out adherent cells, and the cell morphology was found to be mesenchyma-like (**Figure 2A**). The positive rate of anti-CD44 on the cell surface detected by flow cytometry was 95.4% while the CD45 was 0.41% (**Figures 2B,C**), which was consistent with the characteristics of adipose stem cells. The CD44 antibody was used for immunofluorescence staining of cell slides, showing >90% positive cells (**Figure 2D**).

Exosomes Derived From EAT of CAHD Patients Inhibit the Adipogenic Differentiation of EASCs

The EAT tissue blocks were cultured with exosome-free serum *in vitro*, and the exosomes were extracted from the medium. As shown in **Figure 3A**, vesicle-like structures with obvious lipid bilayers could be observed via electron microscope. The total proteins of vesicles were extract, and the expression levels of the exosome markers CD9 and CD81 and the endoplasmic reticulum-specific molecule Calnexin were detected by Western blotting. Total proteins of EASCs were used as a control. As shown in **Figure 3B**, the expressions of CD9 and CD81 were detected in the total proteins of vesicles, whereas Calnexin was negatively expressed, suggesting that the vesicle structures are exosomes.

The adipogenic induction medium was used to induce adipogenic differentiation of EASCs and no significant difference in the level of adipogenic differentiation of EASCs in EAT was observed between CAHD patients and non-CAHD patients according to the results of oil red O staining (data not shown). However, intervention with exosomes derived from the EAT of CAHD patients could significantly inhibit the adipogenic differentiation of EASCs (**Figure 4**, $P < 0.05$ vs. non-AS), suggesting that EAT-derived exosomes are a key regulatory factor in the adipogenic differentiation of EASCs.

MiR-3064-5p Is a Key miRNA in EAT-Derived Exosomes and Targets Nnat

The total RNAs of exosomes derived from EAT of CAHD patients and non-CAHD patients were extracted and subjected to miRNA sequencing. As shown in **Figure 5**, 63 miRNAs (fold-change ≥ 2) with significant differences in expression levels were screened. The mimics or inhibitors of these abovementioned miRNAs were synthesized and transfected into EAT-derived exosomes of CAHD patients to observe the effects of modified exosomes on the adipogenic differentiation of EASCs. The results showed that the knock-down of miR-3064-5p, which was obviously up-regulated in the exosomes derived from the EATs of CAHD patients compared with those derived from non-CAHD patients, in exosomes could significantly improve the adipogenic differentiation of EASCs (**Figures 6A–D**). TargetScan 7.2 was employed to determine the predicted target genes of miRNA-3064-5p, and it showed 562 target genes for miRNA-3064. Among these, we focused on Nnat, with a target score of 95. Furthermore, protein expression of Nnat was detected to be significantly up-regulated in induced EASCs after intervention with miR-3064 knock-down exosomes (**Figures 6E,F**). The luciferase reporter assay showed no effect on luciferase activity in cells transfected with wtNnat 3′ UTR or empty vectors (**Figure 7A**). In addition, compared with the cells transfected with Ctrl mimics, those transfected with miR-3064-5p mimics showed significant down-regulation in mRNA and protein expression levels of Nnat (**Figures 7B–D**).

Nnat Is Silent in EASCs and Shows Low-Level Expression in EAT of CAHD Patients

The immunofluorescence double-labeling method was used to detect protein expression levels of GAPDH and Nnat in EASCs cell slides. As shown in **Figure 8A**, extensive expression of GAPDH was shown in EASCs, but Nnat did not show significant fluorescence, indicating silenced expression of Nnat in EASCs. Immunofluorescence staining was further performed to observe the expression level of Nnat in EAT of CAHD patients and non-CAHD patients. As shown in **Figures 4B–D**, the expression level of Nnat in EAT of CAHD patients was significantly down-regulated comparing with that in EAT of non-CAHD patients ($P < 0.05$). Western blotting further verified this result as shown in **Figures 8E,F**.

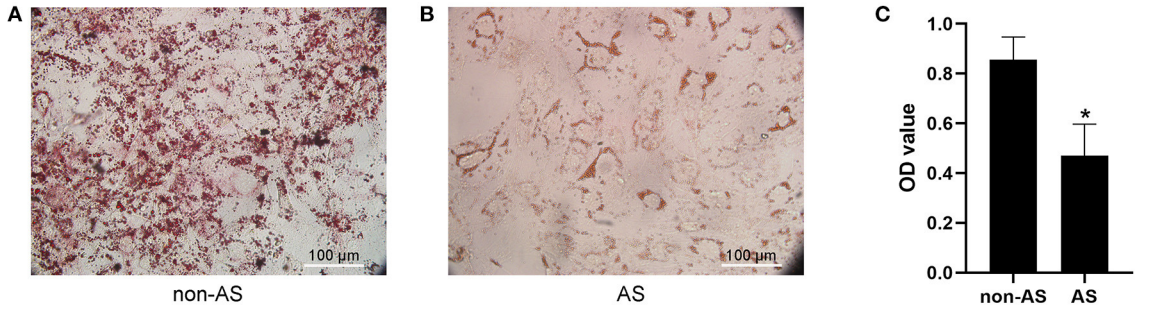

FIGURE 4 | Effect of EAT-derived exosomes on adipogenic differentiation of EASCs. Here the EASCs were derived from the EAT of CAHD patients. **(A)** The representative image of oil red O staining in non-AS group. **(B)** The representative image of oil red O staining in AS group. **(C)** Oil red O was extracted and absorbance was determined spectrophotometrically at 450 nm to quantify adipogenic differentiation. non-AS: the group of EASCs intervened with the EAT-derived exosomes of non-CAHD patients. AS: the group of EASCs intervened with the EAT-derived exosomes of CAHD patients. Data are presented as the means ± standard error of the mean for six independent experiments. $^{*}P < 0.05$ vs. non-AS.

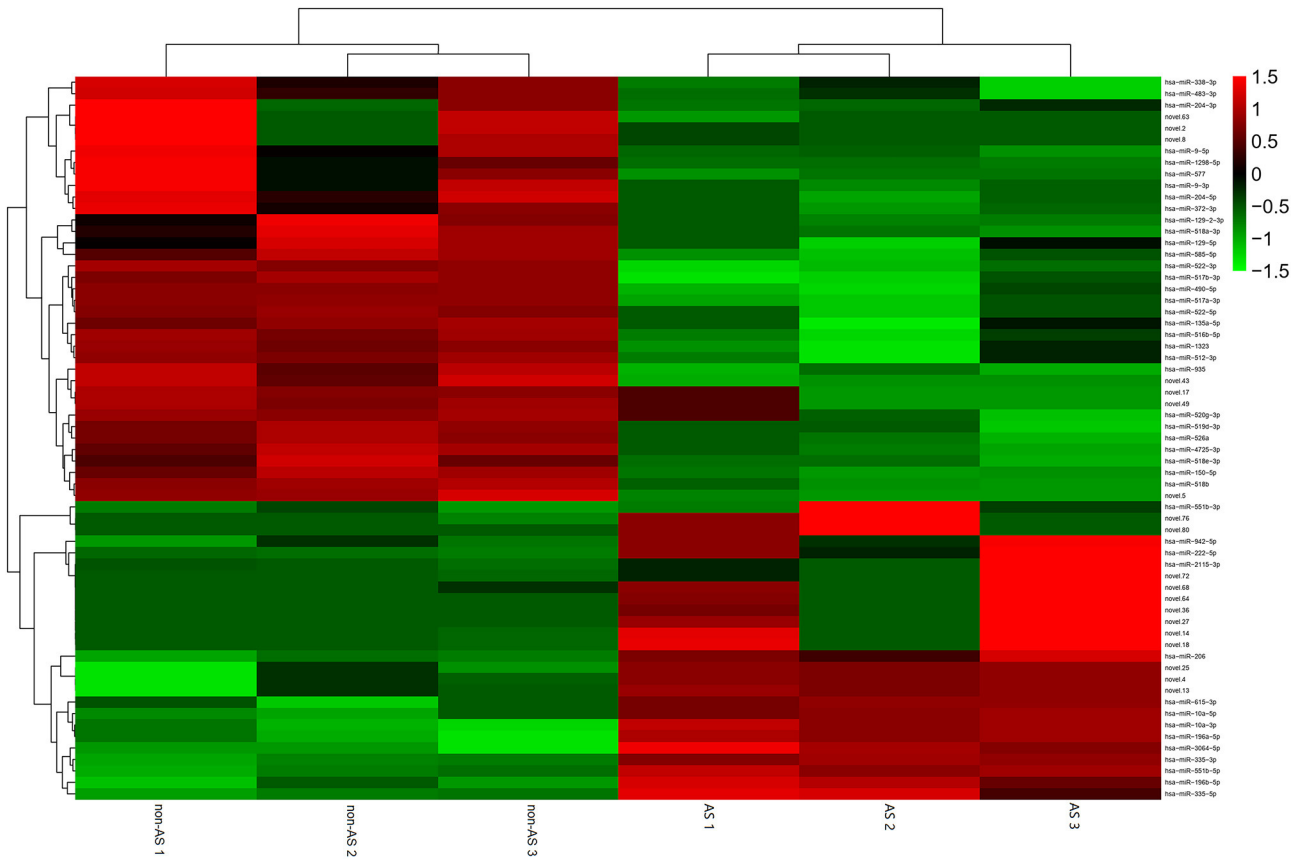

FIGURE 5 | Heatmap of differential miRNAs in EAT-derived exosomes. non-AS: EAT-derived exosomes of non-CAHD patients. AS: EAT-derived exosomes of CAHD patients.

DISCUSSION

For a long time, adipose tissue has been considered to be an energy storage site and endocrine organ. However, in the past few decades, adipose tissue has also been found to be a rich source of mesenchymal stem cells, and is currently a research hotspot in the field of induced spontaneous regeneration and cell therapy. ASCs are easy to obtain and have a strong ability to proliferate *in vitro* and differentiate into other cell types, such as adipocytes, osteoblasts, cardiomyocytes, and hepatocytes (5, 8). Similar to the adipose tissue, abundant ASCs were also found in EAT. In this study, we found that the abundance of EASCs in EATs of CAHD patients was significantly higher than that in EATs of non-CAHD patients. We isolated human

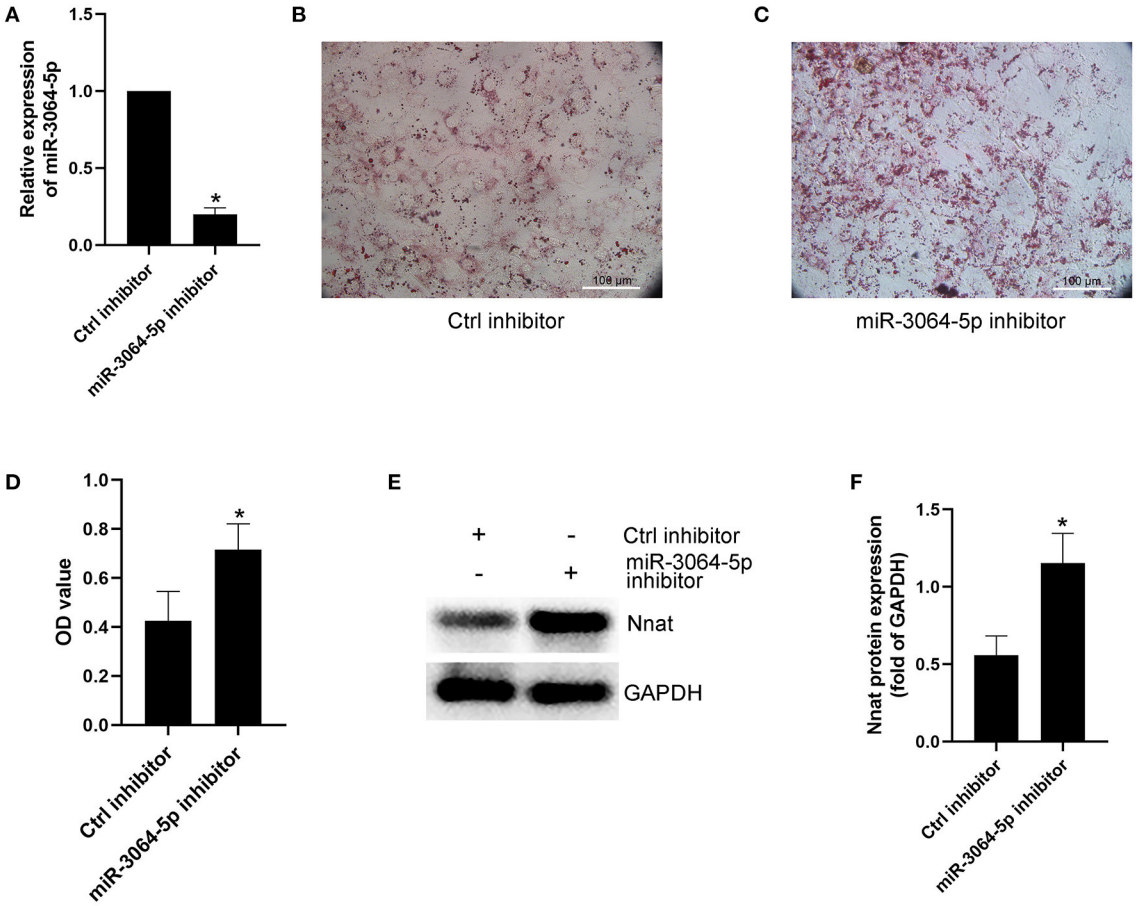

FIGURE 6 | Effect of miR-3064-5p on adipogenic differentiation of EASCs. Here the EASCs were derived from the EAT of CAHD patients. **(A)** Inhibition of miR-3064-5p after adding exosome-shuttling miR-3064-5p inhibitors to the cell culture medium of EASCs. 24 h after incubation, the expression of miR-3064-5p in EASCs was detected using qPCR. **(B)** The representative image of oil red O staining in Ctrl inhibitor group. **(C)** The representative image of oil red O staining in miR-3064-5p inhibitor group. **(D)** Oil red O was extracted and absorbance was determined spectrophotometrically at 450 nm to quantify adipogenic differentiation. **(E)** The protein expression level of Nnat in groups of Ctrl inhibitor and miR-3064-5p inhibitor. **(F)** Semi quantitative analysis of western blotting in **(E)**. Data are presented as the means ± standard error of the mean for six independent experiments. $^{*}P < 0.05$ vs. Ctrl inhibitor.

EASCs and cultured them *in vitro*, and found that there was no significant difference in the adipogenic differentiation ability of EASCs between CAHD and non-CAHD patients. Thus, we speculated that the abnormal microenvironment of EAT in CAHD patients resulting from various triggers (such as inflammation and insulin resistance) inhibited the normal differentiation of EASCs into mature adipocytes. As a result, the normal metabolism of EAT would be disturbed, leading to the dysfunction of EAT, and its autocrine and paracrine cytokines would further deteriorate the EAT microenvironment, forming a vicious circle, and aggravating the formation and progress of AS through the fat-vascular axis. However, there is a fact that CAHD patients have a thicker EAT than non-CAHD patients. One possibility is that when EASCs are induced to differentiate into adipocytes, the differentiation of EASCs into other cell types can be inhibited to a certain extent, and these cell types may be the key to CAHD. This is worth exploring in the further experiments.

Exosomes are a type of extracellular vesicle produced by cellular exocytosis, with a diameter of 30–100 nm, and are composed of lipid bilayers. These vesicles contain a wide range of degradable molecules such as proteins, lipids, and RNA, among others, which can be directly be endocytosed by target cells affecting their biological behavior (9). MicroRNA (miRNA) is the most abundant component among the contents of exosomes. MiRNA is a type of short non-coding RNA, which regulates the transcription and synthesis of proteins by interfering with mRNA transcription and translation. In adipocytes, miRNA can act on multiple targets, affecting adipocyte differentiation and metabolic homeostasis (10, 11). In EAT, miRNA may be involved in the regulation of inflammatory responses and can affect the occurrence and development of coronary artery disease (12). In this study, we cultured EAT blocks *in vitro* and isolated EAT-derived exosomes from the culture medium. We found that the adipogenic differentiation ability of EASCs after intervention with EAT-derived exosomes obtained from CAHD patients

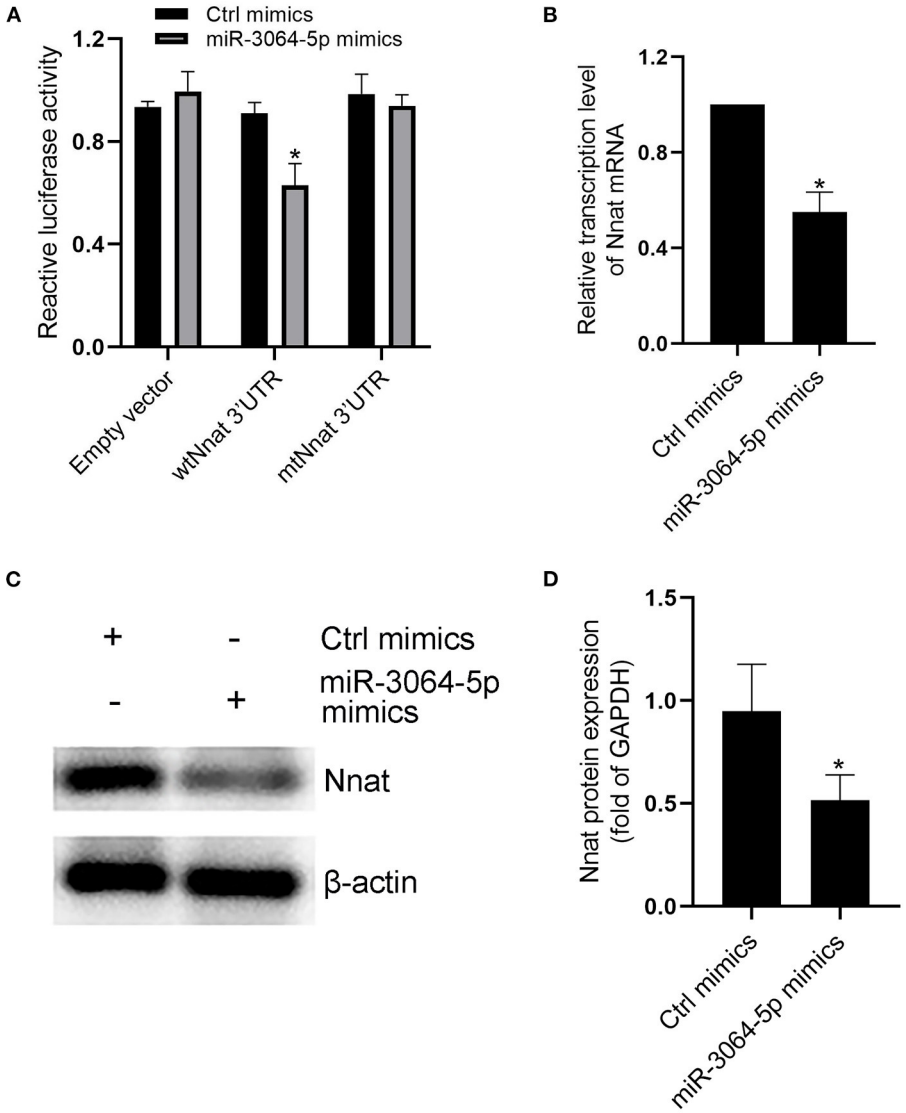

FIGURE 7 | Evidences that miR-3064-5p targeting Nnat. **(A)** The reporter constructs containing the WT or Mut Nnat 3′ UTR regions were co-transfected with control mimics or miR-3064 mimics plasmid into mature adipocytes. After 24 h, firefly luciferase activity in each sample was measured and normalized to control luciferase activity. **(B)** The mRNA transcription level of Nnat after intervention of miR-3064-5p mimics. **(C)** The protein expression level of Nnat after intervention of miR-3064-5p mimics. **(D)** Semi quantitative analysis of western blotting in **(C)**. Data are presented as the means ± standard error of the mean for six independent experiments. $^{*}P < 0.05$ vs. Ctrl mimics.

was significantly attenuated compared with that of EASCs after intervention EAT-derived exosomes from non-CAHD patients. Thus, we hypothesized that EAT-derived exosomes were involved in the regulation of adipogenic differentiation of EASCs. When a pathological microenvironment is formed in EAT, the composition and quantity of miRNAs carried by the exosomes produced by EAT changes accordingly. After being endocytosed by EASCs, the miRNAs in exosomes interfere with the expression levels of key proteins, which ultimately leads to abnormal adipogenic differentiation of EASCs.

To further explore which miRNAs play a major regulatory role, miRNA sequencing was performed to analyze the differential expression of miRNAs in EAT-derived exosomes. At present, there are relatively few research reports on miR-3064-5p, and the studies mainly focus on tumor-related fields. In our study, we noticed that miR-3064-5p was significantly up-regulated in EAT-derived exosomes from CAHD patients, and its inhibitor could obviously improve the inhibitory effect of CAHD-derived exosomes on the adipogenic differentiation of EASCs (13, 14). Further analysis showed that Nnat is the target gene of miR-3064-5p in EASCs. Exosomes modified with the miR-3064-5p inhibitor showed significant up-regulation in the expression of Nnat protein in induced EASCs. The abovementioned preliminary experimental results suggest that miR-3064-5p

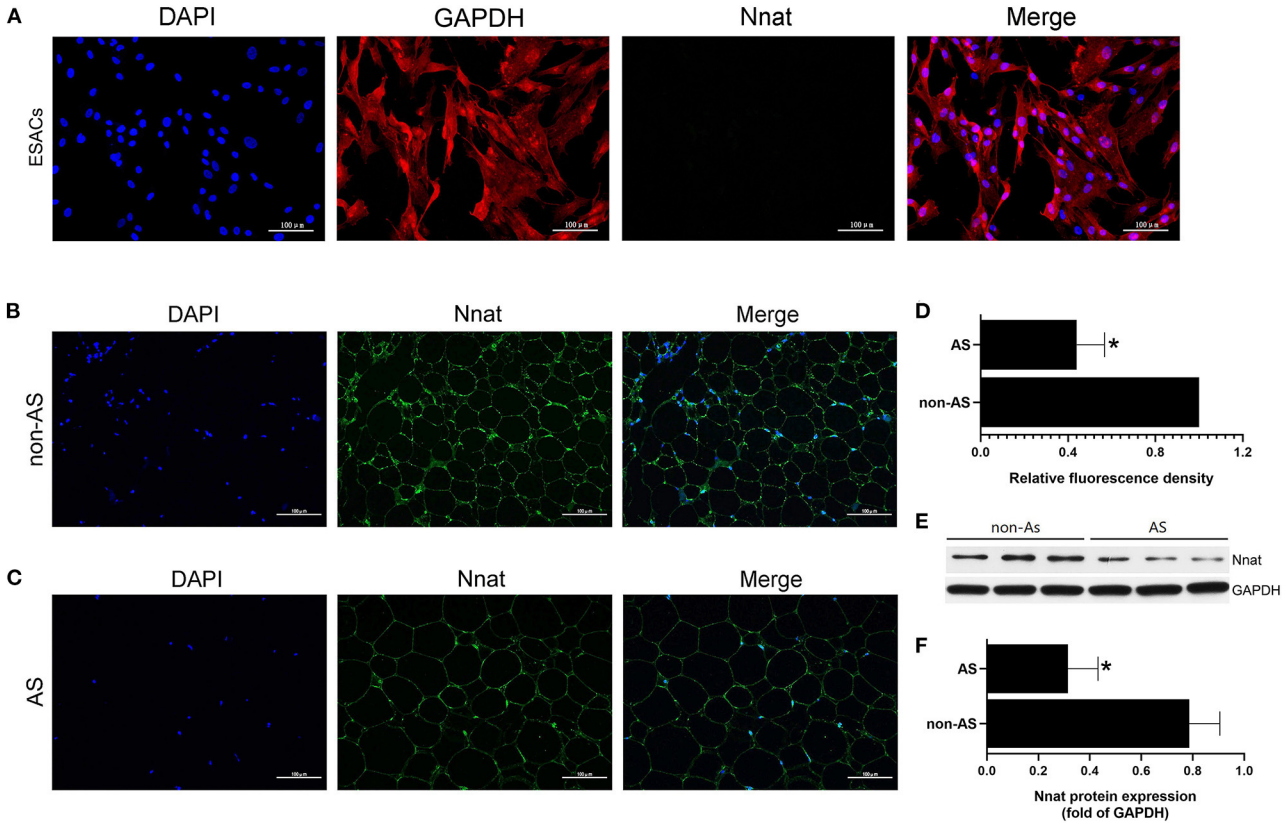

FIGURE 8 | The protein expression level of Nnat in EASCs and EAT. **(A)** Immunofluorescence staining of Nnat in EASCs climbing slices. **(B)** Immunofluorescence staining of Nnat in EAT of non-CAHD patients. **(C)** Immunofluorescence staining of Nnat in EAT of CAHD patients. **(D)** Relative fluorescence density analysis of Nnat in EAT of CAHD and non-CAHD patients. **(E)** The representative western blotting bands of Nnat in EAT of CAHD and non-CAHD patients. **(F)** Semi quantitative analysis of western blotting in **(E)**. non-AS: the EAT of non-CAHD patients. AS: the EAT of CAHD patients. Data are presented as the means ± standard error of the mean for six independent experiments. $^{*}P < 0.05$ vs. non-AS.

in EAT-derived exosomes participates in the regulation of adipogenic differentiation of EASCs by targeting Nnat.

Nnat is a gene related to neurodevelopment, and is involved in pathophysiological processes such as neurodevelopment and metabolism (15). Nnat mainly expresses in adult cerebral cortex, endocrine tissue, placenta, and adipose tissue, and its abnormal expression is associated with diabetes, obesity, and Lafora disease, which may be caused by an Nnat-mediated abnormality in Ca signaling abnormality, inflammation response, glucose exchange, or Nnat misfolding (16, 17). Yang et al. previously reported that the knockdown of Nnat expression reversed the effects of adiponectin on promoting the differentiation of 3T3-L1 cells into mature adipocytes, and inhibiting the release of inflammatory factors and oxidative stress through NF-κB signaling pathway (7). Inflammation and oxidative stress affect adipose metabolism and participate in the incidence and development of various diseases, such as AS, obesity, hypertension, and diabetes (18–21). Analysis of clinical EAT samples in this study showed that the expression of Nnat protein in EAT of CAHD patients was significantly down-regulated compared that in EAT of non-CAHD patients. Interestingly, in isolated EASCs, Nnat protein was almost not expressed and was in a silent state. We speculated that the silent state of Nnat may be the key for EASCs to maintain the characteristics of stem cells without adipogenic differentiation. Nnat activates when EASCs receive an adipogenic signal in the microenvironment, thus promoting the adipogenic differentiation of EASCs. However, the exosomes produced in the metabolically disordered EAT show miR-3064-5p over-expression, which can inhibit the protein transcriptional expression of Nnat after acting on EASCs, in turn resulting in the inability of EASCs to normally differentiate into mature adipocytes even after receiving adipogenic differentiation signals. Nevertheless, this is only a reasonable speculation, and further experimental research is still needed to verify this.

CONCLUSION

In conclusion, this study found that CAHD patients contained higher levels of EASCs in EAT, and no difference in the adipogenic differentiation ability of EASCs *in vitro* was reported regardless of whether the EASCs were CAHD or non-CAHD derived. This suggested that the microenvironment of EAT was affecting the normal adipogenic differentiation of EASCs.

We isolated exosomes from EAT, and confirmed that EAT-derived exosomes from CAHD patients inhibited the adipogenic differentiation of EASCs. We further reported that miR-3064-5p may be the key miRNA for EAT-derived exosomes to regulate the adipogenic differentiation of EASCs, which may play a regulatory role by targeting Nnat. In contrast, Nnat was shown to have a low expression in EAT of CAHD patients and was not expressed in EASCs, suggesting that Nnat is a key regulatory protein for the adipogenic differentiation of EASCs. However, the exact mechanism of Nnat in the regulation of adipogenesis of EASCs still needs to be further explored. In addition, future studies should also focus on the cell types derived from exosomes showing a high expression of miR-3064-5p to have a deeper understanding of the influence of the EAT microenvironment on the occurrence and development of CAHD.

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 studies involving human participants were reviewed and approved by Central People's Hospital of Zhanjiang Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WY and HT designed the study, interpreted data, and wrote the manuscript. WY, HT, KT, and HH performed laboratory measurements and analyzed data. KT and HH interpreted data and critically revised the manuscript. SO and JW collected clinical samples and assisted in the experiments. 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/fcvm.2021.709079/full#supplementary-material

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Perivascular Adipose-Derived Exosomes Reduce Foam Cell Formation by Regulating Expression of Cholesterol Transporters

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Background: Accumulating evidence demonstrates that perivascular adipose tissue (PVAT) plays an important role in maintaining vascular homeostasis. The formation of macrophage foam cells is a central feature of atherosclerosis. This study aimed to evaluate the effect of PVAT-derived exosomes (EXOs) on the lipid accumulation of macrophages and verify the anti-atherogenic characteristics of PVAT.

Methods and Results: We extracted EXOs from the PVAT and subcutaneous adipose tissue (SCAT) of wild-type C57BL/6J mice. After coincubation, the EXOs were taken up by RAW264.7 cells. Oil Red O staining revealed that macrophage foam cell formation and intracellular lipid accumulation were ameliorated by PVAT-EXOs. Flow cytometry showed that PVAT-EXOs significantly reduced macrophage uptake of fluorescence-labelled oxidised low-density lipoprotein (ox-LDL). In addition, high-density lipoprotein-induced cholesterol efflux was promoted by PVAT-EXOs. Western blot analysis showed the downregulation of macrophage scavenger receptor A and the upregulation of ATP-binding cassette transporter A1 and ATP-binding cassette transporter G1, which could be mediated by the overexpression of peroxisome proliferator-activated receptor γ and was independent of liver X receptor α.

Conclusion: Our findings suggest that PVAT-EXOs reduce macrophage foam cell formation by regulating the expression of cholesterol transport proteins, which provides a novel mechanism by which PVAT protects the vasculature from atherosclerosis.

Keywords: cholesterol transport proteins, cholesterol, perivascular adipose tissue, exosome, macrophage foam cell formation

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INTRODUCTION

Atherosclerosis is a fibrofatty lesion of the artery wall that contributes to stroke, coronary artery disease (CAD), and disabling peripheral artery disease and causes high morbidity and mortality worldwide (1, 2). The formation of macrophage foam cells plays a crucial role in the pathogenesis of atherosclerosis. The lipid homeostasis of macrophages depends on the dynamic balance of

cholesterol uptake, efflux and endogenous synthesis. Excessive oxidised low-density lipoprotein (ox-LDL) uptake mediated by macrophage scavenger receptor A (SR-A), the class B scavenger receptor CD36 (3), low-density lipoprotein receptor (LDL-R) and lectin-like ox-LDL receptor-1 (LOX-1), as well as reduced cholesterol efflux via ATP-binding cassette transporter A1 (ABCA1) or ATP-binding cassette transporter G1 (ABGA1) (4) contributes to lipid accumulation.

Perivascular adipose tissue (PVAT) has been shown to have numerous paracrine and endocrine functions and releases a variety of adipocytokines and chemokines (5). Given the adjacent vessel wall, PVAT could have a significant impact on the pathogenesis of atherosclerosis (6). Ren et al. (7) demonstrated that compared with the transplantation of thoracic PVAT from ApoE-/- mice, the transplantation of PVAT from wild-type mice significantly reduced plaque macrophage levels and the messenger RNA expression of inflammatory cytokines. In addition, Terada et al. (8) showed that the transplantation of thoracic PVAT induces the TGF-β1-mediated anti-inflammatory response, which exerts an anti-atherogenic effect. Based on these studies, it can be concluded that under physiological conditions, PVAT possesses anti-inflammatory characteristics and inhibits the development of atherosclerosis. By contrast, under pathological conditions, such as obesity and diabetes, PVAT becomes dysfunctional and secrets pro-inflammatory adipokines that induce endothelial dysfunction and inflammatory cell infiltration, thus contributing to atherosclerosis (9, 10). In this study, we focus on the association between healthy PVAT and macrophage foam cell formation.

Adipose tissue is the main source of exosomes, which are extracellular vesicles with sizes ranging from 40 to 160 nm in diameter (11). Exosomes contain multiple biological factors, such as cell-surface proteins, lipids, metabolites, RNA and DNA (11). Through endocrine or paracrine pathways, exosomes participate in intercellular communication, thereby regulating numerous physiological and pathological processes (12). Flaherty et al. showed that adipocytes release exosomes to modulate macrophage differentiation and function (13). Moreover, Xie et al. suggested that exosomes from the visceral adipose tissue of obese mice regulate macrophage foam cell transformation and polarisation, which promotes the progression of atherosclerosis (14). In this study, we investigated the role of PVAT-derived exosomes in modulating macrophage foam cell formation.

Abbreviations: PVAT, perivascular adipose tissue; EXOs, exosomes; SCAT, subcutaneous adipose tissue; ox-LDL, oxidised low-density lipoprotein; CAD, coronary artery disease; SR-A, scavenger receptor A; LDL-R, low-density lipoprotein receptor; LOX-1, lectin-like ox-LDL receptor-1; ABCA1, ATP-binding cassette transporter A1; ABGA1, ATP-binding cassette transporter G1; PBS, phosphate-buffered saline; TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; FBS, foetal bovine serum; DAPI, 4',6-diamidino-2-phenylindole; BSA, bovine serum albumin; Dil-oxLDL, fluorescence-labelled ox-LDL; HDL, high-density lipoprotein; LXRα, liver X receptor α; PPARγ, peroxisome proliferator-activated receptor γ; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

MATERIALS AND METHODS

Isolation of Adipose Tissue-Derived Exosomes

Twelve-week-old male C57BL/6J mice were purchased from the Animal Centre of Capital Medical University (Beijing, China). The mice were fed a standard diet. The procedure was approved by the Animal Care and Use Committee of Capital Medical University. The mice were anaesthetized intraperitoneally. PVAT of aorta and inguinal subcutaneous adipose tissue (SCAT) were harvested and washed with phosphate-buffered saline (PBS). The tissue was minced into small pieces (1 mm^3) and cultured in DMEM-F12 medium supplemented with 100 U/ml penicillin-streptomycin at 5% CO_2 and 37°C for 24 h. The media was collected and centrifuged at 3,000 g for 10 min to remove the cells. The supernatant was then filtered through a 0.22 μm syringe-driven philtre to remove any remaining cellular debris. The exosomes were isolated through ultracentrifugation and resuspended in PBS. After measurement of concentration with BCA Protein Assay Kit (Beyotime, Shanghai, China), the isolated exosomes were diluted to 1 mg/ml and stored at −80°C until use.

Identification of Exosomes

Exosomes were visualised with transmission electron microscopy (TEM; JEM-1220, Jeol, Tokyo, Japan). In addition, nanoparticle tracking analysis (NTA; Malvern Instruments, Malvern, UK) was performed to estimate the size distribution of the exosomes. For further identification, specific exosome markers were analysed by western blotting, including CD9, CD63 and TSG101.

Exosome Trafficking Assay

RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS) and 100 U/ml penicillin-streptomycin at 5% CO_2 and 37°C. The exosomes were labelled with PKH26 Red Fluorescent Cell Linker Kit (Sigma-Aldrich, MO, USA) according to the manufacturer's protocol, and washed 5 times with PBS. The last PBS supernatant collected after exosome labelling was used as control. The cells were incubated with exosomes (10 μg/ml) for 12 h. After being washed with PBS, the cytoskeleton was stained with phalloidin (YEASEN Biotech, Shanghai, China) and the nucleus with 4',6-diamidino-2-phenylindole (DAPI; Invitrogen, Carlsbad, CA). Fluorescence was analysed by a LEICA TCS-SP2 laser confocal microscope (Leica Microsystems, Wetzlar, Germany).

Flow Cytometry

RAW264.7 cells were incubated in RPMI 1640 medium with 10 μg/ml SCAT-EXOs, 10 μg/ml PVAT-EXOs or equal amounts of PBS for 24 h, and then 50 ng/ml lipopolysaccharide (LPS, Sigma-Aldrich) was added for another 24 h. After being washed twice with cold PBS, the cells were harvested and incubated for 30 min at 4°C in the dark with antibodies including APC-anti-mouse F4/80 (Biolegend, CA, USA), PE-anti-mouse CD80 (Biolegend) and FITC-anti-mouse CD206 (Biolegend). The cells were analysed by flow cytometry (BD Pharmingen, NJ, USA) and FlowJo 7.5 (FlowJo, OR, USA).

Enzyme Linked Immunosorbent Assay

RAW264.7 cells were pretreated with or without EXOs and stimulated with LPS as described for flow cytometry. The supernatants were collected after centrifugation, and the concentrations of TNF-a and IL-6 were measured according to manual of Mouse ELISA Kit (Dakewe Biotech, Shenzhen, China).

Oil Red O Staining and Cholesterol Quantification

RAW264.7 cells were incubated in RPMI 1640 medium containing 0.5% bovine serum albumin (BSA) and 100 U/ml penicillin-streptomycin with or without 10 μg/ml exosomes for 12 h. Then, the cells were treated with 50 μg/ml ox-LDL (Yiyuan Biotech, Guangzhou, China) for an additional 48 h. After being washed with PBS three times, the cells were fixed with 4% paraformaldehyde for 20 min and dehydrated with 60% isopropanol. The cells were stained with filtered Oil Red O solution (Solarbio, Beijing, China) for 10–20 min, and the cell nuclei were counterstained with Mayer's haematoxylin (Solarbio) for 1–2 min. Images were captured with light microscopy. The total cholesterol and free cholesterol levels were measured by assay kits (Solarbio) according to the manufacturer's instructions. The level of cholesterol esters was obtained as total cholesterol minus free cholesterol. In addition, the ultrastructure of foam cells was detected with TEM.

Cholesterol Uptake Assay

RAW264.7 cells were cultured in RPMI 1640 medium supplemented with 0.5% BSA and 100 U/ml penicillin-streptomycin with or without 10 μg/ml exosomes for 2 h. Then, 10 μg/ml fluorescence-labelled ox-LDL (Dil-oxLDL; Yiyuan Biotech) was added to each group and incubated for an additional 4 h. After being washed three times, the cells were resuspended in 0.5 ml of PBS. The Dil-oxLDL uptake was visualised with a fluorescence microscope and further analysed by flow cytometry.

Cholesterol Efflux Assay

RAW264.7 cells were cultured in RPMI 1640 medium containing 0.2% BSA, 1% penicillin-streptomycin, and 1 μg/ml 3-hexanoyl-NBD cholesterol (Cayman Chemical, MI, USA) for 24 h. After being washed with PBS, the cells were cultured in medium with or without 10 μg/ml exosomes for 2 h. Cholesterol efflux was stimulated by 50 μg/ml high-density lipoprotein (HDL; Peking Union-Biology, Beijing, China). The supernatant and cell lysates were collected and transferred to 96-well plates. The fluorescence was measured with a microplate reader (Varian Australia,

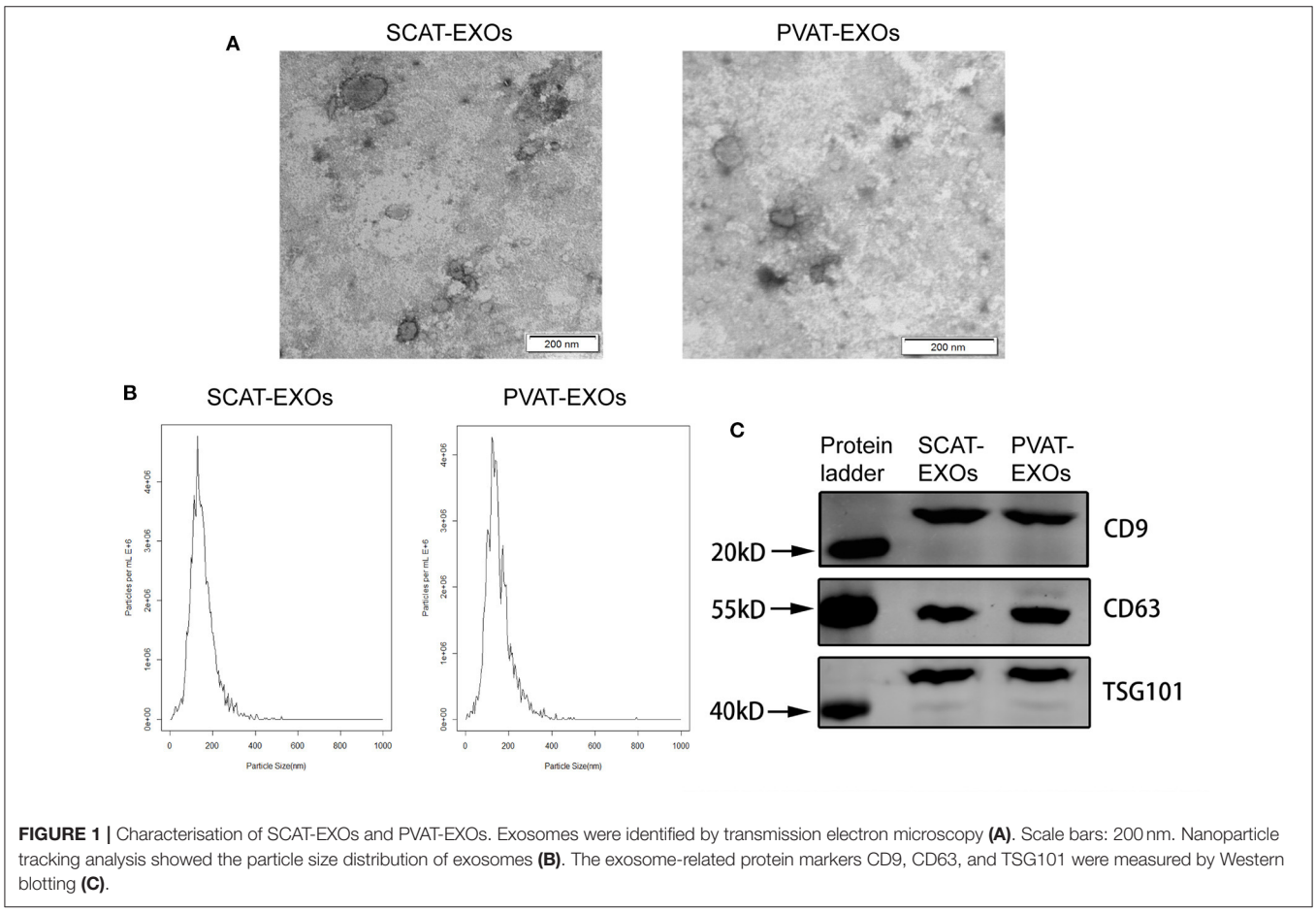

FIGURE 1 | Characterisation of SCAT-EXOs and PVAT-EXOs. Exosomes were identified by transmission electron microscopy **(A)**. Scale bars: 200 nm. Nanoparticle tracking analysis showed the particle size distribution of exosomes **(B)**. The exosome-related protein markers CD9, CD63, and TSG101 were measured by Western blotting **(C)**.

VIC, Australia) at excitation/emission maxima of 473/536 nm. Cholesterol efflux was calculated by the following formula: Media fluorescence intensity/(Cell fluorescence intensity + Media fluorescence intensity) × 100%.

Western Blotting

The proteins from exosomes and cells were extracted with RIPA lysis buffer (Solarbio) supplemented with proteinase inhibitors (Solarbio). Each well was loaded with 30 μg of protein. The proteins were transferred to polyvinylidene fluoride membranes (Millipore, Schwalbach, Germany), and the membranes were blocked with 5% skimmed milk for 1 h. Then, the membranes were probed with primary antibodies (1:1,000) overnight at 4°C. Antibodies against CD9, CD63, TSG101, SR-A, CD36, LDL-R, LOX-1, ABCA1, ABCG1, liver X receptor α (LXRα), and peroxisome proliferator-activated receptor γ (PPARγ) were purchased from Abcam (Cambridge, UK). β-actin (Cell Signalling Technology) was used as the loading control. In addition, the membranes were incubated with the corresponding HRP-conjugated secondary antibodies in the dark at room temperature for 1 h. The immunoreactivity was visualised with a ChemiDoc MP Imaging System (Bio-Rad) and analysed with ImageJ software (MA, USA).

Real-Time Polymerase Chain Reaction

Total RNA was extracted from exosomes or macrophages with TRIzol reagent (Invitrogen). The isolated RNA was reverse transcribed using EasyScript All-in-One First-Strand cDNA Synthesis SuperMix for qPCR (Transgen Biotech, Beijing, China). Quantitative PCR was performed on a Bio-Rad iQ5 Real-Time PCR Detection System with PerfectStart Green qPCR SuperMix (Transgen Biotech, Beijing, China). The following primers were used: CD36, forward 5′- GGACATTGAGATTCT TTTCCTCTG-3′ and reverse 5′- GCAAAGGCATTGGCTGGA AGAAC-3′; SR-A, forward 5′- CTGAGACCTCTGGAACAGG CAT-3′ and reverse 5′- TGCACTAGCAGTGCCATCCTCT-3′; LDL-R, forward 5′- GAATCTACTGGTCCGACCTGTC-3′ and reverse 5′- CTGTCCAGTAGATGTTGCGGTG-3′; LOX-1, forward 5′- GTCATCCTCTGCCTGGTGTTGT-3′ and reverse 5′- TGCCTTCTGCTGGGCTAACATC-3′; ABCA1, forward 5′- GGAGCCTTTGTGGAACTCTTCC-3′ and reverse 5′- CG CTCTCTTCAGCCACTTTGAG-3′; and ABCG1, forward 5′- GACACCGATGTGAACCCGTTTC-3′ and reverse 5′- GCATG ATGCTGAGGAAGGTCCT-3′. The gene expression level was normalised to that of glyceraldehyde 3-phosphate dehydrogenase (GAPDH). All samples were repeated three times, and the data were calculated using the 2- ΔΔCt method.

Statistical Analysis

Statistical analyses were conducted using GraphPad Prism 8.2.1 (GraphPad Software, CA, USA). The data are presented as the mean ± standard deviation. One-way analysis of variance (ANOVA) with Tukey's *post-hoc* adjustment was performed to identify significant differences among groups. P-values < 0.05 were considered statistically significant.

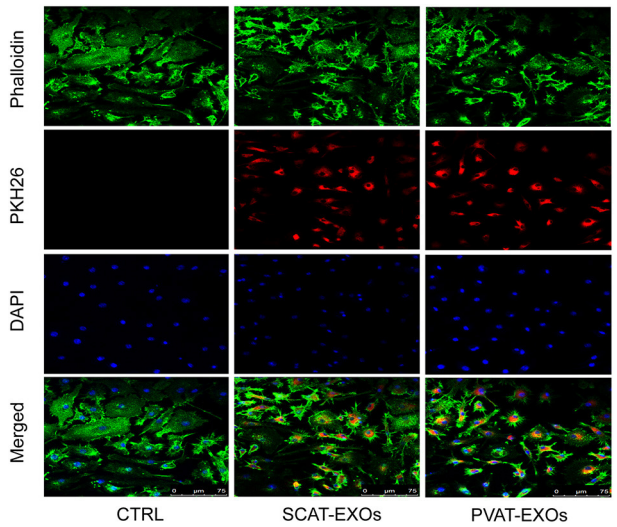

FIGURE 2 | SCAT-EXOs and PVAT-EXOs were transferred into macrophages. Exosomes were labelled with PKH26 (red) and incubated with RAW264.7 cells for 12 h. The cells were then stained with phalloidin (green) and DAPI (blue). Fluorescence signals were analysed by laser confocal microscopy. Ctrl: control group. Scale bars: 75 μm.

RESULTS

Characterisation and Endocytosis of EXOs

We performed three assays to identify the characteristics of the EXOs. First, the EXOs were verified by TEM as cup- or sphere-shaped in morphology (**Figure 1A**). In addition, NTA identified EXOs 50–150 nm in diameter (**Figure 1B**). Moreover, western blot analysis revealed the presence of the EXO-related protein markers CD9, CD63, and TSG101 (**Figure 1C**). We labelled exosomes with PKH26 and incubated the exosomes with macrophages for 12 h. Under a laser confocal microscope, we verified that both SCAT-EXOs and PVAT-EXOs were taken up by macrophages (**Figure 2**).

PVAT-EXOs Reduce the Formation of Macrophage Foam Cells

To verify the effect of PVAT-EXOs on macrophage foam cells, we cultured macrophages with EXOs and treated the cells with ox-LDL. Oil Red O staining showed that SCAT-EXOs had no significant effect on macrophage foam cell formation. Conversely, compared with that in the ox-LDL group, lipid accumulation was significantly reduced by PVAT-EXO pretreatment (**Figure 3A**). To further determine the level of intracellular cholesterol, we measured the total and free cholesterol in each group and calculated cholesterol esters by subtraction. Compared with those of the ox-LDL group, PVAT-EXO incubation significantly reduced total cholesterol (**Figure 3B**), cholesterol esters (**Figure 3C**) and free cholesterol (**Figure 3D**) in macrophages. In addition, there was no significant difference between the groups with or without SCAT-EXOs. As shown in **Figure 3E**, the ultrastructure of foam cells was observed

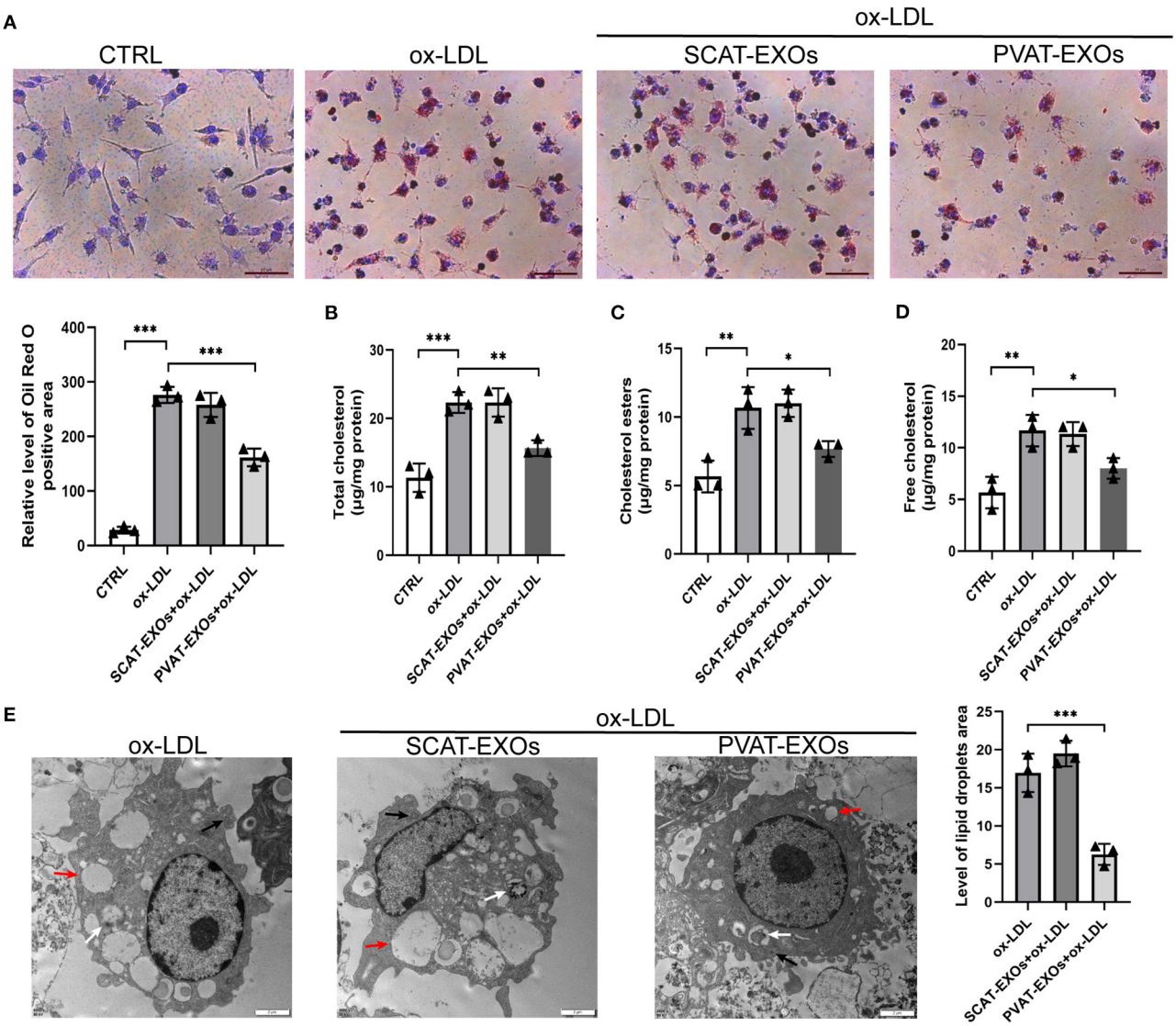

FIGURE 3 | PVAT-EXOs reduced macrophage foam cell formation. RAW264.7 cells were incubated with SCAT-EXOs, PVAT-EXOs or equal amounts of PBS and then incubated with or without ox-LDL. Oil Red O staining indicated that PVAT-EXOs reduced macrophage foam cell formation **(A)**. Scale bars: 50 μm. Lipid accumulation in macrophages was confirmed by determining the levels of total cholesterol **(B)**, cholesterol esters **(C)** and free cholesterol **(D)**. Ctrl: control group. The ultrastructure of foam cells was detected with transmission electron microscopy **(E)**. Black arrows indicate phagosomes, white arrows indicate lysosomes, and red arrows indicate lipid droplets. Scale bars: 2 μm. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the ox-LDL treated group.

by TEM, including phagosomes, lysosomes and lipid droplets. Compared with macrophages treated with ox-LDL, PVAT-EXOs significantly reduced the amount of lipid droplets, while it was not significantly changed by SCAT-EXOs. Furthermore, the flow cytometry and ELISA did not show an influence of exosomes on the phenotypic transition and cytokine production of macrophages (**Figure 4**).

PVAT-EXOs Regulate Cholesterol Uptake and Efflux in Macrophages

We incubated macrophages with PBS, SCAT-EXOs and PVAT-EXOs for 12 h and then treated the cells with Dil-oxLDL for 6 h. As shown in **Figure 5A**, compared with that in the Dil-oxLDL group, there was a substantial reduction in fluorescence in the PVAT-EXO group. We further quantified the flow cytometry results, which revealed that the cholesterol uptake by macrophages was significantly diminished by PVAT-EXOs and was significantly increased by SCAT-EXOs (**Figure 5B**). Additionally, the cholesterol efflux of macrophages was measured via the fluorescence intensity of NBD cholesterol. We found that PVAT-EXOs significantly promoted cholesterol efflux by macrophages, while the fluorescence intensity in the SCAT-EXO group was not distinctly different from that of the control group (**Figure 5C**).

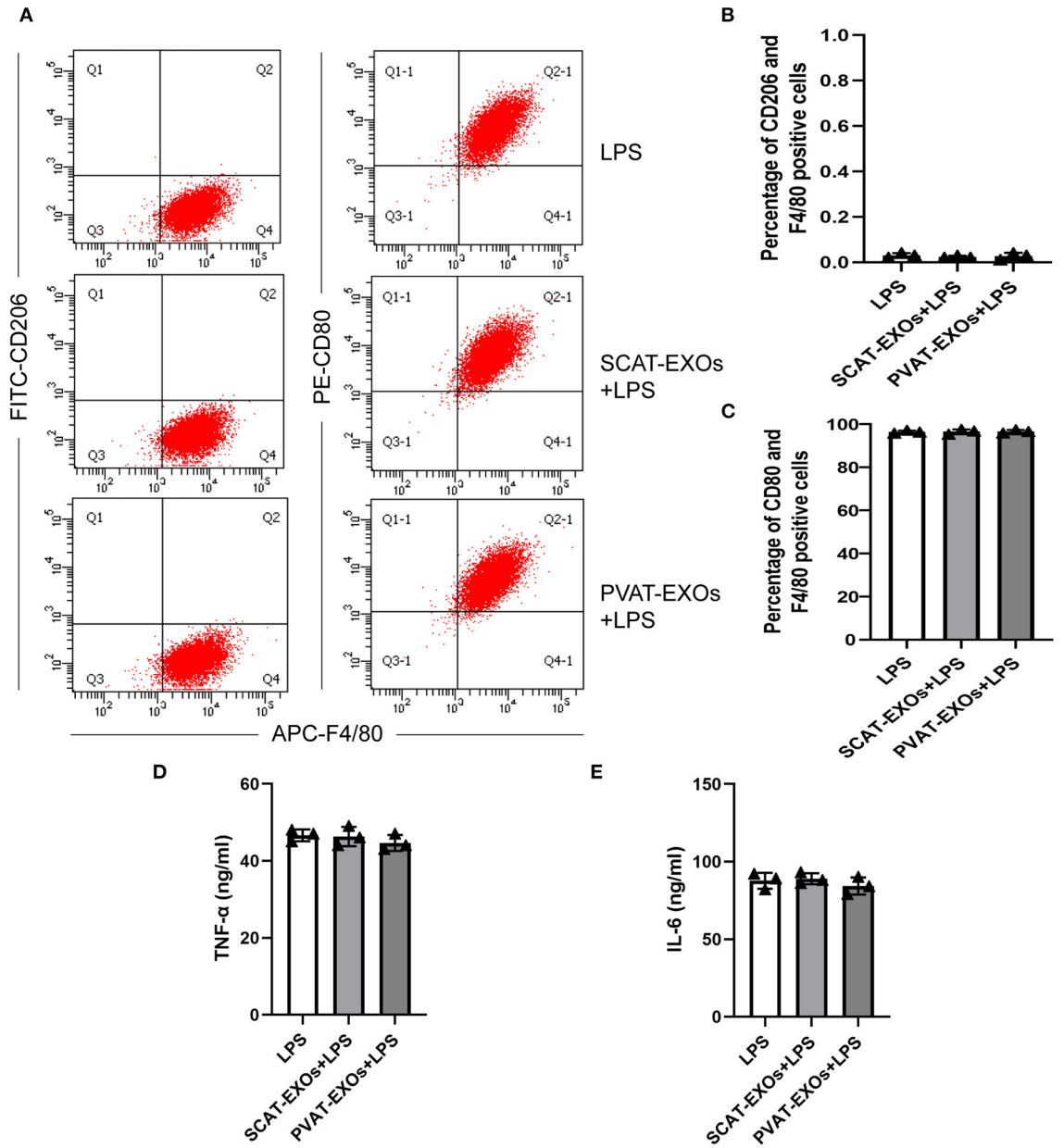

FIGURE 4 | Macrophage polarisation was not affected by SCAT-EXOs or PVAT-EXOs. RAW264.7 cells were incubated with SCAT-EXOs, PVAT-EXOs or equal amounts of PBS and then incubated with lipopolysaccharide (LPS). The phenotypes of cells were analysed by flow cytometry **(A)**. There was no significant difference in the percentages of CD206+/F4/80+ cells or CD80+/F4/80+ cells between the groups **(B,C)**. The production of TNF-a **(D)** or IL-6 **(E)** was not affected by EXOs.

PVAT-EXOs Alter the Expression of Cholesterol Transporters in Macrophages

SR-A, CD36, LDL-R, and LOX-1 are cholesterol uptake transporters, while ABCA1 and ABCG1 mediate cholesterol efflux. To explore the effects of EXOs on cholesterol transporter expression, we incubated macrophages with EXOs for 24 h and treated the cells with ox-LDL for another 6 h. Our results showed that SR-A expression was significantly reduced by PVAT-EXOs, while CD36 and LOX-1 expression were significantly increased by SCAT-EXOs (**Figure 6A**). Both ABCA1 and ABCG1 were upregulated by PVAT-EXOs. There was no significant difference of LDL-R between each group. Furthermore, PPARγ/LXRα are upstream regulatory proteins of ABCA1 and ABCG1. We found that the expression of PPARγ was enhanced by PVAT-EXOs and SCAT-EXOs, and the change in the former was more significant than that of the latter. In contrast, LXRα did not differ after

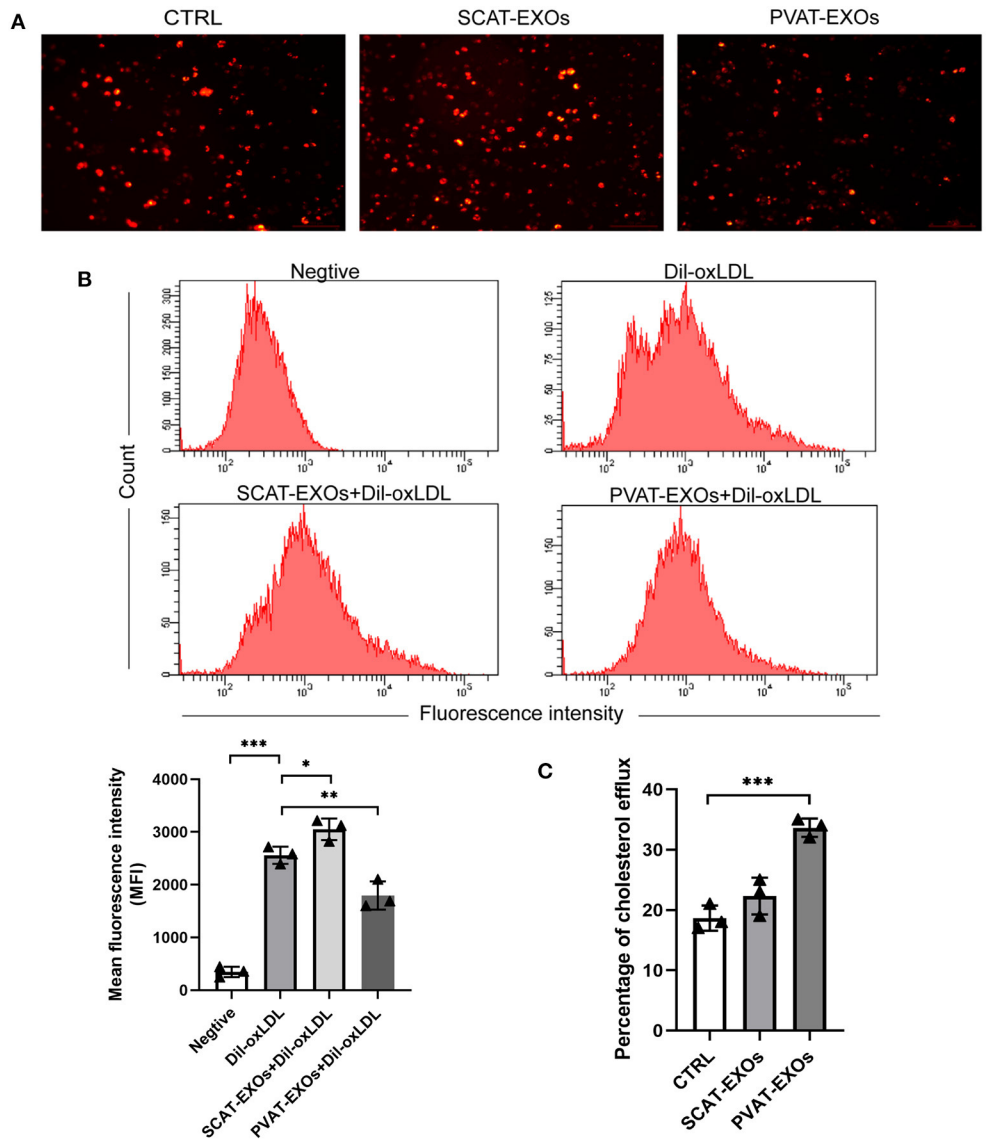

FIGURE 5 | PVAT-EXOs reduced Dil-oxLDL uptake and promote cholesterol efflux in macrophages. After treatment with SCAT-EXOs, PVAT-EXOs or PBS, Dil-oxLDL uptake by RAW264.7 cells was analysed by fluorescence microscopy **(A)**. In addition, the fluorescence intensities were quantified by flow cytometry **(B)**. HDL-mediated cholesterol efflux was significantly promoted by PVAT-EXOs **(C)**. Ctrl: control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the Dil-oxLDL group or control group.

EXO stimulation. The transcriptional levels of the corresponding mRNAs were confirmed by RT-PCR (**Figure 6B**). PVAT-EXOs significantly enhanced the mRNA expression of ABCA1 and ABCG1. CD36, ABCG1, and LOX-1 mRNA levels were increased by SCAT-EXOs.

DISCUSSION

In the current study, we found that PVAT-derived exosomes significantly downregulated the expression of the cholesterol influx transporter SR-A and upregulated the expression of the cholesterol efflux transporters ABCA1 and ABCG1, thereby reducing cholesterol accumulation in macrophages and the formation of foam cells.

In human, visceral adipose tissue, SCAT and most PVAT are white adipose tissue, which stores energy, secrets adipokines and vasoactive factors and causes cardiometabolic disorders after excess accumulation (15). While in mice, SCAT is beige adipose tissue, which could be induced to brown adipose tissue, and is related with thermogenesis, anti-inflammatory properties and cardioprotective effect (15). The differences between PVAT and SCAT have been addressed. Compared with pericarotid adipose tissue, the MCP-1 gene expression was significantly higher in SCAT, while in patients with carotid stenosis the

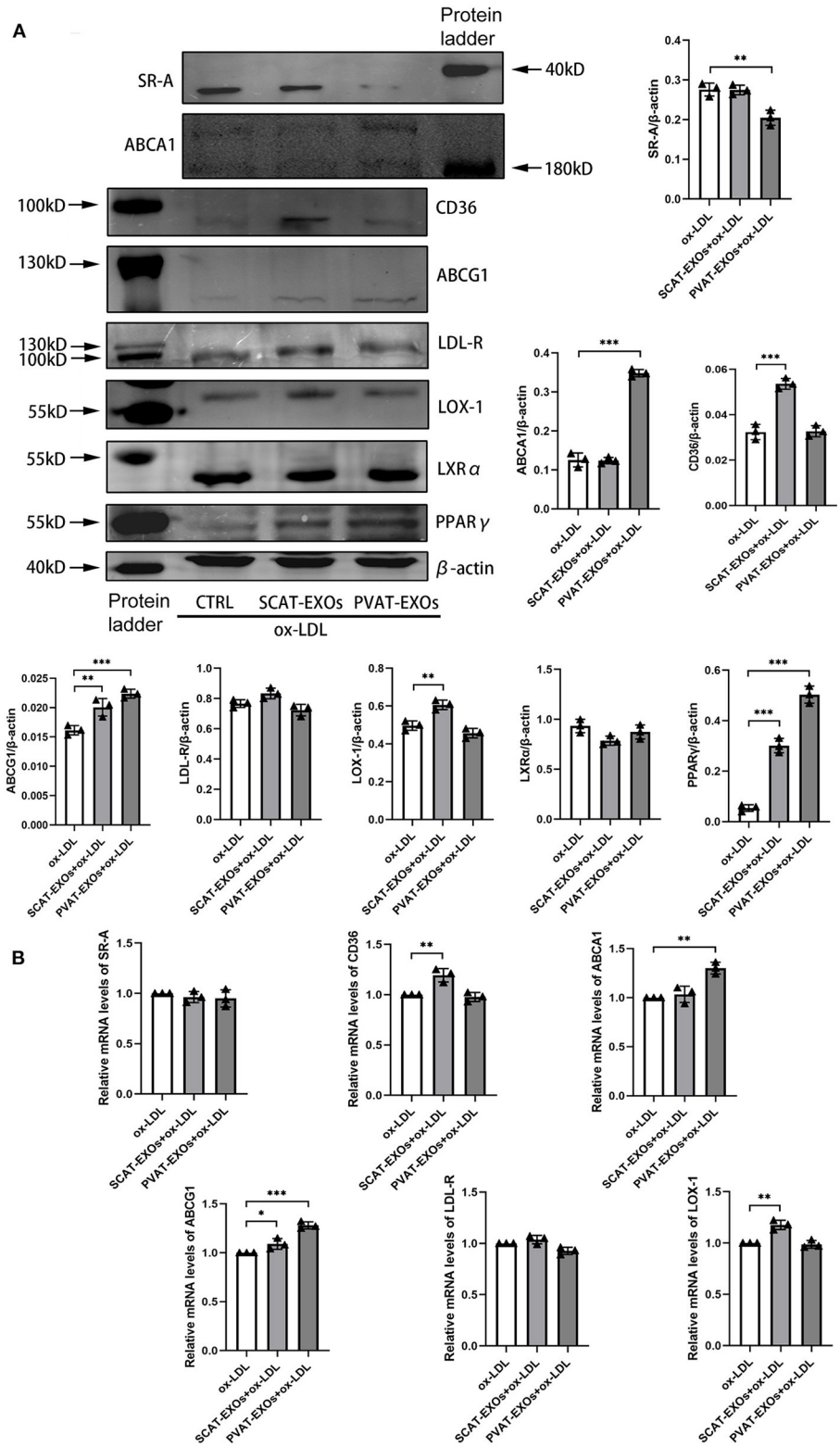

FIGURE 6 | The expression of cholesterol transporters in macrophages was regulated by SCAT-EXOs and PVAT-EXOs. RAW264.7 cells were incubated with SCAT-EXOs, PVAT-EXOs or equal amounts of PBS and then incubated with ox-LDL. Western blot analysis showed the expression of the cholesterol transport proteins SR-A, CD36, LDL-R, LOX-1, ABCA1, and ABCG1 and the upstream proteins LXRα and PPARγ **(A)**. The expression levels were normalised to β-actin. The mRNA expression levels of SR-A, CD36, LDL-R, LOX-1, ABCA1 and ABCG1 were analysed by real-time PCR and normalised to GAPDH **(B)**. Ctrl: control group. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the ox-LDL group.

expression was lower in SCAT (16). The pro-inflammatory mediator IL-6, TNF-α, MCP-1 and adiponectin were found more abundant in SCAT vs. PVAT (17), suggesting the pro-inflammatory phenotype is attenuated in PVAT. In recent years, accumulating evidence suggests that PVAT is a novel factor that modulates vascular biology (18, 19). PVAT releases a wide range of biologically active molecules that participate in inflammatory responses (20, 21), endothelial function (22) and vascular smooth muscle proliferation (23). Under physiological conditions, PVAT was shown to protect the vasculature from atherosclerosis. Adiponectin is an important adipokine that participates in anti-atherogenic processes (24). Margaritis et al. reported that adiponectin regulated eNOS coupling to improve the redox state in vessels, and adiponectin gene expression in PVAT was upregulated by peroxidation products via a PPARγ-dependent mechanism (25). By promoting eNOS phosphorylation and reducing PVAT inflammation, adiponectin improves endothelial-dependent vascular relaxation (22). In addition, omentin was shown to negatively regulate atherosclerosis development by modulating foam cell formation and the inflammatory response in macrophages (26). Consistent with previous studies, we verified that PVAT-derived exosomes were taken up by macrophages and acted as protective factors against foam cell formation. Research on PVAT-derived exosomes is quite rare. Li et al. showed that extracellular vesicles from obese mouse PVAT were taken up by neighbouring smooth muscle cells, leading to phenotypic switching and arterial remodelling (27). Our study is the first to reveal the adversarial association between PVAT-EXOs and the formation of macrophage foam cells. Considering its anatomical location, PVAT could play a fundamental role in the development of atherosclerosis.

Studies on exosomes from different types of adipose tissue and their effects on macrophages have been published recently. Wei et al. indicated that a high-fat diet changed the miRNA profile of mouse visceral adipose tissue-derived exosomes into a proinflammatory phenotype that promoted M1 macrophage polarisation (28). Similarly, Pan et al. showed that miR-34a in epididymal white adipose tissue-derived exosomes was positively associated with obesity and drove the polarisation of macrophages towards the M1 phenotype (29). In contrast, in diet-induced obese mice, inflammation reduction, alternatively activated M2 macrophage polarisation, and the beiging of white adipose tissue were facilitated by exosomes from adipose-derived stem cells (30). However, in this study, we failed to identify the effect of wild-type mouse PVAT-EXOs on M1 macrophage polarisation and inflammatory cytokine production. The high-fat diet-related characteristics of PVAT, which may be proinflammatory, warrant further exploration. Xie et al. reported that exosomes from visceral adipose tissue significantly promoted macrophage foam cell transformation via the suppression of ABCA1- or ABCG1-mediated cholesterol efflux but not SCAT-EXOs (14). Consistently, SCAT-EXOs did not affect the lipid accumulation of macrophages in our study. Furthermore, PVAT-EXOs were shown to markedly reduce the generation of foam cells through the downregulation of SR-A and upregulation of cholesterol efflux transporters, which was accompanied by exacerbated PPARγ expression.

LXRα and PPARγ are ligand-activated nuclear receptors that regulate lipid metabolism in macrophages (31). Moore et al. indicated that PPARγ regulates SR-A expression through posttranscriptional mechanisms, as PPARγ activation leads to a reduction in SR-A protein without altering SR-A mRNA expression (32). Consistent with the previous study, our data revealed a significant reduction in SR-A protein in PVAT-EXO-treated macrophages, which may be mediated by an increase in PPARγ. On the other hand, the expressions of cholesterol uptake protein CD 36, LDL-R, and LOX-1 were not significantly changed by PVAT-EXOs, suggesting the cholesterol uptake decrease should be attributed to SR-A. Activation of the PPARγ-LXRα-ABCA1/ABCG1 pathway has been shown to enhance cholesterol efflux (33). Claudel et al. revealed that PPARγ agonists and LXR/retinoid X receptor ligands induce ABC-1-mediated cholesterol efflux, which significantly reduces atherosclerotic lesions in ApoE–/– mice (34). In addition, Ruan et al. suggested a similarity between PPAR and LXRα in regulating the expression of ABCA1 and the presence of an additive effect when used together (35). In the present study, we suggested that PVAT-EXOs induce increased expression of PPARγ, which accompanied with the upregulation of ABCA1 and ABCG1, without a significant change in LXRα. Earlier research provided similar results. Li et al. demonstrated that a PPARγ agonist promoted ABCG1 expression in LXR double-knockout and wild-type macrophages (36). Additionally, a PPARγ agonist significantly enhanced the expression of ABCG1 in hypercholesterolaemic LDLR–/– mice (36). Therefore, these results suggest that PVAT-EXOs induce cholesterol efflux via the PPARγ-ABCA1/ABCG1 pathway, which is independent of LXRα.

CONCLUSIONS

Our results suggest that PVAT-derived exosomes reduce the formation of macrophage foam cells, which could be protective factors against atherosclerosis. PVAT-derived exosomes downregulate the expression of SR-A to reduce cholesterol uptake in macrophages. In addition, the overexpression of ABCA1 and ABCG1 was induced to promote cholesterol efflux. This anti-atherogenic effect might be mediated by upstream regulation of PPARγ. The use of PVAT-derived exosomes as a promising prevention and therapeutic strategy for atherosclerosis warrants further investigation.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by The Animal Care and Use Committee of Capital Medical University.

AUTHOR CONTRIBUTIONS

YL performed this study, collected data, drafted this article including drawing figures and revising the article. YS and XL helped to perform the study, and revised the article. DZ, CH, JL, YZ, and AG collected and analysed part of data and made revisions. HH, MC, JZ, YZho, and YZha made adjustments to the experimental design and revised the figures. All authors read and approved the final version of the article.

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A Bibliometric Analysis of Exosomes in Cardiovascular Diseases From 2001 to 2021

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Background: Exosomes in cardiovascular diseases (CVDs) have become an active research field with substantial value and potential. Nevertheless, there are few bibliometric studies in this field. We aimed to visualize the research hotspots and trends of exosomes in CVDs using a bibliometric analysis to help understand the future development of basic and clinical research.

Methods: The articles and reviews regarding exosomes in the CVDs were culled from the Web of Science Core Collection, and knowledge maps were generated using CiteSpace and VOSviewer software.

Results: A total of 1,039 articles were included. The number of exosome articles in the CVDs increased yearly. These publications came from 60 countries/regions, led by the US and China. The primary research institutions were Shanghai Jiao Tong University and Nanjing Medical University. *Circulation Research* was the journal and co-cited journal with the most studies. We identified 473 authors among which Lucio Barile had the most significant number of articles and Thery C was co-cited most often. After analysis, the most common keywords are myocardium infarction, microRNA and mesenchymal stem cells. Ischemic heart disease, pathogenesis, regeneration, stem cells, targeted therapy, biomarkers, cardiac protection, and others are current and developing areas of study.

Conclusion: We identified the research hotspots and trends of exosomes in CVDs using bibliometric and visual methods. Research on exosomes is flourishing in the cardiovascular medicine. Regenerative medicine, exosome engineering, delivery vehicles, and biomarkers will likely become the focus of future research.

Keywords: cardiovascular disease, CiteSpace, VOSviewer, bibliometrics, cardiovascular diseases

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INTRODUCTION

Exosomes are a subset of nanosized extracellular vesicles of 40–160 nm in diameter. They possess the same lipid bilayer structure as the origin cells and are rich in bioactive substances, including DNA, RNA, lipid, proteins, metabolites, and other molecules. Nearly all types of living cells release exosomes (1–3). Exosomes were once considered vehicles or "garbage bags," responsible for removing cell debris, including redundant intracellular organelles, for retaining cellular

homeostasis (4). In the mid-1990's, exosomes secreted by immune cells were thought to be related to immune regulation. Over time, exosomes widely present in various body fluids came to be viewed as functional membrane vesicles that mediate intercellular communication by transferring bioactive materials in normal physiology and various diseases and acting as signaling molecules in homeostatic processes or as a result of pathological progression (5–10).

Several studies showed that exosomes participate in cardiovascular diseases (CVDs) (11–14). Several animal experiments demonstrated that exosomes carrying specific functional substances inhibit cardiomyocyte apoptosis and promoting angiogenesis to improve ventricular function and reduce the area of myocardial infarction (MI) (15, 16). Fu et al. found that exosomes rich in miR-338 derived from MSCs reduced cardiomyocyte apoptosis and improved cardiac function in rats with MI by regulating the MAP3K2/JNK signaling pathway (17). Another study showed that HIF-1α overexpression in exosomes mediated cardioprotection in MI by enhancing angiogenesis (18).

Various contents of exosomes represent their cell sources and reflect the physiological and pathological conditions of the origin cells; these cargos can be used as non-invasive diagnostic biomarkers. After MI, the components of circulating exosomes change significantly. A study showed that miRNAs are released when cardiomyocytes are injured, and circulating miR-133a derived from the MI area and the marginal region elevate at an early stage after MI, which can be detected earlier than creatine phosphokinase and cardiac troponin T (19).

As regenerative medicine becomes clinically feasible, there has been extensive interest in stem cell-based therapies for CVDs. However, numerous side effects and low survival of implanted stem cells limited their therapeutic potency (20–26). Recently, evidence suggested that stem cells exert therapeutic effects in a paracrine manner through exosomes (27). Exosomes protect substances from degradation and transport material to recipient cells without causing toxicity or adverse immune responses (28).

Bibliometric analysis focuses on the literature systems and characteristics and has been widely used to understand the knowledge structure and explore developmental trends using qualitative and quantitative analysis of the scientific literature (29, 30). The bibliometric analysis method allows quantitative measurement of the domain outline distribution and the relationship and clustering of a study. In addition to describing and predicting the future development of a particular research area, the contributions of various authors, institutions, countries, and journals can be compared. This analytical approach plays a vital role in developing guidelines, understanding research hotspots, and evaluating research trends (31). Digestive system diseases (32), cancer (33), rheumatic system diseases (34), and nervous system diseases (35), have been studied using this method.

The present study explores the hotspots and developmental trends of exosome research in cardiovascular science over the past 20 years and draws maps of scientific knowledge using CiteSpace and VOSviewer software. The aim was to provide a basis for scientific research into CVDs.

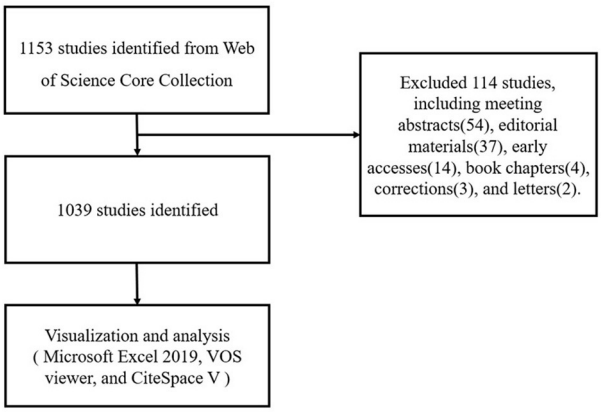

FIGURE 1 | Flowchart of literature selection.

METHODS

Data Collection

Literature was extracted from the Science Citation Index Expanded and Social Science Citation Index of Web of Science Core Collection and was downloaded within 1 day on June 5, 2021. The search terms were as follows: TS = ("cardiovascular" OR "heart") AND TS = (exosomes), and the dates of the search were June 5, 2001, to June 5, 2021, resulting in 1,153 records. We eliminated invalid documents, including meeting abstracts (54), editorial materials (37), early accesses (14), book chapters (4), corrections (3), and letters (2). A total of 1,039 records retrieved were divided into eight document types, of which articles (639) accounted for 55.42% of the total, followed by reviews (400, 34.70%). The retrieved papers were exported and saved as plain text files, and stored in download_txt format (**Figure 1**).

Data Analysis

All valid documents retrieved from Web of Science Core Collection were converted to Microsoft Excel 2019, VOSviewer, and CiteSpace to perform visual analysis.

VOSviewer is a scientometrics network analysis software developed by the Center for Science and Technology Research at Leiden University in the Netherlands. It provides visual analysis and creates maps based on network data. It can construct network diagrams of academic publications, scientific journals, authors, research institutions, countries, and keywords. The items in these networks can be connected by co-citation links, co-occurrence, citation, and bibliographic coupling. VOSviewer software provides three visualization maps: network, overlay and density visualizations (36). The core idea of the software design is co-occurrence clustering, which indicates that they are related. There are several correlations with varying intensities and directions. Based on the measurement index clustering of relationship intensity and direction, various groups can be found. Although VOSviewer is primarily used for bibliometrics, it may also create virtually any type of map of web data. Its most

prominent feature is displaying graphics and is suitable for large-scale data (37).

CiteSpace software is a citation visualization analysis software developed by Professor Chen Chaomei of Drexel University using Java language based on scientometrics and data visualization (38). It presents the structure, laws, and distribution of scientific knowledge using data mining, information analysis, and atlas drawing. Knowledge mapping is a new sub-field of information technology. It is used to visualize research hotspots and evolution processes intuitively and forecast the developmental trends of each field. It is an effective method to analyze big data (37, 39).

We used Microsoft Office Excel 2019 to analyze the articles. We used CiteSpace and VOSviewer software to analyze the distribution of countries/regions visually, authors and co-cited authors, journals and co-cited journals, co-cited references, keyword cluster analysis, and timelines.

RESULTS

The Trend of Publication Outputs

The number of publications in a specific period reflects the developmental trends of research in a field (**Figure 2**). From 2001 to 2020, the number of studies published on exosomes in cardiovascular research showed an overall upward trend. From 2007 to 2009, the number of articles was relatively low, and the research and development of exosomes in cardiovascular science were in an embryonic stage. From 2010 to 2016, the annual growth rate of the number of published papers increased steadily. From 2017 to 2020, the number of articles published on exosomes in cardiovascular medicine increased significantly, and the total number of outputs in 2020 reached 251. It can be seen that a growing number of scholars have begun to pay much attention to the potential of exosomes in cardiovascular fields.

Distribution of Countries/Regions and Institutions

A total of 1,039 articles were published by 351 institutions in 60 countries/regions. As shown in **Table 1**, the most significant number of publications came from the US (343, 33.01%) and China (333, 32.05%), followed by Italy (77, 7.41%), England (62, 5.97%), and Germany (51, 4.91%). The total number of articles from these two highest-ranked countries was more than half of the total. Several countries and institutions, such as France (0.38), England (0.21), Netherlands (0.19), Tongji Univ (0.23), and Nanjing Med Univ (0.19), showed high centrality, circled in purple in **Figures 3**, **4**. This finding suggests that the study of exosomes in these countries and institutions may have played a critical role in cardiovascular research. Each node represents a country, and the size of the node is proportional to the number of articles published. The lines between nodes represent cooperation between countries; denser lines correspond to closer cooperation. **Figure 3** shows 60 nodes and 85 connections with a network density of 0.048, and **Figure 4** shows 351 nodes and 396 connections with a network density of 0.0064. There is active cooperation among countries and institutions, including China, Russia and Switzerland, Nanjing Med Univ., Tongji Univ., and Capital Med Univ.

Journals and Co-cited Academic Journals

We found that 1,039 articles related to exosomes in the cardiovascular science were published in 428 academic journals. The journal of *Circulation Research* (46, 4.43%) had the highest number of outputs, followed by *International Journal of Molecular Sciences* (34, 3.27%), *Circulation* (28, 2.69%), *Frontiers in Physiology* (25, 2.41%), and *Scientific Reports* (22, 2.12%). Among the top 15 journals, *Circulation* has the highest impact factor (IF: 29.690), followed by *Circulation Research* with an IF of 17.367. The analysis of the distribution of the source of published articles is helpful to identify core journals.

Co-citation analysis is designed to measure the degree of relationship between articles. The impact of a journal depends on its co-citation frequency, which reflects the influence of a journal in a specific research field. Among 4,380 co-cited journals, 11 journals were cited over 1,000 times. As is shown in **Table 2**, *Circulation Research* (3,597) was the most frequently cited journal, followed by *Circulation* (2,299) and *Plos one* (2,221). Among the top 15 journals, *Nature* had the highest IF (49.962), followed by *Cell* with an IF of 41.582. According to the journal citation reports partition in 2020, almost all the co-cited journals were distributed in the Q1 region among the top 15 journals, except for *The Journal of Molecular and Cellular Cardiology*.

The dual-map overlay of journals shows the distribution of relationships between journals, citing journals on the left and cited journals on the right. The colored paths between them indicate the cited relationships. As is shown in **Figure 5**, there are three main citation paths, including two orange paths and one green path. The orange path indicates that studies published in Molecular/Biology/Genetics journals and Health/Nursing/Medicine journals are cited for studies in Molecular/Biology/Immunology journals. The green path means that the studies published in Molecular/Biology/Immunology journals are generally cited by Medicine/Medical/Clinical journals.

Authors and Co-cited Authors

A total of 473 authors published articles on exosomes in cardiovascular medicine (**Table 3**). Lucio Barile from Cardiocentro Ticino Laboratory for Cardiovascular Theranostics had the highest number of published papers (12, 1.15%), followed by Abdelnaby Khalyfa (10, 0.96%), Ke Cheng (9, 0.87%), Yaoliang Tang (9, 0.87%), and Costanza Emanueli (9, 0.87%). It is worth noting that the centrality of the authors is relatively low ($\leq$0.03), suggesting that the influence of the authors on exosomes in cardiovascular science needs to be improved. Each node represents an author, with larger nodes representing more published articles. Thicker lines represnt closer cooperation between authors. Different colors refer to clusters of close cooperation. As shown in **Figure 6**, there was a communication and cooperation network among authors in this research area. Two or more authors that are cited simultaneously are called co-cited authors (**Figure 7**). Of the 803 co-cited authors, only four had a citation frequency of more than 200 times.

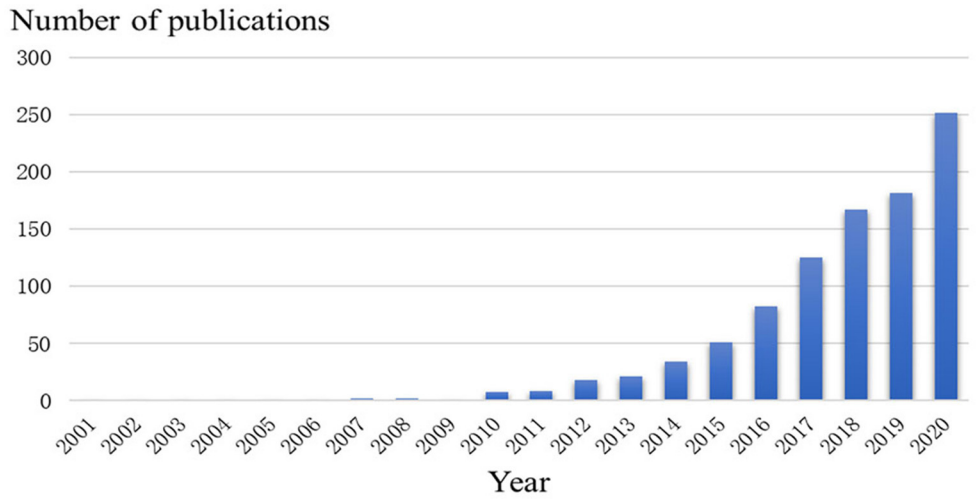

FIGURE 2 | Trends of exosomes published in cardiovascular studies over the past 20 years.

TABLE 1 | Distribution of publications from different countries and institutions.

No.	Country	Year	Centrality	Count (%)	Institution	Year	Centrality	Count (%)
1	US	2009	0.14	343 (33.01%)	Shanghai Jiao Tong Univ (China)	2017	0.06	21 (2.02%)
2	Peoples R China	2012	0.00	333 (32.05%)	Nanjing Med Univ (China)	2017	0.19	20 (1.92%)
3	Italy	2010	0.03	77 (7.41%)	Temple Univ (US)	2016	0.06	20 (1.92%)
4	England	2008	0.21	62 (5.97%)	Univ Alabama Birmingham (US)	2018	0.06	17 (1.64%)
5	Germany	2007	0.03	51 (4.91%)	Soochow Univ (China)	2015	0.10	15 (1.44%)
6	Netherlands	2007	0.19	51 (4.91%)	Fudan Univ (China)	2018	0.01	15 (1.44%)
7	Spain	2008	0.18	47 (4.52%)	Harvard Med Sch (US)	2017	0.05	14 (1.35%)
8	France	2003	0.38	42 (3.90%)	Zhejiang Univ (China)	2017	0.04	13 (1.25%)
9	Canada	2013	0.00	36 (3.46%)	Tongji Univ (China)	2017	0.23	13 (1.25%)
10	Iran	2011	0.06	34 (3.27%)	Natl Univ Singapore (Singapore)	2017	0.01	12 (1.15%)

Co-cited References and References Burst

Co-citation analysis indicated that two references appeared in the reference list of a third citation article, and then the two references formed a co-citation relationship. We listed the 12 most frequently cited references related to research on exosomes in cardiovascular medicine. Among the 834 cited references, 12 references were cited more than 100 times, and the references listed in the top three were all cited more than 150 times (**Table 4**). The most frequently cited reference topic was *Exosomes as Critical Agents of Cardiac Regeneration Triggered by Cell Therapy*, the basic experiment describing exosomes as critical factors for cardiac regeneration and cardiac protection in the paracrine pathway of stem cells.

Figure 8 shows the top 50 references with the most robust citation bursts. It can be seen that the first reference with citation bursts was in 2010. Almost all references on the exosomes in cardiovascular science focused on the reliable citation power in the past 10 years, suggesting that this research may continue to expand in the future.

The Analysis of Hotspots and the Frontiers

Keywords summarize research topics. Through the analysis of keywords, we can understand the research hotspots in specific fields. **Table 5** shows the high-frequency keywords. Among these keywords, MI and microRNA occurred over 200 times, suggesting that exosomes in CVDs may hold substantial research potential.

We used VOSviewer software to cluster the keywords. The circle and label form an element, the color of which identifies the cluster to which it belongs. **Figure 9** displays the clusters of red, blue, and green, indicating three research directions. Green clusters are composed of exosome, CVD, atherosclerosis, biomarkers, and inflammation. The keywords of the red cluster are MI, MSCs, progenitor cells, therapy, repair, regeneration, angiogenesis, cardiac function, and *in vitro*. The keywords of the blue cluster are heart failure, microRNA, cells, expression, activation, apoptosis, and mechanism.

The timeline view is designed based on the interaction and mutation relationship between keywords in a particular field, and it helps to explore the evolutionary track and stage characteristics

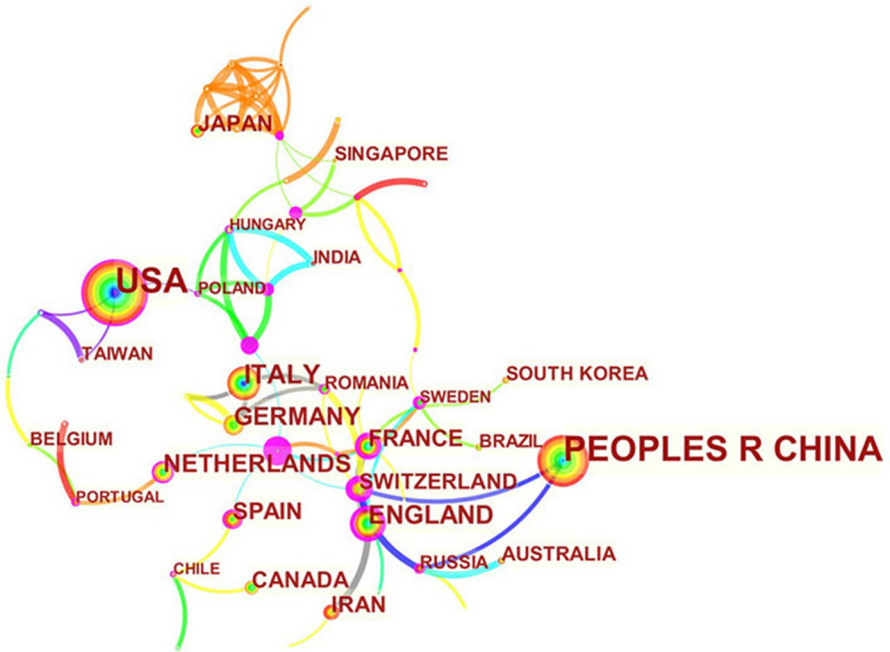

FIGURE 3 | Distribution of publications from different countries.

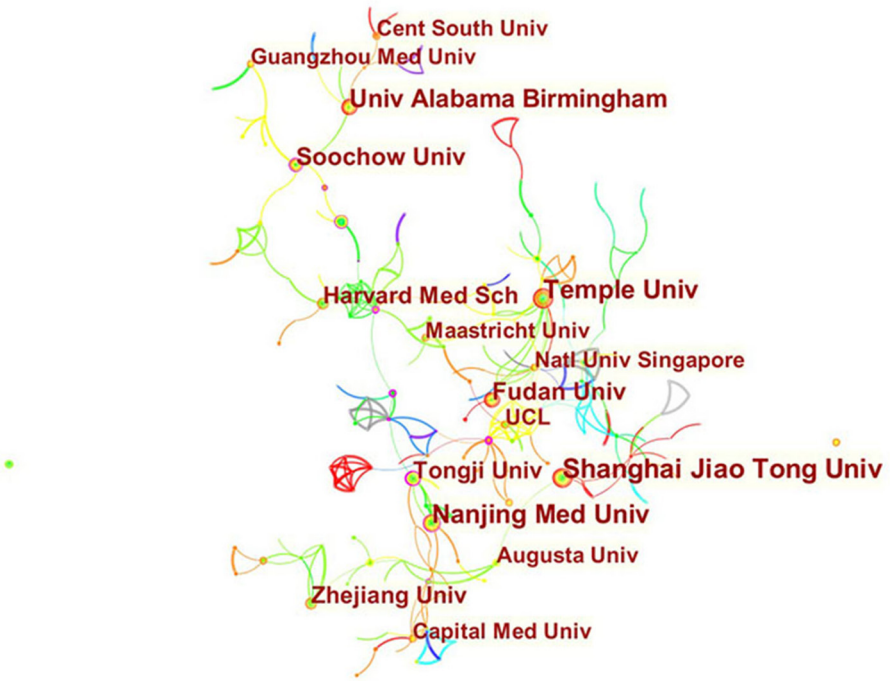

FIGURE 4 | Distribution of publications from different institutions.

TABLE 2 | Top 10 journals and co-cited journals related to exosomes in CVDs.

No.	Journal	Count (%)	IF (2020)	JCR	Co-cited journal	Citation	IF (2020)	JCR
1	Circulation research	46 (4.43%)	17.367	Q1	Circulation research	3,597	17.367	Q1
2	International journal of molecular sciences	34 (3.27%)	5.923	Q2	Circulation	2,299	29.690	Q1
3	Circulation	28 (2.69%)	29.690	Q1	Plos one	2,221	3.240	Q1
4	Frontiers in physiology	25 (2.41%)	4.566	Q2	Proceedings of the national academy of sciences of the United Stated of America	1,387	9.580	Q1
5	Scientific reports	22 (2.12%)	4.379	Q1	Journal of extracellular vesicles	1,338	25.841	Q1
6	Plos one	21 (2.02%)	3.240	Q1	Cardiovascular research	1,293	10.787	Q1
7	Journal of cellular and molecular medicine	21 (2.02%)	5.310	Q1/Q2	Nature	1,107	49.962	Q1
8	Theranostics	19 (1.83%)	11.556	Q1	Journal of biological chemistry	1,106	5.157	Q1
9	Stem cells research and therapy	18 (1.73%)	6.832	Q1/Q2	Scientific reports	1,069	4.379	Q1
10	Cardiovascular research	17 (1.64%)	10.787	Q1	Journal of the American college of cardiology	1,029	24.094	Q1
11	Journal of cardiovascular translational research	17 (1.64%)	4.132	Q2	Journal of clinical investigation	1,000	14.808	Q1
12	American journal of physiology-heart and circulatory physiology	17 (1.64%)	4.733	Q2	Blood	981	22.113	Q1
13	Frontiers in cell and developmental biology	13 (1.25%)	6.684	Q1/Q2	The journal of molecular and cellular cardiology	941	5.000	Q2
14	Frontiers in cardiovascular medicine	13 (1.25%)	6.050	Q2	Cell	874	41.582	Q1
15	Journal of molecular and cellular cardiology	12 (1.15%)	5.000	Q1/Q2	European heart journal	859	29.983	Q1

IF, Impact Factor; JCR, Journal Citation Reports.

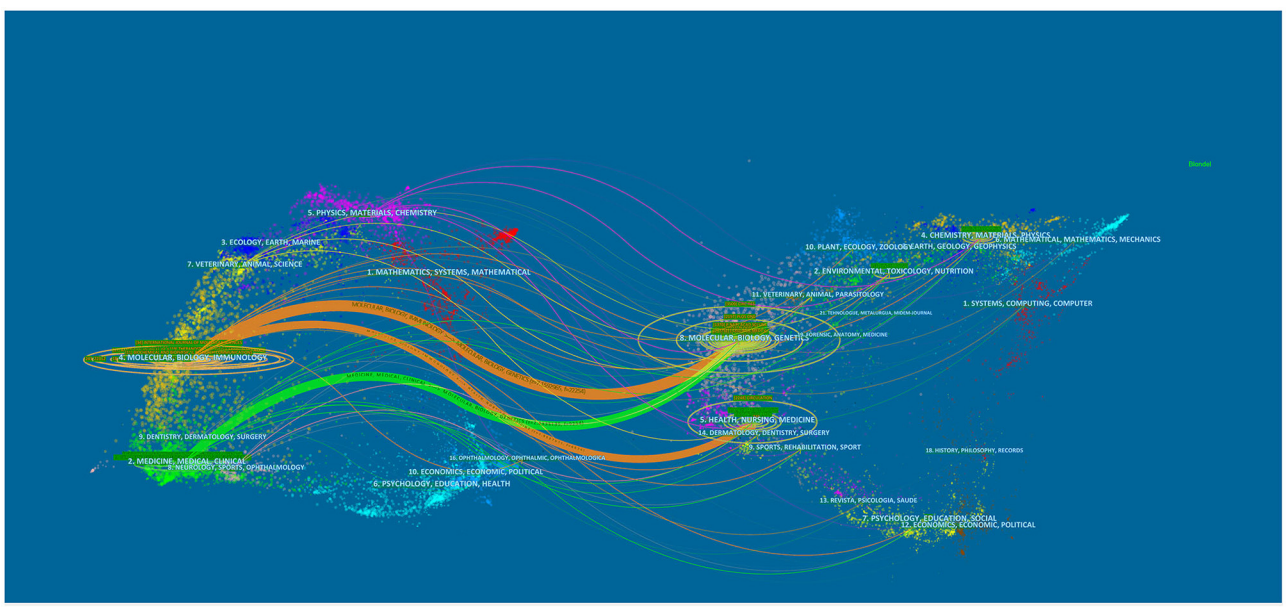

FIGURE 5 | The dual-map overlay of journals on exosomes in CVDs.

TABLE 3 | Top 10 authors and co-cited authors related to exosomes in CVDs.

No.	Author	Count (%)	Centrality	Co-Cited Author	Citation	Centrality
1	Lucio Barile	12 (1.15%)	0.02	Thery C	268	0.09
2	Abdelnaby Khalyfa	10 (0.96%)	0.00	Lai RC	244	0.05
3	Ke Cheng	9 (0.87%)	0.00	Valadi H	238	0.05
4	Yaoliang Tang	9 (0.87%)	0.01	Barile L	237	0.03
5	Costanza Emanueli	9 (0.87%)	0.01	Sahoo S	179	0.03
6	Jianyi Zhang	8 (0.77%)	0.03	Ibrahim AGE	175	0.03
7	Raj Kishore	8 (0.77%)	0.01	Arslan F	161	0.01
8	Eduardo Marban	8 (0.77%)	0.00	Raposo G	159	0.06
9	Susmita Sahoo	7 (0.67%)	0.01	Bang C	158	0.02
10	Giuseppe Vassalli	6 (0.58%)	0.00	Wang XH	156	0.01

FIGURE 6 | CiteSpace visualization map of authors involved in exosomes in CVDs.

of the field. **Figure 10** shows a timeline chart of exosomes in CVDs drawn based on CiteSpace software; it visually reflects the phased hotspots and developmental path of exosomes in CVD research from the time dimension. From 2003 to 2010, research on exosomes in cardiovascular science had not yet garnered attention. The research during this period was focused on the cell and extracellular vesicle levels. The main keywords were induction, platelet microparticle, expression, oxidative stress, and smooth muscle cell. From 2010 to 2019, research on exosomes in cardiovascular science increased, and related mechanism research accelerated. The main keywords were biomarker, MI, MSCs, angiogenesis, cancer, diabetes mellitus, peripheral blood, apoptosis, pathway, miRNA, diagnosis, inflammation, heart failure, hypertrophy, cardioprotection, ischemia/reperfusion injury, cardiac fibrosis, and gene expression. In the past 2 years, scholars began to explore the potential and value of exosomes in clinical research. The main keywords were a clinical trial, targeted delivery, stroke, poor prognosis, translation, isolation, and cardiotoxicity.

DISCUSSION

General Information

The trend of annual publications published from 2001 to 2006 demonstrated that the studies during this period were lacking, suggesting that research on exosomes in CVDs was not in-depth and lacked a research basis between exosomes and disease. From 2007 to 2010, small numbers of articles began to appear; however the research during this period was embryonic. From 2010 to 2016, the number of articles began to increase, and a growing number of investigators began to pay attention to the role of exosomes in cardiovascular science (40–45). In the past 5 years, the number of published articles grew rapidly, suggesting that the research of exosomes is becoming more mature and its research in cardiovascular science is likely to become a hot topic and research direction in the future.

Using visual analysis of the distribution of countries and institutions, we can see that the US and China are the leading countries where research on exosomes of CVDs is occurring.

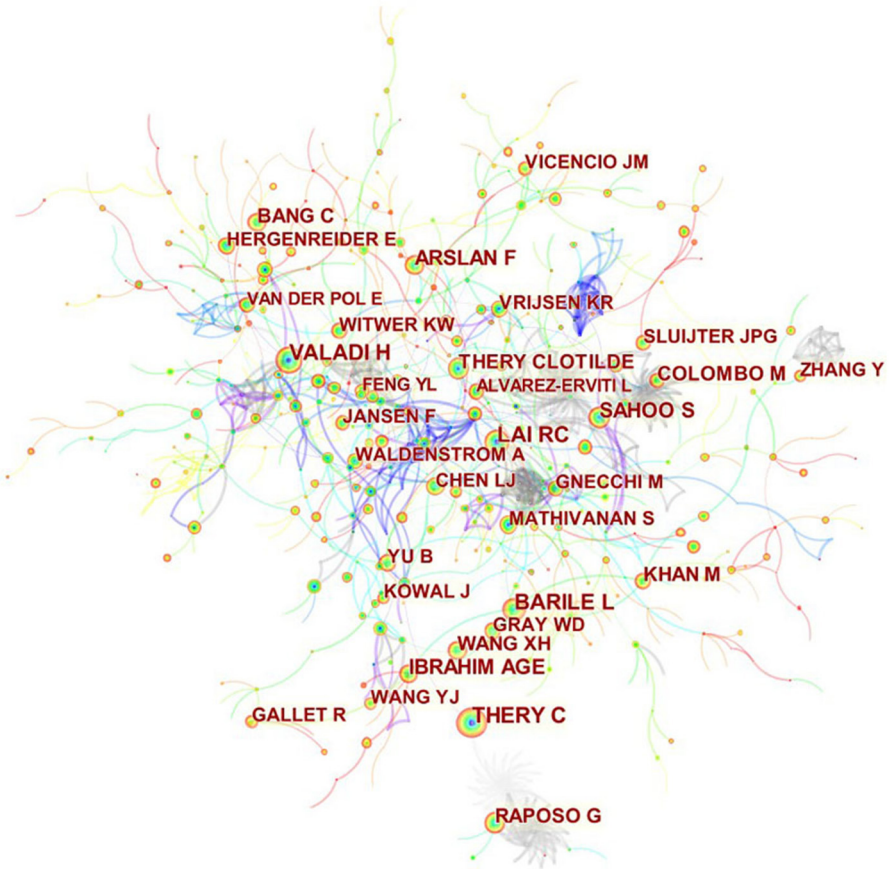

FIGURE 7 | CiteSpace visualization map of co-cited authors involved in exosomes in CVDs.

TABLE 4 | Top 12 co-cited references related to exosomes in CVDs.

No.	Reference	Citation	Year	Centrality
1	Exosomes as critical agents of cardiac regeneration triggered by cell therapy	175	2014	0.03
2	Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury	159	2013	0.03
3	Extracellular vesicles from human cardiac progenitor cells inhibit cardiomyocyte apoptosis and improve cardiac function after myocardial infarction	158	2014	0.01
4	Cardiac fibroblast–derived microRNA passenger strand-enriched exosomes mediate cardiomyocyte hypertrophy	146	2014	0.02
5	Embryonic stem cell-derived exosomes promote endogenous repair mechanisms and enhance cardiac function following myocardial infarction	141	2015	0.02
6	Exosome secreted by MSC reduces myocardial ischemia/reperfusion injury	123	2010	0.08
7	Extracellular vesicles: Exosomes, microvesicles, and friends	118	2013	0.02
8	Cardiac progenitor-derived exosomes protect ischemic myocardium from acute ischemia/reperfusion injury	118	2013	0.04
9	Identification of therapeutic covariant microRNA clusters in hypoxia treated cardiac progenitor cell exosomes using systems biology	114	2015	0.03
10	Exosomes secreted by cardiosphere-derived cells reduce scarring, attenuate adverse remodeling, and improve function in acute and chronic porcine myocardial infarction	106	2017	0.02
11	Plasma exosomes protect the myocardium from ischemia-reperfusion injury	102	2015	0.01
12	Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles	100	2014	0.00

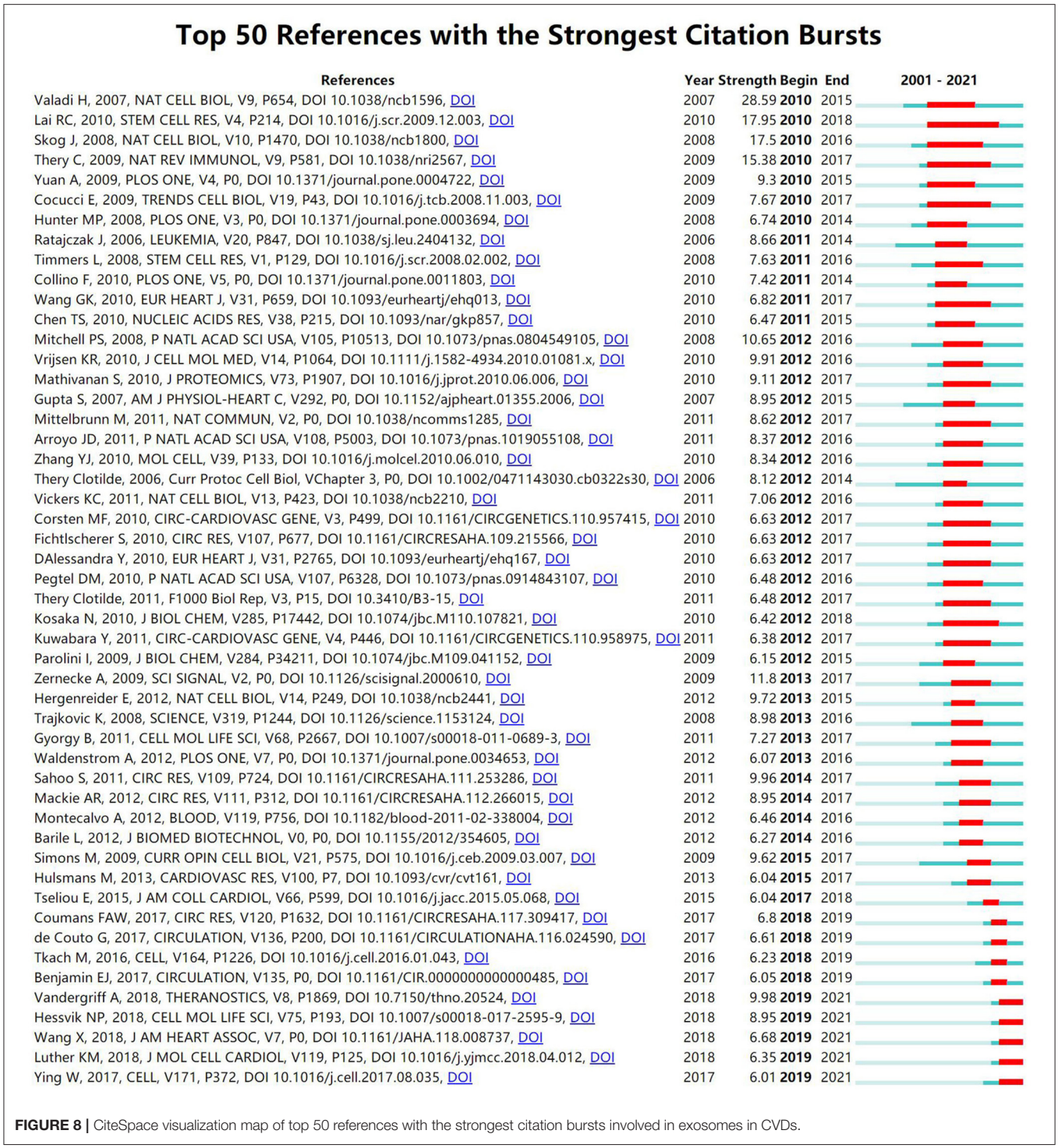

FIGURE 8 | CiteSpace visualization map of top 50 references with the strongest citation bursts involved in exosomes in CVDs.

Among the top 10 study institutions, nearly all research institutions were in these two countries, including Shanghai Jiao Tong University, Nanjing Med University, Temple University, and the University of Alabama Birmingham. As shown in **Figures 3**, **4**, although countries possess their cooperation networks, the breadth, and intensity of cooperation were not ideal. For example, the US and China do not cooperate or communicate. From the perspective of research institutions, most cooperating institutions are limited to internal connections, and there is substantially less transnational cooperation and exchange of findings. This situation hinders the development of this research field. Therefore, it is strongly suggested that the

TABLE 5 | Top 20 keywords related to exosomes in CVDs.

No.	Keywords	Count	Centrality	No.	Keywords	Count	Centrality
1	Exosome	721	0.13	11	Stem cell	128	0.01
2	Extracellular vesicle	332	0.01	12	Angiogenesis	118	0.03
3	Myocardial infarction	266	0.08	13	Heart failure	115	0.02
4	MicroRNA	222	0.03	14	Therapy	102	0.03
5	Microvesicle	184	0.04	15	Mechanism	101	0.03
6	Mesenchymal stem cell	167	0.05	16	Endothelial cell	100	0.03
7	Heart	147	0.03	17	Cell	91	0.03
8	Cardiovascular disease	147	0.10	18	Inflammation	86	0.02
9	Biomarker	139	0.02	19	*In vitro*	75	0.04
10	Expression	136	0.09	20	Regeneration	71	0.04

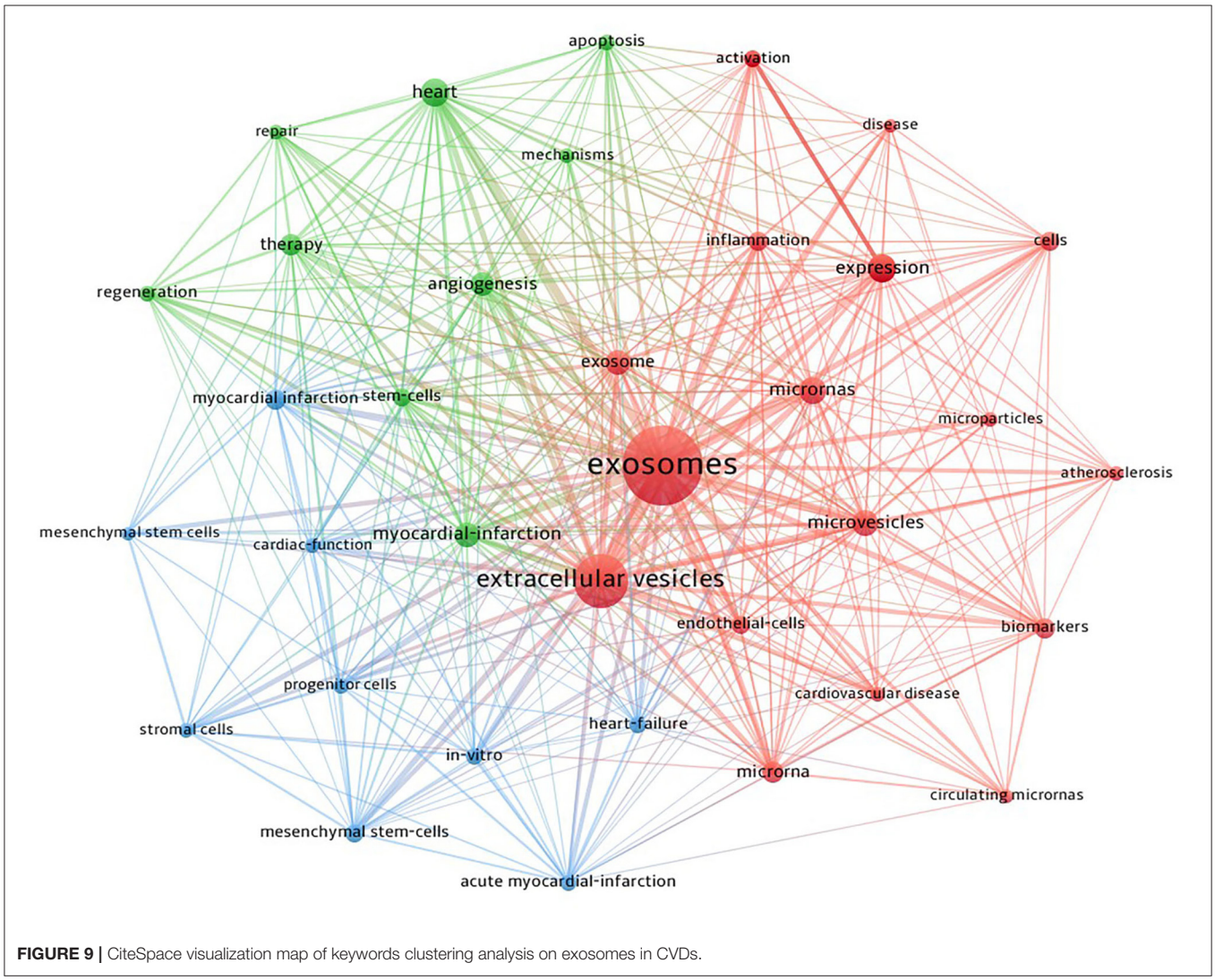

FIGURE 9 | CiteSpace visualization map of keywords clustering analysis on exosomes in CVDs.

US and China and research institutions from other countries should remove academic barriers, cooperate, and communicate to promote the research and development of exosomes in cardiovascular science.

Several studies of exosomes were published in influential cardiovascular journals such as *Circulation Research*, and *Circulation;* however, exosomes also have potential value based on other journals. For example, in addition to research on

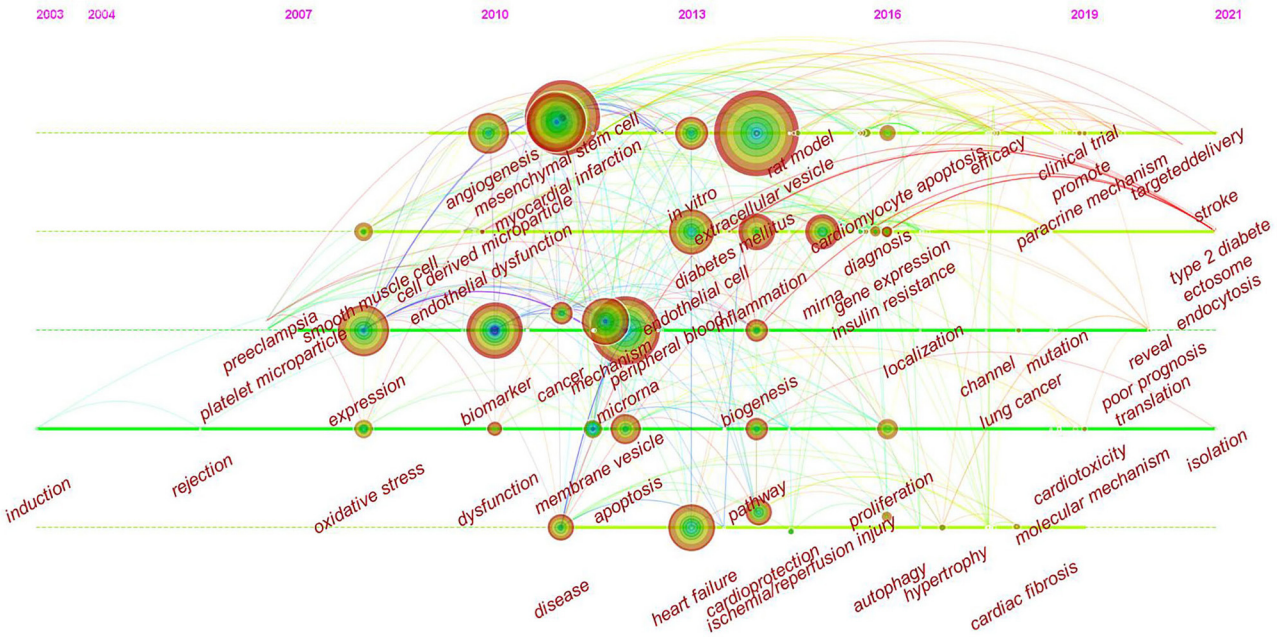

FIGURE 10 | CiteSpace visualization map of timeline viewer related to exosomes in CVDs.

CVDs, exosomes in cardiovascular science also involve molecular science (46), pharmacology (47), therapeutics (48), stem cells (49), and heart transplantation (50). Regarding co-cited academic journals, we can see that the majority of studies are from high-impact journals. These journals have a significant influence on the international community and affect the research direction of their respective academic fields. In **Figure 5**, the dual-map overlay of journals indicates the subject distribution of academic journals. Considering the two main pathways in the diagram, research on exosomes in CVDs is limited to basic research and begins to convert primary research results into clinical research.

From the perspectives of author contributions and co-cited authors, Lucio Barile, a Swiss scholar, made the most contributions with 12 published articles, followed by Abdelnaby Khalyfa from the University of Chicago with 10 articles. It should be noted that Barile had the most significant number of published articles and ranked fourth among the co-cited authors. Barile's team studies exosomes, stem cells (51) and cardiac protection (52). In a frequently cited animal study, this group showed that exosomes derived from cardiac-resident progenitor cells might have a better cardioprotective effect than exosomes originated in marrow-derived MSCs, and the exosomes can play a role by binding related active proteins on its surface to ligands, which will provide a basis for future research on the cardioprotective effects of stem cell-derived exosomes (53). In 2020, Barile showed that during heart transplantation, plasma-derived circulating exosomes combined with endocardial, myocardial biopsy could be used as biomarkers to diagnose allograft rejection according to the differential protein spectrum on the membrane surface (13). These articles suggest that exosomes are likely to become physiological or pathological diagnostic markers in clinical practice in addition to their cardioprotective therapeutic effect. Among co-cited authors, Thery (268) was the most frequently cited, followed by Lai (244), Valadi (238), and Barile (237). One point that is often overlooked is that, although the number of articles and citations of contributing authors and co-cited authors is significant, their influence in this research field remains insufficient. Therefore, in the future, we should pay attention to the number of articles and improve the impact of the results.

There were more than 100 top 12 co-cited references. The top three co-cited references (15, 54, 55) focused on the effects of stem cell-derived exosomes on MI. These authors found cardioprotective effects of exosomes on MI, including reducing cardiomyocyte apoptosis, promoting revascularization, and reducing the size of MI. The most explosive reference was published in *Nature Cell Biology* by Valadi's group (54). The authors showed that exosomes could be used as signal carriers to mediate cell communication by transporting bioactive substances such as RNA and proteins. In recent years, the references with the highest citation frequency focused on exosome engineering (56), targeted therapy (57), and regenerative medicine (58). It can be seen that some breakthroughs have been made in the research of exosomes in cardiovascular science using advanced technology.

The Hotspots and Frontiers

Keywords summarize research topics and core content. Based on keyword co-occurrence analysis, the distribution and development of various research hotspots in a particular field can be understood. In addition to exosomes and CVDs, the keywords that frequently appear in **Table 5** are MI, microRNA, MSC,

biomarker, and expression. Clustering was performed based on keyword co-occurrence analysis to obtain a clustering map of exosome keywords in CVDs (**Figure 9**), and finally, the clusters of three colors were formed.

According to clustering analysis of keywords and the timeline viewer (**Figure 10**), research on exosomes in the cardiovascular science mainly focused on the following aspects:

Role of Exosomes Derived From Stem Cells After MI

As regenerative medicine is approaching clinical applicability, to identify cardioprotection strategies to regenerate lost myocardium and restore cardiac function, cellular therapy for CVDs has become an active area of research (59). Stem cells potentially proliferate and differentiate, with self-renewal and replication, producing highly differentiated functional cells (60, 61). However, some obstacles to therapy include immunological incompatibility, complex operation, high cost, cancers risks, and the potential to form ectopic tissue (62). Several studies reported that the transfusion of stem cells could cause arrhythmias, pulmonary embolism, and vascular obstruction to varying degrees, resulting in the death of animals or patients. Poor engraftment and biosafety of stem cells directly affect the clinical application, becoming a significant obstacle to its commercial development (63, 64). Recently, evidence has suggested that stem cells exert their therapeutic effects in a paracrine manner, primarily through exosomes to enhance cell survival, differentiation, and adaptive immune responses. Exosomes without self-replicating capabilities have a lower risk of ectopic differentiation, tumor formation, genetic instability, and immune system rejection.

MI refers to the local myocardial ischemia and anoxic necrosis caused by coronary artery occlusion and blood flow interruption. Restoration of blood supply is the hallmark of the treatment of ischemic diseases, and a critical treatment is the promotion of angiogenesis. Several lines of evidence demonstrated that stem cells such as cardiosphere-derived cells, embryonic stem cells, MSCs, induced pluripotent stem cells (iPSCs), stem cells derived from adipose tissue, and derived exosomes could promote angiogenesis. Stem cell-derived exosomes contain pro-angiogenic factors or angiogenic-related regulatory factors to promote angiogenesis. Investigators can manipulate exosomes such as cargo carriers to transport biological molecules to target cells to promote angiogenesis by exosomes engineering or treating parent cells differently (65). Gao et al. found that exosomes derived from human iPSCs promote endothelial cell tube formation and microvessel sprouting from mouse aortic rings and protect human iPSCs-cardiomyocytes by reducing apoptosis, maintaining intracellular calcium homeostasis increasing adenosine 5′-triphosphate (66). Another animal study showed that exosomes derived from embryonic stem cells enhanced cardiac functional recovery after MI and promoted angiogenesis, improving myocardial cell survival and reducing myocardial fibrosis. Notably, 8 weeks after *in vivo* transplantation, embryonic stem cell-derived exosomes enhanced the survival and proliferation of cardiac progenitor cells to form new cardiomyocytes in ischemic areas (67). These findings suggest that stem cell-derived exosomes promote angiogenesis, exert anti-inflammatory and cardioprotective effects, reduce apoptosis, and inhibit fibrosis.

Although exosomes provide therapeutic opportunities for ischemic diseases, especially MI, several limitations restrict their clinical application. First, the production, isolation, storage of exosomes, and culture conditions of their parent cells lack optimal protocols and remains a lack of reliable potency assays to evaluate the efficacy of exosomes therapy. Researchers have used several methods to isolate exosomes; the most common are ultracentrifugation-based techniques and ultrafiltration; however, it appears that the isolation method affects experimental results, at least to some extent (68). Second, researchers need to pay attention to the health and physiological status of the parent cells that secrete exosomes. Rezaie et al. reported MSCs and their exosomes kinetic are affected by aging and other aged exosomes. Exosomes from aged MSCs lose their regeneration potential, accelerating biological development and negatively impacting the function of recipient cells (69). Choosing an appropriate pathway for exosomes to access target sites is another issue that needs to be addressed. Biomaterials such as hydrogels have been developed to deliver large doses of exosomes to target tissues to induce angiogenesis to overcome the disadvantages of faster clearance by the intravenous injection route, considered the most widely used. Optimizing the administration method to obtain high efficacy and specificity to treat specific diseases is critical to the clinical application of exosomes (70). Finally, the uptake ability of target cells may also affect the treatment effects of exosomes.

Regarding these, exosomes originating from stem cells have attracted considerable attention in tissue engineering and regeneration. Nevertheless, there is a paucity of large-scale clinical data to verify this. Further research is needed to overcome these limitations in the future (65).

Function and Mechanism of Exosomes in CVDs

Nearly all cell types can spontaneously create or produce exosomes under certain stimuli, and the generation of exosomes involves double invagination. The first invagination of exosomes occurs *via* inward budding of the plasma membrane to form a cup-shaped intracellular endosomal compartment that contains cell-surface proteins, fluid, and extracellular constituents such as proteins, lipids, and metabolites (71). Further invagination of the intracellular endosomes forms multivesicular bodies (MVBs) that contain specific intraluminal vesicles of various sizes and specific content. MVBs are either degraded by fusing with lysosomes or autophagosomes or fusing with the plasma membrane to release their contents into the extracellular microenvironment as exosomes (72, 73). These findings suggest that exosomes promote cell-to-cell communication by carrying cargos of origin to transfer signals from one cell to another. The heart is a complex of different cells, including cardiomyocytes, cardiac progenitor cells, endothelial cells, fibroblasts, vascular smooth muscle cells, and immune cells. Intercellular communication and crosstalk maintain the homeostasis and function of the heart and accelerate the pathological process of various types of CVDs (74, 75). Zheng et al. found that vascular smooth muscle cell-derived exosomes mediate the transfer of Krüppel-like factor 5-induced miR-155 from smooth muscle cells to endothelial cells,

damaging the tight junction of endothelial cells and the integrity of barriers leading to increased endothelial cell permeability and accelerating atherosclerosis (76). Macrophage-derived exosome miR-21-3p, which has been treated with nicotine, the principal component of cigarette smoke, may accelerate the development of atherosclerosis by increasing the migration and proliferation of vascular smooth muscle cells through its target protein (77). Exosomes mediate the occurrence and development of CVDs *via* upstream or downstream signaling pathways *in vivo*, and the relevant mechanisms are being elucidated (78–80).

Exosomes are used to develop and deliver drugs for therapeutic purposes. Many synthetic drug delivery systems have been developed and introduced to the market over the past few decades; however, their application has been limited due to inefficiency, cytotoxicity, or immunogenicity. Natural drug carrier systems have developed rapidly, especially involving exosomes. Compared with synthetic drug delivery systems, exosomes can be distributed throughout the body and cross the blood-brain barrier by using natural intracellular transport capacity and biocompatibility. Akbari and Rezaie found that exosomes can be used as drug delivery systems for treating severe acute respiratory syndrome coronavirus 2 pneumonia. Exosomes have advantages over other nanocarriers in that they are phospholipid vesicles that are derived from cells. They are relatively safe with low immunogenicity and can pass through physiological barriers. In this respect, exosomes can be constructed by direct or indirect engineering. Direct engineering refers to loading therapeutic agents such as biomolecules or drugs directly into exosomes, and then these exosomes are delivered to target tissues. Indirect engineering refers to co-culture or genetic modification of parent cells and therapeutic agents to produce artificial/drug-loaded exosomes (62). Sun et al. demonstrated that encapsulating curcumin in exosomes enhanced anti-inflammatory activity (81). Tang and colleagues reported that inoculating chemotherapeutic drugs with tumor cells allows the drugs to be packaged as exosomes, and collecting these exosomes and using them in a mouse tumor model killed tumor cells with no significant side effects (82). Nevertheless, more efforts are needed to realize the transformation and application of exosomes. Clinically, it is necessary to overcome the difficulties in the production, isolation, and storage of exosomes and explore the mechanism of exosomes production, cargo classification, internalization, and transportation.

Application of Exosome as a Biomarker in CVDs

The gold standard for diagnosing coronary atherosclerosis and viral myocarditis remains invasive examination (83, 84), while MI and acute heart failure require specific and rapid diagnosis (85). Exosomes are secreted by endothelial cells, cardiac progenitor cells, cardiac fibroblasts, and cardiomyocytes, suggesting that exosomes are involved in CVDs. Exosomes secreted by damaged or diseased hearts can carry intracellular substances that may reflect cell origin and pathophysiological states as signatures or fingerprints of donor cells. In various clinical environments, substances such as RNA and proteins carried by exosomes may serve as prognostic and diagnostic markers of CVDs (86–89).

In the updated ExoCarta, several contents were identified in exosomes, including ∼9,769 proteins, 3,408 mRNA, 2,838 miRNA, and 1,116 lipids. In CVDs, the content of exosomes can be altered according to the severity of the disease. A study showed that exosomes and circulating miRNAs were significantly increased after myocardial ischemia-reperfusion injury in pigs. Myocardial and muscle-specific miRNAs rapidly increased in plasma 2.5 h after ischemia, while the number of exosomes was increased as early as 1 h after ischemia. It was found that accumulated exosomes were enriched with miRNA-133b, miRNA-208b, and miRNA-499 (90). Cargo RNAs of exosomes contain various biotypes, including mRNA, miRNA, lncRNA, and circRNA. This diversity of RNAs, the heterogeneity of exosomes, and the overall low concentration of RNAs in exosomes complicate the characterization of RNA cargo of exosomes. More than 200 miRNAs exist in the heart, and cardiac-derived miRNAs participate in heart development and function regulation. In recent years, miRNAs have been shown to function as micromodulators in cellular communications and are involved in cell signaling and microenvironment remodeling. Exosomes containing miRNAs serve as biomarkers for the diagnosis and outcome prediction of CVDs (91–96). Studies showed that the cardiac specificity of miRNAs appears to be better than conventional diagnostic tests such as troponin because miRNAs can be detected more rapidly and are more sensitive and specific than myocardial troponin in the context of acute MI. Several studies showed that the miRNAs of heart-derived exosomes could be detected in urine, which opens the way for liquid biopsy in cardiovascular medicine (97–99).

Exosome as biomarkers have several advantages. They can travel in several body fluids and are involved in various pathophysiological processes throughout disease progression. They are also convenient to sample. More importantly, the lipid bilayer membrane of the exosome protects the miRNA from decomposition (100–103).

Research on exosomes in cardiovascular science is increasing exponentially, and the accumulated research contributes to the potential of exosomes as non-invasive clinical biomarkers. However, given further development of precision medicine, it is critical to consider individual differences' molecular characteristics, lifestyle, and environmental impact (99).

LIMITATIONS

CiteSpace and VOSviewer cannot wholly replace system retrieval. The uneven quality of collected literature data can reduce the credibility of atlas drawing, and the differences of data update also cause the retrieval results to differ from the actual number of included articles. Therefore, more accurate literature analysis should be based on the knowledge map constructed by CiteSpace and VOSviewer combined with specific literature. Nevertheless, literature analysis based on visualization undoubtedly lays the foundation for scholars to

quickly understand the research hotspots and development trends of exosomes in cardiovascular science.

CONCLUSION

Exosomes possess essential research value and application prospects in cardiovascular science. Using CiteSpace and VOSviewer software for visual analysis, research on exosomes in CVDs demonstrated a substantial development trend. Increasing numbers of articles published in international core journals suggest a significant impact. The leading countries are the US and China; however, there is a need for enhanced cooperation and exchange between countries and institutions. All scholars should increase the number of articles and strengthen the influence of articles. In addition to focusing on basic research, we should focus on the transformation of results and the study of exosomes in patients with CVDs. At present, the research on exosomes in cardiovascular science focuses on ischemic heart disease, pathogenesis, regeneration, stem cells, targeted therapy, biomarkers, and cardiac protection, and these will serve as the focus of future research.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

DM and SL conceived the study. BG, LS, and TW collected the data. DM, LZ, and ZZ re-examined the data. BG, QL, and YF analyzed the data. DM wrote the manuscript. ZG, SL, and HX reviewed and revised the manuscript. All authors contributed to the article and approved the submitted version.

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Blockade of Exosome Release Suppresses Atrial Fibrillation by Alleviating Atrial Fibrosis in Canines With Prolonged Atrial Pacing

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Background: Clinical studies have shown that exosomes are associated with atrial fibrillation (AF). However, the roles and underlying mechanisms remain unclear. Hence, this study aimed to investigate the function of exosomes in AF development.

Methods: Twenty beagles were randomly divided into the sham group ($n = 6$), the pacing group ($n = 7$), and the pacing + GW4869 group ($n = 7$). The pacing and GW4869 groups underwent rapid atrial pacing (450 beats/min) for 7 days. The GW4869 group received intravenous GW4869 injection (an inhibitor of exosome biogenesis/release, 0.3 mg/kg, once a day) during pacing. Electrophysiological measurements, transmission electron microscopy, nanoparticle tracking analysis, western blotting, RT-PCR, Masson's staining, and immunohistochemistry were performed in this study.

Results: Rapid atrial pacing increased the release of plasma and atrial exosomes. GW4869 treatment markedly suppressed AF inducibility and reduced the release of exosomes. After 7 days of pacing, the expression of transforming growth factor-β1 (TGF-β1), collagen I/III, and matrix metalloproteinases was enhanced in the atrium, and the levels of microRNA-21-5p (miR-21-5p) were upregulated in both plasma exosomes and the atrium, while the tissue inhibitor of metalloproteinase 3 (TIMP3), a target of miR-21-5p, showed a lower expression in the atrium. The administration of GW4869 abolished these effects.

Conclusions: The blockade of exosome release with GW4869 suppressed AF by alleviating atrial fibrosis in a canine model, which was probably related to profibrotic miR-21-5p enriched in exosomes and its downstream TIMP3/TGF-β1 pathway.

Keywords: exosomes, atrial fibrillation, fibrosis, MiR-21-5p, TIMP3, canine

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INTRODUCTION

Atrial fibrillation (AF) is the most commonly sustained tachyarrhythmia in the clinic, and its incidence is increasing markedly with the aging population (1). Previous studies have shown that cellular Ca^{2+}-handling abnormalities and ectopic/triggered activity led to the initiation of AF, while cardiac structural remodeling provides substrates for the maintenance of AF (2). Fibrosis is the

main pathological process of structural remodeling. Although inflammation, oxidative stress, and microRNAs (miRNAs) partly account for it (3), the molecular mechanisms of atrial fibrosis during AF remain to be elucidated.

Exosomes are 40–150-nm small extracellular vesicles secreted by most cells. RNA (including mRNA, miRNA, and other non-coding RNA), proteins, and lipids are selectively incorporated into exosomes and then released into the extracellular space (4). In recent years, growing evidence has shown that exosomes mediate cardiac fibrosis by protein interactions or post-transcriptional translation regulation (5). The renin–angiotensin system is believed to be a major contributor to cardiac fibrosis. Pironti et al. found that heart tissue could release functional angiotensin II type I receptor-enriched exosomes in a mouse model with cardiac pressure overload (6). miRNAs, another major component of exosomes, have also been shown to be involved in the pathogenesis of cardiac fibrosis. Yang et al. identified that cardiomyocyte-derived exosomes containing miR-208a promoted cardiac fibroblast (CF) proliferation and differentiation into myofibroblasts (7). Nevertheless, several studies have confirmed that exosomal miRNAs suppress CF proliferation and antagonize fibrosis progression (8). Exosomes can function differently under different stimulation conditions and microenvironments. What roles exosomes play in the progression of AF remain unknown.

To date, evidence regarding associations between exosomes and AF has mainly focused on clinical research. Some scholars found that plasma exosomes differentially expressed miRNAs between AF and sinus rhythm (9, 10). Bioinformatics analysis showed that these miRNAs and their targets possibly contribute to atrial fibrosis (11). Moreover, it has been demonstrated that, compared with the control group, the epicardial adipose tissue of patients with AF secretes more exosomes, and the expression of pro-inflammatory and pro-fibrotic factors is higher in exosomes (12). In addition, a recent study reported that myofibroblast-derived exosomes reduced the expression of voltage-gated L-type calcium channel subunit α1c ($Ca_v1.2$) and increased the vulnerability to AF (13). Based on these studies, we hypothesized that the increased secretion of exosomes was likely to promote AF.

In this study, we aimed to observe the change in exosome release as well as the effect of exosomes on AF inducibility and atrial fibrosis in a canine model of AF. Furthermore, we also tried to investigate the possible mechanism of cardiac fibrosis mediated by exosomes.

MATERIALS AND METHODS

This study was approved by the animal studies subcommittee of our institutional review board and was in accordance with the guidelines of the National Institutes of Health for the care and use of laboratory animals.

Animal Model Preparation

All male beagle canines were maintained under the same conditions in the Animal Experimental Center of Renmin Hospital at Wuhan University. Canines aged 8–12 months with body weights of 8–10 kg were randomly assigned to three groups. The sham group ($n = 6$) received pacemaker implantation under sterile conditions without atrial pacing. The pacing group ($n = 7$) received pacemaker implantation with continuous rapid atrial pacing (450 beats/min) for 7 days. The pacing + GW4869 group ($n = 7$) underwent the same pacing model as the pacing group while administered with a slow intravenous injection (0.3 mg/kg, once a day) of GW4869 (MedChemExpress, USA). Each canine received an intravenous injection of 30 mg/kg pentobarbital sodium before the operation. After successful anesthesia, intubation and ventilation with room air supplemented with oxygen from a respirator (MAO01746, Harvard Apparatus Holliston, USA) and continuous ECG monitoring were performed.

Cardiac Pacemaker Implantation

Under fluoroscopic guidance, the atrial electrode was implanted in the right atrial appendage for stimulation through the right external jugular vein and then connected to the high-frequency pacemaker placed in the pouch made under the subclavian skin. The pacemaker was programmed to stimulate the right atrium at a frequency of 450 beats/min. After the right atrium showed successful pacing, the electrode was fixed, and the pouch was sutured. Before prolonged pacing started, the canines received post-operative recovery for 3 days, and four million units of penicillin were intramuscularly injected twice a day.

Electrophysiological Measurements

Multielectrode catheters were guided to the four pulmonary veins and the left and right atrium. The atrial effective refractory periods (AERPs) were measured using the S_1S_2 programmed stimulation protocol (including eight S_1 regular train stimuli and one S_2 pre-mature stimulus at twice the pacing threshold intensity). The cycle length (CL) of S_1 was 250 ms, whereas S_1S_2 intervals started as 180 ms and were first decreased by 10 ms and then reduced by 2 ms with each stimulus cycle until the AERP was reached. The longest S1S2 interval without atrial pacing induced by S_2 pre-mature stimulus was recorded as AERP (**Figures 1A,B**). The dAERP was calculated by the maximum AERP minus the minimum AERP at all recording sites. The inducibility and duration of AF were assessed using programmed S_1S_1 stimulation (a 5-s burst at CL of 120, 100, 75, and 60 ms, three times at every frequency). AF was defined as an irregular atrial rate >500 lasting for more than 5 s (**Figures 1C,D**). AF inducibility and AF duration were determined by the number of episodes and the maximum duration induced by all bursts of every canine, respectively. Data were recorded by a computerized electrophysiology system (Lead 7000, China). During these operations, canines were supplied with 5 mg/kg pentobarbital sodium to maintain anesthesis every 2 h.

Histology and Immunohistochemistry

At the end of the experiment, the canines were euthanized with an intravenous injection of excess pentobarbital sodium. The hearts were quickly excised and washed with phosphate-buffered saline (PBS). Then, the atrial tissues were fixed with 4% paraformaldehyde and paraffin-embedded. Deparaffined sections

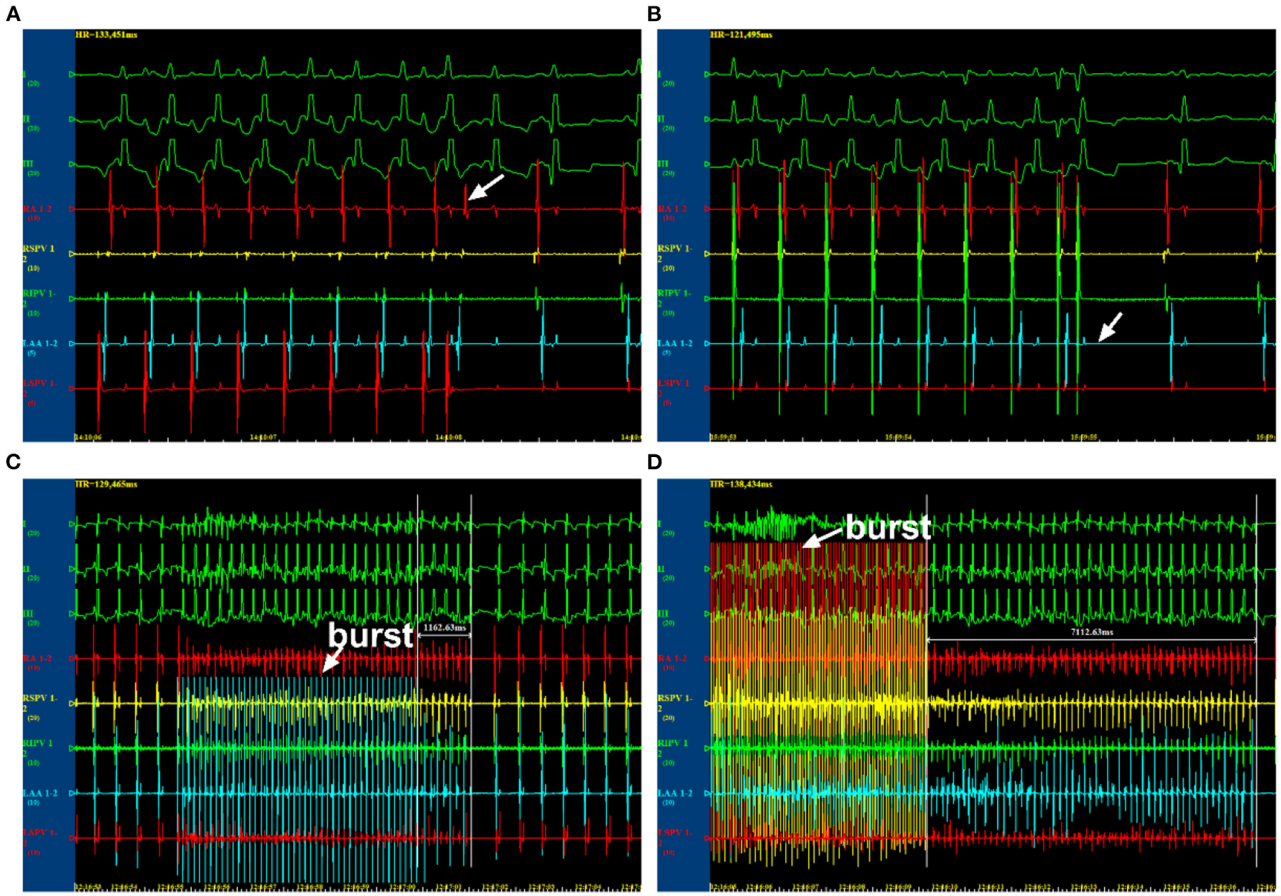

FIGURE 1 | Electrophysiological examination by programmed stimulations. **(A)** The state at which AERP was not reached. **(B)** The state at which AERP was reached. **(C)** The state at which atrial fibrillation (AF) was unable to be induced. **(D)** The state at which AF was induced. AERP, atrial effective refractory period.

(4 μm in thickness) were stained with Masson's trichrome reagent. The degree of atrial fibrosis was calculated as the percentage of collagen area. For immunohistochemistry staining, the deparaffined sections were subjected to heat-mediated antigen retrieval. The primary antibody anti-CD63 (Sanying Biotechnology, China) was used to incubate atrial samples, followed by secondary antibody HRP-labeled goat anti-rabbit IgG (ASPEN Biotechnology, China). Image-Pro Plus 6.0 software (Media Cybernetics, USA) was used to analyze images. Three visual fields of the right atrium at ×200 were tested randomly in each sample.

Exosome Isolation

Blood samples were obtained from the jugular vein and centrifuged at 3,000 rpm for 10 min to remove cellular debris. The supernatants were transferred to fresh tubes and stored at −80°C until analysis. Exosomes were isolated using Total Exosome Isolation Reagent (from serum) (Invitrogen, USA) according to the instructions of the manufacturer. A one-quarter volume of isolation reagent was added to each serum sample, and then the samples were mixed and kept at 4°C for 30 min. Each mixture was centrifuged at 10,000 g for 10 min, and the supernatants were removed. The pellet was resuspended in 100–150 μl of 0.22-μm filtered cold PBS and stored at −80°C for subsequent analysis. The volumes of serum were recorded to calculate the concentration of exosomes.

Exosome Characterization

Exosomes were characterized by transmission electron microscopy (TEM), nanoparticle tracking analysis (NTA) and western blotting. For TEM, 100 μl of isolated exosomes was fixed in 25% glutaraldehyde overnight, dropped onto a copper net, and stained with 2% phosphotungstic acid oxalate for 2 min. Images were acquired by a transmission electron microscope (HT-7700, Japan). For NTA, briefly, 50 μl of isolated exosomes were washed and diluted to the appropriate concentration with 1X PBS buffer. The volumes and dilution ratios of exosomes were recorded. The size range and particle concentration of the exosomes were examined using a Zeta View instrument (PMX 110, Germany). The expression of exosome marker proteins (CD63, CD81, and TSG101) was determined by western blotting. The antibody information is listed in the western blotting section.

Real-Time Fluorescent Quantitative PCR

Total RNA was extracted from the isolated exosomes and atrial tissues using TRIpure Total RNA Extraction Reagent (ELK Biotechnology, China) according to the protocols of the manufacturers. The relative levels of miR-21-5p and U6 were detected by Stem-Loop RT-qPCR using an Enturbo™ SYBR Green PCR Supermix Kit (ELK Biotechnology, China) with a StepOne™ Real-Time PCR instrument (Life Technology, USA). The data were analyzed by $2^{-\Delta\Delta CT}$ method.

Western Blotting

Total proteins were extracted from atrial samples using radioimmunoprecipitation assay buffer plus phosphoprotease inhibitors (ASPEN Biotechnology, China). The same amount (40 μg) of extracted protein was separated by electrophoresis in SDS-PAGE gels and transferred to polyvinylidene difluoride membranes. Membranes were blocked with 5% blocking buffer for 1 h at room temperature and incubated overnight at 4°C with primary antibodies against CD63 (Biorbyt, England), CD81 (Abcam, USA), TSG101 (Sigma-Aldrich, Germany), Rab27a (Sanying Biotechnology, China), collagen I (Novusbio, USA), collagen III (Abcam, USA), matrix metalloproteinase (MMP)-2 (Bioss, USA), MMP-9 (Bioss, USA), tissue inhibitor of metalloproteinase 3(TIMP3) (Lsbio, USA), and transforming growth factor-β1 (TGF-β1) (Sanying Biotechnology, China). The membranes were washed three times with tris-buffered saline with 0.1% Tween® 20 and then incubated with horseradish peroxidase-conjugated anti-rabbit secondary antibody (ASPEN Biotechnology, China) for 1 h at room temperature. The blots were exposed with an ECL Detection Kit (ASPEN Biotechnology, China). The expression levels of the proteins were determined and normalized to the relative intensity of GAPDH using image analyzer software (AlphaEase FC, USA).

Statistical Analysis

All data are expressed as mean ± standard deviation. Two-sample independent Student's *t*-test was performed to compare the means of two groups. ANOVA, followed by Newman–Keuls tests, was used to compare the mean values of continuous variables among multiple groups, and any significant differences

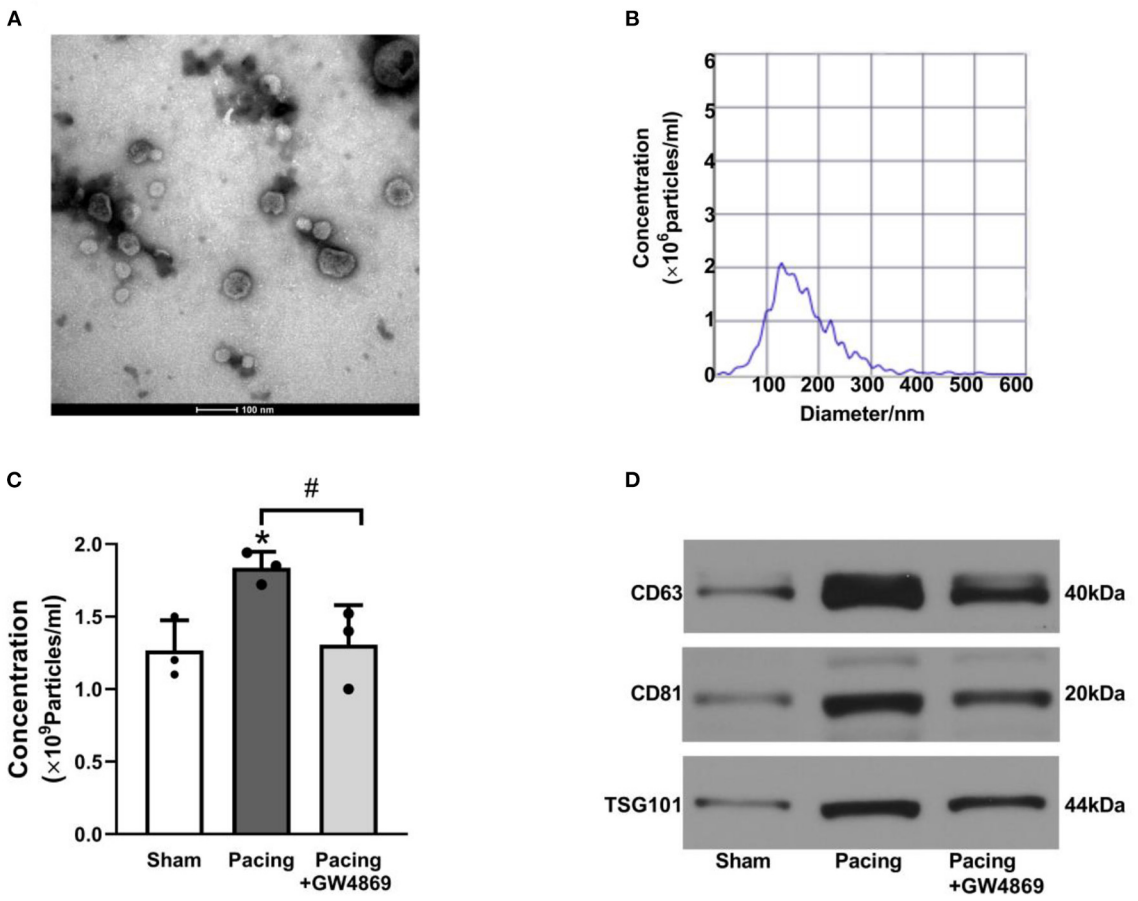

FIGURE 2 | Characteristics of plasma exosomes in a canine model of AF. **(A)** Representative electron microscopy image of exosomes isolated from plasma ($n = 3$, scale bar, 100 nm). **(B)** Representative NTA picture of the exosome size range and concentration ($n = 3$; dilution ratio, 1:500). **(C)** The concentration of plasma exosomes according to NTA analysis and plasma volume. **(D)** Representative western blotting images of the exosome markers CD63, CD81, and TSG101. $^{*}P < 0.05$ vs. the sham group. $^{\#}P < 0.05$ vs. the pacing group. AF, atrial fibrillation; NTA, nanoparticle tracking analysis.

were further analyzed using the Tukey–Kramer test. All statistical tests were two-sided, and a probability value <0.05 was considered statistically significant. All data were analyzed using GraphPad Prism 8 (GraphPad, USA).

RESULTS

Prolonged Rapid Atrial Pacing Increased the Release of Plasma Exosomes

As shown in **Figure 2**, TEM, NTA, and western blotting were used to characterize the exosomes isolated from plasma. TEM showed that isolated exosomes were extracellular vesicles with a diameter of ∼30–150 nm (**Figure 2A**). NTA further measured the size distribution and approximate concentration of the vesicles. The size of the vesicles ranged between 30 and 300 nm, most of which were 100–150 nm in diameter (**Figure 2B**). Plasma exosome concentrations were calculated according to NTA results and plasma volume. After rapid atrial pacing, the concentration of plasma exosomes rose to $(1.84 \pm 0.11) \times 10^9$ vesicles/ml from $(1.27 \pm 0.21) \times 10^9$ vesicles/ml, which was decreased to $(1.31 \pm 0.27) \times 10^9$ vesicles/ml by GW4869 treatment (both $P < 0.05$; **Figure 2C**). Western blotting showed that the vesicles expressed the exosome markers CD63, CD81, and TSG101 (**Figure 2D**). The results suggested that prolonged rapid atrial pacing increases the release of plasma exosomes, which was dampened by GW4869 treatment.

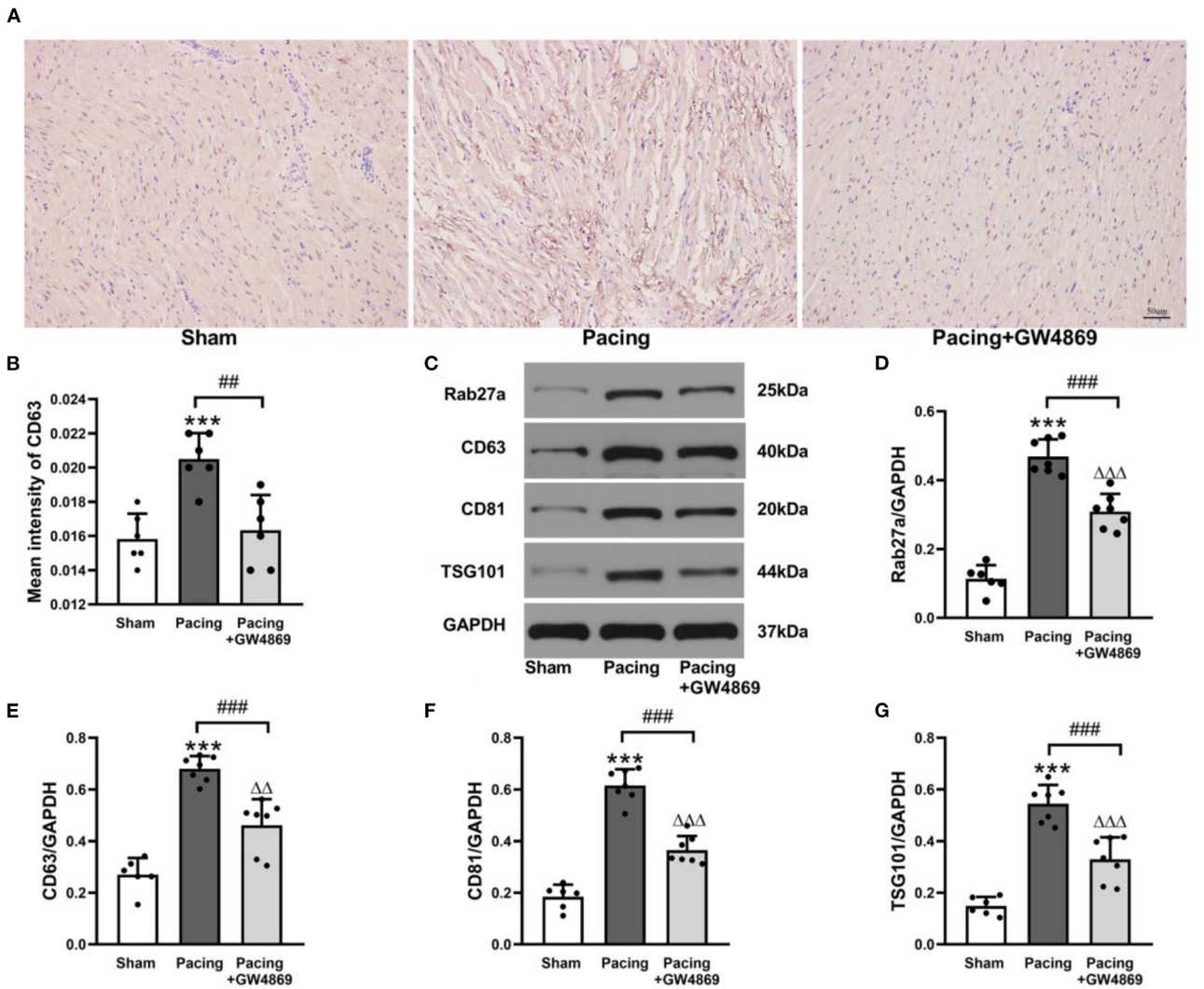

FIGURE 3 | Analysis of exosomal marker proteins in the atria from a canine AF model. **(A,B)** Immunohistochemistry staining of CD63 and mean values of CD63 intensity in the atria ($n = 6$, ×200). **(C)** Representative western blotting images of Rab27a, CD63, CD81, and TSG101 in the atria ($n = 6$ for the sham group and $n = 7$ for the pacing and GW4869 groups). **(D–G)** The mean expression levels of Rab27a, CD63, CD81, and TSG101 in the atria ($n = 6$ for the sham group and $n = 7$ for the pacing and GW4869 groups). $^{***}P < 0.001$ vs. the sham group. $^{\#\#}P < 0.01$ vs. the pacing group, $^{\#\#\#}P < 0.001$ vs. the pacing group. $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ vs. the sham group. AF, atrial fibrillation.

Prolonged Rapid Atrial Pacing Increased the Release of Atrial Exosomes

As shown in **Figure 3**, immunohistochemistry staining and western blotting were used to analyze the expression of exosome marker proteins in the atria. Immunohistochemistry staining showed that the mean density of the exosome marker CD63 in the pacing group was distinctly higher than that in the sham group and the pacing + GW4869 group (both $P < 0.01$; **Figures 3A,B**). To further investigate the change in atrial exosome secretion, Rab27a (an important membrane protein associated with exosome secretion) and exosome markers, such as CD63, CD81, and TSG101, were examined by western blotting. Compared to the sham group, the levels of Rab27a, CD63, CD81, and TSG101 in the atrium were greatly higher in the pacing group (Rab27a: 0.47 ± 0.05 vs. 0.11 ± 0.04; CD63: 0.68 ± 0.05 vs. 0.27 ± 0.06; CD81: 0.62 ± 0.06 vs. 0.18 ± 0.05; TSG101: 0.54 ± 0.07 vs. 0.15 ± 0.04; all $P < 0.001$) and were reduced in the pacing + GW4869 group (all $P < 0.001$). The expression levels of exosome marker proteins remained different between the sham group and the pacing + GW4869 group (all $P < 0.01$; **Figures 3C–G**). These results indirectly showed that prolonged rapid atrial pacing could increase the release of atrial exosomes, which were reduced by GW4869 treatment.

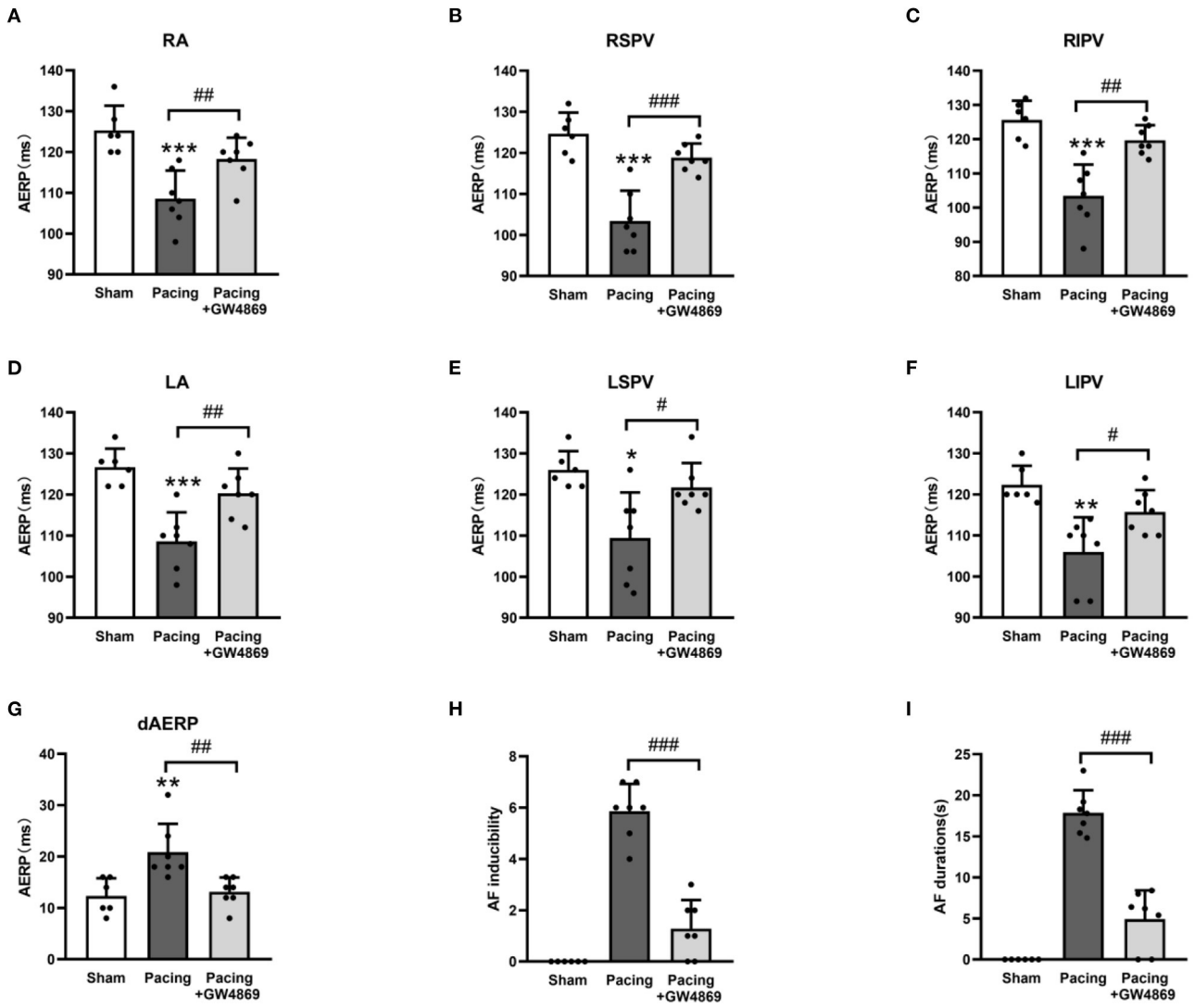

FIGURE 4 | Electrical remodeling in a canine model of AF. **(A–F)** Differences in AERPs at the RA, RSPV, RIPV, LA, LSPV, and LIPV ($n = 6$ for the sham group and $n = 7$ for the pacing and GW4869 groups). **(G)** Difference in the dAERP ($n = 6$ for the sham group and $n = 7$ for the pacing and GW4869 groups). **(H)** Difference in AF inducibility, shown by the number of episodes ($n = 6$ for the sham group and $n = 7$ for the pacing and GW4869 groups). **(I)** Difference in mean AF durations ($n = 6$ for the sham group and $n = 7$ for the pacing and GW4869 groups). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. the sham group. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the pacing group. AERP, atrial effective refractory period; dAERP, dispersion of atrial effective refractory period; RA, right atrium; RSPV, right superior pulmonary vein; RIPV, right inferior pulmonary vein; LA, left atrium; LSPV, left superior pulmonary vein; LIPV, left inferior pulmonary vein; AF, atrial fibrillation.

Inhibition of Exosome Release Lightened AERP Shortening Caused by Prolonged Rapid Atrial Pacing

To evaluate the effect of exosome inhibition on cardiac electrophysiology, AERPs at different sites were recorded. As shown in **Figure 4**, compared with the sham group, AERPs at the six recorded sites were significantly decreased in the pacing group (all $P < 0.05$), which were mitigated by the administration of GW4869 (all $P < 0.05$; **Figures 4A–F**)—for example, the AERP at RA was 125 ± 6 ms in the sham group, 109 ± 7 ms in the pacing group ($P < 0.001$ vs. the sham group), and 118 ± 5 ms in the pacing + GW4869 group ($P < 0.01$ vs. the pacing group). In addition, the dAERP was markedly increased in the pacing group and was reduced in the pacing + GW4869 group (both $P < 0.01$) (**Figure 4G**). There were no significant differences in the AERP and dAERP between the sham group and pacing + GW4869 group.

Inhibition of Exosome Release Dramatically Reduced AF Inducibility and Persistence After Prolonged Rapid Atrial Pacing

The inducibility and duration of AF were assessed through S1S1 programmed stimulus. AF inducibility was increased in the pacing group and was dramatically lessened by GW4869 treatment ($P < 0.001$; **Figure 4H**). In addition, the prolonged AF durations after rapid atrial pacing were significantly shortened by GW4869 (4.91 ± 3. 51 vs. 17.87 ± 2. 75 s, $P < 0.001$;

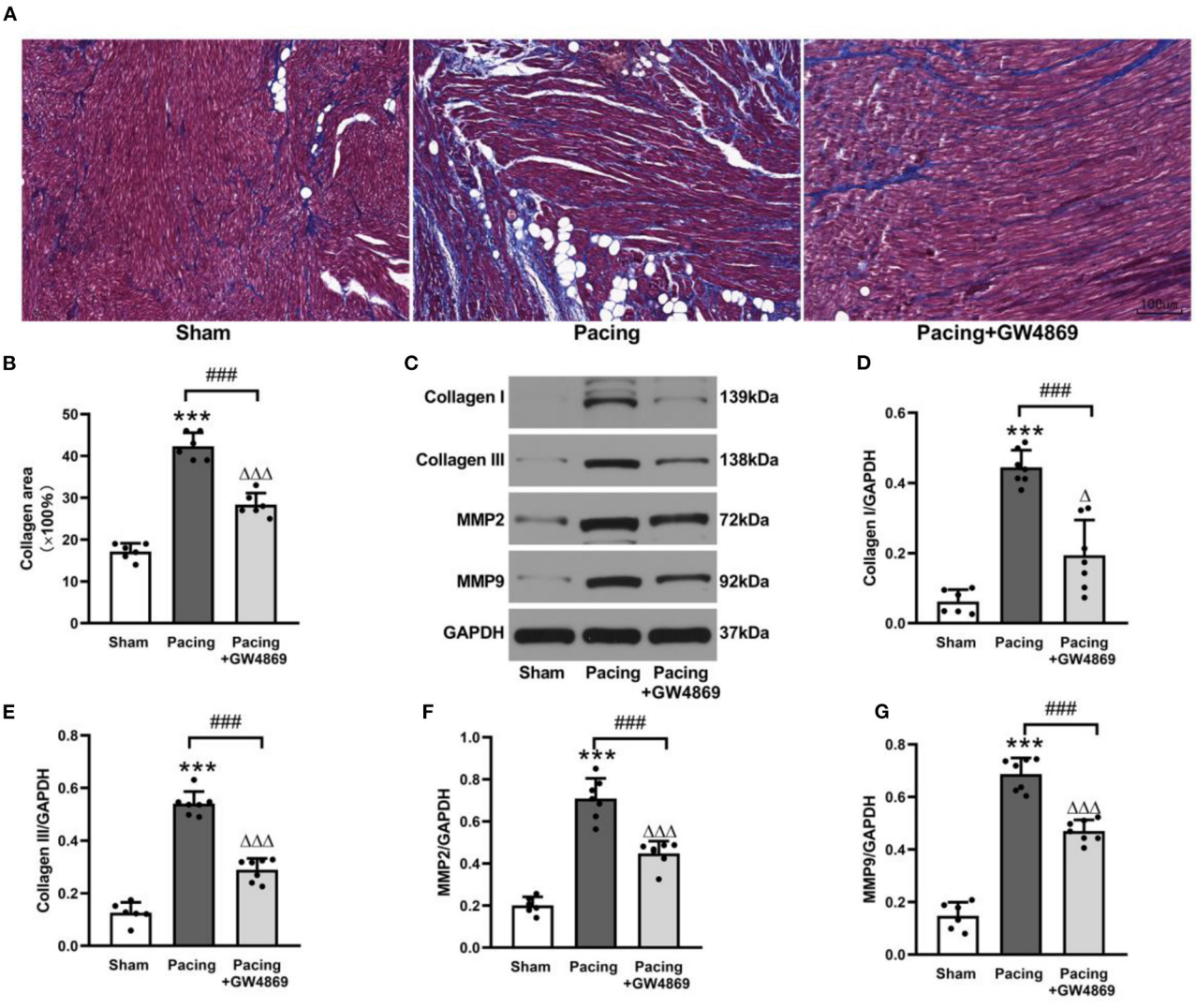

FIGURE 5 | Structural remodeling in a canine model of AF. **(A,B)** Masson's trichrome staining of the atria and mean values of collagen area in the atria ($n = 6$, ×200). **(C–G)** Representative Western blotting images and the mean expression levels of collagen I, collagen III, MMP-2, and MMP-9 in the atria ($n = 6$ for the sham group and $n = 7$ for the pacing and GW4869 groups). $^{***}P < 0.001$ vs. the sham group. $^{\#\#\#}P < 0.001$ vs. the pacing group. $^{\Delta}P < 0.05$, $^{\Delta\Delta\Delta}P < 0.001$ vs. the sham group. MMP-2, matrix metalloproteinase-2; MMP-9, matrix metalloproteinase-9; AF, atrial fibrillation.

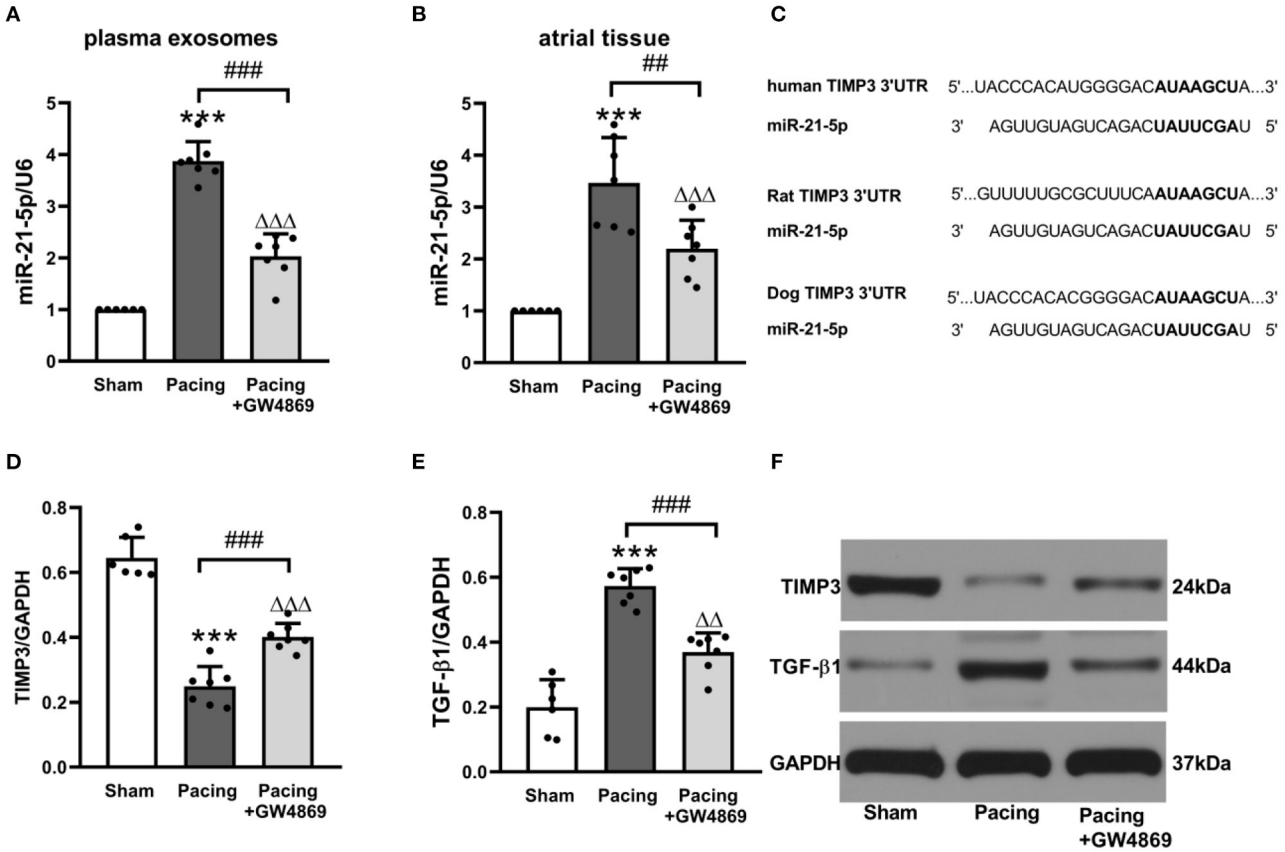

FIGURE 6 | Possible mechanism of atrial fibrosis mediated by exosomes. **(A,B)** Relative levels of miR-21-5p and U6 in plasma exosomes and atrial tissue ($n = 6$ for the sham group and $n = 7$ for the pacing and GW4869 groups). **(C)** Predicted miR-21-5p target sequences in the 3′UTR of TIMP3 among humans, rats, and dogs. **(D–F)** Representative Western blotting images and the mean expression levels of TIMP3 and TGF-β1 in the atria ($n = 6$ for the sham group and $n = 7$ for the pacing and GW4869 groups). $^{***}P < 0.001$ vs. the sham group. $^{\#\#}P < 0.01$, $^{\#\#\#}P < 0.001$ vs. the pacing group. $^{\Delta\Delta}P < 0.01$, $^{\Delta\Delta\Delta}P < 0.001$ vs. the sham group. miR-21-5p, microRNA-21-5p; TIMP3, tissue inhibitor of metalloproteinase 3; TGF-β1, transforming growth factor-β1; AF, atrial fibrillation.

Figure 4I). These results showed that inhibition of exosome release exerted a potential inhibitory effect on the inducibility and persistence of AF.

Inhibition of Exosome Release Suppressed AF by Ameliorating Atrial Fibrosis

As shown in **Figure 5**, the degree of atrial fibrosis was evaluated by Masson's trichrome staining and western blotting. Compared with that in the sham group, collagen deposition in the atrium was observably increased in the pacing group (42.33 ± 3.20% vs. 17.17 ± 1.94%, $P < 0.001$) and was reduced by GW4869 treatment (28.33 ± 2.81% vs. 42.33 ± 3.20%, $P < 0.001$) (**Figures 5A,B**). Consistent with this result, western blotting showed that the expression of collagen I, collagen III, MMP-2, and MMP-9 in the atria was greatly enhanced in the pacing group (all $P < 0.001$) and repressed in the pacing + GW4869 group (all $P < 0.001$; **Figures 5C–G**)—for example, after rapid atrial pacing, the relative level of collagen I was increased from 0.06 ± 0.03 to 0.44 ± 0.05, and GW4869 treatment restored it to 0.19 ± 0.10 (both $P < 0.001$). These data indicated that the increased release of exosomes caused by prolonged rapid atrial pacing primarily played a pro-fibrotic role; therefore, inhibition of exosome release ameliorated atrial fibrosis in a canine model of AF.

Exosomes Mediated Atrial Fibrosis Through Exosomal Pro-fibrotic miR-21-5p and Its Downstream Pathway

To further explore the relationship between exosomes and atrial fibrosis, qRT-PCR was used to verify pro-fibrotic miRNA expression in plasma exosomes and atrial tissues. As shown in **Figure 6**, compared with the sham group, plasma exosomal miR-21-5p was significantly increased in the pacing group ($P < 0.001$), and administration of GW4869 reversed this result ($P < 0.001$). Moreover, the expression pattern in atrial tissue was consistent with that in plasma exosomes (**Figures 6A,B**). Bioinformatics analyses indicated that miR-21-5p targeted TIMP3, which participated in fibrosis *via* the TIMP3/TGF-β pathway (**Figure 6C**). As anticipated, the expression level of TIMP3 in the pacing group was lower than that in the sham group (0.25 ± 0.06 vs. 0.64 ± 0.06, $P < 0.001$) and in the pacing

+ GW4869 group (0.25 ± 0.06 vs. 0.40 ± 0.04, $P < 0.001$). The expression of TGF-β1 was inversely correlated with TIMP3 (**Figures 6D–F**). These data illustrated that exosome-mediated atrial fibrosis partly resulted from exosomal pro-fibrotic miR-21-5p and its downstream TIMP3/TGF-β1 pathway in a canine model of AF.

DISCUSSION

This study was the first attempt to explore the effect of exosomes on AF inducibility and maintenance in a canine model of rapid atrial pacing. We provided novel evidence as follows: (1) rapid atrial pacing increased the release of plasma and atrial exosomes in canines; (2) increased exosomes mainly played a pro-fibrotic role in AF; thus, blockade of exosome release with GW4869 suppressed AF by alleviating atrial fibrosis; and (3) this pro-fibrotic effect of exosomes partly resulted from miR-21-5p enrichment in exosomes and its downstream pathway of TIMP3/TGF-β1.

Exosomes, as important mediators of cellular signal transduction and intercellular communication, have been demonstrated to participate in some cardiovascular diseases, such as coronary atherosclerosis, myocardial infarction, and heart failure (14, 15). GW4869, a non-competitive neutral sphingolipase (N-SMase) inhibitor, has been used as an inhibitor of exosome synthesis and release in some studies (16–18). A recent study verified that coronary artery ligation increased the local release of large and small extracellular vesicles in a murine model of MI (19). Blocking the release of exosomes with GW4869 alleviated the lipopolysaccharide-induced myocardial inflammatory response and improved the cardiac function (20).

As the most commonly sustained arrhythmia, the mechanism and treatment of AF remain suboptimal. Several studies have shown that miRNAs in exosomes are more stable and sensitive than those in blood and thus have a potential role as clinical biomarkers for AF (9). Similarly, due to the vesicular structure, the paracrine mode of action, and fusion with recipient cells, exosomes can be modified specifically to carry specific substances to recipient cells for AF treatment (21). Nevertheless, little is known regarding the association between exosomes and the pathological processes of AF in animal models. In the present study, we established an AF model by rapid atrial pacing in canines. The concentration analysis of plasma exosomes showed that exosome release was increased in the pacing group. Meanwhile, western blotting confirmed the higher expression of vesicle secretion-associated protein Rab27a and exosome marker proteins CD63, CD81, and TSG101 in the paced atria. The release of exosomes was increased after prolonged rapid atrial pacing. The shortening of the AERPs in specialized heart tissues caused differential excitability and conductivity at different heart sites, contributing to AF inducibility and persistence after rapid atrial pacing. By electrophysiological measurements, we observed that shortened AERPs and increased AF inducibility after rapid atrial pacing were alleviated by GW4869 treatment. Treatment with GW4869 also significantly shortened the AF durations. These results indicated that increased exosomes probably played a vital role in the development of AF. The changes in AERPs could be related to some ion channel-related miRNAs contained in exosomes. A previous study also showed that Ang II-treated cardiac fibroblast-derived exosomes contained miR-21-3p, which could regulate the expression of Cav1.2 and contribute to the development of a susceptible substrate for atrial fibrillation (13). However, we did not further explore the mechanism by which inhibition of exosome release directly changed AERPs.

Atrial fibrosis is an important contributor to AF maintenance, which is not only related to AF mechanisms but also increases the risk of complications and therapeutic failure. It involves a series of complicated processes, including the accumulation of extracellular matrix proteins and the imbalance of enzyme activation and inactivation (22). Previous studies have shown that exosomes participate in fibrosis progression. However, most studies were carried out *in vitro*, which was not enough to justify the overall effect of exosomes on fibrosis and provide insights for clinical transformation. Moreover, the function of exosomes depends strongly on stimulation conditions and microenvironments—for example, Działo et al. found that Wnt5a-enriched exosomes activated the ERK1/2 and JNK pathways, induced the production of IL-6, and promoted fibrosis (23). Wang et al. showed that miR-107 in vascular endothelial cell-derived exosomes could alleviate fibrosis through the HIF-1α/Notch1/PDGFRβ/YAP1/Twist1 pathway (24). Thus, we investigated the impact of exosomes on atrial fibrosis in a canine model of AF. Our results showed that the exosomes increased by rapid atrial pacing markedly exacerbated collagen deposition in the atria and elevated the expression of collagen I, collagen III, MMP-2, and MMP-9, which could be reversed by the administration of GW4869. These results suggested that the enhanced release of exosomes primarily played a pro-fibrotic role in a canine model of AF. Inhibition of exosome release likely provided a novel treatment method for atrial fibrillation by mitigating atrial fibrosis.

Proceeding with this study, we further explored the mechanism of exosomes in pro-fibrotic effects. On account of the inclusion of lipid bilayers, miRNAs in exosomes are generally thought to stay biologically stable (25). Some scholars even consider exosomes to be the main form of miRNAs present in serum. Moreover, abundant studies have unveiled the critical impact that miRNAs exert on the activation of fibrosis signals (26). These findings guided us to investigate the role of miRNAs enriched in exosomes in facilitating atrial fibrosis. Emerging evidence has suggested a role for miR-21 in the development of cardiac fibrosis (27, 28). More importantly, a recent study uncovered that the level of circulating miR-21 was relevant to the echocardiographic parameters of atrial remodeling and prediction of AF (29). Therefore, we sought to validate the expression of miR-21-5p in plasma exosomes and atrial tissue. The data showed that rapid atrial pacing elevated the miR-21-5p expression levels in both plasma exosomes and the atrium. GW4869 treatment antagonized these effects. Thus, we speculated that miR-21-5p enriched in exosomes was related to atrial fibrosis in a canine model of AF.

Subsequently, bioinformatics analysis was performed by TargetScan 7.2 to search the target genes of miR-21-5p. Among

the target genes with high correlation, TIMP3 has been demonstrated to be involved in fibrosis progression in an MMP-dependent or MMP-independent manner (30). Loss of TIMP3 increased myocardial fibrosis and elevated the expression of MMP2 and MMP9 to a greater extent in iron-overloaded mice (31). TGF-β plays a central role in cardiac fibrosis and CF function. Another study showed that TIMP3 could modulate the TGF-β expression in Ang II-treated CFs (32). In our study, we explored whether higher levels of exosomal miR-21-5p exacerbated TIMP3 degradation and increased TGF-β1 expression. As expected, the pacing group showed a lower TIMP3 expression and a higher TGF-β1 expression, which were reversed by GW4869 treatment. Moreover, a recent study also showed that a miR-21-5p antagomir could repress myocardial fibrosis by targeting TIMP3 after myocardial infarction (33), which provided an important foundation for our research. These results indicated that the increased release of exosomes induced by prolonged rapid atrial pacing probably promoted atrial fibrosis through the miR-21-5p/TIMP3/TGF-β1 signaling pathway.

CONCLUSIONS

We demonstrated for the first time in a canine model of AF that prolonged rapid atrial pacing increased exosome release and that the blockade of exosome release with GW4869 suppressed AF by alleviating atrial fibrosis, which was related to pro-fibrotic miR-21-5p enriched in exosomes and its downstream TIMP3/TGF-β1 signaling pathway. This study provides a new insight into the mechanism underlying AF maintenance.

Limitations

In our experiment, we confirmed that GW4869 treatment reduces the release of plasma and atrial exosomes and suppresses AF by alleviating atrial fibrosis. However, the quantitation standard of exosomes has still not reached a consensus in recent years. We assessed the release of exosomes in plasma by NTA analysis, while that in the atrium was assessed by exosome marker protein expression. They were not necessarily perfectly correlated with the exosome number. In addition, we failed to evaluate the precise pharmacokinetics of GW4869 and its potential adverse effects on other organ systems. Finally, we only provided a possible mechanism by which exosomes participate in atrial fibrillation by regulating atrial fibrosis. Whether some other miRNAs exist in exosomes induced by rapid atrial pacing and how they function in AF remains unknown. The miRNA sequencing analysis and specific mechanism still need further study.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Laboratory Animal Welfare and Ethics Committee of Renmin Hospital of Wuhan University.

AUTHOR CONTRIBUTIONS

YY and QZ conceived and designed the research. YW, YY, and SH performed the experiments. SH, ZC, and DL analyzed the experiment results. YY drafted and edited the manuscript. YF, HC, and XW revised the manuscript. All authors contributed to the article and approved the submitted version.

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REVIEW
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Exosomes and Exosomal Non-coding RNAs Are Novel Promises for the Mechanism-Based Diagnosis and Treatments of Atrial Fibrillation

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Atrial fibrillation (AF) is the most common arrhythmia worldwide and has a significant impact on human health and substantial costs. Currently, there is a lack of accurate biomarkers for the diagnosis and prognosis of AF. Moreover, the long-term efficacy of the catheter ablation in the AF is unsatisfactory. Therefore, it is necessary to explore new biomarkers and treatment strategies for the mechanism-based AF. Exosomes are nano-sized biovesicles released by nearly all types of cells. Since the AF would be linked to the changes of the atrial cells and their microenvironment, and the AF would strictly influence the exosomal non-coding RNAs (exo-ncRNAs) expression, which makes them as attractive diagnostic and prognostic biomarkers for the AF. Simultaneously, the exo-ncRNAs have been found to play an important role in the mechanisms of the AF and have potential therapeutic prospects. Although the role of the exo-ncRNAs in the AF is being actively investigated, the evidence is still limited. Furthermore, there is a lack of consensus regarding the most appropriate approach for exosome isolation and characterization. In this article, we reviewed the new methodologies available for exosomes biogenesis, isolation, and characterization, and then discussed the mechanism of the AF and various levels and types of exosomes relevant to the AF, with the special emphasis on the exo-ncRNAs in the diagnosis, prognosis, and treatment of the mechanism-based AF.

Keywords: exosome, non-coding RNAs, atrial fibrillation, diagnosis, treatment

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INTRODUCTION

Atrial fibrillation (AF) is a most common type of cardiac arrhythmia and a global burden with significant morbidity, mortality, and socioeconomic problem (1, 2). The AF affects 1–1.5% of the population worldwide, the frequency of the condition is closely related to advancing age, and its prevalence is expected to more than double over the next 40 years (3, 4). Catheter ablation is an established treatment for AF, especially for paroxysmal AF (PAF). However, the success rate for the persistent AF (PsAF) is not ideal because the procedure is often accompanied by risks and other pathological complications. Moreover, there is a lack of effective upstream management for the AF (5–7).

Extracellular vesicles (EVs) include exosomes [diameter range (DR): 30–150 nm], microvesicles (DR: 50–1,000 nm) and apoptosomes (DR: 50–5,000 nm) (8). Exosomes are found in almost

all body fluids (9–11). They normally contain lipids, proteins, and various RNAs, depending on the cells type and the cellular microenvironment (12, 13). Initially, exosomes were believed to be excretory vehicles to discard the metabolic waste but are now regarded as intercellular communicators that shuttle genetic information and proteins between cells (14, 15). The exosomal cargoes not only reflect the disease state, but also the physiological process of the receptor cells. Therefore, they can serve as unique biomarkers of developmental processes and prognostics/diagnostics of the disease states (16). Recently, the role of exosomes in cardiovascular diseases has been extensively studied, mainly in in the acute myocardial infarction (AMI), congestive heart failure (CHF), and coronary atherosclerotic disease (CAD), however, comprehensive elucidations on arrhythmia, especially on the AF are limited (17, 18). This review aimed to analyze the current knowledge regarding the exosomes' formation, isolation, biological functions, and advancements in the medical application, including potential diagnostic and therapeutic use in the AF.

EXOSOME

Exosome Biogenesis

Exosome biogenesis and generation depend on the cell types or cellular microenvironments (19, 20). The exosome biogenesis is schematized in **Figure 1**.

Exosomes are formed by two invaginations of the plasma membrane. The first invagination generates early endosomes in the cytoplasm. The early endosomes mature into late endosomes, whose secondary invagination forms intraluminal vesicles (ILVs). Late endosomes and then finally form multivesicular bodies (MVBs). However, not all ILVs are released as exosomes and some of them would fuse with lysosomes and undergo degradation (21, 22). The exosome formation is tightly regulated by the endosomal sorting complex required for transport (ESCRT) and ESCRT-independent pathways. Exosome cargoes include proteins, lipids, and nucleic acids (23). In addition, nucleic acids especially non-coding RNAs (ncRNAs) serve as important cargoes and mediate cells communication (24–26). At present, the mechanisms underlying the cargoes sorting remain unclear.

The detailed mechanism of the MVBs intravesicular trafficking and fusion with the plasma membrane remains elusive. The known proteins involved are SNAREs, Rabs family (RAB27, RAB11, and RAB35), and Ras GTPase (27–30). The intravesicular trafficking may be mediated by the calcium-dependent Rabs family and the fusion process may be mediated by the SNAREs proteins (20, 25, 31). Exosomes are released into the extracellular space after fusion with the membrane. The release of the MVBs occurs in a calcium-dependent manner.

Exosome Uptake

Possible consequences following the exosomes release include: (1) capture by the neighboring cells or re-absorption by their secretory cells; (2) remote relocation, recognition and fusion with the recipient cells membrane; (3) entry into the circulation and translocation to the other organs (32). However, the underlying mechanisms of the exosome uptake by the recipient cells remain debatable. As reported, there are three suggested mechanisms of uptake: internalization, direct fusion and receptor-ligand mediated uptake (33–40). Thereby, although the precise mechanism of exosome uptake is unclear, one fact remains obvious: the exosomes participate in cells communication through a complex intercellular exchange of

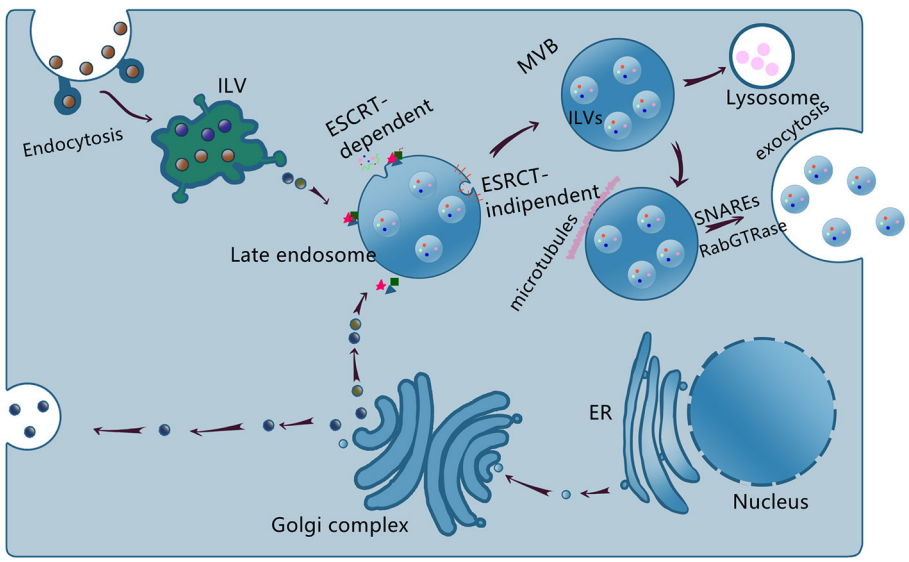

FIGURE 1 | Schematic representation of exosome biogenesis, sorting, and release. The endosome membrane invaginates and sprouts to form intraluminal vesicle (ILV), the early endosome, and then matures to form multivesicular body (MVB) via ESCRT-dependent and ESCRT-independent, the late endosome. Some MVBs reach lysosome and the contents are degraded, others transported to the cell membrane to release exosomes via SNAREs and RabGTPase.

biologically active molecules, modulating the function and behavior of the recipient cells. There is compelling evidence of this process occurring in a variety of diseases including cardiovascular diseases (11, 18, 20, 27, 32, 33, 41).

Exosome Isolation

The isolation of pure exosomes is a critical step to understand their structures and physio-pathological roles in diseases. Nevertheless, there are no currently reliable protocols to isolate absolute pure exosomes. Although several methods have been used to isolate exosomes, each approach exhibits advantages and disadvantages (**Table 1**).

Ultracentrifugation

Ultracentrifugation is the most commonly used method for exosome isolation. The process consists of a series of centrifugation cycles at different centrifugal forces and durations to separate exosomes from other components (42–45).

Density Gradient Centrifugation

Density gradient centrifugation exploits differences in vesicle size and density through discontinuous density gradient layers with progressively decreased density from the bottom to the top (46–48).

Cushion Combined With Density Gradient Ultracentrifugation

In this protocol, the exosomes are firstly concentrated using a 60% iodixanol cushion to recover a maximum number of exosomes with their property preserved. Then, the concentrated exosomes are separated through the density gradient ultracentrifugation to remove the non-exosomes contaminants (22).

Size-Based Isolation Methods

Size-based isolation methods use filters (ultrafiltration) or chromatography columns and merely depend on size or weight. Size exclusion chromatography (SEC) is also a size-based separation technique that uses a stationary phase consisting of resin particles of known porous size to isolate exosomes (49–51).

Immune-Affinity Purification of Exosomes

Exosome membranes contain large quantities of proteins. These proteins can be tagged by their specific corresponding antibodies to identify and isolate exosomes (45, 52–54).

Polymer-Based Precipitation

Precipitation methods are easy and fast approaches for isolating exosomes, which use commercial kits. The exosomes are precipitated by altering their solubility in the solution (50, 55).

Microfluidics-Based Isolation Techniques

Recently, microfluidics-based technologies have been introduced to identify and isolate exosomes. This technique exploits both physical and biochemical properties of exosomes, such as acoustic, electrophoretic, and electromagnetic characteristics (56, 57).

Other Isolation Methods Using Commercial Kits

An increasing number of commercial kits are presently available for exosome isolation. Girijesh et al. analyzed these commercial kits regarding yield, purity, and downstream applications. They determined that the isolation kit by Invitrogen could isolate more exosomes from the culture supernatant than the IZON gel-filtration chromatography kit, 101-Bio PureExo kit, and affinity-based MagCapure kit. However, exosomes extracted using the Invitrogen kit contained cytotoxic chemicals, which may inhibit cell growth (58).

Exosome Characterization

The characterization of exosomes has been a challenge due to their nano-scale size. So far, several techniques were employed for exosome characterization. The detail advantages, disadvantages and procedure are summarized in **Table 2** (12, 59–71).

MECHANISMS OF AF

The mechanisms of the AF are complex and multi-factorial, and the pathophysiology includes three phases: initiation, maintenance, and progression (3, 72). Conceptually, these components link to the triggers and substrates. A trigger can act as an initiator, and the maintenance and progression generally require a substrate (73). Changes in substrate usually cause electrical and structural remodeling (74–76). In addition, a progression occurs over time from the trigger-driven disease, through the progress of atrial substrate, to the structural remodeling. These phases correspond to the clinical observation that about 5% of the patients with pAF progress to the persistent form each year, and 35–40% of PsAF patients may develop permanent AF within < 1 year (77, 78).

Triggers for AF

Three main mechanisms causing focal triggers are: enhanced atrial automaticity, early after-depolarization, and delayed after-depolarization (77, 79). In this regard, cellular calcium homeostasis may play an important role, which may cause heterogeneous electrophysiological properties, and then induce a vulnerable substrate formation (72, 74, 79, 80). These changes causing electrophysiological heterogeneity can result in initiation and sustenance of arrhythmia (72, 81).

Substrate Changes for AF

Many theories about electrical remodeling have been proposed, and their common pathophysiological notion is reentry or micro-reentry (6). In myocardial AF, altered electrical property causes a shortening of the refractory period or slower conduction and thereby provides an anatomical substrate for reentry (5). Moreover, the structural changes such as dilatation and fibrosis of the atrium also affect the conduction and then maintain the reentry circuits (81, 82). Further, the calcium current is reduced by the inactivation and downregulation of the gene expression of calcium channels, which may lead to a shortening of the action potential (5, 83, 84).

TABLE 1 | Current available exosomes isolation techniques.

Method		Pros	Cons	Procedure and application
Ultracentrifugation		(1) The most commonly used method (2) Suitable for large sample capacity	(1) Time-consuming, costly instrumentation (2) Un-efficiently (3) Loss of large amount and damage of exosomes (4) Unsuitable for small amounts of samples or rare samples	It consists of a series of centrifugation cycles of different centrifugal force and duration to separate exosomes. Centrifugation is initially performed at a low speed, followed by ultracentrifugation at 100,000 to 120,000 × g. Finally, the isolated exosomes are resuspended in the appropriate medium. It is suitable for sample such as urine, ascites, and supernatant culture medium
Density gradient centrifugation	(1) Two step method	(1) High purity (2) Structure and function integrity	(1) Low yield and time-consuming (2) Unsuitable for large amounts of sample	The sample is usually layered onto the top of the density gradient medium and subjected to an extended round of ultracentrifugation. The vesicles travel through the gradient until they reach the point at which their density matches the one of the surrounding solution. The separated exosomes are then conveniently recovered by simple fraction collection. The process is suitable for scale analysis of exosomes
	(2) Single step method	(1) Integrity (2) Higher recovery yield	(1) Unsuitable for large amounts of sample	The conditioned medium containing exosomes was directly loaded on 30% sucrose gradient and centrifuged at 100,000 × g, 4°C for 90 min
	(3) Cushion combined with density gradient ultracentrifugation	(1) High purity (2) Preservation properties	(1) Time-consuming	Firstly concentrated by using 60% iodixanol cushion to maximize exosomes recovery. Then, the concentrated exosomes are separated through density gradient ultracentrifugation to remove non-exosomes contaminates
Size-based isolation methods [ultrafiltration, Size exclusion chromatography (SEC)]		(1) Rapid (2) No requiring centrifuge equipment	(1) Isolation of exosomes larger than the pore size of the matrix of the stationary phase used (2) Low yield and the purified sample is diluted (3) Significant hands-on time for column preparation, washing, and equilibration (4) Manual collection of fractions may introduce operator-dependent variability	It uses a stationary phase consisting of resin particles with known porous size. Similarly to density gradient centrifugation, SEC has been shown to allow reduction of contaminant proteins. The process is suitable for small scale analysis of exosomes
Immune-affinity purification		(1) High purity (2) Highly efficient (3) Maintaining exosomes specific morphology, biological activity, and molecular profiles	(1) Multiple steps in sample preparation, making the isolation prone to errors (2) PH value and salt concentration of the buffer might affect the biological activity of exosomes	Magnetic beads are widely used in this method for capturing anti-CD9, anti-CD63, and anti-CD81 antibodies and isolating exosomes
Polymer-based precipitation		(1) Easy, does not require any specialized equipment (2) High recovery rate (3) It is scalable for large sample sizes	(1) It contains lots of contaminating proteins (2) Polymer present in the sample may interfere with the downstream analyses	The sample is mixed with water excluding polymers, that tie up water molecules and force less soluble components out of solution. Generally, the biological fluid is incubated with a precipitation solution and, after incubation, the precipitate containing exosomes is isolated by low speed centrifugation. It is scalable for large sample sizes
Microfluidics based isolation Techniques	(1) Microfluidic based immunoaffinity capture approach (ExoChip)	(1) Highly efficient	(1) Not suitable for large volume, lack of method validation	Microfluidic devices exploit sample-bead interactions and subsequent separation of the beads. The sample is incubated with capture beads off-chip, and only downstream bead separation step takes place on-chip.
	(2) Microfluidics based membrane filtration approach	(1) Highly efficient and low cost	(1) Not suitable for large volume, lack of method validation	Devices use the micro-fluidics based membrane filtration approach isolating exosomes by their size.

(Continued)

TABLE 1 | Continued

Method	Pros	Cons	Procedure and application
			(1) the first such device is a nanoporous membrane with an adjustable pore size that inserted in a microfluidic chip; (2) a multiscale filtration device, which ciliated nanowire-on-micropillar structure that traps specifically sized liposomes (3) a type of microfluidic device based on pillar-array that can sort particles in a continuous flow through nano-deterministic lateral displacement.
Commercial kits	The Invitrogen isolation kit could isolate more and a broad size distribution of exosomes from the culture supernatant than the iZON gel-filtration chromatography kit, 101Bio PureExo kit, and affinity-based MagCapure kit. The quantity and quality of RNA isolated from exosomes showed no significant differences among these isolation kits. However, exosomes extracted using the Invitrogen kit appear to contain cytotoxic chemicals, which inhibit cell growth		

TABLE 2 | Current exosomes characterization techniques.

Method	Advantages	Disadvantages	Detectable size range
TEM	High resolution, discriminate exosomes from other similar-size contaminants, immunostaining	Sample preparation may change the morphology of exosomes, potential damage by electron beam	>5 nm
NTA	Easy sample preparation, fast analysis, high resolution, vesicles are directly observed	Possible overlaying effect of larger vesicles, fail to distinguish exosomes from other nano-contaminants	50–1,000 nm
AFM	Minimal sample preparation without any destructive procedure	Scan speed, temperature and state of the tip may influent the analysis	>5 nm
DLS	High resolution	Fail to distinguish exosomes from other nano-contaminants	>5 nm
FACS	Able to identify specific EV subpopulations	Low detection sensitivity for EV	>300 nm
SEM	High-resolution imaging	Complex sample preparation Requires fixation and drying	>5 nm
TRPS	Information about surface charge of vesicles	Pores may be easily blocked by particles, generate a signal higher than the background noise of the system	>5 nm
Exoview platform	Small volume, low purification biases	Expensive instrumentation, time consuming	>5 nm
Flow cytometry	Fast analysis	Relate low resolution	Not available

TEM, transmission electron microscopy; NTA, nanoparticle tracking analysis; AFM, atomic force microscopy; DLS, dynamic light scattering; FACS, fluorescence-activated cell sorting; SEM, scanning electron microscopy; TRPS, tunable resistive pulse sensing.

Atrial Fibrosis in AF

Extensive evidence shows that structural remodeling, particularly interstitial fibrosis, critically contributes to the substrate formation for the AF (6). Angiotensin-II mediates cardiac fibrosis in a variety of cardiac pathologies (85–87). The angiotensin II induces the TGF-β1 synthesis, which potently stimulates fibroblast activity. Moreover, the platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF) can also stimulate fibroblast proliferation and differentiation (74, 88–90).

Atrial Apoptosis in AF

All cellular lineages undergo programmed cell death, but the fibrillating atria are more prone to apoptotic activation (91). It is likely that the apoptotic process begins relatively early in the AF and causes tissue remodeling (88, 92). Evidence from experimental models suggests that apoptosis, leukocyte infiltration, and increased cell death occur early and precede the arrhythmogenic structural remodeling (93).

Immune Response in AF

The relationship between immune response and the AF is multiplex (94). Recently, several elucidations have shown that higher levels of inflammatory mediators and immune cells infiltration and are closely related to the AF (95). Inflammation could regulate calcium homeostasis and connexin expression, which in turn change the atrial substrates and cause AF initiation, and maintenance (96). The TNF not only could induce abnormal Ca^{2+} handling and arrhythmogenicity in pulmonary vein and cardiomyocytes, but also could activate the TGF-β signaling pathway in the myofibroblasts and increase the matrix metalloproteinase (MMP)-2 and MMP-9 secretion (97). The IL-2 can change the amplitude of electrically stimulated and caffeine-induced Ca^{2+} transients in myocytes. Inflammation also could alter the atrial conduction properties and increase the conduction heterogeneity by affecting the expression or distribution of the gap junction protein connexin (Cx) (Cx40 and Cx43), thereby inducing and maintaining AF (98). The leucocyte activation

and increased levels of myeloperoxidase could increase the MMP-2 and MMP-9 activity, which then mediate atrial fibrosis and remodeling (99). Moreover, inflammatory mediators are associated with atrial electrical properties. The CD36 levels are positively correlated with the atrial voltage (100). Low levels of the HSP27 or CRP are associated with low atrial voltage (101).

Atrial Myocardial Ischemia for AF

Acute myocardial infarction (AMI) is often accompanied by AF (102). The incidence of new-onset AF among AMI events varied from 4.5 to 10.9% in clinical settings (103). The mechanism of new AF in AMI is multi-factorial, among which acute atrial ischemia (AAI) caused by AMI plays an important role (104). AMI would cause electrical instability of ventricular cardiomyocytes, causing ventricular tachycardia or ventricular fibrillation (105). Similarly, AAI can also easily cause electrical conduction disorders in atrial cardiomyocytes, thereby increasing the susceptibility to atrial fibrillation (106). Therefore, increasing the blood supply after AAI may have a positive effect on preventing and reducing the occurrence of atrial fibrillation in these patients.

DIFFERENT EXPRESSION OF EXOSOME IN AF

In the AF, the cardiomyocytes and their microenvironment in the atria are in diverse pathological states. Because the biogenesis and secretion of exosomes significantly depend on the cellular conditions of the cardiomyocytes, the AF may cause changes in the exosomes profile and their cargoes in the atrial tissue and circulation (107).

Comparing the profile of circulating microparticles (MPs) between the AF patients and individuals with normal sinus rhythm (SR), Siwaponanan et al. found that the AF patients had significantly higher levels of cMPs (92). In addition, the EVs were measured in 836 patients with AF and in a cohort of control individuals in a study by Thulin et al. They showed that higher EVs were seen in anticoagulated patients with AF and a higher risk of stroke than the control population, possibly due to the high burden of AF (108). Moreover, Wang et al. found that the PsAF patients had a significantly increasing number of circulating microvesicles. Therefore, AF can cause different levels of circulating exosomes, especially PsAF (109).

Therefore, patients with AF have significant differentially expressed (DE)-exosomes, and the exosomes cargoes may be related to pro-inflammation, pro-fibrosis and apoptosis, which are important mechanisms of AF. Therefore, the exosomes may play a role in facilitating AF.

CLINICAL AND BIOMEDICAL VALUES OF EXOSOME IN AF

As stated previously, exosomes have been suggested as novel vehicles for intercellular communication in the cardiovascular system (71). Non-coding RNAs (ncRNAs) have emerged as important regulators of cardiac functions and diseases (110). So, the ncRNAs as important cargoes of exosomes, the exosomal ncRNAs (Exo-ncRNAs) should play an important role in the AF pathological process and can be used as diagnostic markers or in the treatment approach (111).

NcRNAs in AF Progression

NcRNAs mainly include miRNAs, long non-coding RNAs (lncRNAs) and circular RNAs ect. MiRNAs are small ncRNAs of 22–24 nucleotides that are capable of regulating gene expression by interacting with the mRNA transcript 3'UTRs and promoting mRNA degradation and/or protein translation blockage (112). LncRNAs are a more diverse group of ncRNAs, providing transcriptional and post-transcriptional roles and subclassified according to their functional properties (113). CircRNAs are a closed continuous loop, function as sponges for miRNAs to regulate the expression of target genes and directly regulate transcription with RNA Pol II or protein coding (110, 114). We summarized current state-of-the-art knowledge on the functional of ncRNAs and their regulatory mechanisms in AF.

miRNAs in AF

Many miRNAs are involved in cardiac remodeling, some of them regulate the ion channels, connexins or other proteins involved in the electrical remodeling, some regulate pro- or anti-fibrotic signaling cascades leading to the structural remodeling.

MiR-1 was down-regulated in the PsAF patients, accompanied by the up-regulation of *KCNJ2* and IK1 density, which was associated with the shortening of the action potential duration (APD) and enabled the reentry and AF maintenance (115, 116). MiR-26 was also down-regulated in the fibrillating atria, causing an up-regulation of transient receptor potential cation 3 (TRPC3) channels, which regulated the calcium influx, cell proliferation, extracellular signal-regulated kinase phosphorylation in the cardiac fibroblasts (117, 118). Recently, down-regulation of miR-29b and miR-106b-25 cluster (miR-25, miR-93, and miR-106b) was found in the AF patients atrial (119–121). MiR-30c and miR-133 down-regulation were accompanied by increased atrial fibrosis, and upregulation of their target gene *CTGF*, a pro-fibrotic mediator (122, 123). Besides, the MiR-133 was significantly down-regulated after the zinc finger homeobox 3 (ZFHX3) was knocked down, which increased the remodeling by targeted pro-fibrosis signaling (124). Additionally, up-/down-regulation of miR-133/miR-590 resulted in down-/ up-regulation of their target gene TGF-β1/TGF-β R II collagen expression (125). MiR-21 was up-regulated in the cardiac fibroblasts, which aggravated the pro-fibrotic ERK-MAP kinase signaling pathway (126–128). MiR-328 was also up-regulated in the AF patients' atrial tissue. The over-expression of miR-328 could lead to L-type calcium current reduction and APD shortening, increasing the AF vulnerability (129, 130). MiR-499 was elevated in fibrillating atrial tissue. A relationship was found between the miR-499 and *KCNN3*, which may have been involved in the AF pathophysiology (131). Moreover, the miR-499 mediated the AF by altering the mitochondrial fission and apoptosis signaling (132). MiR-208 can target the gene *GJA5* encoding the cardiac Cx40, and therefore mediate the pro-arrhythmogenic remodeling (133–136).

Long Non-coding RNAs in AF

LncRNAs are involved in gene expression and cellular activity through a variety of mechanisms. Dysregulation of lncRNAs may be associated with cardiac diseases.

Based on competing endogenous RNAs' (ceRNAs) hypothesis, RP11-296O14.3 may participate in the AF pathological process (137). The lncRNA TCONS_00106987 was found increased in a rabbit AF model, which promoted the electrical remodeling by sponging miR-26 to regulate the *KCNJ2* (138). The lncRNA MIAT/TCONS_00202959 had an increase/decrease in fibrillating atrial tissues. The MIAT may target the miR-133a-3p to regulate the atrial fibrosis, and TCONS_00202959 may elongate the atrial effective refractory period (AERP) to decrease the AF inducibility (139, 140). Xu et al. (141) found that the lncRNA NONHSAT040387 and NONHSAT098586 were the most DE-lncRNAs in the AF patient blood samples. In another study, 19 DE-lncRNAs were identified from the AF patient monocytes, and the lncRNA HNRNPU-AS1 was the highest positive correlated one. Further, GO and KEGG analyses showed that these DE-lncRNAs were mainly involved in the metabolic, biosynthetic, RNA binding, NF-kappa B, and cytokine-cytokine receptor interaction signaling pathways (142). Additionally, the lncRNA GAS5 was found downregulated in the AF patients, and the change of the GAS5 occurred prior to the left atrial enlargement. Moreover, the GAS5 was negatively correlated to the ALK5, which could enhance the AF progression (143, 144). Besides, the lncRNA VDAC2P2, PVT1, NEAT1, PCAT-1, LICPAR, and NRON were increased in the AF patients, which were positively correlated with the collagen production and fibroblasts proliferation (145–151). However, the lncRNA LINC00472 and HOTAIR were downregulated. The LINC00472 could regulate the AF progression via modulating the miR-24/JP2/RyR2 signaling pathway, and HOTAIR could function as a ceRNAs in the Cx43 expression by sponging MiR-613 (151–154). In addition, NRON could alleviate atrial fibrosis through the suppression of M1 macrophages, promoting the M2 macrophage polarization. The lncRNA TCONS_00075467 could modulate the electrical remodeling by sponging miR-328 to regulate the *CACNA1C* expression (155). The lncRNA AK055347 may accelerate the AF pathogenesis by dysregulating the mitochondrial energy production via the regulation of Cyp450, ATP synthase, and MSS51 (156). Microarray and RNAs sequencing (RNA-seq) were employed in the lncRNAs analysis. The lncRNAs microarray of cardiac fibroblasts cells showed that the lncRNA AF159100, BC086588, and MRNR026574 were up-regulated while the MRAK134679, NR024118, and AX765700 were down-regulated (157). Another analysis showed that the lncRNA ENST00000559960/ uc004aef.3 was up-regulated/down-regulated in the AF patients' leukocytes (158). The RNA-seq analysis of lncRNAs in the AF canine cardiac fat pads showed that the TCONS_00032546 and TCONS_00026102 could shorten the AERP and increase the AF inducibility (159). The RNA-seq analysis in the AF patients showed that several DE-lncRNAs were involved in the signaling pathways associated with the PI3K/Akt, TGF-β, calcium, inflammation, oxidative stress, autophagy, apoptosis, and collagen synthesis (160, 161). Moreover, another RNA-seq data by Ke et al. identified that the lncRNA RP11-99E15.2 and RP3-523K23.2 participated in the AF pathogenesis via regulating the extracellular matrix binding and the transcription of the HSF2 (162).

Circular RNAs in AF

Recently, studies showed a potential role of circRNAs in myocardial fibrosis and thus initiation and progression of the AF.

The circRNA-miRNA networks showed extensive interaction among DE-circRNAs and the AF-related miRNAs and mRNAs (163). The circRNAs microarray found 120 DE-circRNAs in the AF patients' monocytes. The circRNA_7571, circRNA_4648, circRNA_4631, and circRNA_2875 had the most binding nodes in the circRNA-miRNA networks and were closely interacted with the miRNAs (142). In addition, Gao et al. found that in the PsAF blood samples, circ_0004104 promoted cardiac fibrosis via the TGF-β pathway. Several other studies identified DE-circRNAs in the atrial tissues of AF patients (164). Zhang et al. identified 147 DE-circRNAs and GO and KEGG analyses indicated that many DE-circRNAs transcribed from the host genes were implicated in the regulation of sequence-specific DNA binding transcription factor activity (165). Zhang et al. (166) recognized 23 DE-circRNAs and circ_0000075 and_0082096 may participate in the AF pathogenesis via the TGF-β pathway. Another RNA-seq analysis found 296 DE-circRNAs and the circRNA-associated with the ceRNAs network may induce the AF through the cardiac muscle contraction alterations. Simultaneously, these DE-circRNAs may be involved in regulating the miR-208b and miR-21 expression (167). Another RNA-seq analysis in the patients with the PAF and PsAF found an increase of circRNAs from PAF transition to PsAF, accompanied by miRNAs down-regulation (168). According to an analysis of DE-circRNAs and ceRNAs network in the AF patients from the GEO database, 376 DE-circRNAs were identified, which were enriched in the cytokine-cytokine receptor interaction, and two ceRNAs pairs were identified (circRNA-100053- miR-455-5p-TRPV1 and circRNA-005843- miR-188-5p-SPON1) (169, 170).

Exosomal-NcRNAs in AF

Exo-NcRNAs as Pathogenic Factors for AF

Many studies have found that exo-ncRNAs are related to the initiation and progression of Af. Myofibroblast-derived exo-miR-21-3p could reduce Cav1.2 expression, by regulating the AKAP/PKC signaling pathway, and then increase AF susceptibility (87, 171). Lu et al. found that exo-miR-328 could target the genes CACNA1C and CACNB1, which encode L-type calcium channels, and then lead to atrial remodeling (172). Shan et al. (125) showed that, in canines atrial fibroblasts, the decreased expression of exo-miR-133 and miR-590 were associated with atrial fibrosis, and then promoted AF. Epicardial fat (eFat) contains amounts of exsomes rich in pro-inflammatory and pro-fibrotic molecules, which can affect the neighboring atria, and induce the initiation and maintenance of AF (173–175). According to these researches, eFat tissues were collected from AF patients and were grown as organ cultures by Shaihov-Teper. eFat-EVs were isolated from the culture medium for further analysis. Moreover, to establish a causal association

between eFat-EVs and vulnerability to AF, the study generated an *in vitro* AF model using induced pluripotent stem cell-derived cardiomyocytes (iCMs). The cultured explants from patients with AF secreted more EVs and harbored greater amounts of pro-inflammatory and pro-fibrotic cytokines, as well as pro-fibrotic miRNAs. Moreover, the eFat-EVs from patients with AF impacted the proliferation and migration of human mesenchymal stem cells (MSCs) and endothelial cells (ECs) and induced sustained reentry in iCMs (1). Some other studies also revealed that cardiomyocytes derived exo-miR-1, -miR-208a, -miR-21, -miR-223, -miR-26, -miR-29b, -miR-328, and -miR-499 could target pathways which involved in myocardial metabolism and remodeling (5, 172, 176). In short, these finding reveal the connection between exo-ncRNAs and the pathogenesis of AF, which may provide a promising alternative strategy to improving AF prevention and treatment.

Exo-NcRNAs as Diagnostic Biomarkers for the AF

Circulating miRNAs hold great promise as new diagnostic and prognostic biomarkers for cardiovascular diseases, but the specificity and sensitivity of the miRNAs could be affected by several factors. Due to the protection by the lipid bilayer membrane, circulating exo-miRNAs would provide stable miRNAs, and therefore, circulating exo-miRNAs may possess higher sensitivity and specificity to use as potential biomarkers for cardiovascular diseases (32). Nowadays, circulating exo-miRNAs as biomarkers were mainly used in the AMI, CHF, and CAD (exo-miR-150, -miR-320a, and -miR-208b ect.) (121, 177). Some studies have also found circulating exo-miRNAs could be used as diagnostic/prognostic biomarkers for AF. A study comparing circulating the exo-miRNAs between the patients with SR, PAF, and PsAF.

Wei et al. identified significant three DE-exo-miRNAs (miR-92b-3p, miR-1306-5p, and miRlet-7b-3p), and these miRNAs and target genes participated in AF pathogenesis, like as energy metabolism, lipid metabolism, inflammation, and enzyme activity (178). Wang et al. found that circulating exo-miRNAs: miR-483-5p, miR-142-5p, miR-223-3p were correlated with the AF and multivariate logistic analysis suggested that the miR-483-5p was independently in correlation with the AF (179). A study by Mun et al. also found that compared with patients with supraventricular tachycardia, the expression level of 45 circulating exo-miRNAs in patients with perAF was significantly increased ($>$ 1.5 times). What's more, the DE circulating exo-miRNAs (miRNA-103a, miR-107, miR-320d, miR-486, and let-7b) were increased by more than 4.5 times in the PsAF. Moreover, these miRNAs and their target genes were involved in the atrial structure and function, oxidative stress, and fibrosis pathways (180). Further, Liu et al. isolated exosomes from pericardial fluid (PF), and found that the miR-382-3p, miR-450a-2-3p, and−3126-5p in the exosomes, and especially the miR-382-3p seemed pivotal in the AF progression (181). Therefore, circulating exo-miRNAs have the potential to serve as biomarkers in assessing the AF severity or prognosis, but more rigorous studies are necessary to confirm the supposition (**Table 3**).

Exo-NcRNAs as Potential Therapeutics Approaches in Pathogenic Mechanism of AF

There has been no research on the application of exosomes to the treatment of AF patients. Even in terms of animal experimental studies, direct data to prove the treatment of atrial fibrillation by exosomes-NCRNA is very limited. However, as mentioned previously, the mechanisms of the AF are closely linked to fibrosis, remodeling, inflammation, and apoptosis. In addition, acute atrial ischemia is always accompanied by AF. Therefore, the intervention on these mechanisms may provide a promising alternative new directions for AF treatment. Growing evidence suggests the role of exo-ncRNAs on these mechanisms, and therefore, the exo-ncRNAs may be used as the potential therapeutic tool for AF (18) (**Table 3**).

Anti-fibrosis

Adipose-derived stem cells (ADSCs)-exo-miR-146 could inhibit myocardial fibrosis by down-regulating the gene *EGR1* (182). The exo-Let-7c originating from the MSCs exhibits antifibrotic property, through regulating the TGF-β/Smad (183). The exo-miR-17 and miR-210 derived from the cardiac progenitor cells (CPCs) could inhibit the TGF-β-induced fibrosis under oxidative stress (184). Bone marrow-derived MSCs (BMMSCs)-exo-miR-22 could target the *Mecp2* to alleviate fibrosis (185). Moreover, exosomes enriched with the miR-290, miR-294, and miR-295 derived from the embryonic stem cells (ESCs) could significantly ameliorate fibrosis (186). Cardiomyocytes-exo-miR-378, miR-29a, miR-29b, and miR-455 could exert an anti-fibrotic effect by reducing the collagen and MMP9 via inhibiting the MAPK and Smad pathways (187). Moreover, the exo-miR-320 derived from diabetic cardiomyocytes could negatively affect the proliferation and migration of ECs (188, 189). Furthermore, $CD133^{+}$-exo-miR-126 could reduce VCAM, SPRED-1, and MCP1, and subsequently decrease the interstitial fibrosis (190). Activated macrophage-exo-miR-155 has been shown to decrease fibroblast proliferation by inhibiting the SOS-1 (191). The miR-126, miR-425, and miR-744 enriched exosomes could inhibit fibrosis by targeting the f TGF-β and collagen I (192–194). Further, exo-miR-26a could blunt the FOXO1 activation and inhibit cardiac fibrosis (195). However, several exo-miRNAs have controversial properties. The exo-miR-21 and miR-181b could reduce or accelerate cardiac fibrosis under different conditions (196, 197). In RHD, the exo-miR-155-5p could reduce valvular fibrosis by inhibiting the SOCS1/ STAT3 pathway (25, 198). Moreover, lncRNA Mhrt was shown to inhibit cardiac fibrosis and cardiac myocyte hypertrophy (199).

Aforementioned, atrial fibrosis plays an important in atrial remodeling. A variety of exo-ncRNAs, especially derived from stem cells, can inhibit and improve myocardial fibrosis through a variety of pathways. Therefore, we believe that the treatment based-on these exo-ncRNAs may be an important strategy to prevent and treat AF by inhibiting fibrosis.

Anti-apoptosis

The exo-miR-320d from the ADSCs negatively regulated STAT3 expression, indirectly inhibited cardiomyocytes apoptosis in AF, and increased survival, providing new insights into treatment

TABLE 3 | Exo-ncRNAs as potential diagnostic biomarkers and therapeutics approaches in pathogenic mechanism of AF.

Exo-ncRNAs	Origination	Effect	Mechanisms	References
Exo-miR-92b-3p/Exo-miR-1306-5p/Exo-miRlet-7b-3p	Plasma	Diagnostic	These miRNAs and target genes were involved in the process of AF through affecting biological processes such as energy metabolism, lipid metabolism, inflammation, and enzyme activity	(178)
Exo-miR-483-5p/Exo-miR-142-5p/Exo-miR-223-3p	Plasma	Diagnostic	Some of the pathways are related with myocardial remodeling (PI3K-Akt signaling pathway, adrenergic signaling in cardiomyocytes, focal adhesion, Wnt signaling pathway, calcium signaling pathway) and oxidative stress (MAPK signaling pathway, oxytocin signaling pathway)	(198)
Exo-miRNA-103a/ Exo-miR-107/Exo-miR-320d/Exo-miR-486/Exo-miR-let-7b	Serum	Diagnostic	These miRNAs were involved in atrial function and structure (e.g., gap junction, adherens junction, adrenergic signaling), oxidative stress (e.g., MAPK, AMPK), fibrosis (e.g.,Wnt, hypoxia inducible factor-1), and other pathways	(180)
Exo-miR-382-3p/Exo-miR-450a-2-3p/Exo-miR-3126-5p	Pericardial fluid	Diagnostic	Implicated in cardiac fibrosis-related pathways, including the hypoxia-inducible factor-1 (HIF1), mitogen activated protein kinase (MAPK), and adrenergic and insulin pathways	(181)
Exo-Let-7c	MSCs	Treatment	Anti fibrosis, regulating the *TGF-β/Smad*	(183)
Exo-miR-17	CPCs	Treatment	Anti fibrosis, inhibit the TGF-β-induced fibrosis under oxidative stress	(184)
Exo-miR-19a	MSCs	Treatment	1) Anti-apoptosis, inhibit oxidative stress-induced apoptosis by targeting three prime untranslated regions in cylindromatosis, subsequently achieving the protective effect. 2) Anti-inflammation, decrease the expression of the inflammatory cytokines, moreover, pro-inflammatory/anti-inflammatory factors were down-regulated/up-regulated. 3) Anti fibrosis, downregulates the expression of the target proteins in CMs, *PTEN*, and *Bcl-2*-like protein, and activates the *Akt* and *ERK* signaling pathways	(71, 200)
Exo-miR-21	CPCs/ iPSCs/MSCs	Treatment	1) Anti-apoptosis, ameliorate the CMs apoptosis, which may relate to the inhibition of *caspase 3/7* mediated apoptosis by the *miR-21/PDCD4* signal axis. 2) Angiogenesis, induce angiogenesis and improve the cardiac cells' survival via inhibiting the *PTEN/Akt* pathway	(196, 197, 209)
Exo-miR-22	BMMSCs	Treatment	Anti fibrosis and anti-apoptosis, target the *Mecp2* to alleviate fibrosis and inhibit apoptosis	(185)
Exo-miR-24-3p	MSCs	Treatment	Anti-apoptosis, decrease apoptosis and promote the CMs proliferation	(201)
Exo-miR-25-3p	BMMSCs	Treatment	1) Anti-inflammation, inhibit the inflammatory cytokines expression. 2) Anti-apoptosis, inhibit apoptosis by *Ezh2/Socs3*	(22)
Exo-miR-26a	Muscle	Treatment	Anti fibrosis, blunt the *FOXO1* activation and inhibit cardiac fibrosis	(195)
Exo-miR-125b	BMMSCs	Treatment	1) Anti-apoptosis and 2) anti-inflammation, had the ability of anti-apoptosis and inhibit the inflammatory cytokines expression	(204)
Exo-miR-126	$CD133^+$ cells/ CFs/ADSCs	Treatment	1) Anti fibrosis, reduce *VCAM, SPRED-1,* and *MCP1*, and subsequently decrease the interstitial fibrosis. 2) Anti fibrosis, inhibit fibrosis by targeting the f *TGF-β* and *collagen I.* Anti-apoptosis, reduce apoptosis in neonatal rats cardiomyocytes and improve cell survival by targeting *ERRFI1*. 3) Angiogenesis, promote the generation of microvascular cells and the migration of endothelial progenitor cells, through enhancing the *VEGF* pathway via the suppression of angiogenesis inhibitors *SPRED1* and *PI3KR2*	(190, 192, 211)
Exo-miR-132	CDCs	Treatment	1) Angiogenesis, inducing capillary-like tube formation and enhancing the migration and proliferation of HUVEC, through suppressing the expression of the *Efna3* and *RASA1*	(221)
Exo-miR-133a	NA	Treatment	1) Anti-apoptosis, inhibits apoptosis in myocardial ischemic postconditioning, prevents the expression of *TAGLN2* and *caspase-9*, and upregulates the expression of antiapoptotic protein *Bcl-2*	(201)
Exo-miR-144	MSCs	Treatment	Anti-apoptosis, target the *PTEN/AKT* pathway, and thus improve the apoptosis of the CMs	(202)
Exo-miR-146a	ADSCs/DCs/ CDCs	Treatment	1) Anti fibrosis, down regulating the gene *EGR1*. 2) Anti-inflammation, regulated the inflammatory response by inhibiting the *IRAK-1*. 3) Anti-apoptosis, targeting of *Irak-1* and *Traf6*, both involved in the toll-like receptor (TLR) signaling pathway	(182, 210, 215, 231)
Exo-miR-155	Macrophage/ECs	Treatment	1) Anti fibrosis, decrease fibroblast proliferation by inhibiting the *SOS-1*. 2) Anti-inflammation, can polarize macrophages to M2 cells, inhibit inflammatory reactions (KLF2/miR-155)	(191, 232)

(Continued)

TABLE 3 | Continued

Exo-ncRNAs	Origination	Effect	Mechanisms	References
Exo-miR-155-5p	Serum	Treatment	1) Anti fibrosis, enhances the *S1PR1* and inhibits the *SOCS1/STAT3* signaling pathway, thereby reducing the 2) Anti-inflammation, reduce the *IL-6* and *IL-17* in the valve tissue and serum	(214)
Exo-miR-181a	MSCs	Treatment	Anti-inflammation, create an anti-inflammatory environment and increase the Tregs polarization	(213)
Exo-miR-185	BMMSCs	Treatment	Anti-apoptosis and anti-inflammation, had the ability of anti-apoptosis targeting *Socs2*	(207)
Exo-miR-210	CPCs/MSCs	Treatment	1) Anti fibrosis, inhibit the TGF-β-induced fibrosis under oxidative stress. 2) Anti-apoptosis, downregulated its known targets, ephrin A3 and PTP1b, inhibiting apoptosis in cardiomyocytic cells. 3) Angiogenesis, inducing capillary-like tube formation and enhancing the migration through suppressing the expression of the *Efna3* and *RASA1*	(184, 205, 219, 220)
Exo-miR-221	MSCs	Treatment	Anti-apoptotic by inhibiting the *P53* and *Bcl-2b* and reducing the methylation of *CpG* binding protein-2	(206)
Exo-miR-223	BMMSCs	Treatment	Anti-inflammation, induce the expression of *ICAM-1* to inhibit the inflammatory reaction	(218)
Exo-miR-320	CMs	Treatment	Anti fibrosis, negatively affect the proliferation and migration of ECs	(188)
Exo-miR-320d	ADSCs	Treatment	Anti-apoptosis, negatively regulated *STAT3* expression, indirectly inhibited CMs apoptosis in AF, and increased survival, providing new insights into treatment strategies of AF	(17)
Exo-miR-423-3p	CFs	Treatment	Anti-apoptosis, improve the viability of the H2C9 and reduce apoptosis by targeting the *RAP2C*	(212, 213)
Exo-miR-290/Exo-miR-294/Exo-miR-295	ESCs	Treatment	Anti fibrosis, anti-apoptosis and angiogenesis, increases neovascularization improves cardiomyocyte survival and reduces fibrosis. Enhances cardiac progenitor cell survival and proliferation, as well as cardiac commitment	(186)
Exo-miR-378/Exo-miR-29a/Exo-miR-29b/Exo-miR-455	CMs	Treatment	Anti fibrosis, reducing the collagen and *MMP9* via inhibiting the *MAPK* and Smad pathways	(187)
Exo-miR-425/Exo-miR-744	Serum	Treatment	Anti fibrosis, inhibit fibrosis by targeting the f *TGF-β* and *collagen I*	(193, 194)
Exo-miR-181b/Exo-miR-182	CDCs/MSCs	Treatment	Anti-inflammation, reduce *PKCδ* transcription. Promoted the polarization of M2 macrophages and thereby alleviated the inflammatory response	(216, 217)
Exo-miR-150-5p/Exo-miR-142-3p/Exo-Let-7d	Tregs	Treatment	Anti-inflammation, reduce the immune reactions, and suppress the Th1 proliferation and secretion of the pro-inflammatory cytokines	(209, 223)
Exo-lncRNA Mhrt	ND	Treatment	Anti fibrosis, inhibit cardiac fibrosis and cardiac myocyte hypertrophy	(199)

CMs, cardiomyocytes; ECs, Endothelial cells; CFs, Cardiac fibroblasts; CDCs, cardiosphere derived cells; MSCs, cardiac progenitor cells; ESCs, Embryonic stem cells; ADSCs, adipose-derived stem cells; BMMSCs, bone marrow derived cardiac progenitor cells; CPCs, cardiac progenitor cells; iPSCs, induced pluripotent stem cells. ND, Not Determined.

strategies of AF (17). The MSCs-exo-miR-19a could inhibit oxidative stress-induced apoptosis by targeting three prime untranslated regions in cylindromatosis (CYLD), subsequently achieving the protective effect (11, 200). Another exo-miRNA derived from the MSCs (exo-miR-24-3p) was also found to decrease apoptosis and promote the cardiomyocytes (CMs) proliferation (201). Under hypoxia, the MSCs-exo-miR-144 could target the PTEN/AKT pathway, and thus improve the apoptosis of the CMs (3, 202). Moreover, the exo-miR-210 and exo-miR-133a could inhibit apoptosis under hypoxia, by preventing transgelin 2 (TAGLN2) and caspase-9, and up-regulating the anti-apoptotic protein Bcl-2b. Simultaneously, it improved the ability to resist oxidative stress and supported the stem cells' survival (203, 204). Exosome-derived miR-210 downregulated its known targets, ephrin A3 and PTP1b, inhibiting apoptosis in cardiomyocytic cells (205). The BMMSCs-exo-miR-22 to reduce the methylation of CpG binding protein-2 and reduce cardiomyocyte apoptosis (186), and BMMSCs-exo-miR-221 could mediate the anti-apoptotic effect by inhibiting the P53 and Bcl-2b (206). Moreover, the BMMSCs-exo-miR-185 and exo-miR-125b had the ability of anti-apoptosis (207, 208). The exo-miR-21 originating from the CPCs and iPSCs was reported to ameliorate the CMs apoptosis, which may relate to the inhibition of caspase 3/7 mediated apoptosis by the miR-21/PDCD4 signal axis (183, 196, 209). The CDCs-exo-miR-146a could reduce scar formation after myocardial infarction in rats, inhibit cardiomyocyte apoptosis, and improve heart function (210). In addition, Wang et al. showed that the exo-miR-126 could reduce apoptosis in neonatal rats cardiomyocytes and improve cell survival (211). Cardiac fibroblasts-exo-miR-423-3p was also found to improve the viability of the H2C9 and reduce apoptosis by targeting the *RAP2C* (212).

Cardiomyocytes apoptosis can occur earlier than atrial remodeling. AF can also aggravate the apoptosis. Cardiomyocytes apoptosis and AF are a mutually deteriorating process. Early intervention for apoptosis may prevent and inhibit the initiation and progression of AF. Previous studies showed that exo-ncRNAs have important significance in improving apoptosis. Therefore,

we believe that exo-ncRNAs with anti-apoptotic functions may have potential prospects in the treatment of AF.

Anti-inflammation

The MSCs-exo-miR-19a could decrease the expression of the inflammatory cytokines. In addition, the pro-inflammatory/anti-inflammatory factors were down-regulated/up-regulated by the treatment with the exo-miR-19a (71). The MSCs-exo-miR-181a could create an anti-inflammatory environment and increase the Tregs polarization (213). Moreover, the exosomes derived from Tregs could transfer the miR-150-5p, miR-142-3p, and Let-7d to dendritic cells (DCs) and T-helper 1 (Th1), reduce the immune reactions, and suppress the Th1 proliferation and secretion of the pro-inflammatory cytokines (214). The exo-miR-146a secreted by the DCs regulated the inflammatory response by inhibiting the IRAK-1 (215). Further, the CDCs-exo-miR-181b and BMMSCs-exo-miR-182 promoted the polarization of the M2 macrophages and thereby alleviated the inflammatory response (216, 217). The BMMSC-exo-miR-25, -miR-185, -miR-125b, and ADSCs-exo-miR-126 were also found to inhibit the inflammatory cytokines expression (207). Moreover, the exo-miR-223 and miR-210 could induce the expression of ICAM-1 to inhibit the inflammatory reaction (25, 32, 218).

The immune response participates in the pathogenesis of a variety of cardiovascular diseases, including AF. Anti-inflammatory has been validated maybe useful for the treatment of AF. EXo-ncRNA, as a new strategy for anti-inflammatory, should have important significance in the treatment of AF, but more researcsh are still needed.

Angiogenesis

The ADSCs-exo-miR-126 was found to promote the generation of microvascular cells and the migration of endothelial progenitor cells, through enhancing the VEGF pathway via the suppression of angiogenesis inhibitors SPRED1 and PI3KR2 (192). The EMSCs-exo-miR-21 could induce angiogenesis and improve the cardiac cells' survival via inhibiting the PTEN/Akt pathway (197). The BMSCs-exo-miR-210 and miR-132 could promote angiogenesis, inducing capillary-like tube formation and enhancing the migration and proliferation of HUVEC, through suppressing the expression of the Efna3 and *RASA1* (219–221). Moreover, several MSCs-exo-miRNAs including miR-30b, miR-30c, miR-424, and let-7 were identified to exert pro-angiogenic properties (178).

Promoting angiogenesis in ischemic areas is one of the important methods to improve MI. As previously stated, AAI can increase the susceptibility to AF, so promoting angiogenesis may be an important method for the treatment and prevention of AF. The exosomes-ncRNA may have an irreplaceable role in promoting angiogenesis.

EXOSOME ENGINNERING FOR AF TREATMENT

Direct Exosome Engineering

In direct encapsulation of cargoes into exosomes by sucrose gradient ultracentrifugation, Sun et al. used sucrose gradient ultracentrifugation successfully to encapsulate curcumin (a hydrophobic reagent) into the EL-4 cells-derived exosomes (222). However, this protocol can only be used for hydrophobic drugs. In order to address this, more active encapsulation techniques were applied, such as loading of catalase along with (1) incubation with and without saponin, (2) freeze-thaw cycles, (3) sonication and extrusion (223). Other processes like lipofection and electroporation have limited transfer efficiency and exosome concentration. As an alternative approach, the EVs-imitating structures were developed (173). Liposomes may be the most promising EV-imitating structure (224). Exosome delivery approaches mainly include intravenous injection or direct injection into the target area. Study found that injection of the liposomes into the infarct zone had significant anti-inflammatory, anti-fibrotic, and pro-angiogenetic effects (223).

Indirect Exosome Engineering

Insufficient retainment in the myocardium is one of the major challenges in using exosomes for clinical applications. Currently, technologies for increasing exosomes retainment are being developed. Many targeting molecules have been developed for the exosome conjugation to enhance the retention and achieve the target delivery to the cardiac tissue. For example, Alvarez-Erviti et al. fused cardiomyocyte-specific binding peptide to the exosomal N-terminus of murine transmembrane protein Lamp2b to improve the cardiac tropism of the exosomes (225). Vandergriff et al. designed the myocardium-targeting exosomes with cardiac homing peptide (CHP) and found increased cells viability and exosomal uptake in the cardiomyocytes (226). The other example of indirect engineering is the manipulation of the loading mechanism to selectively load cargoes into the exosomes. Moreover, an attractive tool for protein delivery by the exosomes, which was based on the integration of a reversible protein interaction module was sensitive to blue light and led to the protein loading into exosomes (227). In addition, through transferring encoding genes to the parent cells, exosomes with enhanced production efficiency, specific packaging ability, and the delivery to target cells were developed, which comprised of a production booster, an active packaging device, and a cytosolic delivery helper (166, 228). The latest advances in biomaterials such as heart patches and hydrogels have made them the new favorites for endogenous repair treatments. Liu et al. loaded the exo-miRNAs into hydrogels and exploited them *in situ* to the rat hearts. This approach made the more sustainable exosomes with higher bioavailability, improved cardiac functions, and decreased CMs apoptosis (229). Studies by Vunjak-Novakovic et al. and Chen et al. reported similar results (166). Moreover, encapsulating the exosomes with the antioxidant peptides could enhance exosome targeting effects. Nevertheless, the targeted exosome delivery approaches with enhanced retention still need to be further explored. Moreover, those delivery approaches can be incorporated with a minimally invasive surgical approach such as CT or ultrasound guide tube pericardiostomy to reduce the risk associated with the treatment (**Table 4**).

Overall, exosomes prepared by exosome engineering may have a wide spectrum of prospects for the treatment of diseases including AF.

TABLE 4 | Current exosomes engineering techniques for Af treatments.

Exosomes engineering technologies	Pros	Cons
Encapsulate cargoes by sucrose gradient ultracentrifugation	Protect drugs from degradation, enhance drugs stability, bioavailability and effect	This protocol can only be used for hydrophobic drugs
Encapsulation cargoes through incubation, freeze-thaw cycles, sonication, and extrusion	Allows loading of both hydrophilic and hydrophobic drugs	Causes exosomal bilayer disruption
EV-imitating structure (liposomes)	Targeting, stable structure and contents	Physiochemical instability Can form unwanted degradants
Fusing cardiomyocyte-specific binding peptide to the exosomes (Cardiac homing peptide)	Enhance exosomes targeting	Displays only protein loading
Manipulation of the loading mechanism to selectively load cargoes into the exosomes (protein loading in exosomes based on integration of light sensitive reversible proteins interaction module)	Enhance exosomes targeting Controllable mechanism of loading	Displays only protein loading
Transfection of a gene encoding exosome-targeting proteins into parent cells.	Enhance production efficiency, specific packaging, and delivery to target cells	Displays only protein loading
Heart patches and hydrogels	Making exosomes release more sustained with higher bioavailability; enhance exosomes effects with better target	The delivery approaches with enhanced retention is unsatisfactory

ADVANTAGES AND DISADVANTAGES OF EXOSOME FOR AF

Since the discovery of exosomes, studies on cardiovascular diseases (CVDs) have attracted extensive attention. In this review, we focused on the potential application of exosomes as diagnostic/prognostic and therapeutic tools in AF. Subsequently, we discussed the pros and cons of the use of exosomes. The application of exosomes has many advantages (32, 193, 230): (1) Alterations in exo-cargoes profile secreted by cardiac cells during AF would reflect the parental cells pathophysiological state with extreme specificity and sensitivity, and therefore they may appear as "fingerprint" of the AF pathogenetic processes; (2) Exosomes can be isolated from nearly all obtainable biofluids such as blood and urine; (3) Exosomes serve as a vehicle that protects cargoes from degradation and targets the cargoes to the recipient cells, with the less traumatic and abnormal modifications. (4) Well-designed engineered exosomes may enhance their therapeutic effects, making them promising tools for clinical application. (5) Exosomes therapy has fewer ethical issues, compared with stem cell therapy. Although the exosomes application for the AF has significant benefits, it also has some limitations (31, 202, 228): (1) Exo-RNAs in the circulating come from different tissues, so the source of exosomes cannot be completely determined, which may affect the specificity of the biomarkers for diagnosing AF. (2) The extraction and purification of exosomes are very complicated without a gold standard, and the efficiency is limited, moreover, the specificity and contents of exosomes are unstable. (3) The safety and toxicity of exosomes cannot be fully established. Although lower immunogenicity was reported, some cases may suffer fever or allergic and hemolytic reactions ect. (4) The delivery methods of the exosomes to the heart are sub-optimal. Moreover, even many techniques have been applied to improve the exosome targeting, but there is still the possibility of "off-target," which may not only reduce efficiency but also cause additional side effects. (5) The dosage regimen of exosomes is not clear, and there are limitations on their pharmacokinetic parameters. (6) The exact exosomes' therapeutic effect is unclear, and how exosomes fulfill their specificity is yet to be fully understood.

Nowadays, exosomes have been extensively investigated in several pathological contexts such as ACS, MI, and HF diseases, but barely in the AF. However, as mentioned previously, as diagnostic biomarkers or treatment for AF, exosomes have many potential benefits, even if there are some limitations. Therefore, we need more elucidations to further clarify the exosomes' clinical value and side effects.

CONCLUSION

In the past decade, research on exosomes biology, pathophysiological function, and potential clinical application has increased exponentially and provided novel knowledge in mechanisms and cargoes of exosomes, thereby providing an opportunity to use in the AF diagnosis and treatment. The review of preclinical and clinical studies concluded that the circulating exosomes containing cardiac-specific cargoes, especially ncRNAs, have great potential for the AF diagnosis/prognosis. Further, exo-ncRNAs have important therapeutic effects on AF pathogenesis. Exosome engineering can improve the distribution and selectivity to control the exosomal cargoes. Encapsulation technology has generated a platform for the effective delivery of synthetic and biopharmaceuticals. Therefore, the application of the exo-ncRNAs in the AF may have a good prospect. However, the exo-ncRNAs research related to the AF is still in its infancy, and many aspects need to be improved: (1) The isolation, characterization, and identification should be standardized and simplified. (2) Nomenclature should be consistent. (3) Exosomes should be quantified. (4) Further elaboration on the exosomes mechanism, improvement of targeting, reducing degradation, increasing retention needs to be elucidated in future research.

In conclusion, this review summarized the current biogenesis, isolation, biological functions, and future applications of the exosomes relevant to AF. Exosomes hold unprecedented opportunities for future applications for the AF either as biomarkers for diagnosis/prognosis or as therapeutic tools. Simultaneously, the challenges in the exosomes' application are also significant. Therefore, more prospective, large-scale, and multi-centered trials are needed before the exosomes can be used clinically in the AF. Undoubtedly, exosome-based application will herald a new chapter in clinical diagnosis/prognosis and treatment of AF.

AUTHOR CONTRIBUTIONS

CC and WZ researched the article and wrote the manuscript. QC, TZ, YP, YL, and YX reviewed and edited the manuscript before submission. All authors provided substantial contribution to the discussion of content.

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REVIEW
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Platelet-Derived Exosomes and Atherothrombosis

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Platelet-derived exosomes (PLT-Exos) are the main subtype of extracellular vesicles secreted by platelets, which carry proteins, nucleotides, lipids, and other substances to acceptor cells, playing an important role in intercellular communication. PLT-Exos increase with platelet activation and are involved in the process of atherothrombosis by delivering cargo to acceptor cells. Atherosclerotic plaque rupture, causing thrombosis and arterial occlusion, is the basic pathological change leading to cardiovascular events. PLT-Exos from different donors have different functions. PLT-Exos secreted by healthy volunteer or mice can inhibit platelet activation and inflammation of endothelial cells, thus exerting an antithrombotic effect, while PLT-Exos derived from some patients induce endothelial apoptosis and an inflammatory response to promote atherothrombosis. Furthermore, increased PLT-Exos reflect platelet activation and their cargoes also are derived from platelets; therefore, PLT-Exos can also be used as a biomarkers for the diagnosis and prognosis of cardiovascular disease. This article reviews the characteristics of PLT-Exos and discusses their role in cell-to-cell communication and atherothrombosis.

Keywords: platelet, exosomes, intercellular communication, atherothrombosis, plaque, thrombus

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INTRODUCTION

The fundamental mechanism of atherothrombosis comprises plaque disruption and subsequent thrombus formation. Atherothrombotic events, such as myocardial infarction are major causes of cardiovascular death (1). Atherosclerosis starts with endothelial dysfunction, followed by neointima formation, lipid accumulation, foam cell formation, and plaque rupture (2–5). After plaque rupture, prothrombotic substances are exposed to the blood, followed by platelets and coagulation cascade activation, resulting in thrombosis (6–8). In this process, platelets are activated by inflammatory cells, collagen, von Willebrand factor (VWF), tissue factors, and thrombin (9, 10). Platelet activation causes more platelet-derived exosomes (PLT-Exos) to be secreted, which play important roles in atherothrombosis.

Platelet-derived exosomes are a type of extracellular vesicles (EVs), comprising a tiny vesicles with a lipid bilayer released by platelets. More than 75% of EVs, including exosomes, in the blood are derived from platelets (11). Exosomes (30–150 nm in diameter) are derived from the nucleosome and are released by the fusion of multivesicular bodies (MVBs) with the plasma membrane. After release from the donor, exosomes can transport various substances, including mRNAs, microRNAs (miRNAs), proteins, lipids, molecules, ceramide, and phosphatidylserine, to acceptor cells. On the one hand, exosomes play a role in cell-to-cell communication (12–14)in many

pathological processes, such as cardiovascular disease (15), body immunity (16), nerve repair (17), aging (18), and cancer (19, 20). On the other hand, exosomal cargoes reflect the status of the parent cells and are important disease diagnostic markers.

Platelets, originating from megakaryocytes in the bone marrow, are an important part of the blood, and are involved in various pathological processes, such as hemostasis, thrombosis, and the immune response (21). During atherothrombosis, platelet activation is accompanied by massive release of PLT-Exos, which in intercellular communication by transporting cargoes such as microRNAs and proteins. Studies have shown that the functions of PLT-Exos from different donors vary. Exosomes secreted by healthy volunteer or mice can inhibit platelet aggregation and endothelial cell inflammation, while PLT-Exos derived from some patients promote endothelial cell apoptosis and the neutrophil-mediated inflammatory response. Based on their important regulatory role, PLT-Exos are expected to be a new method or target for the prevention and treatment of atherothrombosis (22–24). This article reviews the mechanisms involved in the regulation of atherothrombosis by PLT-Exos.

CHARACTERISTICS OF PLATELET-DERIVED EXOSOMES

The surface of exosomes comprises different types of surface proteins, such as quad transmembrane proteins, integrins, and immunomodulatory proteins (25). Exosomes can be recognized by most cells and transport proteins, RNAs, cytokines, lipids and other cargoes to acceptor cells to exert multiple regulatory roles (26). Therefore, PLT-Exos usually carry intra platelet substances for information transmission to regulate their target cells. In addition, platelets contain three main types of granules: α granules, dense granules, and lysosomes. Among them, α granules are the most abundant organelles, containing immunoinflammatory regulators, cell adhesive molecules (e.g., fibrinogen, VWF, and multimerin 1), and coagulation factors (e.g., factor V, IX, and XIII) (21). Platelet dense granules are released into the extracellular space directly or through the open canalicular system (OCS) (27) after platelet activation (28). Similar to α molecules, PLT-Exos are also rich in proinflammatory and immunochemokines, such as C-X-C motif chemokine ligand (CXCL)3, C-C motif chemokine ligand 5 (CCL5), CXCL7, platelet factor 4 (PF4), glycoprotein Ib platelet subunit alpha (GP1B), complements C3 and C5, and the platelet activation marker selectin P (CD62p) (29). In addition, platelets can also selectively release RNA into exosomes, prompting exosomes to exert specific functions after entering the acceptor cells (30).

The functions of PLT-Exos are also closely related to causes of platelet activation. Platelet activation caused by different factors results in the release different PLT-Exos in terms of number, size, and, content (31, 32). Platelets produce vesicles with different properties under mechanical forces, such as high shear forces, or in the presence of biochemical reagents, such as thrombin (33, 34). For example, activation of platelets by ADP, thrombin, or collagen results in noticeable differences in terms of the proteins in exosomes (35). Therefore, platelets activated in different diseases can release specific exosomes that can be used for both disease diagnosis and prognostic evaluation, and are directly involved in disease progression.

INTERCELLULAR COMMUNICATION OF PLATELET-DERIVED EXOSOMES

The Biogenesis of Platelet-Derived Exosomes

The biogenesis of PLT-Exos is complex. Firstly, various proteins, such as exosomal membrane proteins, lipid-anchored outer membrane proteins, and peripheral surface proteins (36), can enter cells *via* endocytosis of the plasma membrane and form early sorting endosomes. Secondly, early sorting endosomes can fuse with nucleosomes containing other intracellular substances to transform them into late sorting endosomes, which can then transport cargoes with the assistance of the endoplasmic reticulum and Golgi apparatus (12, 36, 37). During the process of late sorting endosome formation, some proteins and lipids are packaged to form intraluminal vesicles (ILVs). Next, ILVs germinate inward (38) to form multivesicular bodies (MVBs). Some MVBs combined with lysosomes or autophagosomes are decomposed, and other MVBs fuse with the plasma membrane under the action of linker proteins to undergo exocytosis. Furthermore, there are many small vesicles in the lumen of MVBs. The MVB lumen contents, including the intact vesicles, are released into the extracellular space. These small vesicles carrying various substances are called exosomes, which reach acceptor cells *via* body fluids, recognize receptors, and enter cells (39, 40) (**Figure 1**).

The biogenesis of exosomes is regulated by a variety of mechanisms, and both the endosomal sorting complex required for transport (ESCRT) pathway and non-ESCRT pathway play important roles in the formation of MVBs from ILVs. ESCRT is mainly composed of four different protein complexes (ESCRT-0, −I, −II, −III) on the MVB membrane, which encapsulates cargoes through microdomains to form small membrane vesicles and further form ILVs (37, 39). ESCRT-0, which gathers in the limiting membrane of MVBs, can recognize ubiquitinated proteins (cargo) and associate with clathrin. Subsequently, ESCRT-I and ESCRT-II together form stable hetero oligomers with ESCRT-0, aggregating the ubiquitinated cargo in the endosomal membrane. The total complex then recruits and combines with ESCRT-III (23). ESCRT-III promotes the production of complexes (41), ultimately enclosing ILVs into endosomes through budding and dividing. Meanwhile, there is also an ESCRT-independent mechanism for the release of exosomes that still form ILVs, even when all four key subunits of the ESCRT-complex are silenced (42). For example, the inhibition of neutral sphingomyelinase can reduce the release of MVBs and promote the release of exosomes *via* the ESCRT independent pathway (43). In addition, Baietti et al. (44) reported that the syndecan–syntenin–ALG-2-interacting protein X (ALIX) axis is an important regulator of membrane trafficking

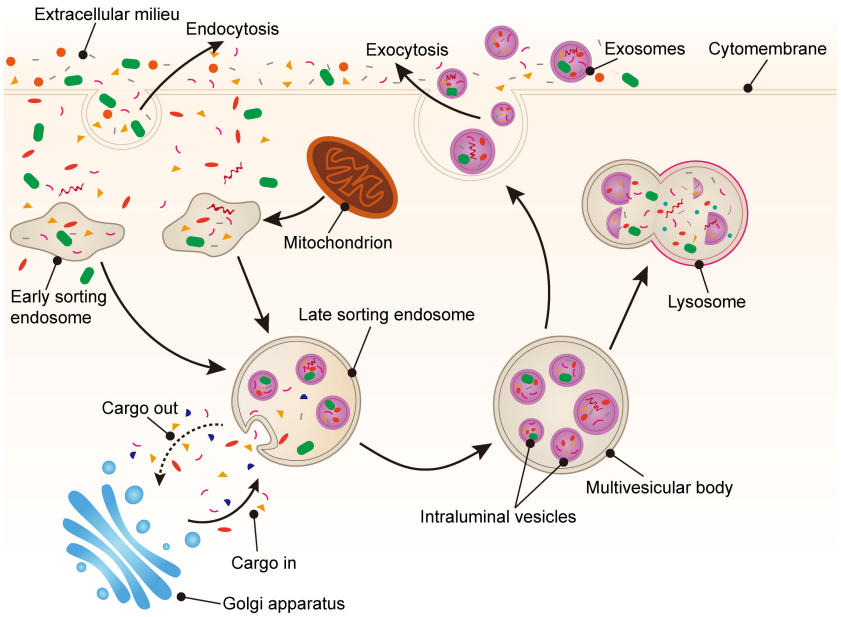

FIGURE 1 | Generation of platelet-derived exosomes. Extracellular proteins, lipids, and metabolites enter the cell through endocytosis. At the luminal side of the cell, the plasma membrane bulge from the outside to the inside to form early sorting endosomes (ESEs). Next, ESEs can fuse with other nucleosomes and transport substances through the Golgi apparatus, gradually forming late sorting endosomes (LSEs). Then, LSEs form intraluminal vesicles (ILVs) through plasma membrane invagination. Finally, the cargoes are further modified *via* the endosomal sorting complex required for transport (ESCRT) pathway or non-ESCRT pathway to eventually form multivesicular bodies (MVBs). MVBs fuse with the plasma membrane and secrete exosomes into the extracellular space *via* exocytosis.

and heparan sulfate-assisted signaling, which can regulate the occurrence of exosomes.

Fate of Platelet-Derived Exosomes in Recipient Cells

Platelet-Derived Exosomes play a role in cell-to-cell communication by entering acceptor cells to release a variety of substances carried from the mother cells. Exosomes enter recipient cells in four main ways, including receptor-dependent endocytosis, phagocytosis, macropinocytosis, and membrane fusion (45, 46). In the process of entering the cell, exosomes bind to cell surface receptors and move in a slow drifting mode on the plasma membrane, and then enter the cell through endocytosis. Then, exosomes diffuse in the local microenvironment of the cytoplasm in a confined mode or move along the cytoskeleton in rapid directed mode (47, 48). Some exosomes undergoing plasma membrane fusion to release their cargo directly into the acceptor cells, and others enter the cells to form MVBs together with ILVs. One part of MVBs are dissolved by lysosomes, and the other part release their exosomal cargoes into the recipient cells (37) (**Figure 2**).

PLATELET-DERIVED EXOSOMES REGULATE ATHEROTHROMBOSIS

Platelets in Atherothrombosis

Platelets are involved in plaque formation and thrombosis, through platelet adhesion, activation, and aggregation. Platelet adhesion mainly occurs after endothelial cell injury. Platelet glycoprotein GPIa (GPIa) and GPIIa on the platelet membrane bind to collagen through VWF, so that platelets adhere to the injury site (34, 49) and become an important component of the plaques. Platelet activation is reflected in three aspects. First, after platelet adhesion, collagen binds to VWF, triggering calcium-mediated intraplatelet signals, after which thromboxane A2 and adenosine diphosphate bind to other soluble agonists (such as α-thrombin and epinephrine) to promote platelet activation. Second, inflammatory cells, such as leukocytes (50), neutrophils (51), B cells, and T cells also activate platelets (10). Third, after plaque rupture, with activation of the coagulation cascade, thrombin binds to the G protein-linked protease-activated receptor of platelets to activate platelets. After platelet activation, α-and δ-granules are released into the blood, and the adhesive glycoproteins and hemostatic molecules carried by them promote platelet aggregation (34). Platelet aggregation results from platelet activation leading to enhanced binding of platelet surface GPIIb/IIIa receptors to other adhesion proteins, particularly fibrinogen (FG), which exacerbates thrombin-mediated conversion of fibrinogen to fibrin, thereby promoting thrombosis (6).

Platelets are fundamental in atherothrombosis. Many exosomes are secreted after platelet activation to participate in this pathological process. Proteomics showed that integrin subunit alpha 2b (ITGA2B) and integrin subunit beat 3 (ITGB3) levels were enhanced in PLT-Exos from patients with burns (52). ITGA2B binds to FG to promote platelet activation and blood coagulation, and ITGB3 binding to VWF exerts a

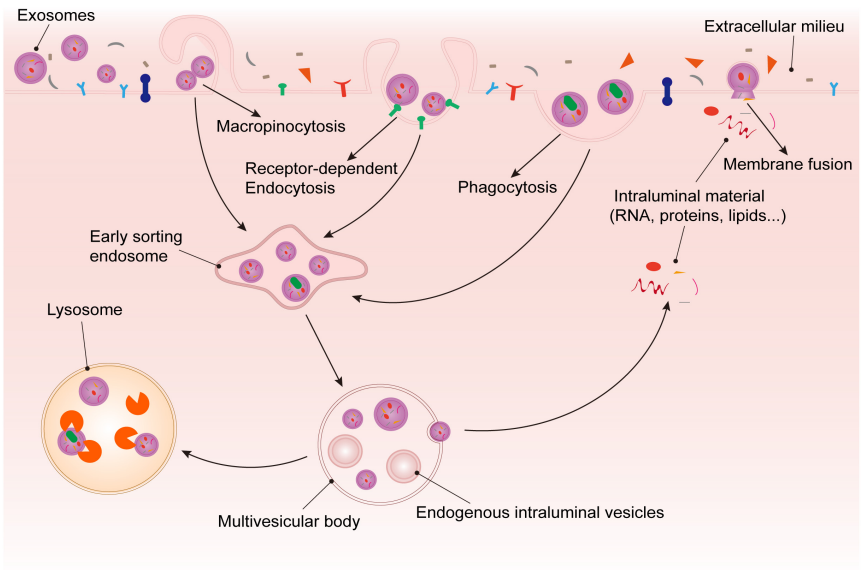

FIGURE 2 | Fate of platelet-derived exosomes in recipient cells. After being recognized by recipient cell surface receptors, exosomes enter cells through *via* phagocytosis, macropinocytosis, membrane fusion, or receptor dependent endocytosis. On the one hand, exosomes that enter *via* membrane fusion release their cargoes into the target cells directly. On the other hand, the exosomes that enter the cell *via* the other methods are internalized to form ESEs, and then combine with ILVs to form MVBs. Some of the exosomal substances are released from MVBs, and the rest would be degraded by lysosomes.

rapid hemostatic effect. A study illustrated that PLT-Exos are both a marker of disease status and also contain potentially pathogenic proteins. Srikanthan et al. found that PLT-Exos could reduce platelet activity and adhesion to collagen, reduce CD36 expression, and inhibit platelet aggregation in an FeCl3-induced carotid artery thrombosis model in mice (53). Therefore, potentially, PLT-Exos can be both pathogenic, because of the inclusion of procoagulant proteins, and resistant to platelet activation and aggregation, which might be related to the source of PLT-Exos and their specific cargo proteins.

Endothelial Cells in Atherothrombosis

The role of endothelial cells in atherothrombosis is divided into two aspects. On the one hand, as the initial factors of plaque formation, endothelial injury and barrier dysfunction, are the basis of pathological changes, such as platelet adhesion, lipid deposition, and foam cell and inflammatory cell aggregation. On the other hand, the healthy endothelium expresses mediators to prevent platelet activation, including nitric oxide (NO), prostacyclin (PGI2), and ectonucleoside triphosphate diphosphohydrolase-1 (E-NTPDase1), and those that inhibit coagulation, such as thrombomodulin, the heparin−antithrombin III system, and tissue factor pathway inhibition (34, 54). Besides, endothelium-derived prostacyclin and platelet-derived thromboxane A2 are considered to be mutually antagonistic components of the dynamic thrombotic balance at the vessel-blood interface, which might regulate atherothrombosis (54, 55). Endothelial cell dysfunction is mainly caused to the activation of endothelial cells, especially type II activation with increased expression of interleukin 1 (IL-1), tumor necrosis factor alpha (TNF-α, and vascular cell adhesion molecule 1 (VCAM-1), which leads to chronic inflammation of the endothelium and accelerates atherothrombosis (56).

Endothelial injury promotes the activation of platelets and secretion of PLT-Exos. PLT-Exos can regulate endothelial cell function by transporting miRNAs. In 2013, Gldlöf et al. found that miR-320b released from activated platelets into endothelial cells inhibited intercellular adhesion molecule 1 (ICAM-1) expression in patients with myocardial infarction; however, the study did not determine whether it entered cells through exosomes (57). Yan et al. demonstrated that thrombin-activated platelets can inhibit ICAM-1 expression in endothelial cells through transporting miRNA-223 in exosomes, and found that miR-223 might inhibit endothelial inflammation by regulating nuclear factor kappa B (NF-κB) and mitogen-activated protein kinase (MAPK) pathways (58). To further explore the role of PLT-Exos in endothelial injury, Wang et al. demonstrated that PLT-Exos inhibited the expression of the target gene ADAM10 (encoding A disintegrin and metalloproteinase domain 10), regulated the NF-κB pathway, downregulated IL-1β, IL-6, TNF-α, triglycerides, and total cholesterol, and inhibited endothelial cell inflammation and lipid deposition by delivering miR-25-3p into endothelial cells (59). Therefore, PLT-Exos can protect endothelial cells *via* miRNA regulation.

Platelet-Derived Exosomes can also synergistically regulate endothelial cells through multiple pathways. For example, PLT-Exos can not only enhance ITGA2B and ITGB3 protein levels (52), but also can activate the Yes1 associated transcriptional regulator (YAP) protein (60). These proteins can all activate the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)/protein kinase B (AKT) signaling pathway, which weakens the inflammation of endothelial cells by inducing autophagy.

Moreover, in the same disease, PLT-Exos can also act through multiple pathways to complete complex regulatory mechanisms. In patients with sepsis, the Janiszewski et al. (61) found that PLT-Exos can produce reactive oxygen and induce endothelial cell apoptosis through nicotinamide adenine dinucleotide phosphate (NADPH). Subsequently, their team further found that exosomes secreted by platelets exposed to NO and bacteria induced caspase-3 activation and apoptosis in endothelial cells by producing superoxide, NO, and peroxynitrite, which resulted in endothelial dysfunction (62). That study also revealed that PLT-Exos can induce endothelial dysfunction in addition to inhibiting endothelial cell inflammation, probably because the PLT-Exos were derived from patients. Similarly, in diabetic retinopathy, PLT-Exo secretion is significantly increased and CXCL10 is upregulated, which activate the toll like receptor 4 (TLR4) signaling pathway and induce retinal endothelial injury (63). In brief, PLT-Exos can protect endothelial cells by inhibiting endothelial cell inflammation, but can also lead to vascular dysfunction by inducing endothelial cell apoptosis, mainly depending on the source of PLT-Exos and their content.

Inflammatory Cells in Atherothrombosis

In atherothrombosis, inflammatory cells are both major participants in plaque formation and can promote thrombosis by activating platelets and the coagulation cascade. On the one hand, plaque formation is a chronic inflammatory process (64), and macrophages and T cells produce a large number of mediators, including proinflammatory cytokines, co-stimulators of immune activation, eicosenoids, reactive oxygen species, and nitrogen species (6, 9). Furthermore, inflammation causes platelet activation and promotes plaque progression. Activated platelets not only secrete PF4, regulated upon activation, normally t-expressed, and presumably secreted (RANTES), macrophage inflammatory protein 1-alpha (MIP-1α), and epithelial-derived neutrophil-activating protein 78 (ENA-78) (65), which promote monocyte aggregation, but also expresses CD40L to regulate the formation of platelet-leukocyte complexes and recruit regulatory T cells (66). In addition, platelet-secreted alpha granules store abundant chemokines, such as CXCL4, which can promote the recruitment and activation of endothelial cells and leukocytes (67). On the other hand, thrombus formation is promoted by inflammatory cells. Leukocytes mediate thrombin activation through the production of tissue factor (TF) and granzyme (68–70), and promote thrombus formation through damage-associated molecular patterns (DAMPs) that promote coagulation system activation (65, 71, 72), platelet activation, and aggregation (73–75).

Inflammatory cells can activate platelets to secrete exosomes and promote thrombosis. A previous study focused on the interaction between neutrophils and PLT-Exos. On the one hand, neutrophils promote thrombosis through procoagulant factors and soluble mediators that induce platelet activation and aggregation in neutrophil EVs. On the other hand, neutrophils promote thrombosis through neutrophil extracellular traps (NETs) that create a scaffold for platelets and other blood cells to attach to Blanch-Ruiz et al. (51). Kuravi et al. found that PLT-Exos can promote neutrophil adhesion to endothelial cells and enhance inflammation through CD62P and CXC-chemokines, which resembles the actions of platelet-derived microvesicles (76). Moreover, excessive activation of immune thrombi during septic shock cause thrombotic inflammation, and PLT-Exos activate the AKT/mechanistic target of rapamycin (mTOR) autophagy pathway to promote the formation of NETs through high-mobility group protein 1 (HMGB1) and/or miR-15b-5p and miR-378a-3p (77). In summary, PLT-Exos promote neutrophil-mediated thrombosis; however, the limited number of previous studies has resulted in a lack direct evidence for a role of PLT-Exos in atherothrombosis.

APPLICATION OF PLATELET-DERIVED EXOSOMES

Potential in the Treatment

The role of PLT-Exos in atherothrombosis is expected to lead to their application in the treatment of coronary heart disease. Previous studies have demonstrated the role of exosomes in coronary heart disease (78, 79) and found that exosomes can be detected in atherosclerotic plaques (80). Unlike other exosomes, PLT-Exos are secreted in large amounts during platelet activation and can regulate thrombosis through multiple pathways, involving platelets, endothelial cells, and inflammation, which have wide potential for therapeutic intervention (**Table 1**). PLT-Exos reduced endothelial cell inflammation in Apoe -/- mice (59), inhibited the entry of oxidized low-density lipoprotein and cholesterol into macrophages, restrained foam cell formation (53), and then slowed the process of atherosclerosis. In acute thrombosis, in addition to directly inhibiting platelet activation and adhesion (53), PLT-Exos could transfer into smooth muscle cells and reduce the expression of platelet-derived growth factor receptor-beta (PDGFRβ) to inhibit smooth muscle cell proliferation and regulate vascular smooth muscle cell injury and repair (81). The above studies provide an experimental basis for the application of PLT-Exos in disease treatment.

Exosomes can be used not only for disease prevention and control, but also as carriers to transport cargo. Exosomes have unique advantages in that they are not easily cleared by immunization and are well tolerated after exosome injection (25). For example, intravenous injection of PLT-Exos inhibited atherosclerosis progression in mice (59). Exosomes act as cargo carriers to deliver miRNAs, siRNAs, and drugs to receptor cells and play a role in regulating target cells to treat diseases (82). For instance, enhanced levels of miR-223 in PLT-Exos inhibited the inflammation involving monocytes (83). In addition, to solve the problem of stent restenosis, Guan et al. immobilized PLT-Exos on the stent surface using electrostatic recheck, which could improve endothelial function, inhibit the macrophage pro-inflammation (M1 phenotype), and promote their conversion to the anti-inflammatory (M2) phenotype (84). This demonstrated the application prospects of PLT-Exo in cardiovascular biomaterials. Another study found that aspirin inhibited the increase in the levels of chemokines and high-mobility group box 1 (HMGB1) in PLT Exos, but the total amount of PLT-Exos was not changed. That study indicated that antiplatelet drugs do not

inhibit exosome secretion, and we expect to further explore the synergistic effect of drugs and exosomes in therapy in a future study (85). In short, PLT-Exos can be applied to the treatment of diseases from multiple perspectives, such as directly in treatment, as cargo carriers, in combination with biomaterials or other drugs; however, research in this area is still in its infancy.

Emerging Diagnostic Markers

Exosomes are widespread in most biological fluids (86), such as blood, saliva, and urine, and are secreted by cells in physiological or pathological conditions. Exosomal cargoes and characteristics are closely related to disease status, and increased attention has been paid to their role in tumor diagnosis and evaluation (87–89). Thus, research has identified them as potential biomarkers for the study of cardiovascular diseases (90, 91). Moreover, clinical diagnosis and treatment would be facilitated through the detection of exosomes in biological fluids, which would reduce the need for invasive operations and computed tomography radiation. For example, Tan M et al. found that miR-223, miR-339, and miR-21, which are associated with platelet activation, were significantly elevated in PLT-Exos before arterial thrombosis, and thus might represent new predictive biomarkers (81). There are still relatively few studies about PLT-Exo, mainly because of the difficulty in extracting PLT-Exos and controlling the experimental conditions. Plasma exosomes are mainly derived from platelets and can be used to replace PLT-Exos to a certain extent, bringing convenience to clinical applications.

TABLE 1 | The role of PLT-Exo in atherothrombosis.

Article(references)	Research target	Functional changes
Qin et al. (52)	Coagulation	Coagulation in burn patients (+)
Srikanthan et al. (53)	Thrombosis	Platelet aggregation (–) CD36 in platelet (–) Occlusive thrombosis (–)
Li et al. (58)	Thrombosis-inflammation response	ICAM-1 (–) NF-κB pathways (–) MAPK pathways (–)
Yao et al. (59)	Endothelial cell inflammation	IL-1β, IL-6, and TNF-α (–) Atherosclerosis (–)
Janiszewski et al. (61)	Endothelial cell apoptosis	NADPH in sepsis (+) Apoptosis rates in sepsis (+)
Gambim et al. (62)	Endothelial cell apoptosis	Caspase-3 activation in sepsis (+) Apoptosis in sepsis (+)
Zhang et al. (63)	Endothelial injury	CXCL10 in diabetic rats (+) TLR4 pathways in diabetic rats (+)
Kuravi et al. (76)	Neutrophil-endothelial cell interactions	Adhesion (+) Inflammatory responses (+)
Jiao et al. (77)	Neutrophil	HMGB1 in septic shock (+) Akt/mTOR pathway in septic shock (+) NETs in septic shock (+)
Poon et al. (83)	Monocytes	IL-6 and NLRP3 during CPB (–) inflammatory responses during CPB (–)
Tan et al. (81)	Vascular smooth muscle cells	PDGFRβ (–)

NADPH, nicotinamide adenine dinucleotide phosphate; CPB, Cardiac surgery with cardiopulmonary bypass; NETs, neutrophil extracellular traps; PDGFRβ, growth factor receptor-beta; (+), increase; (–), decrease.

CONCLUSIONS AND PERSPECTIVE

Atherothrombosis is the pathological basis of acute cardiovascular events, and platelet activation is an important condition for thrombosis (92). Therefore, how exosomes released by activated platelets function in thrombosis has become the focus of research attention. Exosomes carry a variety of information from platelets into acceptor cells and function in intercellular communication, which is expected to lead to new therapeutic approaches. Therefore, we discussed the role played by PLT-Exos in atherothrombosis and their mechanisms. By specifically delivering different miRNAs and proteins, PLT-Exos can inhibit platelet activation and aggregation, and reduce endothelial cell inflammatory injury. However, different sources of PLT-Exos act differently, and PLT-Exos from some patients would promote endothelial apoptosis and neutrophil-mediated inflammatory response. Hence, flexible applications and modifications of PLT-Exos have great potential to prevent and treat atherothrombosis (93).

Platelet-derived exosomes can be obtained from different sources, leading to significant differences in their cargoes and functions. Exosomes secreted by platelets in disease states often contain pathogenic factors that can be used as biomarkers for disease diagnosis, but do not necessarily act on receptor cells. For example, PLT-Exos are rich in proinflammatory factors and chemokines, reflecting the activation of platelets, while PLT-Exos may play an antiphlogistic and antithrombotic role in receptor cells. In addition, because PLT-Exos are rich in a variety of cargoes, they can play different roles by carrying different regulators. In previous studies, PLT-Exos secreted in disease states often showed high levels of pathogenic factors and can enter target cells to promote disease progression, while exosomes obtained from healthy volunteer or mice can inhibit platelet activation and endothelial inflammation. Of course, these results are only a summary of the current studies on atherothrombosis and are not absolute. For instance, in patients undergoing cardiac surgery with cardiopulmonary bypass (CPB), increased miR-223 in PLT-Exos can downregulate the expression of IL6 and NLRP3 (encoding NLR family pyrin domain containing 3) in monocytes to inhibit the inflammation induced by CPB (84). Thus, the functions of PLT-Exos depend mainly on their source and cargoes.

Although the mechanisms of PLT-Exos in multiple pathological processes, such as platelet activation and endothelial inflammation injury, have been reported, there are still many problems that need further study because of the complex mechanisms of atherothrombosis and the variety of PLT-Exo cargoes. First, thrombosis is mainly caused by platelet activation and the coagulation cascade (94); however, the mechanism by

which PLT-Exos regulate platelet activation is unclear, and there is also a lack of studies on the role of the coagulation system. Second, previous studies have affirmed the therapeutic effect of PLT-Exos by intervening in endothelial cells using PLT-Exos in healthy volunteers; however, there is a lack of intervention experiments with PLT-Exos in patients, which makes it difficult to explain the regulatory mechanism of PLT-Exos in disease. Third, experimentally, PLT Exos are obtained by activating platelets using different protocols, which caused differences in exosomal cargoes and thus introduced experimental errors. Recent research has focused on the function of exosomes and the role of their mediated miRNAs and proteins, which are still some distance away from clinical application. On the one hand, we should develop specifications for obtaining PLT-Exos, study the functions of PLT-Exos, then perform genomics analysis to validate miRNAs and proteins that play a major role, and finally apply PLT-Exos in clinical treatment. On the other hand, we can modify PLT-Exos and use them as carriers to deliver specific drugs or cytokines into receptor cells to exert their functions (95).

Encouragingly, previous studies demonstrated the key role of PLT-Exos in atherothrombosis and revealed part of the mechanism, laying the foundation for next step of research. As exosome research progresses, we look forward to the future application of PLT-Exos as diagnostic markers and intervention mediators in the clinical treatment of cardiovascular diseases, ultimately bringing benefits to patients.

AUTHOR CONTRIBUTIONS

KW, HH, and ML structured the manuscript giving contribute to figures and text editing. DS and XM revisited the article implementing the final manuscript form. All authors contributed to the manuscript production and in the final revision.

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Size Distribution of Microparticles: A New Parameter to Predict Acute Lung Injury After Cardiac Surgery With Cardiopulmonary Bypass

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Background: Acute lung injury (ALI) is a common complication after cardiac surgery with cardiopulmonary bypass (CPB). No precise way, however, is currently available to predict its occurrence. We and others have demonstrated that microparticles (MPs) can induce ALI and were increased in patients with ALI. However, whether MPs can be used to predict ALI after cardiac surgery with CPB remains unknown.

Methods: In this prospective study, 103 patients undergoing cardiac surgery with CPB and 53 healthy subjects were enrolled. MPs were isolated from the plasma before, 12 h after, and 3 d after surgery. The size distributions of MPs were measured by the LitesizerTM 500 Particle Analyzer. The patients were divided into two subgroups (ALI and non-ALI) according to the diagnosis of ALI. Descriptive and correlational analyzes were conducted between the size distribution of MPs and clinical data.

Results: Compared to the non-ALI group, the size at peak and interquartile range (IQR) of MPs in patients with ALI were smaller, but the peak intensity of MPs is higher. Multivariate logistic regression analysis indicated that the size at peak of MPs at postoperative 12 h was an independent risk factor for ALI. The area under the curve (AUC) of peak diameter at postoperative 12 h was 0.803. The best cutoff value of peak diameter to diagnose ALI was 223.05 nm with a sensitivity of 88.0% and a negative predictive value of 94.5%. The AUC of IQR at postoperative 12 h was 0.717. The best cutoff value of IQR to diagnose ALI was 132.65 nm with a sensitivity of 88.0% and a negative predictive value of 92.5%. Combining these two parameters, the sensitivity reached 92% and the negative predictive value was 96%.

Conclusions: Our findings suggested that the size distribution of MPs could be a novel biomarker to predict and exclude ALI after cardiac surgery with CPB.

Keywords: microparticles, cardiac surgery, acute lung injury, cardiopulmonary bypass, size distribution

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INTRODUCTION

Cardiac surgery with cardiopulmonary bypass (CPB) is a traumatic procedure and prone to postoperative acute lung injury (ALI) (1–3). The incidence of ALI after CPB surgery is about 15–60%, and the incidence of ALI in children with CPB surgery is as high as 60%. ALI-related mortality in the general population reaches 40%, and may go up to 80% in the patients after cardiac surgery with CPB, which makes ALI a major cause of increased mortality after cardiac surgery (4, 5). The mechanism by which cardiac surgery induces ALI remains unclear but may involve inflammation, activation of coagulation, increased permeability of the alveolocapillary barrier, epithelium dysfunction (6), and alveolar epithelium cell apoptosis (7). More importantly, no precise method is currently available to predict ALI after cardiac surgery.

Circulating microparticles (MPs) are a group of membrane vesicles generated from a variety of sources including endothelial cells, neutrophils, platelets, etc. upon activation or apoptosis. We and other researchers have demonstrated that circulating MPs are elevated in cardiovascular diseases including valve heart disease, acute coronary syndromes, congenital heart disease, and cardiac surgery (8–15). MPs have been proved to have important physiological and pathophysiological functions and have been suggested as a promising biomarker to diagnose and predict different stages of diseases (11, 16–26). We also demonstrated that MPs were significantly increased, impairing endothelial function and vasodilation which may cause hemodynamic instability after cardiac surgery (9, 10, 18, 27). We previously found that endothelial microparticles were increased in patients after cardiac surgery and could induce ALI (10, 17). In the early stage of ALI, alveolar macrophage-derived MPs contain activated TNF-α, which may arouse a strong effect on inflammation and immunomodulation (28). Procoagulant tissue factor-bearing MPs may lead to lung injury through activated coagulation factors X and Xa and induce pulmonary fibrosis in interstitial lung diseases (29). In the rat model of ischemia-reperfusion-induced lung injury, elevated circulating MPs carrying miR-155 increase the pulmonary vascular permeability, leading to lung injury (30). Moreover, caspase-1 contained in monocyte-derived MPs mediates the apoptosis of alveolar epithelium cells (31). Recently, we found that MPs from cardiac surgery with CPB contained many pro-inflammatory proteins such as C-reactive protein, myeloperoxidase, serum amyloid A, S100 calcium-binding protein A8, and S100 calcium-binding protein A9, which may induce severe inflammatory response and ALI (32). Indeed, the circulating angiotensin-converting enzyme-positive endothelial MPs were increased in ALI (33). However, it is unclear whether MPs can be used to predict ALI after cardiac surgery.

Abbreviations: ALI, Acute lung injury; CPB, Cardiopulmonary bypass; MPs, Microparticles; AUC, Area under the curve; IQR, Interquartile range; NTA, Nanoparticle Tracking Analysis; NYHA, New York Heart Association; OI, Oxygenation index; PPV, Positive predictive value; NPV, Negative predictive value; DLS, Dynamic light scattering.

In this study, we measured the number and size distribution of MPs using a new technique in the patients who underwent cardiac surgery with CPB. We found that the number of MPs in the patients notably increased compared with healthy subjects. However, the number of MPs did not differ in patients with ALI and without ALI. More importantly, the size at peak and interquartile range (IQR) of MPs in patients with ALI are significantly smaller than that of patients without ALI. Our findings suggest that the size distribution of MPs can be used to predict and exclude ALI after cardiac surgery with CPB.

MATERIALS AND METHODS

Patient Selection

The patients who underwent cardiac surgery CPB in the First Affiliated Hospital, Sun Yat-sen University were selected. Inclusion criteria were patients older than 18 years who underwent cardiac surgery with CPB through a mid-sternal incision and with general anesthesia under endotracheal intubation. Exclusion criteria were patients with previous cardiac surgery, previous central nervous system disease, renal failure, hepatic dysfunction, and previous lung disease such as thoracic trauma, pulmonary infection, and chronic pulmonary diseases before operation. The enrolled 103 patients were divided into two groups: patients with ALI ($n = 25$) and patients without ALI (non-ALI, $n = 78$). In addition, 53 healthy subjects were recruited as a control group whose age, gender, as well as other baseline characteristics, were matched with those of the patients included. The study was approved by the Ethics Review Board of the First Affiliated Hospital, Sun Yat-sen University. Prior informed consent was obtained from all subjects who were enrolled in this study.

Isolation and Size Distribution of MPs

The isolation of MPs followed the methodological guidelines for studying extracellular vesicles (34). All patients and healthy subjects fasted overnight. Blood samples were collected from the peripheral vein about 2.7 ml into a tube with sodium citrate at the time before, 12 h, and 3 d after surgery. The blood samples were centrifuged at 2,000 g for 20 min at 4°C to get platelet-rich plasma. The platelet-rich plasma then was centrifuged at 11,000 g for 2 min at 4°C to obtain platelet-poor plasma. All procedures mentioned above were done 2 h after blood collection. The samples were then stored at −80°C. Platelet-poor plasma was melted at 37°C and then centrifuged at 13,000 g for 45 min to prepare MPs for size distribution detection. MPs were resuspended in phosphate buffer solution to 1.0 ml. The size distribution of MPs was measured by the LitesizerTM 500 Particle Analyzer (Anton Parr, Ashland, VA, United States).

Nanoparticle Tracking Analysis

The quantification of MPs was determined by the Nanoparticle Tracking Analysis (NTA) system (NS300; Malvern, United Kingdom) as described previously (9). Briefly, the samples were gently inserted into the detection channel followed by setting an ideal resolution and brightness for the observation of MPs' motions. The movement tracks of MPs were recorded

for 60 s with a detection threshold optimized for each sample and repeated three times. Data processing was performed by the NTA 3.3 software (Malvern, United Kingdom).

Data Collection

Data collection included demographics, history, type of cardiac disease; preoperative data such as blood type, blood routine, biochemistry parameter, myocardial enzyme, coagulation, New York Heart Association (NYHA) classification; intraoperative data such as duration of surgery and CPB; postoperative data such as duration of mechanical ventilation, ICU stay, arterial blood gas analysis, and the corresponding data at 12 h and 3 d after surgery when extracting MPs.

Diagnosis of ALI

The patients after cardiac surgery with CPB were divided into two subgroups (ALI and non-ALI) to further analyze the correlation between size distributions of MPs and acute lung injury. The diagnosis of ALI complies with the standard proposed by the American-European Consensus Conference Committee in 1994 (35): 1. Acute onset. 2. The partial pressure of oxygen /fraction of inspiration $O_2 \leq 300$ mmHg, regardless of positive end-expiratory pressure level. 3. Bilateral infiltrates seen on frontal chest radiograph. 4. < 18 mmHg when measured or no clinical evidence of left atrial hypertension.

Statistical Analyzes

Statistical analyzes were performed using SPSS 23.0 software (SPSS Inc, Chicago, Ill). For comparison between patients and healthy subjects, an independent 2-sample *t*-test was conducted. To compare the difference before and after surgery (at various time points), repeated measures analyses with Bonferroni Correction were applied. A $\chi 2$ analysis was conducted to compare proportions between different groups. The relationship between different indexes of MPs' size distribution was evaluated by Spearman's correlation analysis. Parametric data were presented as mean ± standard deviation (untransformed data) or 95% CI (transformed data). $P < 0.05$ was considered statistically significant.

RESULTS

Clinical Characteristics

A total of 103 patients were enrolled and 25 (24.3%) of them developed ALI after cardiac surgery (**Figure 1**). The demographic data and basic perioperative parameters are shown in **Table 1**. There were no significant differences between the ALI and non-ALI groups in demographic and perioperative characteristics except age ($P = 0.023$). The ALI group tended to have longer CPB time, clamping time, and operation time. The operation data are shown in **Table 2**.

Concentration of Circulating MPs in Patients and Healthy Subjects

The number of MPs in the patients notably increased compared with healthy subjects (**Figure 2A**). However, the ALI group and non-ALI group did not differ in the number of MPs (**Figure 2B**).

Comparison of Post-Operative Oxygenation Index (OI) Between ALI and Non-ALI Groups

As shown in **Figure 3**, OI in the ALI group decreased over time after surgery and reduced to around 240 mmHg at 12 h after surgery. OI in non-ALI patients remained around 390 mmHg. OI between the ALI and non-ALI groups differed significantly at 3 h after operation, and the differences became more pronounced with the extension of time, especially at 12 h after the operation when it showed the steepest drop compared with the non-ALI group. However, OI between 12 h and 3 d after surgery remained steady in the ALI and non-ALI groups. Moreover, OI at 0 h after the operation did not differ between the ALI and non-ALI groups.

The Size Distribution of MPs in Patients and Healthy Subjects

There were two peaks of the size distribution curve of MPs in **Figures 4A,B**. One was located < 100 nm and was considered to be exosomes, and the other was located between 100 and 1,000 nm and was considered to be MPs. The diameters in 80% of the MPs (from the first decile to the last decile) in the patients were between 125 and 280 nm at 12 h after surgery, 150 and 400 nm at pre-operation, and 140 and 360 nm at 3 d after surgery. First, we compared the MPs size distribution of all patients with that of healthy subjects at different time points (**Table 3**, **Figures 4A,B**). We found that the MP size at peak of the patients before surgery was slightly < in the healthy subjects (**Table 3**, **Figure 4C**). The MP size at peak at 12 h after surgery dramatically decreased to 231.43 nm compared with 260.55 nm at the pre-operation level, which increased to 252.05 nm at 3 d after surgery (**Table 3**, **Figure 4C**). The IQR of the MP size in patients before surgery was increased compared with healthy subjects. However, the IQR at 12 h after surgery also dramatically reduced to 117.42 nm compared with 163.29 nm at the pre-operation level, which elevated to 138.98 nm at 3 d after surgery (**Table 3**, **Figure 4D**). However, the peak intensity, which means the percentage of MPs at peak, showed no significant difference between healthy subjects and patients before surgery (**Table 3**, **Figure 4E**). The peak intensity at 12 h after surgery increased to 7.78% compared with 6.25% in pre-operation, which elevated to 7.24% at 3 d after surgery (**Table 3**, **Figure 4E**). The changes in peak intensity are opposite to the changes in IQR after cardiac surgery. In addition, the MP size at the peak was positively correlated with the IQR and negatively correlated with the peak intensity at 12 h after surgery (**Figures 4F,G**).

The Distribution of MPs Between the ALI and Non-ALI Groups

The perioperative size distribution of MPs is shown in **Table 4**, **Figure 5**. **Figure 5A**, **Table 4** show that the size at the peak of MPs in patients with ALI at 12 h and 3 d after surgery were < those of the patients without ALI. The preoperative size at the peak of MPs in patients with ALI was also < those of the patients without ALI, but there was no statistical significance. The peak intensity did not differ between the ALI and non-ALI groups except at 12 h,

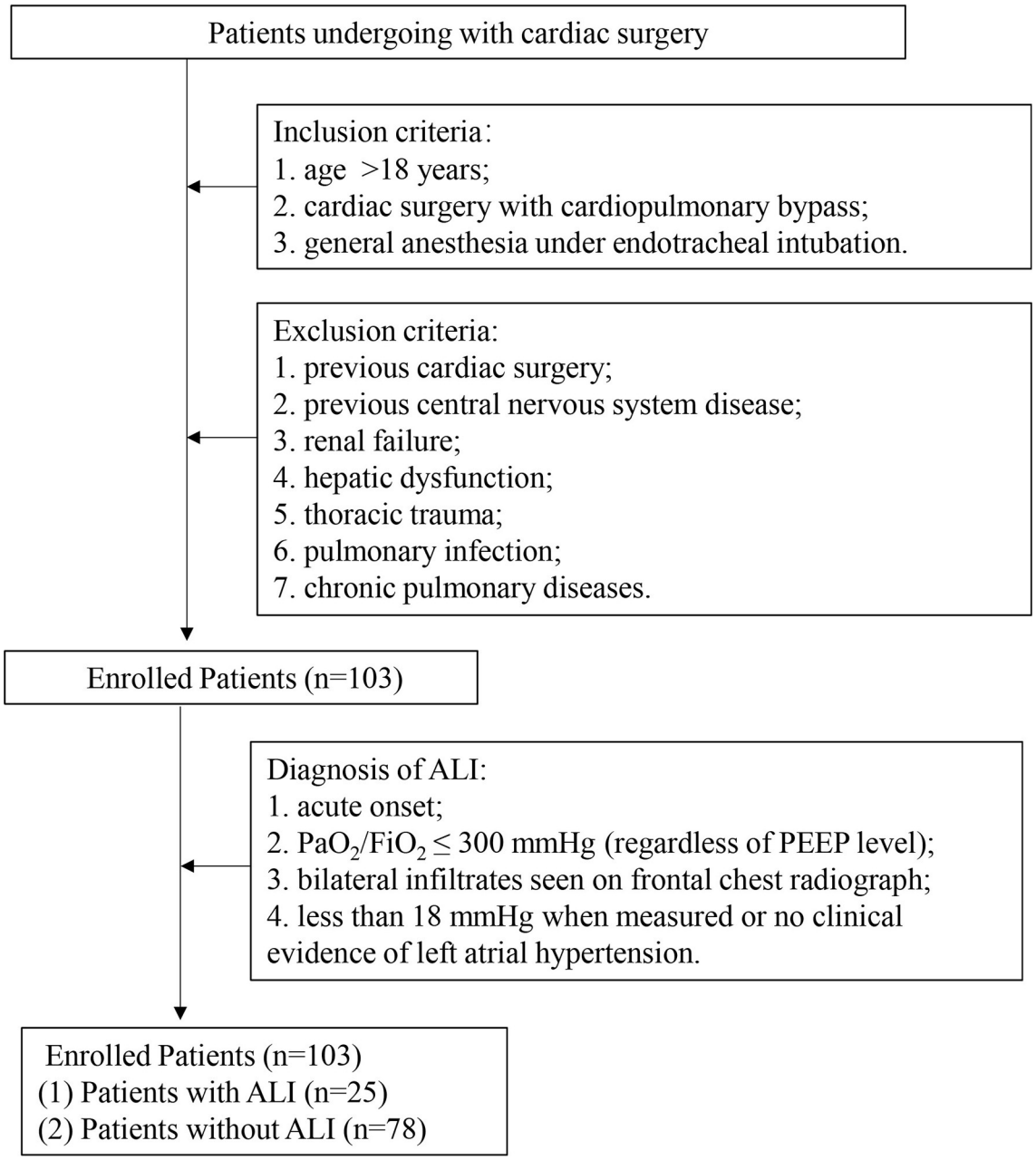

FIGURE 1 | Schematic flow chart of the study design. ALI: acute lung injury; PaO_2, partial pressure of oxygen; FiO_2, fraction of inspiration O_2 and PEEP, positive end-expiratory pressure.

when the peak intensity in patients with ALI was elevated than those in patients without ALI at 12 h (**Figure 5B**, **Table 4**). As for the IQR, there were significant differences between the ALI and non-ALI groups at 12 h and 3 d after surgery, when the values in the patients with ALI were < those in the patients with non-ALI. Meanwhile, there was no statistical significance for the IQR between the ALI and non-ALI groups before surgery (**Figure 5C**, **Table 4**).

Correlations Between Perioperative Measurements and ALI

Since the index of the size distribution of MPs at 12 h after surgery dramatically and repeatedly changes, which might indicate the occurrence of ALI at a very early stage, we further performed the univariate analysis of the size at peak, the IQR, and the peak intensity at 12 h after surgery as well as preoperative clinical parameters potentially associated with ALI as shown in **Table 5**,

TABLE 1 | Perioperative clinical parameters between the ALI and non-ALI groups.

	ALI ($n = 25$)	non-ALI ($n = 78$)	*P*
Age (yr)	59.16 ± 12.67	54.57 ± 10.80	0.023*
Female (%)	48.00	30.77	0.874
BMI (kg/m^2)	25.82 ± 5.49	23.85 ± 3.43	0.107
Drinking (%)	12.00	10.25	0.951
Smoking (%)	20.00	28.21	0.422
Hypertension (%)	40.00	26.92	0.215
Diabetes (%)	12.00	5.13	0.368
Hyperlipidemia (%)	28.00	28.21	0.185
Atrial fibrillation (%)	16.00	23.08	0.457
Coronary disease (%)	52.00	33.33	0.096
Previous myocardial infarction (%)	4.00	1.28	0.396
NYHA classification (I/II/III/IV, n)	1/14/9/1	6/40/28/4	0.922
LVEF (%)	64.12 ± 13.45	63.03 ± 12.92	0.716
CPB time (min)	209.32 ± 106.53	163.88 ± 77.73	0.061
Clamp time (min)	117.48 ± 59.00	95.51 ± 51.83	0.074
Operation time (min)	424.88 ± 171.16	357.22 ± 133.34	0.079

Data are presented as mean ± standard deviation or number of patients (n, %). ALI, acute lung injury; BMI, body mass index; NYHA, New York Heart Association; LVEF, left ventricular ejection fraction; CPB, cardiopulmonary bypass.

TABLE 2 | The operation data between the ALI and non-ALI groups.

Surgery	ALI ($n = 25$)	non-ALI ($n = 78$)	*P*
Valve	10	43	0.188
CABG	4	9	0.811
Congenital	1	3	1.000
Great artery	2	5	1.000
Valve + CABG	3	5	0.632
Valve + Congenital	1	4	1.000
Others	4	9	0.811

Data are presented as number of patients (n). ALI, acute lung injury; CABG, coronary artery bypass grafting.

Figure 6. There are five main parameters associated closely with ALI: size at peak (12 h), IQR (12 H), peak intensity (12 H), BMI, and CPB time. We put these 5 parameters together to perform a multivariate analysis. The results showed that the size at peak of MPs at post-operative 12 h and CPB time are the independent risk factors for the occurrence of ALI after surgery.

Receiver Operating Characteristic Curve Analysis

The value of MP size distribution in predicting the occurrence of ALI was further evaluated using the receiver operating characteristic curve (**Figure 7**). The area under the curve (AUC) of peak diameter at 12 h was 0.803. **Table 6** shows that the best cutoff value for the size at peak at 12 h after surgery to diagnose ALI was 223.05 nm with a sensitivity of 88.0% and a specificity of 66.7%. The positive predictive value (PPV) and negative predictive value (NPV) were 0.458 and 0.945, respectively. As for the IQR of 12 h after surgery, the AUC was 0.717. The best cutoff value for the IQR to diagnose and exclude ALI was 132.65 nm with a sensitivity of 88.0% and a specificity of 47.4%. The PPV and NPV are 0.349, and 0.925, respectively. Combining these two parameters (size at peak and IQR) as a combined diagnostic factor for analysis, the AUC was 0.809, the sensitivity went up to 0.92, and the NPV reached 0.96.

When the size at peak at 12 h after surgery reached 239.2 nm, the sensitivity was 100% with a specificity of 51.3% and 1 of NPV, whereas when the size at peak at 12 h after surgery reached 194.745 nm, the specificity was 84.6% with a sensitivity of 40% and 0.455 of PPV. When the IQR at 12 h after surgery increased to 166.7 nm, the sensitivity was 100% with a specificity of 23.1% and 1 of NPV, whereas when the IQR at 12 h after surgery decreased to 69.2 nm, the specificity was 87.2% with a sensitivity of 36% and 0.474 of PPV. Combining these two parameters as a combined diagnostic factor, we found that when the value was 251.873 nm, the sensitivity was 100% with a specificity of 52.6% and 1 of NPV, whereas when the value was 201.078 nm, the specificity was 84.6% with a sensitivity of 40% and 0.364 of PPV (**Table 6**).

DISCUSSION

ALI is a severe complication after cardiac surgery with CPB and the incidence is up to 60%. Unfortunately, there is no parameter available to predict or exclude ALI after cardiac surgery with CPB. In the past decade, we and other researchers focused on the role of MPs in ALI, the different origins of MPs (endothelial microparticles, monocyte microparticles, platelet-derived microparticles, leukocyte microparticles), especially the concentration, types, and compositions of MPs, and demonstrated that MPs may participate in the development of ALI (11, 12, 16–18, 28, 30, 32, 33, 36–40). Thus, MPs may be a diagnostic marker and treating target in the early stage of ALI. In this study, we found that the size distribution of MPs can be used to predict and exclude ALI after cardiac surgery with CPB.

MPs have been described as cell-derived membrane vesicles of 100–1,000 nm diameter containing proteins, DNAs, and cytosolic materials in extracellular spaces (41). MPs were analyzed with a conventional flow cytometer in many studies. Previously, we found the endothelial microparticles detected by the flow cytometer are increased after cardiac surgery (10). The levels of endothelial microparticles seem to have no correlation with acute lung injury after cardiac surgery probably as a result of inadequate detection for microparticles with diameters $<$ 300 nm. Because of the inherent limitations of the conventional flow cytometer, as these instruments were traditionally developed to measure whole cells, which are orders of magnitude larger and express far more molecules of identifying epitopes, this approach cannot accurately measure the MPs smaller than 300 nm in diameter. Although electron microscopy and atomic force microscopy can detect the MPs with a diameter $<$ 300 nm (42), it is unpractical in clinical measurement since it is time-consuming. Some studies used the conventional flow cytometry plus polystyrene beads of 110, 200, 500 nm, and 1 mm diameter to set up the MP size gate in two small angle light scatter detectors to analyze MPs smaller than 300 nm in diameter. However, it was

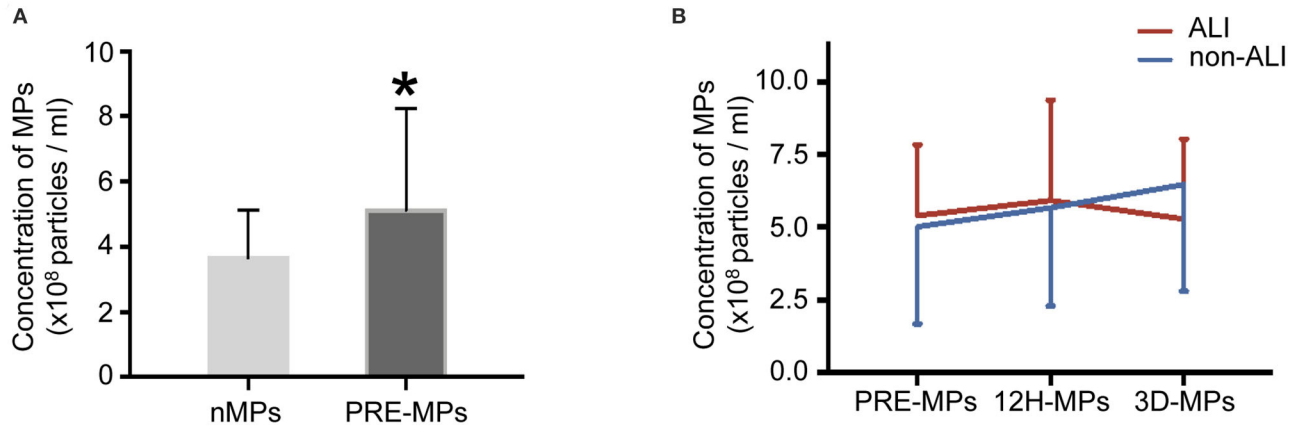

FIGURE 2 | Concentrations of circulating microparticles (MPs) in all patients and healthy subjects. **(A)** The concentrations of MPs in healthy subjects and patients. **(B)** The concentrations of MPs at different perioperative times between ALI and non-ALI groups. *, vs. nMPs, $P < 0.05$. nMPs, MPs from healthy subjects; PRE-MPs, MPs from pre-operation; 12H-MPs, MPs from 12 h after operation; 3D-MPs, MPs from 3 d after the operation.

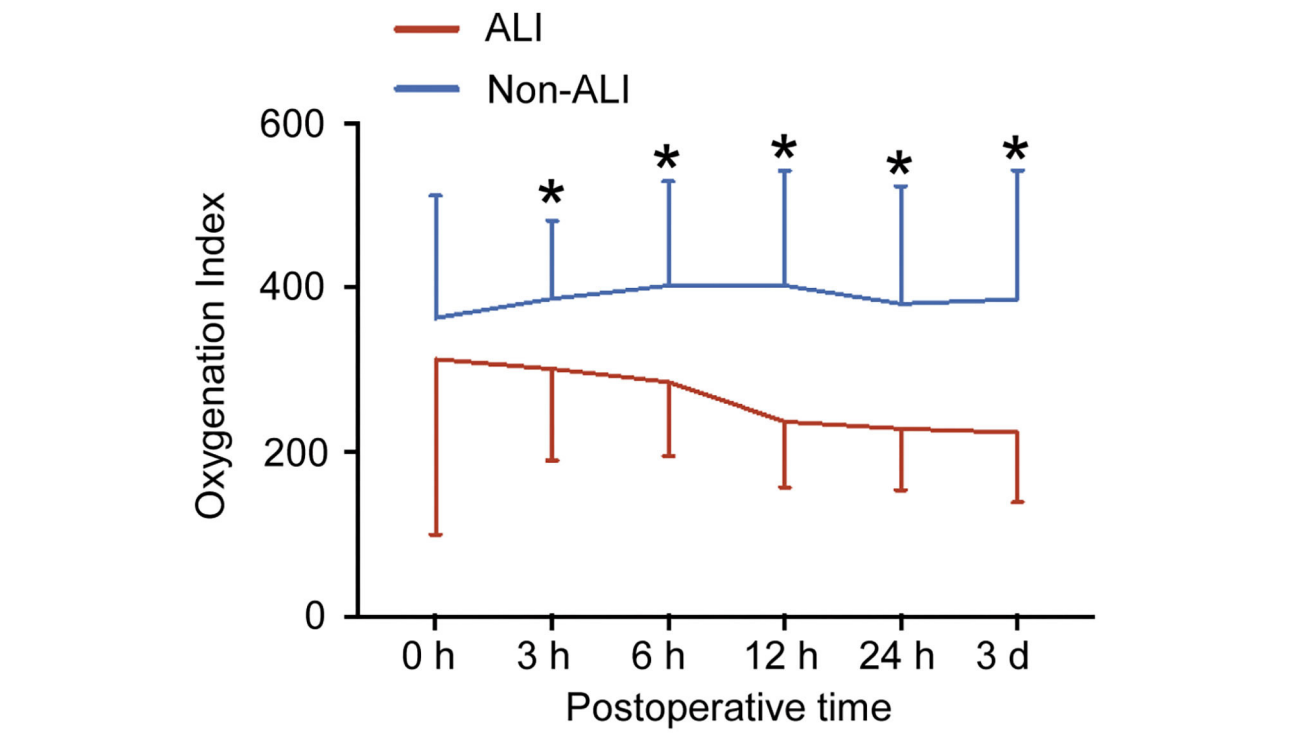

FIGURE 3 | Postoperative oxygenation index (OI) between the ALI and non-ALI groups. The figure showed the OI in patients with and without ALI at different times after surgery. *, vs. ALI group at the same time point, $P < 0.05$.

difficult to separate the background noise with the MPs smaller than 300 nm and effectively measure the size of MPs by different diameter groups, so it was not able to precisely calculate the size of each MP (43, 44). Recently, NTA has been applied to mathematically calculate the concentration and size distribution of MPs, which records the path of each particle to determine the mean velocity and diffusivity, since the particles undergo Brownian motion (45). We adopted this approach to analyze the concentration of MPs in the present study. However, for accurate measurement of the size distribution of MPs, the NTA procedure requires accurate optimization of camera and analysis settings. Separate detections with different settings may be needed to

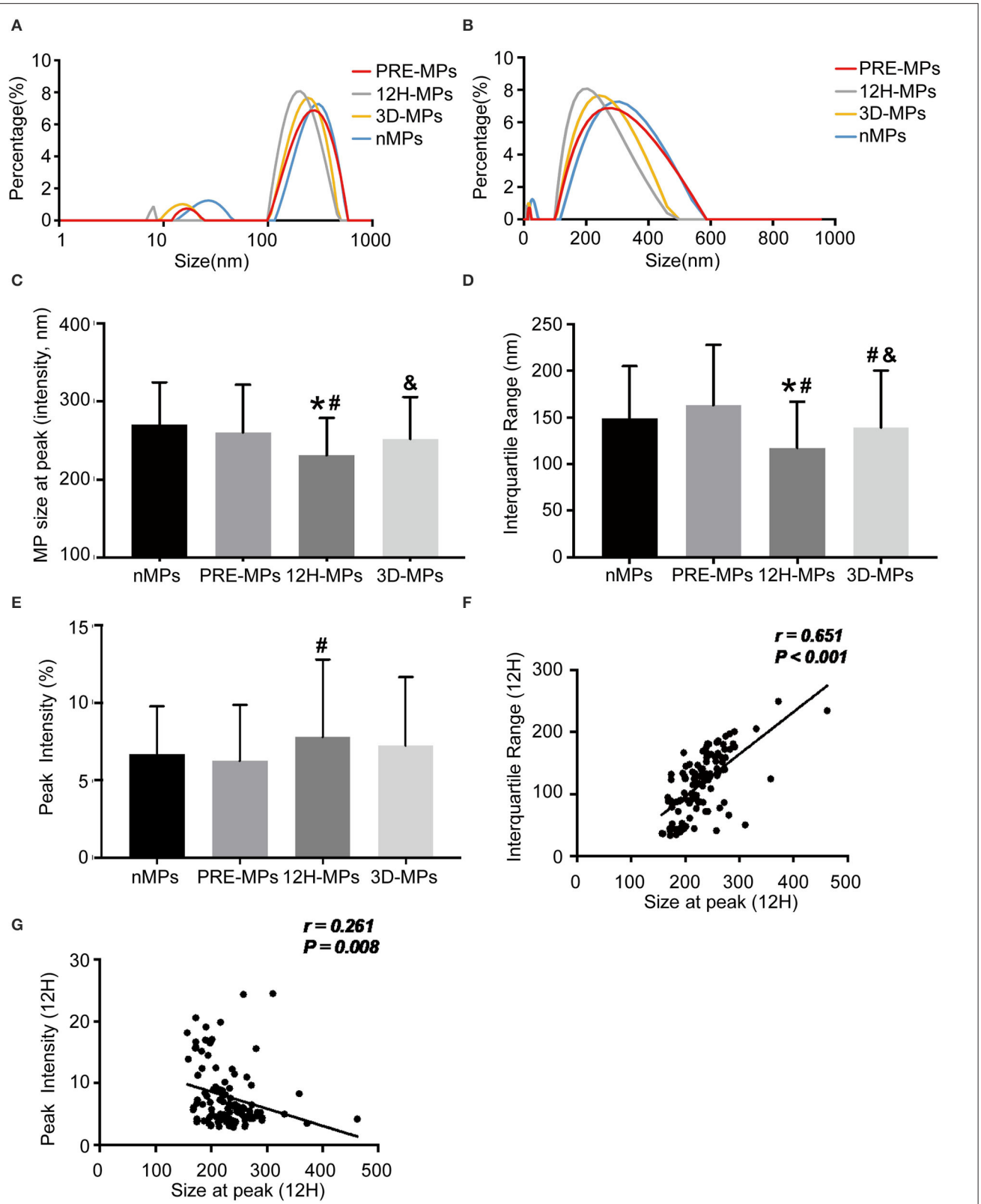

FIGURE 4 | The size distribution of microparticles (MPs) in patients and healthy subjects. **(A,B)**, Representative size distribution curve of MPs in patients **(A,B)** from the same patients using different X-axis. **(C)** The MPs size at peak between patients and healthy subjects. **(D)** The interquartile range (IQR) between patients and healthy subjects. **(E)** The peak intensity between patients and healthy subjects. **(F)** Correlation between size at peak and IQR at 12 h after operation. **(G)** Correlation between size at peak and peak intensity at 12 h after operation. *, vs. nMPs, $P < 0.05$; #, vs. PRE-MPs, $P < 0.05$; &, vs. 12H-MPs, $P < 0.05$. nMPs, MPs from healthy subjects; PRE-MPs, MPs from pre-operation; 12H-MPs, MPs from 12 h after operation; 3D-MPs, MPs from 3 d after operation; 12H, 12 h after operation.

TABLE 3 | The size distribution of MPs.

	nMPs	PRE-MPs	12H-MPs	3D-MPs
Size at peak (nm)	270.92 ± 53.50	260.55 ± 60.88	231.43 ± 47.37*#	252.05 ± 53.95&
Peak intensity (%)	6.69 ± 3.08	6.25 ± 3.61	7.78 ± 5.01#	7.24 ± 4.42
IQR (nm)	149.22 ± 55.64	163.29 ± 64.75	117.42 ± 49.49*#	138.98 ± 61.05#&

*Data are presented as mean ± standard deviation. *, vs. nMPs, P < 0.05; #, vs. PRE-MPs, P < 0.05; &, vs. 12H-MPs, P < 0.05. nMPs, MPs from healthy subjects; PRE-MPs, MPs from pre-operation; 12H-MPs, MPs from 12 h after operation; 3D-MPs, MPs from 3 d after operation; IQR, interquartile range.*

TABLE 4 | Size distribution of MPs between the ALI and non-ALI groups.

Size distribution of MPs	ALI ($n = 25$)	non-ALI ($n = 78$)	*P*
Size at peak (PRE)	243.76 ± 64.15	265.93 ± 59.21	0.114
Peak intensity (PRE)	7.37 ± 4.89	5.89 ± 3.04	0.162
IQR (PRE)	143.57 ± 75.35	169.61 ± 60.14	0.080
Size at peak (12H)	198.49 ± 23.81	241.99 ± 48.27	<0.001
Peak intensity (12H)	9.59 ± 5.27	7.20 ± 4.82	0.037
IQR (12H)	89.40 ± 41.04	126.40 ± 48.82	0.001
Size at peak (3D)	225.84 ± 46.07	260.45 ± 53.84	0.005
Peak intensity (3D)	8.41 ± 5.02	6.87 ± 4.17	0.129
IQR (3D)	113.54 ± 58.15	147.13 ± 60.05	0.016

Data are presented as mean ± standard deviation. ALI, acute lung injury. PRE, pre-operation; 12H, 12 h after operation; 3D, 3 d after operation; IQR, interquartile range.

obtain accurate readings for MPs subsets in heterogeneous mixtures (45). Therefore, in this study, we used a LitesizerTM 500 to measure the size distribution of MPs by dynamic light scattering, a technique to calculate the characteristic time of the fluctuations originating from the Brownian motion of MPs in scattered light intensity, and obtained the results using the Einstein-Stokes equation (46–54). This technique can detect the average particle diameter and particlediameter distribution of nano-sized particles dispersed in a liquid. Using this technique, we found that the diameters in most of the MPs were around 170–300 nm at 12 h after surgery, 175–500 nm before surgery, and 175–500 nm at 3 d after surgery, indicating that using a conventional flow cytometer to measure MPs can only detect a small part of MPs previously, and most of the MPs cannot be detected, especially at 12 h after surgery.

Using this technique, we found that the size of MPs is smaller in patients than in healthy subjects. More importantly, the size of MPs is dramatically reduced after cardiac surgery with CPB, especially at postoperative 12 h. The same happened in the IQR, which is smaller after surgery, especially at postoperative 12 h. On the contrary, the peak intensity increases after the surgery, especially at postoperative 12 h. The curve of the size distribution of MPs seems to be able to shift from right to left (becomes small) with the shape becoming high and narrow (sharp shape) after cardiac surgery with CPB, especially at postoperative 12 h. Why the size distribution of MPs after cardiac surgery is altered remains unclear. One speculation is larger MPs may be filtered by the cardiopulmonary bypass membrane during cardiac surgery. There are a few reports on the changes in sizes or diameters of circulating microparticles under pathological conditions (55–57). The circulating extracellular vesicles (also called microparticles) derived from the ALI patients showed smaller sizes compared with control healthy subjects, but with no significant difference, which may be the result of the insufficiency of enrolled patients (57). The diameters of circulating microparticles isolated from the patients with chronic fatigue syndrome were significantly smaller than those derived from non-fatigued healthy controls (55). In the pig model of the metabolic syndrome, the mesenchymal stromal cells-derived extracellular vesicles were on average smaller in size compared with the extracellular vesicles from the pig control (56). The sizes of circulating MPs are altered under different pathological states and the correlation between the changes and ALI after cardiac surgery is unclear. When further comparing the peak diameter and the IQR of MPs at 12 h after surgery, we found that the peak diameter and the IQR of MPs decreased more significantly in patients with ALI than in patients without ALI, suggesting that the MPs' generation is different before and after the operation, and between the ALI and non-ALI groups. It seems that cardiac surgery with CPB and ALI can stimulate the production of small-sized MPs, which may be used as a biomarker. Indeed, it has been reported that there were increased levels of small-sized MPs in patients with psoriasis (58), which is consistent with our findings. Thus, small-sized MPs may be generated under disaster stress and numerous inflammations, and the true mechanisms need to be further investigated in the future. Another finding in this study is the size distribution of MPs also shifts from right to left (being smaller) with the shape becoming high and narrow (sharp shape) before surgery in patients with ALI compared to the non-ALI group, although there is no statistical significance (may get statistically significant when increasing the sample size), indicating that patients with the small-size and sharp shape distribution of MPs are prone to ALI after surgery.

As mentioned above, the peak diameter and the IQR of MPs at 12 h after surgery changed most significantly. We further analyzed the relationship between these parameters and ALI. We found a certain relationship between peak diameter at 12 h after surgery and ALI in the present study (AUC = 0.803). Although the diagnostic specificity of peak diameter at the best cutoff value is about 66.7%, the diagnostic sensitivity is very high, which reached 88% with a 0.945 negative predictive value. More importantly, the peak diameter of MPs can be used as

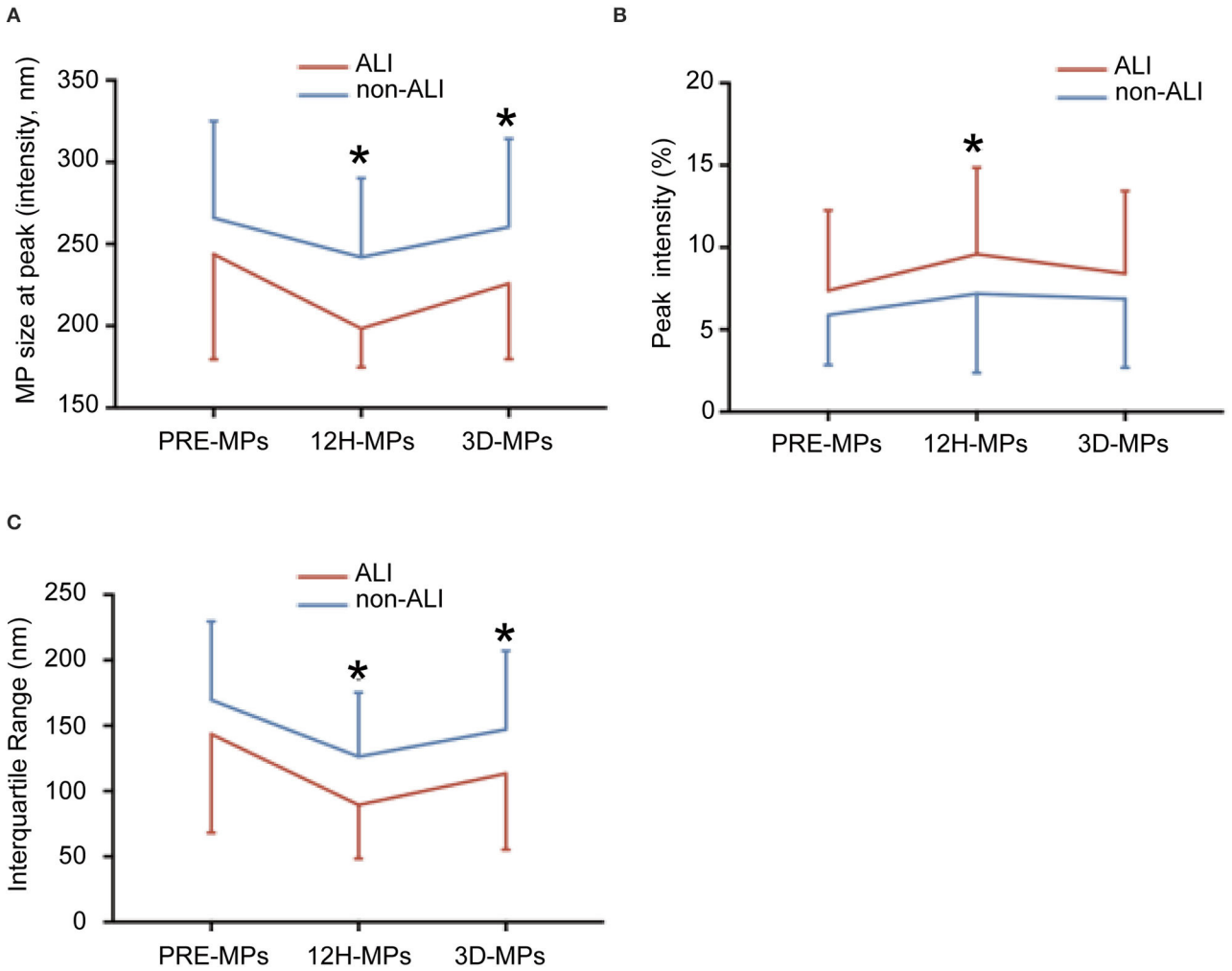

FIGURE 5 | The size distribution of microparticles (MPs) at different times between ALI and non-ALI groups. **(A)** The MPs size at peak between ALI and non-ALI groups. **(B)** The IQR between ALI and non-ALI groups. **(C)** The peak intensity between ALI and non-ALI groups. *, vs. ALI group at the same time point, $P < 0.05$. PRE-MPs, MPs from pre-operation; 12H-MPs, MPs from 12 h after operation; 3D-MPs, MPs from 3 d after the operation.

an exclusive index of ALI when the sensitivity reaches 100%, and the peak diameter at 12 h after surgery is 239.2 nm. In other words, the diagnosis of ALI could be excluded if the peak diameter at 12 h after surgery is larger than 239.2 nm. We can also increase the diagnostic specificity to 84.6% with a negative predictive value of 0.815 by setting a peak diameter smaller than 194.745 nm. Moreover, the IQR of postoperative 12 h also has a certain value in the prediction of ALI. Combining peak diameter and IQR through regression equation at the best cutoff value, the diagnostic sensitivity can reach 92% with a negative predictive value of 0.96. We can also set a certain value of IQR and the combined peak diameter and IQR at 12 h after surgery to reach 100% of sensitivity and more than 85% of specificity to exclude and diagnose ALI. The question is how to diagnose ALI if the peak diameter is between 194.745 and 239.2 nm, the IQR is between 69.2 and 166.7 nm, or the combined factor is between 201.078 and 251.873 nm, which accounts for about 40% of the patients. In this situation, we can combine clinical manifestations and other diagnostic methods to predict ALI. Indeed, further logistic regression analyses suggested that the size of MPs at postoperative 12 h was an independent risk factor for the occurrence of ALI. This is very important since ALI is a severe complication with a great impact on cardiopulmonary function, and postoperative 12 h is a very early stage after cardiac surgery, and doctors can distinguish suspicious ALI patients and conduct further inspection and intervention to prevent the occurrence of ALI or treat patients at an earlier stage of ALI. Therefore, it is of great value to use the size distribution of MPs to predict and exclude ALI after cardiac surgery with CPB.

In this study, the OI of the two groups did not significantly differ at postoperative 0 h. However, the OI of ALI patients decreased gradually with time after

TABLE 5 | Univariate and multivariate analyses of ALI.

	Univariate		Multivariate	
	OR (95 % CI)	***P***	**OR (95 % CI)**	***P***
Size at peak (12H) [a)]	0.700 (0.587, 0.835)	< 0.001	0.687 (0.557, 0.846)	< 0.001
IQR (12H) [a]	0.842 (0.756, 0.938)	0.002		
Peak intensity (12H)	1.091 (1.002, 1.187)	0.044		
Age	1.041 (0.995, 1.089)	0.084		
BMI	1.121 (1.001, 1.255)	0.048		
Smoking	0.636 (0.212, 1.906)	0.419		
Hyperlipidemia	5.045 (0.792, 32.134)	0.087		
Hypertension	1.374 (0.920, 2.051)	0.120		
CPB time	1.006 (1.000, 1.011)	0.036	1.008 (1.001, 1.014)	0.015
Clamp time	1.007 (0.999, 1.016)	0.093		

[a] *Measurement unit: ×10 nm. ALI, acute lung injury; OR, odds ratio; CI, confidence interval; 12H, 12 h after operation; IQR, interquartile range; BMI, body mass index; CPB, cardiopulmonary bypass.*

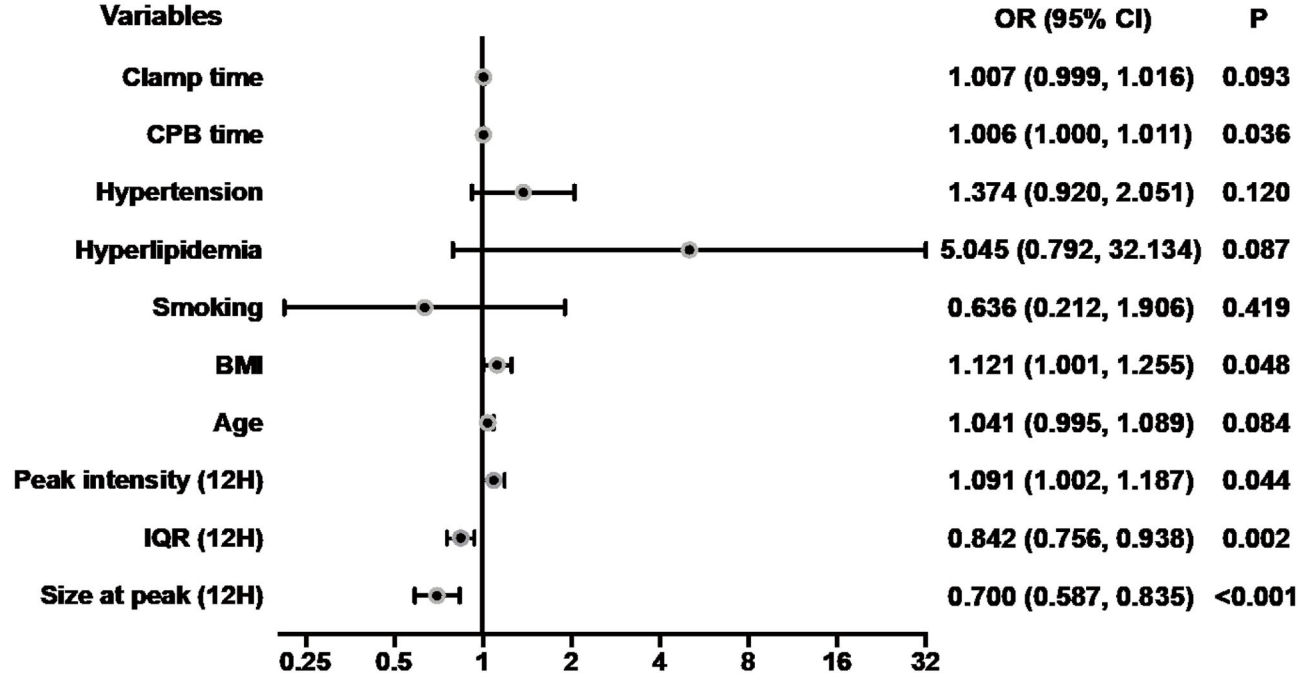

FIGURE 6 | Forest plot for logistic regression analysis. The odds ratios for acute lung injury according to size at peak (12 H) and selected risk factors. CPB, cardiopulmonary bypass; BMI, body mass index; IQR, interquartile range; Size at peak (12H), the size at peak at 12 h after operation; OR, odds ratios; CI, confidence interval.

surgery. It seemed that the pathological changes of the lung had appeared in the early period after the operation, and earlier than the measurement indexes detected by existing methods, such as artery blood gas analysis and chest radiography. The OI of ALI patients decreased apparently in the first 12 h, and then remained stable until 3 d. This is also the reason the three time points were chosen (pre, 12 h, 3 d) in this study.

Limitation: 1. This is a single-center study and the sample size of 103 patients is relatively small. Multicenter researches and an enlarged sample are needed in future studies. 2. The preoperative OI of patients was not obtained, since arterial blood gas analysis was not performed regularly before the operation. If there was no significant difference in preoperative OI between the ALI and non-ALI groups, MPs will be of higher value in predicting postoperative ALI. 3. We chose three time points (pre, 12 h, 3 d) in this study. If we can monitor frequently or even dynamically,

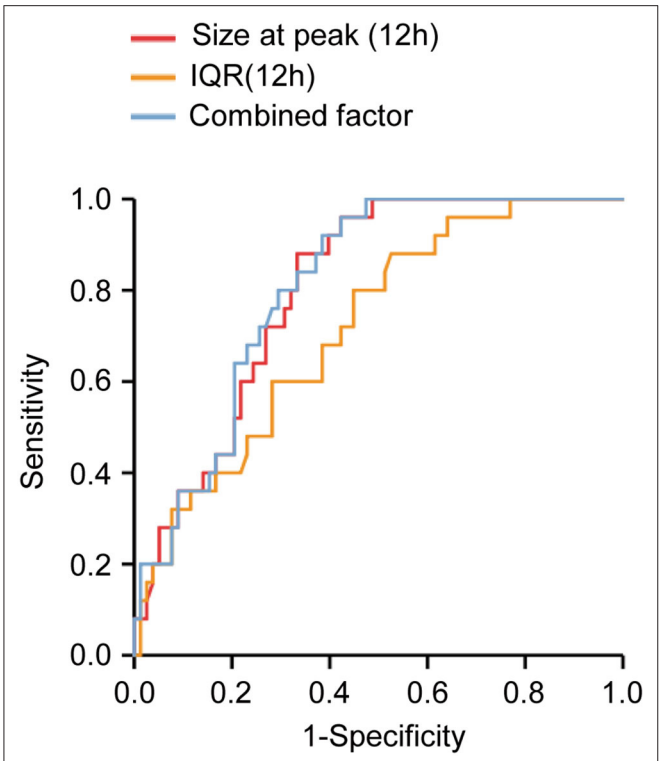

FIGURE 7 | Receiver operating characteristic curve analysis of microparticles (MPs) size distribution determined at 12 h after operation. The areas under the curves were 0.803, 0.717, and 0.809 for size at peak (12H), IQR (12H), and the combined factor, respectively. Size at peak (12H), the size at peak at 12 h after operation; IQR (12H), the IQR at 12 h after operation; combined factor, the combined factor of size at peak (12H) and IQR (12H).

TABLE 6 | Receiver operating characteristic curve.

	AUC	Cutoff value	At cutoff value			
		(True positive cases)	Sensitivity	Specificity	NPV	PPV
Size at peak (12H)	0.803	223.050 (22) [a]	0.880	0.667	0.945	0.458
		194.745 (10) [b]	0.400	0.846	0.815	0.455
		239.200 (25) [c]	1.000	0.513	1.000	0.397
IQR (12H)	0.717	132.650 (22) [a]	0.880	0.474	0.925	0.349
		69.200 (9) [b]	0.360	0.872	0.810	0.474
		166.700 (25) [c]	1.000	0.231	1.000	0.294
Combined factor [d]	0.809	241.532 (23) [a]	0.920	0.615	0.960	0.434
		201.078 (8) [b]	0.400	0.846	0.790	0.364
		251.873 (25) [c]	1.000	0.526	1.000	0.403

[a] Best cutoff value. [b] Cutoff value with specificity about 85%. [c] Cutoff value with sensitivity up to 100%. [d] The combined factor of size at peak (12H) and IQR (12H). AUC, area under the curve; NPV, negative predictive value; PPV, positive predictive value; 12H, 12 h after operation; IQR, interquartile range.

the relationship between ALI and the size distribution of MPs will be more explicit.

Conclusion: Our data showed the peak diameter and the IQR of MPs at postoperative 12 h are smaller in patients with ALI than those in the non-ALI group after cardiac surgery with CPB. This is the first demonstration of the relationship between the size distribution of MPs and ALI after cardiac surgery with CPB. Our findings suggested that the size distribution of MPs could be a novel biomarker to predict and exclude ALI after cardiac surgery with CPB.

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 studies involving human participants were reviewed and approved by Ethics Review Board of the First Affiliated Hospital, Sun Yat-sen University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

J-SO, YL, and Z-JO: conception and design. H-XY, CC, Y-QL, X-JL, Y-TC, K-FL, Y-PJ, J-SL, and Y-QX: acquisition of data. CC and Y-QL: analysis and interpretation of data. J-SO, YL, Z-JO, H-XY, K-FL, CC, and Y-QL: drafting or revising of the article. All authors contributed to the article and approved the submitted version.

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REVIEW
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Cellular Crosstalk in the Vascular Wall Microenvironment: The Role of Exosomes in Vascular Calcification

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Vascular calcification is prevalent in aging, diabetes, chronic kidney disease, cardiovascular disease, and certain genetic disorders. However, the pathogenesis of vascular calcification is not well-understood. It has been progressively recognized that vascular calcification depends on the bidirectional interactions between vascular cells and their microenvironment. Exosomes are an essential bridge to mediate crosstalk between cells and organisms, and thus they have attracted increased research attention in recent years. Accumulating evidence has indicated that exosomes play an important role in cardiovascular disease, especially in vascular calcification. In this review, we introduce vascular biology and focus on the crosstalk between the different vessel layers and how their interplay controls the process of vascular calcification.

Keywords: exosomes, vascular calcification, vascular smooth muscle cells, endothelial cells, immune cells

AN INTRODUCTION TO EXOSOMES

Exosomes, with a diameter of 30–150 nm, are a type of extracellular vesicle (EV) released from intracellular stones called multivesicular bodies (MVBs). They were first reported by Johnstone (1) and simply considered a pathway to expel waste to regulate membrane recycling during cell proliferation. Exosomes have been shown to play a role in communicating B lymphocytes and dendritic cells (2). Secreted exosomes were able to regulate cellular functions locally or even over long distances. Besides, exosomes could act as messengers that transfer lipids, messenger RNAs (mRNAs), microRNAs (miRNAs), and proteins between cells (3). It has become clear that the conditioned culture media as well as body fluids including blood, urine, and cerebrospinal fluid contain numerous cell-derived vesicles. Exosomes played prominent roles in tumor metastasis, bone diseases, diabetes, obesity, and thyroid disease, among other conditions. Thus, there is growing interest in understanding the functional role and regulatory mechanisms of exosomes (4–8).

Exosomes are derived from plasma membrane invagination into an early endosome. Then, the mature endosome can evolve into MVBs with different cargo, followed by the budding of selective intracellular vesicles from intraluminal vesicles (ILVs). However, there is no consensus regarding whether these vesicles are involved in general or specific physiological secretion because different types of membrane vesicles could be generated in different conditions (9, 10).

Ectosomes/microparticles (MPs), and apoptotic bodies (ABs) are other common EVs, which have often been confused with exosomes (11, 12). However, their diameter, assembly, subcellular origin, sedimentation force, and biogenetic mechanisms are different than exosomes (11). We described the characteristics of different types of EVs in a previous publication (5). In addition, some researchers have proposed using the term "extracellular vesicles" rather than "exosomes," because the components, classification, and biological functions of these vesicles are unclear (13).

Cardiovascular disease remains the leading cause of morbidity and mortality throughout the world. In recent years, exosomes haves gained increasing attention in the cardiovascular field, largely owing to their ability to transmit biological information between adjacent and distant cells (14). Previous reviews mostly focused on the underlying mechanisms of exosomes in cardiovascular diseases (15, 16), yet ignore the cellular crosstalk among different vessel layers. Recent studies have found that endothelial cells, VSMCs, fibroblast, macrophages, and even circulation blood cells around vascular wall environments respond actively to stimulus and participate in vascular calcification *via* transdifferentiating, cytokine secretion, extracellular vesicle synthesis, angiogenesis regulation, and hemodynamics. In this article, we aim to summarize the complex interactions between exosomes derived from multiple cell populations in the cardiovascular microenvironment.

DEFINITION AND CLASSIFICATION OF VASCULAR CALCIFICATION

Vascular calcification (VC) is a life-threatening complication characterized by ectopic calcium-phosphate deposition in the blood vessel walls. VC has been reported as an independent predictor of all-cause mortality and cardiovascular mortality in patients with uraemia, and it is inextricably associated with cardiovascular events including myocardial infarction, cerebral embolism, and amputation (17, 18). The clinical classification of VC depends on the area of calcification, mainly involving intimal calcification, medial calcification, adventitial calcification, and valve calcification (17, 19, 20). Intimal calcification is common in patients with atherosclerosis, also known as atherosclerotic calcification. Under the forces of inflammation, oxidative stress, mechanical stress, and other factors, the intima often proliferates with lipid deposits and macrophage infiltration, which present as focal spots or plaques (21). Vascular medial calcification, also known as Mönckeberg's sclerosis, occurs in elderly individuals as a result of aging, while it is often accelerated in patients with metabolic disorders, such as diabetes, chronic kidney disease (CKD), and heritable mineralization disorders (22). Medial calcification can occur independently of atherosclerotic lesions, resulting in increased arterial stiffness, reduced compliance of the blood vessels, and elevated risk of adverse cardiovascular events and mortality (23). In turn, calcification in different locations have their own distinctive features, with intimal and medial calcification being most pronounced.

As we illustrate above, VC is able to occur in different layers of the vessel wall and highly correlated with major adverse cardiovascular events, so several treatments aim to target anti-VC. However, effective therapy is still lacking. A meta-analysis showed that statin has a strong association with coronary stenoses rather than coronary calcification (24). Besides, Pi binders didn't work in reducing VC in the CKD population (25). Because there is no specific treatment available for VC currently, a better understanding of the cellular crosstalk that occurs during VC could provide new biomarkers for diagnosis and prognosis as well as novel targets for treatment.

MECHANISMS OF VASCULAR CALCIFICATION

Early studies had suggested that VC is a passive pathologic process in the vascular system due to imbalanced calcium and phosphorus metabolism (26). However, since the late 1990s researchers have gradually recognized that VC is an active, multifaceted, and dynamic process that is similar to bone development (27). The phenotype transition of vascular smooth muscle cells (VSMCs) into osteoblastic cells is the key step that contributes to VC progression. Oxidative stress, inflammation, apoptosis, autophagy, matrix remodeling, and microRNAs (28, 29) have also been reported to play important roles in regulating VC. Exosomes are of great significance in regulating cardiovascular homeostasis and bone biology (30). Under physical states, vascular cells secret exosomes to modulate the normal function of the vascular wall. However, certain exosomes gain calcification potential after the microenvironment is disrupted, and thus contribute to VC procession through transport and loading (31). Latest studies have shown that EVs derived from lipopolysaccharide (LPS-EK)-treated macrophages can propagate inflammation and oxidative stress in VSMCs, thus facilitating the progression of VC (32). Moreover, it is reported that bone marrow mesenchymal stem cell-derived exosome (BMSC-Exo) acts as an anti-calcification effector in CKD by delivering enclosed miR-381-3p. In summary, exosome plays an irreplaceable role in vascular calcification.

Abbreviations: EV, extracellular vesicle; MVBs, multivesicular bodies; mRNAs, messenger RNAs; miRNAs, microRNAs; ILVs, intraluminal vesicles; MPs, microparticles; ABs, apoptotic bodies; VC, Vascular calcification; CKD, chronic kidney disease; VSMCs, vascular smooth muscle cells; ECs, Endothelial cells; VEGF, vascular endothelial growth factor; PDGF, platelet-derived growth factor; bFGF, basic fibroblast growth factor; IGF-1, insulin-like growth factor 1; NO, nitric oxide; PGI2, prostacyclin; ET, endothelin; EndMT, endothelial–mesenchymal transition; SM22alpha, smooth muscle 22 alpha; OPN, osteopontin; OCN, osteocalcin; BMP-2, bone morphogenetic protein-2; ALP, alkaline phosphatase; RUNX2, Runt-related transcription factor 2; MVs, matrix vesicles; MGP, matrix Gla protein; ROS, reactive oxygen species; TANK, TRAF family member associated NFkappaB activator; CXCL12, Chemokine 12; EMPs, endothelial microparticles; TNF-α, tumor necrosis factor-alpha; HUVECs, human umbilical vein endothelial cells; mTOR, mammalian target of rapamycin; TSG101, Tumor Susceptibility Gene 101; HMGB1, high mobility group box 1; BMDCs, bone marrow dendritic cells; MSCs, Mesenchymal stromal cells; HSCs, hematopoietic stem cells; cMPs, Circulating microparticles; pMPs, platelet-derived microparticles; AC, coronary artery calcification; ACE, angiotensin-converting enzyme; ncRNAs, non-coding RNAs; siRNA, small interfering RNA; ECM, extracellular matrix; Treg, regulatory T; LPS-EK, lipopolysaccharide; BMSC-Exo, bone marrow mesenchymal stem cell-derived exosome.

CELL BIOLOGY IN THE VASCULAR WALL MICROENVIRONMENT

Anatomically, blood vessel walls can be divided into three layers: tunica intima, tunica media, and tunica adventitia. However, the vessel wall is not so divisive as it seems, it is more inclined to function as an integrated whole with cellular crosstalk, thus we regard it as "vascular wall microenvironment." The vascular wall microenvironment comprises various cell types, including cells embedded into vessel walls, blood cells, stromal cells, and immune cells. These cells function together to convey blood throughout the body, bringing nutrients, removing wastes (33), and modulating vascular tone and blood pressure. Hence, maintaining normal metabolic crosstalk among multiple cell types in different layers is critical for vascular wall microenvironment stabilization and remodeling. In this section, we comprehensively summarize the cell biology in different vessel layers and the evidence that they act as key mediators and regulators of VC in the vascular wall microenvironment (**Figure 1**).

Tunica Intima: Endothelial Cells

Endothelial cells (ECs) are the principal cells of the thinnest intimal layer. The long axis of ECs is consistent with the direction of blood flow, providing a smooth surface for blood flow and building a barrier between the central lumen and the surrounding vessel wall. Hence, it is not surprising that ECs have the ability to initiate both haemostasis and inflammation (34) and to modulate blood pressure and angiogenesis. More importantly, ECs are the first cells of the vascular system exposed to harmful stimuli. ECs can transfer messages *via* secretion of pro-calcification cytokines, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PDGF), basic fibroblast growth factor (bFGF), insulin-like growth factor 1 (IGF-1), nitric oxide (NO), prostacyclin (PGI2), and endothelin (ET), among others. Furthermore, ECs initiate the endothelial–mesenchymal transition (EndMT) in the microvasculature mineralization process by altering its EC-specific markers into mesenchymal cell-specific markers (35). In fact, ECs are far more than just a physical barrier: they are extensively involved in VC (36). The exosome-mediated crosstalk between ECs and VSMCs, through a paracrine mechanism, likely plays a regulatory role in VC pathogenesis.

Tunica Media: VSMCs

The tunica media is an elastic membrane mostly composed of VSMCs. These cells are required for optimal functioning of arteries, primarily facilitating vessel dilation, and constriction to direct blood flow into various organs and tissues (37–39). VSMCs are arranged in a spiral form around the vessel lumen and display great phenotypic plasticity (40). Under pathological conditions, such as vascular injury, mechanical stretch, and other stimulation (41–43), a physical contractile phenotype could differentiate toward a synthetic phenotype to remodel extracellular matrix components. VSMCs lose the contractile phenotype-specific markers α-smooth muscle actin and smooth muscle 22 alpha (SM22 alpha) and progressively remodel themselves into osteoblast-like cells that express specific osteogenic genes in VC progression, such as Runt-related transcription factor 2 (RUNX2), osteopontin (OPN), osteocalcin (OCN), bone morphogenetic protein-2 (BMP-2), alkaline phosphatase (ALP), and osterix (44, 45). Activation of downstream Msx2 and Wnt signaling initiates VSMC calcification (46, 47). Recent studies have even indicated that there was great heterogeneity in VSMC phenotypes, which possessed a variety of features, including adipocytic, senescent, foam, and osteochondrogenic phenotypes (48–50). Epigenetic regulation (51) and microRNAs also contribute to VSMC differentiation under calcifying conditions (30, 52). These novel findings that link VSMC and tunica media VC have important implications for the design of novel diagnostic and therapeutic strategies. However, these studies have only focussed on the direct effects of factors on VSMCs and have largely ignored the combined effects of the vascular wall microenvironment. An interesting perspective on triggered exosome excretion within the vascular wall microenvironment has received more attention (22, 46).

Tunica Adventitia

The tunica adventitia mainly consist of fibroblasts, myofibroblasts, immune cells (macrophages, dendritic cells, T cells, B cells, and mast cells), stem/progenitor cells, vascular pericytes, smooth muscle cells, adipocytes, among others; hence, the adventitia is the most complex layer of blood vessels (53). The cells in the adventitia can regulate the structure and function of all three layers of the vascular wall (54).

There is an intriguing hypothesis that adventitial cells are "sentinel cells" of vascular dysfunction, carrying out a passive secondary response to monitoring the vascular wall microenvironment (55). Adventitial fibroblasts and their subpopulations, which are recognized as adipogenic progenitors, could transdifferentiate into myofibroblasts (56). Researchers have shown that in diabetic mice feed with a high-fat diet, myofibroblasts in the aortic adventitia participate in osteogenesis, targeting vascular Msx1 and Msx2 (57). Besides, adventitial fibroblasts respond quickly to the release of growth factors, inflammatory cytokines, angiogenic factors, adhesion molecules, and reactive oxygen species when faced with vascular injury and stress (53). Moreover, the adventitial progenitor cells contribute to both vascular injury repair and VC (53), confirming their indispensable role in VC. However, it is unclear whether adventitial stem cells only proliferate and differentiate to maintain vascular wall microenvironment homeostasis, or whether they also have the ability to reprogram vascular development under pathological conditions.

EXOSOME-MEDIATED CROSSTALK IN THE VASCULAR WALL MICROENVIRONMENT

Numerous studies have demonstrated that exosomes mediate changes in the vascular wall microenvironment, including facilitating hydroxyapatite crystal deposition (56), regulating

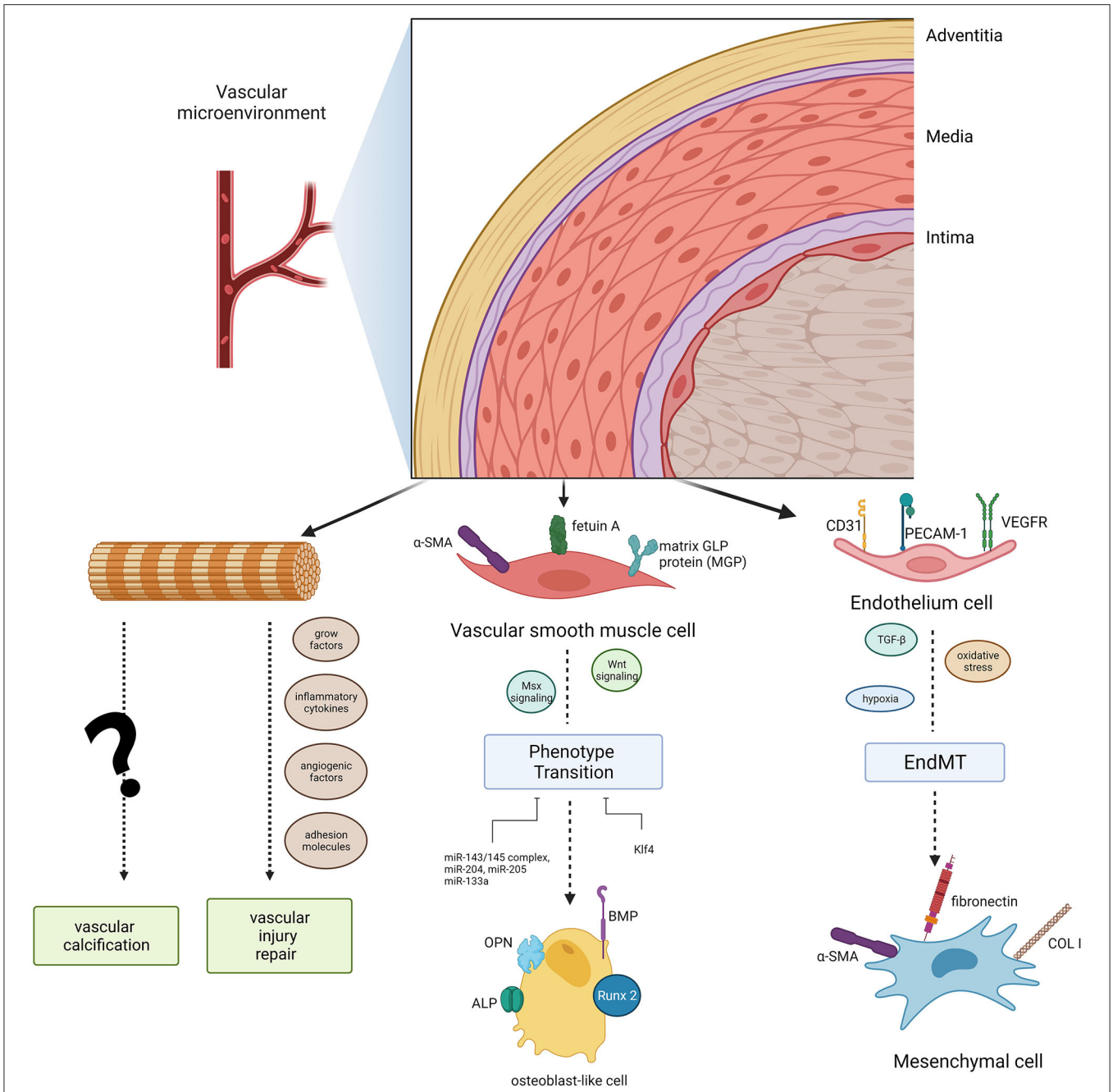

FIGURE 1 | Diagram of the cell populations in the vascular microenvironment and their roles in vascular calcification. Endothelial cells (ECs) and vascular smooth muscle cells (VSMCs) can induce phenotype transitions (EndMT, endothelial- mesenchymal transitions, a specific transition of ECs) in the microvasculature mineralization process *via* several signaling pathways. Adventitial fibroblasts could function in vascular calcification as well as vascular injury repair.

phenotype transition (58), or interfering in underlying signaling. In the vascular system, exosomes can be released by several types of cells, and their biological effects on the recipient cells depend on exosomes and the microenvironment where the recipient cells reside. In this section, we summarize exosome-mediated crosstalk among cells during VC in the following paragraphs.

VSMCs–VSMCs Crosstalk

VSMCs are major effector cells of VC. It has long been recognized that VSMCs can transfer pro-calcification signals among themselves by releasing matrix vesicles (MVs). MVs in blood vessels resemble bone MVs, which are the initial sites for mineralization (59–62). Bone MVs are rich in calcium phospholipid-binding proteins, which transform

excess extracellular calcium into calcified MVs. Calcified MVs trigger hydroxyapatite crystal deposition in the extracellular matrix and then interact with collagen, which promotes bone mineralization (63–65).

Similarly, MVs played an important role in the initiation of vascular mineralization by regulating calcium and phosphate homeostasis in the extracellular matrix (66, 67). Reynolds et al. (68) demonstrated that MVs derived from VSMCs contain mineralization inhibitors such as fetuin-A, an endogenous calcium binder, to prevent VC under normal conditions (58). This was consistent with our previous research that plasma vesicles derived from patients with uremia and renal transplant recipients with VC had different contents of endogenous calcification inhibitors or promoters (69). High circulating endogenous calcium and phosphate might contribute to enhancing VC by decreasing the mineralization inhibitor matrix Gla protein (MGP) (68, 70). Utilization of the L-type calcium channel blocker verapamil could inhibit vascular smooth muscle mineralization and MVs activity (71). This evidence suggested that MVs mainly take function *via* regulating calcium and phosphate metabolism.

Given that MVs share similar compositional characteristics to exosomes, new discoveries are shedding light on the working mechanisms of exosomes (66). Exosomes can act as a specific signal transporter participating in cell proliferation, migration, apoptosis, calcification, and differentiation through VSMC–VSMC crosstalk. Notably, VSMC calcification was mediated by regulating the volume of exosomes secreted from donor VSMCs in response to environmental factors. For example, environmental calcium stress could increase exosome release from VSMCs, which subsequently promotes VC (66). Bhat et al. (72) also indicated that *Mcoln1* gene deletion in mice increases exosome secretion from VSMCs, which contributes to arterial tunica media calcification and stiffening. Other evidence showed that excessive reactive oxygen species (ROS) production, increased the number of exosomes released from VSMCs, therefore increasing the calcification of recipient VSMCs (67). Taken together, calcification of arteries was extended from calcified VSMCs to adjacent normal cells by regulating exosome excretion, and the detailed mechanisms should be elucidated further.

Exosomes are able to carry important cargo such as mRNAs, miRNAs, lipids, and proteins from donor cells and thus regulate the function of recipient cells (30). Hence, besides modifying the number of released exosomes, the progression of calcification could be controlled by altering the content of functional molecules in exosomes. Pan et al. (73) illustrated that microRNA profiles were significantly altered in exosomes from VSMCs undergoing calcification induction. Nine hundred eighty-seven and 92 significantly upregulated and downregulated miRNAs were identified, respectively. Other research had revealed that miRNA expression in MVs during VC altered the expression of osteogenic markers (Smad1, Runx2, ALP, and osterix) (74–77), providing strong support that exosomes could transport vital information during VC. In a recent study, we showed that melatonin treatment diminished both calcification and senescence of VSMCs. The anti-calcification role was mediated by decreased miR-204/miR-211 in exosomes released from calcified VSMCs (78). Clinically, circulating as well as cell-derived exosomes with a low fetuin-A level play a detrimental role in VC (58, 79). While the GFOGER peptide, a six amino-acid repeat present in type 1 collagen, alleviated VC by reducing the content of osteogenic switching–related proteins, such as TANK-binding kinase 1 and casein kinase II, in VSMC-derived EVs (80). These pieces of evidence remind us that the cargo loaded by exosomes secreted from VSMCs changes dynamically with the progression of VC, which provides potential diagnostic biomarkers and targets for treatment. However, more in-depth exploration needed to be conducted before applying in clinical.

A number of studies have focussed on the roles of exosomes in mineral deposition during VC. This focus has limited discoveries of how exosomes function as information transporters among cell populations (66). Nevertheless, there was a view that exosomes were just erroneously trapped in the extracellular matrix, which adds a new dimension to EV-mediated calcium deposition (81). It seemed that exosomes have more effects than what has been reported; thus, greater attention is needed regarding their role in VC. Clarifying how exosomes mediate cell-to-cell communication and the composition or phenotype of exosomes in physiological and pathological vascular conditions will be crucial to expanding our understanding of how exosome-mediated actions in vessels contribute to VC. This information could facilitate the discovery of new therapeutic targets and preventive strategies.

ECs–VSMCs Crosstalk

Exosome-mediated crosstalk between ECs and VSMCs is widely recognized to be involved in regulating the formation of calcific plaque. Similar to VSMC–VSMC crosstalk, EC-derived EVs can mediate intercellular communication through distinct cargo and bioactive ligands. Exosomal miRNAs can convey information from the donor to the recipient to promote the phenotype transition of VSMCs in cardiovascular disease. Zernecke et al. (82) indicated that EC-derived membranous microvesicles convey paracrine alarm signals to recipient vascular cells to trigger the miR-126-mediated production of CXCL12 during atherosclerosis. Moreover, shear stress–stimulated EC-derived EVs containing miR-143/miR-145 controlled target gene expression in co-cultured VSMCs and led to atherosclerotic lesion diminishment in the aorta of $Apoe^{-/-}$ mice (83). In elderly patients with CKD, endothelial microparticles (EMPs) from tumor necrosis factor-alpha (TNF-α)–stimulated human umbilical vein endothelial cells (HUVECs) loaded with BMP-2 could be actively taken up by VSMCs, thus inducing osteogenesis and calcification (84). Besides, Cavallari et al. (85) found that among the majority of circulating EVs derived from ECs, platelets, and monocytes/macrophages, only endothelial EVs showed notable enhancement in patients with CKD compared with healthy people, indicating that ECs are the major origin of circulating EVs in patients with CKD (85). Interestingly, compared with young subjects, only EVs from senescent ECs and the plasma of elderly people could induce calcification. These findings provide a foundation for elucidating the relationship between aging and EC-derived EVs (86). Furthermore, a

recent study used fluorescence microscopy and confirmed the intracellular uptake of EVs between valvular interstitial cells to valvular ECs through the endosomal pathway, once again revealing the key role of EVs in cardiovascular cell-to-cell communication (81).

A series of studies have focussed on how exosome activity connects diabetes and VC. In one study, high glucose-induced diabetes-related VC/aging by increasing lactate dehydrogenase activity and the product of lipid peroxidation (denoted by the malondialdehyde content) through HUVEC-Exos (87). In another study, high glucose-stimulated ECs showed activation of the exosomal Notch3/mammalian target of rapamycin (mTOR) signaling pathway, and thus promoted VSMCs calcification/aging (88). These findings could lead to novel strategies for the prevention of diabetes induced VC. In summary, the investigation of exosomes from ECs revealed the relationship between endothelial cells and vascular calcification, thus providing innovative ideas for the intervention and treatment of vascular calcification. Of course, more in-depth exploration was required to be conducted.

Immune Cells–VSMCs Crosstalk

Immune cells represent multiple cell populations that mediate a large part of the progression of various cardiac immune responses, such as atherosclerosis (89) and myocardial infarction. The immune response consists of innate and adaptive immunity, which has been considered a major contributor to the occurrence of cardiovascular disease in the past few years (89). Regulatory T (Treg) cells, a kind of cells that can negatively regulate the immune response, play a critical role in immune response. Exosomes are proven to contribute significantly to the homeostasis modulation of Treg cells by transmitting exosomal contents to recipient cells in diseased/healthy tissue (90). Previous studies have shown that Treg-cell-derived exosomes are suppressive effectors in the immune response by delivering miR-155 (91, 92). The key point is that miR-155 is of great significance in VC procession (77), and exosomal miR-155 derived from Treg cells might serve as another key source of miRs during VC. Therefore, exosomes are emerging as new biomarkers between immune response and VC procession, and such cellular crosstalk mostly depends on the miRs transportation.

Exosomes derived from immune cells and their parent cells have been shown to enhance pleiotropic dimensions of intercellular communication in some specific pathological states (93). New et al. (94) found that macrophage-derived MVs with exosomal markers (CD9 and TSG101) contributed directly to the early calcification of atherosclerotic plaques through the phosphatidylserine–annexin V-S100A9 membrane complex; this pathway could also be activated in diabetes mellitus (95). A novel mechanism is that high mobility group box 1 (HMGB1), a cytokine associated with biomineralization, promoted the secretion of MVs from macrophages and induced ectopic mineralization *in vitro* in a medium with high calcium and phosphorus *via* the RAGE/p38 MAPK/nSMase2 signaling pathway (96). An *in vivo* study indicated exosomes from cultured bone marrow dendritic cells (BMDCs) could be absorbed by aortic ECs of mice through intravenous injection (97). Furthermore, mature dendritic cell-derived exosomes activated endothelial inflammation and induced atherosclerosis *via* TNF-α-mediated activation of the NF-κB signaling pathway in a manner similar to that of lipopolysaccharide (97).

In addition to the direct cell-to-cell approach, immune cells can interfere in exosome-mediated information transport among other cells. In a macrophage/VSMC co-culture system, macrophage galectin-3 regulated the migration of VSMC-derived exosomes and induced diabetic vascular intimal/medial calcification translocation, which may provide a potential method for early intervention in diabetic VC (98). These findings have extended our knowledge of how exosomes derived from immune cells regulate immune responses during VC. This information has provided insights into searching for novel approaches to prevent VC.

Stem Cells–VSMCs Crosstalk

Bone marrow-derived stem/progenitor cells are pluripotent, meaning they can differentiate into multiple stromal cell types, such as astrocytes, adipocytes, myocytes, fibroblasts, and osteoprogenitors (99, 100). Mesenchymal stromal cells (MSCs) play an essential role in vascular regeneration and development under physiological conditions because they can promote angiogenesis, mediate immunity, and restrain inflammation (101). Previous studies have mainly focussed on how cytokines released from MSCs modulate VC, and there have been only a few studies concerned with MSC-derived exosomes. Several compelling trials have focussed on how exosomes from bone marrow-derived MSCs are involved in VC. Exo treatment diminished the calcium content and alkaline phosphatase activity in the presence of high phosphorus due to exosomal microRNAs (102), such as miR-146a (103) and miRNA-126/miRNA-145 (104). Similar to other stem cells, hematopoietic stem cells (HSCs) are capable of secreting EVs, including exosomes and microvesicles. Based on the available data, the cardioprotective action of injected HSCs might be explained by paracrine communication of HSC-derived exosomes and microvesicles between donor and recipient cells. Moreover, the indirect cardioprotective functions of HSCs in cardiovascular disease are mainly due to the EVs produced in HSC-derived cells, such as dendritic cells and endothelial stem cells (105). Therefore, in-depth research of the molecular mechanism of the exosomes derived from stem cells in the vascular wall microenvironment is critical to take full advantage of the promising prospects of effective treatment.

Other Cell Populations

Circulating microparticles (cMPs) also play essential roles in cellular crosstalk and act as messengers to activate cells. Suades et al. (106) reported that platelet-derived microparticles (pMPs) are able to stimulate thrombosis. They also found that high levels of TSP1+/CD142+ platelet-derived microparticles distinguish young patients with high cardiovascular risk and subclinical atherosclerosis from matched healthy volunteers ($P < 0.0001$) (106). Women with a history of pre-eclampsia show a high risk for future coronary artery calcification (CAC) and metabolic disorder, which may be associated with a larger group of specific blood-borne, cell-derived antigen-positive microvesicles, including stem/progenitor cell antigen CD117 and tissue factor,

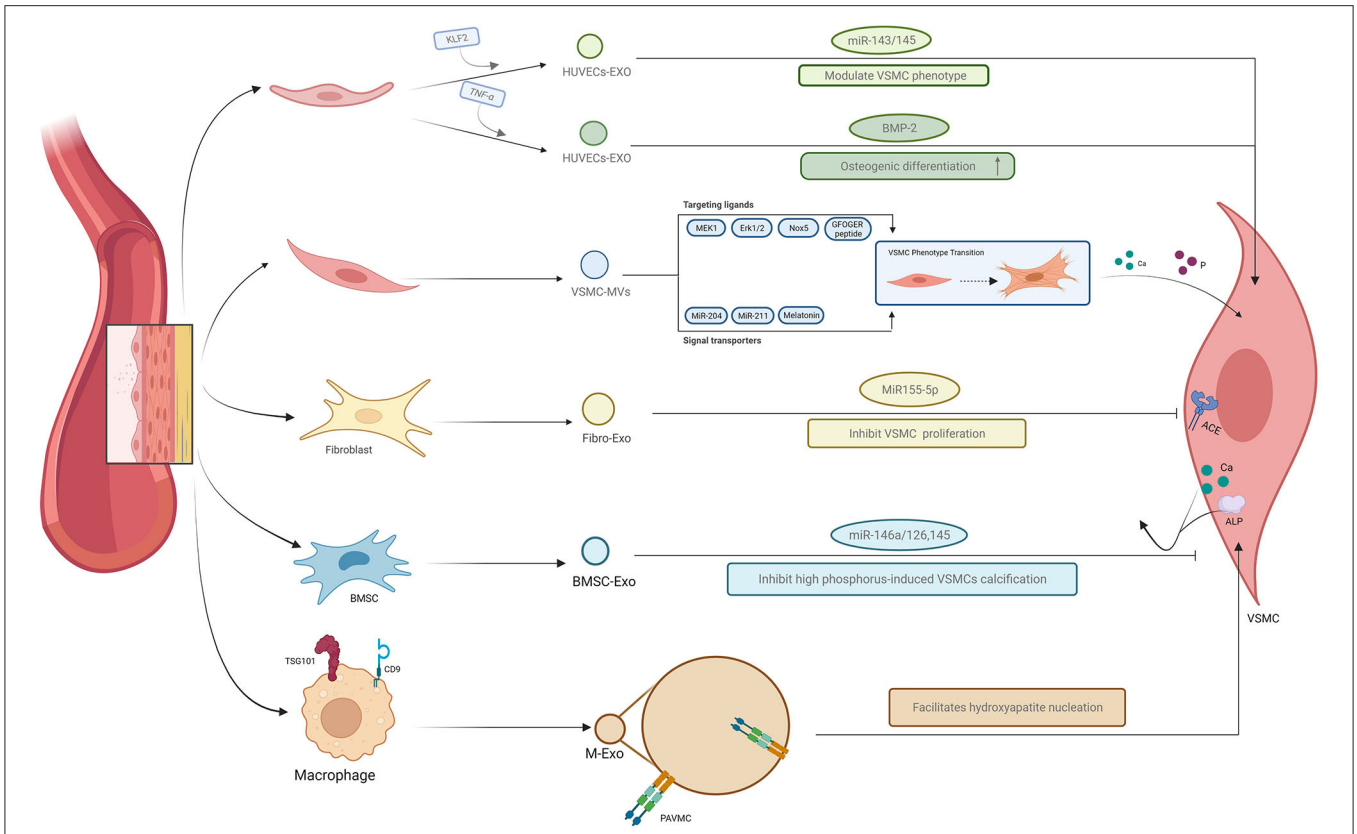

FIGURE 2 | Exosome-mediated crosstalk in the vascular microenvironment. The exosomes derived from different cell populations can facilitate or inhibit vascular calcification *via* exosome-mediated crosstalk between VSMCs and other cell populations through different molecules, such as miRNAs and bone morphogenetic protein-2 (BMP-2), among others.

ICAM-1abundent microvesicles (107). Another interesting trial showed that EVs isolated from aortic adventitial fibroblasts in Wistar–Kyoto rats can deliver miR-155-5p to attenuate VSMC proliferation and vascular remodeling by suppressing angiotensin-converting enzyme (ACE) expression (108). These findings indicate the tremendous diagnostic and therapeutic potential of EVs-based cardiac regenerative therapies. However, their diagnostic potential for CAC remains to be determined.

Collectively, it is easy to understand that the exosomes mediate the communication between different cells within the vascular wall microenvironment. A brief explanation of the relationship was summarized in **Figure 2**. And the summary of bioactive cargo in different exosomes and their regulatory pathway is presented in **Table 1**.

THE DIAGNOSTIC AND THERAPEUTIC VALUE OF CIRCULATING EXOSOMES IN VC

Great Diagnostic Potential

Cytologic content of exosomes indicates the biological status of the original cell. As exosomes could modulate their surface signaling molecules to encapsulate selective cargos in different stages of diseases, they may be the most direct and specific non- or semi-invasive targets for early diagnosis of VC. Dynamic expression profiles of exosomal miRNAs can provide clues to the development and progression of cardiovascular disease, including VC. Several studies have reported that miRNA were loaded selectively into EVs (83, 109) since there were distinguished differences in the miRNA profiles of SMCs and their EVs. Moreover, miRNAs were concentrated in exosomes, making up 50% of the total RNA inside. It has been reported that when compared with plasma total miRNAs, EV-derived miRNAs have higher diagnostic efficacy in colon cancer (110). Additionally, exosomes are considered stable and survive in an extreme environment without loss of potency (111). However, direct evaluation of circulation exosomal miRNAs as VC diagnostic or prognostic biomarkers needs to be completed before exosomes be exploited as clinical tools (109).

Possible Therapeutic Targets

In recent years, researchers have emphasized the great potential of exosomes in therapeutics (82), The exosomes derived from stem cells and MSC have shown cardiovascular protection effects in several disease models, involving the promotion of therapeutic effects in myocardial infarction (112, 113), reduction of myocardial ischemia/reperfusion injury (114, 115),

TABLE 1 | Role of exosomes in mediating vascular calcification in vascular wall microenvironment.

Exosome origin	Cargo	Involved pathway	Effects on vascular calcification	References
Vascular smooth muscle cells (VSMCs)	Mineralization inhibitors (fetuin-A)	Mitogen-activated protein kinase (MEK1 and ERK1/2) signaling	Decrease intracellular Ca^{2+} of recipient VSMCs	(79)
	Ca^{2+}	NADPH oxidase 5 (Nox5) mediated reactive oxygen species (ROS) production	Increase cytosolic Ca^{2+} and VSMC phenotypic switching	(67)
	Proteins involved in vascular calcification (such as osteogenic markers, TANK-binding kinase 1, and casein kinase II)	GFOGER peptide (a specific, six amino-acid repeat in type 1 collagen)	Decrease osteogenic switching in VSMCs	(80)
	miR-204/miR-211 cluster	Melatonin-mediated paracrine action	Attenuate the osteogenic differentiation and senescence of VSMCs	(78)
Endothelial cells	Apoptotic bodies	miR-126-mediated CXCL12 production	Confer features of plaque stability in different mouse models of atherosclerosis	(82)
	miR-143/miR-145	Krüppel-like factor 2 (KLF2)-transduced pathways	Reduce atherosclerotic lesion formation	(83)
	Bone morphogenetic protein 2 (BMP-2)	Upregulation of Cbfa1 and downregulation of SM22α	Enhance VSMC osteogenesis and calcification	(84)
Macrophages	S100A9 and annexin V	Facilitate hydroxyapatite nucleation	Accelerate microcalcification in chronic renal disease	(95)
	HMGB1	RAGE/p38 MAPK/nSMase2 signaling pathway	Lead to mineral deposition	(96)
	Pro-inflammatory cytokines and CAD, PAI-1, and Saa3 proteins	Inflammatory and oxidative responses.	Aggravate the VC process	(32)
Mesenchymal stromal cells (MSCs)	miR-146a	TXNIP-dependent action	Diminish VSMC calcification	(103)
	miR-126 and miR-145	Transition of macrophages from a pro-inflammatory and atherogenic phenotype (M1) to an anti-inflammatory and anti-osteogenic phenotype (M2c)	Inhibit thrombosis and calcification	(104)
Fibroblasts	miR155-5p	Reduced vascular angiotensin-converting enzyme, angiotensin II, and proliferating cell nuclear antigen levels	Promote VSMC proliferation and vascular remodeling	(108)

inhibition of pulmonary hypertension (116), and improvement in neurovascular function and plasticity (117). Although these advanced efforts have become one of the key focus in drug development, strong evidence of exosomal therapeutics on VC is still lacking. A number of *in vitro* studies have demonstrated that exosomes exert anti-calcifying effects by decreasing the VSMC phenotype transformation and EndMT, and thus negatively affect hydroxyapatite mineral deposition. And the anti-calcifying effect was mediated by functional cargos such as miR-29b (118), miR-30b/c (118), miR-125b (75), miR-34b/c (119), miR-135a (120), and miR-712 (120).

Those exosomes as well as cargos might be a potential target for gene therapy in VC. However, exosomal miRNAs appear to vary in their ability to promote or inhibit processes depending on the pathological microenvironment. Ulbing et al. (121) found that circulating miR-223 was decreased in patients with CKD. The reduced miR-223 expression has been recognized as a risk factor for VC morbidity. In contrast, when there is elevated inorganic phosphorus, the expression of exosomal miR-223 is upregulated in VSMCs (122). Such a contradiction implies that further studies *in vivo* and clinical use were needed to evaluate the value of exosome-based therapies for VC patients.

It is worth mentioning that although direct evidence is illegible, some studies provide clues that exosomes might offer therapeutic effects on calcifying cardiovascular tissue. Imipramine, an inhibitor of acid sphingomyelinase, can reduce the production of osteoblast-derived calcifying microvesicles (123), and the Ca^{2+} channel blocker verapamil was proved to block calcifying EV biogenesis, matrix vesicle activity, and extracellular matrix (ECM) mineralization, as well as disrupting atheroma formation in the rat aorta (71). Dimerization of the aforementioned sortilin protein also participates in calcification regulation through EV assembling and trafficking, thus inhibiting sortilin homodimer formation is another auspicious EV-associated therapeutic method (124).

Overall, exosomes presenting in the vascular wall microenvironment are not only conceivable candidate biomarkers for VC and its related diseases, but also serve as therapeutic targets.

CONCLUSIONS AND FUTURE PERSPECTIVES

In this paper, we analyzed the structure of the vascular wall and demonstrate that various cells in the vascular microenvironment are not merely physical structural barriers, but regulators with anti/pro-calcification functions that mediate transcellular regulation by exosomes. Exosomes within the cardiovascular microenvironment also have the capacity of biomarkers and therapeutic targets for VC.

What makes us exciting was that exosomes have been employed as potential carriers for gene therapy (3). Their nano-size and flexibility allow them to cross major biological barriers easily, makes them excellent nanocarriers for delivering chemical drugs and gene drugs, such as siRNAs, miRNAs and antagonist (125). Compared with traditional liposomes, viral vectors, and inorganic nanoparticles, exosomes have relatively low toxicity and immunogenicity. In addition, inherent homing ability of exosomes suggests their potential utility in drug delivery (126, 127). Specifically, exosomes surface could easily be modified, by conjugated with a cell or tissue specific aptamer and cell penetrating peptide which accomplish the goal of precision treatment (128, 129). However, using exosomes as nano-drug delivery implements is currently unable to achieve since its low rate of drug encapsulation (125, 130), how to engineer exosomes so that they are stably expressed once introduced into the recipient body and to avoid off-target effects are important issues to resolve. A big step in the analysis of the formation of exosome compartments would improve understanding of the roles of exosomes and their function in VC. The past decades have witnessed huge advances in molecular imaging, which may provide additional diagnostic methods and technical tools utilizing exosomes to further improve anti-VC therapeutics and diagnostics.

AUTHOR CONTRIBUTIONS

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

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Reporter Systems for Assessments of Extracellular Vesicle Transfer

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Extracellular vesicles (EVs) are lipid bilayer particles naturally released from most if not all cell types to mediate inter-cellular exchange of bioactive molecules. Mounting evidence suggest their important role in diverse pathophysiological processes in the development, growth, homeostasis, and disease. Thus, sensitive and reliable assessments of functional EV cargo transfer from donor to acceptor cells are extremely important. Here, we summarize the methods EV are labeled and their functional transfer in acceptor cells are evaluated by various reporter systems.

Keywords: exosomes, cell–cell communication, reporter, EVs visualization, recipient cells, extracellular vesicle

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INTRODUCTION

Extracellular vesicles (EVs) are lipid-bilayer membrane-enclosed structures released by most if not all cell types in physiological and pathological environments. They are classified into subtypes, including exosomes, ectosomes, and apoptotic bodies, based on the origin of biogenesis, and have different yet overlapping sizes and compositions (1–6). Exosomes (50–150 nm) are formed as intraluminal vesicles of endosomes and released into extracellular space upon endosome and cell membrane fusion, whereas ectosomes (0.1–1 μm) bud out directly from the plasma membrane (6, 7). Apoptotic bodies (50–5000 nm), on the other hand, dislodge from dying and disintegrating cells (8, 9). Loaded with a large spectrum of bioactive agents (i.e., proteins, RNAs, lipids, and metabolites) from donor cells (3, 4), EVs mediate effective transfer of cargos locally to neighboring cells or remotely *via* blood circulation to cells of other organs, thereby impacting the functional states of acceptor cells, tissue homeostasis, and disease pathophysiology (4, 10–16). Upon interacting with acceptor cells, EVs can affect their function *via* triggering membrane-mediated intracellular signaling, fusing with cell membrane to release bioactive cargos into the cytoplasm, or being endocytosed into endosomal system to evade lysosomal degradation through largely unknown mechanisms to regulate cellular activities. For example, exosome-derived ectopic mRNA or miRNA can translate into functional proteins or suppress mRNA translation, respectively, in acceptor cells. Given the often low abundance of exosome-delivered molecules in acceptor cells relative to endogenously expressed biomolecules and the complex intracellular feedback networks, accurate measurements of EV-mediated effects is vital to EV research. Here we summarize a number of reported methods EVs are labeled and their functional transfer are evaluated.

EXTRACELLULAR VESICLE LABELING

Labeling of Isolated Extracellular Vesicles With Chemical Dyes

Labeling of EVs with detectable molecules is widely used for tracking EV biodistribution and uptake into target cells. Various components of EV membrane, particularly lipids

and proteins, can be labeled with chemical dyes. Available lipophilic fluorescent dyes include PKH26 (excitation/emission wavelength maxima, λex/λem = 551/567 nm), PKH67 (λex/λem = 490/502 nm), DiO (λex/λem = 484/501 nm), DiI (λex/λem = 549/565 nm), DiR (λex/λem = 750/780 nm), and FM 4–64 (λex/λem = 558/734 nm). While these dyes insert into the membrane lipid bilayer of EVs, generating stable and long-lasting fluorescence signal (17–23), it was reported that at least PKH dyes can trigger EV enlargement as the result of membrane fusion or intercalation (24). Protein fluorescent membrane dyes include carboxyfluorescein succinimidyl diacetate ester fluorescent (25, 26) and maleimide flours (27), which may overcome certain limitations associated with other lipophilic (DiI, PKH67), non-lipophilic (ExoGlow-Vivo), and RNA (SYTO) dyes (27–29).

Labeling of Isolated Extracellular Vesicles With Aptamers

An aptamer is a short single-stranded DNA or RNA molecule with unique structural features that ensure binding to specific molecular target with known or unknown identity (30). Aptamers are usually selected from synthetic libraries using systematic evolution of ligands by exponential enrichment (SELEX). LZH8, an aptamer selected by using whole HepG2 hepatocytes (31), demonstrates impressive binding affinity to HepG2 cell-derived exosomes (32). Wan et al. linked LZH8 to a "trigger" sequence, so that the LZH8-trigger was able to bind to HepG2-exosomes meanwhile extending by base pairing between the "trigger" sequence and fluorescein (FITC)-conjugated oligos to amplify the FITC signal and enlarge the overall structure, allowing direct flow cytometry analysis of these modified exosomes (32). Nevertheless, whether LZH8 aptamer conjugation affects exosome tropisms or uptake by target cells remain to be elucidated.

Labeling of Isolated Extracellular Vesicles With Radioisotope or Magnetic Resonance Imaging Contrast Agents

Recently, evidence suggests that radionuclides or magnetic resonance imaging (MRI) contrast fluid can be loaded into the isolated EVs, enabling imaging of administered EVs *in vivo* by nuclear and MRI approaches (33–36). This method is particularly beneficial for deep tissue imaging with the potential of clinical application.

Tagging of Extracellular Vesicle Surface Protein *via* Genetic Engineering

Exosome membrane contains abundant tetraspanins (CD63, CD81, CD9) and lactadherin, which are often used as exosome biomarkers (37). These molecules were genetically engineered to fuse with fluorescent proteins/bioluminescence-generating enzymes, so the exosomes can be visualized under fluorescent microscope or by supplementation with bioluminogenic substrates (38–41). For example, CD63 fused with pHluorin, a pH-sensitive form of green fluorescent protein (GFP), permits tracking of endogenous EVs in the transparent zebrafish with high spatiotemporal accuracy, leading to the finding that the yolk syncytial layer-derived exosomes are endocytosed by macrophages and endothelial cells of the caudal vein plexus (CVP) in a scavenger receptor- and dynamin-dependent manner, thereby providing the trophic support for CVP growth (42).

Among different bioluminescence-generating enzymes, it appears that NanoLuc or ThermoLuc, when tethered to CD63, are preferred for sensitive imaging and tracking of EVs *in vivo* and *in vitro* (43). Luo et al. generated a transgenic mouse line that expresses CD63-NanoLuc fusion protein specifically in cardiomyocytes, thus cardiomyocyte-derived EVs, and found NanoLuc signals in thymus, testis, lung, and kidney, supporting the notion that cardiomyocyte-derived EVs mediate molecular exchange between heart and other organs (44). Besides CD63, lactadherin was also engineered, such as by fusion with Gaussia luciferase to visualize and track exosomes *in vivo* (45, 46). In a sophisticated system, EV surface was tagged with a membrane-bound biotin acceptor peptide linked outward to *Gaussia* luciferase fusion protein and biotin ligase, thus the EVs can be labeled with supplemented biotin and visualized *in vivo* with duo modal imaging, bioluminescence (with luciferin) and fluorescence (with streptavidin-fluorophore) (47). Lastly, genetic engineering can also achieve radiolabeling. Takakura et al. engineered lactadherin–streptavidin fusion protein on exosomes and used iodine-125 (^{125}I)-labeled biotin to label exosomes (48, 49).

Genetic engineering of donor cells to molecularly tag EV surface proteins avoids additional labeling processes and has the advantage of achieving cell-specific EV labeling *in vivo*. Also, it is likely that the engineered proteins, displaying on EV membrane, have significantly lesser untoward effects on the biochemical property of EVs, compared to chemical dyes. However, it should be aware that EVs are diverse in the expression of biomarkers, therefore these methods may be limited by labeling only a certain subgroup of EVs. In addition, although reports were mostly focused on EV membrane proteins, intraluminal soluble proteins, such as exosome-enriched ALIX and TSG101, may also have the potential for tagging, which would indicate the uptake and intracellular trafficking of exosome-derived non-membranous proteins. Lastly, the results obtained from these experiments may still need to be interpreted with care, as the signals are subject to the processing of individual fusion proteins and may not indicate the function of the entire EV proteome.

Bio-Conjugation of Extracellular Vesicle Proteins

Azide–alkyne cycloaddition (click chemistry) is a powerful tool that permits covalent conjugation, thus tagging, of exosomes. Azide or alkyne group can be incorporated into EVs by supplementing azide or alkyne bearing amino acids (e.g., AHA) (50, 51) or glycans/proteoglycans (52) to EV-producing cells, *in vitro* or *in vivo*, or by directly adding azide or alkyne bearing chemicals to the isolated EVs (53). The click reaction is catalyzed by Cu, but also can occur in Cu-free physiological fluids permitting *in vivo* labeling (54, 55). Diverse imaging modalities

(fluorescence, luminescence, radioactive imaging, and MRI) can be adapted to the system for *in vivo* tracking of the labeled EVs. Interestingly, click reactions seem not to affect the size of the exosomes, nor exosome adhesion or internalization in target cells (53).

Notably, David Tirrell group has identified a methionyl-tRNA synthetase L274G mutant (MetRS*), which utilizes the non-canonical azide-bearing amino acid azidonorleucine (ANL) as surrogate of methionine to incorporate into newly synthesized proteins (56). Engineered expression of MetRS* in the neuron enabled ANL-labeling and click-reaction based identification of neuronal specific proteomes *in vivo* (57). Our group introduced MetRS* into mesenchymal stem cells (MSCs) and administered these cells into the ischemic heart of mice supplemented with ANL, followed by serial isolation of azide-labeled (i.e., MSC-derived) proteins from total cardiac protein lysates. MSCs are believed to exert beneficial effects *via* paracrine mechanisms, and our study for the first time revealed MSC proteome real-time *in situ* in the injured cardiac tissue, revealing new insights into MSC mediated cardiac protection and repair (58). We also isolated EVs from ANL-treated MSCs and administered these EVs to mice with surgically induced myocardial infarction. The ANL-labeled (i.e., MSC exosome-derived) proteins were isolated with click-catalyzed alkyne-agarose capture from various organs at different time points and identified with mass spectrometry; the MSC exosomal proteins were also localized histologically *via* fluorescent non-canonical amino-acid tagging (FUNCAT) *in situ*. We found that MSC exosomal proteins distributed in different organs are highly diverse, that ischemic injury significantly augments the tissue intake of exosomes, and that in the injured tissue, the exosomal proteins are predominantly associated with cytosol vs. membrane (51).

Collectively, labeling of EVs has considerably facilitated assessments of their biodistribution and cellular uptake (**Table 1**), however, it does not allow discrimination between non-functional uptake (lysosomal degradation) and functional transfer or delivery (protein-mediated signaling, mRNA translation, or miRNA repression of target mRNA).

TABLE 1 | Labeling and reporter systems for evaluation of EV transfer.

Labeling and reporter systems	EV subtype	*In vivo* detection and biodistribution	Functional transfer	Subcellular resolution
Chemical dyes	All EVs	+	–	+
Aptamers	Specific EVs to aptamer	–	–	+
Radioisotope or MRI dye	All EVs	++	–	–
Genetic labeling	Biomarker positive EVs	+	–	+
Cre-loxP	Cre^+ EVs	–/+	++	++
miRNA targeting reporters	Certain $miRNA^+$ EVs	–/+	++	++
CRISPR/Cas9-gRNA reporters	$sgRNA^+$ EVs	–/+	++	++

–, no applicability; –/+, low applicability; +, medium applicability; ++, high applicability.

REPORTERS OF EXTRACELLULAR VESICLE TRANSFER IN ACCEPTOR CELLS

While EVs carry various types of bioactive cargos, their mRNA and miRNA activities in the recipient cells are most frequently used to evaluate EV functional transfer (47, 59). An ideal reporter system entails low basal reporter activity in the absence of EVs but highly induced reporter activity when EVs are present. Frequently used reporters are based on the Cre-loxP system, miRNA recognition size on target mRNA 3′UTR, and CRISPR/Cas9 system (**Table 1**).

Cre-loxP

The reporter gene is placed downstream of a floxed stop codon in the expression cassette introduced in recipient cells and activated when the cells are transduced with EVs from Cre-expressing donor cells (60). Ridder et al. observed LacZ reporter activation in neurons after taking up EVs carrying functional Cre messenger RNA from immune cells, establishing a unique EV-mediated immune cells to neuron crosstalk (61, 62). In another study, by comparing three Cre reporter mice, Rosa26-LacZ, Rosa26-EGFP, and Rosa26-EYFP, the authors found that Gr1+CD11b+ myeloid-derived suppressor cells (MDSCs) is a major cell population targeted by tumor-released EVs, and that EV transfer augments the immunosuppressive phenotype of these cells (63).

Zomer et al. optimized this Cre-loxP system where the transfer of Cre-bearing EVs induces a switch of DsRed to eGFP expression in the Cre-reporter cells (60). Interestingly, Cre mRNA but not Cre protein was detected in EVs, suggesting that Cre mRNA transfer was primarily responsible for the DsRed-to-eGFP switch (60). Furthermore, the authors applied this Cre-mediated DsRed to eGFP conversion system *in vivo* and found that the recipient tumor cells taking up EVs from highly malignant tumor cells display an augmented migratory and metastatic activity, suggesting that EVs are able to transfer the malignant property of tumors (64). Interestingly, Sterzenbach et al. engineered the expression of Cre protein fused with a WW tag (WW-Cre), which can be recognized by the evolutionarily conserved late-domain L-domain-containing protein Ndfip1, leading to increased Cre protein ubiquitination, thus packing into exosomes; using this system, the authors demonstrated that administered exosomes *via* nasal route reached brain cells, leading to Cre-mediated recombination in mT/GFP mice (65). This study, however, indicated functional delivery of Cre protein through exosomes.

Taken together, the Cre-loxP based reporters have proven that exosomes can mediate functional transfer of Cre mRNA and protein. It should be noted that Cre-loxP system has been used extensively in lineage tracing studies in the past and the Cre mediated reporter expression is rarely in no-Cre expressing cells. Given that exosomes pack predominantly small RNAs or

mRNA fragments (66), it is likely that the Cre-loxP system, without enhancement of Cre loading into exosomes, would underestimate EV transfer.

miRNA Targeting Reporters

miRNAs are predominantly enriched in EVs (59) and well recognized as important effectors responsible for EV induced biological response (66). miRNA target mRNA at specific sequence primarily located in the 3′UTR, inhibiting mRNA translation and inducing mRNA degradation. Dual-luciferase reporter systems are engineered by placing wild-type 3′UTR or miRNA-targeting-sequence mutated 3′UTR downstream of a firefly luciferase reporter; overexpression of miRNA decreases luciferase expression and activity with wild-type 3′UTR but not with mutated 3′UTR (67). Using this system, Thomou et al. engineered an adenoviral vector containing 3′UTR luciferase reporter for human-specific miRNA hsa_miR-302f and transduced mouse liver. They found that expression of pre-hsa_miR-302f but not control miRNA in the brown adipose tissue (BAT) led to >95% reduction of luciferase activity in the liver, indicating EV transfer of hsa_miR-302f from BAT to liver (68). In the mice with adipose-specific knockout of the miRNA-processing enzyme Dicer (ADicerKO), which has a markedly reduced mature miRNAs, the authors found that serum EVs from wild-type mice significantly decreased the level of luciferase activity with a FGF21 mRNA 3′UTR reporter in the ADicerKO liver, further confirming that BAT-derived EVs transfer miRNA to affect gene regulation of the liver (68).

CRISPR/Cas9 – gRNA Reporters

Recently, de Jong et al. generated an elegant CRISPR/Cas9 based reporter system (69). It is known that Cas9 nuclease mediates non-homologous end joining (NHEJ) repair that creates frameshifts. The reporter construct is driven by CMV promoter; the expression cassette contains genes of mCherry and two alternative reading frames of F2A (a cleavable peptide) fragment and eGFP, which are linked by a "linker-STOP" sequence that contains the specific recognition site of a short single guide RNA (sgRNA) and generates +1 or +2 nt frameshift (eGFP transactivation) at a total frequency of ∼80%. The recipient cells were transduced permanently with the reporter construct, and EV transfer was assessed by co-culture with sgRNA expressing donor cells (close contact or no close contact) or by directly adding donor-derived EVs. The EV-mediated sgRNA transfer permanently activates the expression of eGFP (i.e., mCherry and eGFP double positive). Thus, the system allow visualization of EV-mediated functional sgRNA delivery in single cell level. In a co-culture experiment with cell number ratio of 50:1 (sgRNA donor cells: reporter-transduced recipient cells), an encouraging transfer rate of ∼0.2% was observed (69). Nevertheless, like Cre-loxP based reporter, CRISPR/Cas9 based reporter also causes permanent activation of the reporter gene, the effects are cumulative and not real-time.

Collectively, the reporter systems based on the Cre-loxP system, miRNA and their recognize sites, and the CRISPR/Cas9 system have been used to verify EV mediated intracellular delivery of functional mRNA, protein, and small RNAs in target cells, which are instrumental to our understanding of EV biology.

OUTLOOK

Conceivably, all direct EV labeling methods (for assessment of EV uptake) and reporter systems (for evaluation of functional transfer) have limitations. Direct EV labeling has the potential of affecting the chemical property of EVs' membrane, which may alter the EV biodistribution or uptake tropism and dynamics. Genetic engineering of donor cells have the advantage of directly studying endogenous EVs in a tissue specific fashion, however, difficult to distinguish between exosomes, microvesicles, and non-EV associated transport, not to mention the effects of transgenic expression on cellular activities. Current reporter systems, on the other hand, primarily indicate the functional transfer of single molecules (mRNA, protein, or small RNA) in recipient cells, thus subject to the unique dynamics of these individual molecules, and the sensitivity may not be at the same scale as the EV-carried biomolecules to be studied. The choices of reporter systems should be based on the biological questions to be addressed in specific studies and corroborated by other functional readouts. For example, the power of these reporter systems would be significantly enhanced if combined with other cutting-edge technologies, such as single cells sequence or spatial transcriptome analysis. For real-time monitoring of functional mRNA, protein, or sgRNA transfer, some sensitive and specific binary systems, such as the TetR-based transactivators (70) or dCas9-VPR transactivators (71), may be used. The sensitivity of the reporter systems may also be enhanced by increasing the loading of bioactive cargos and/or the membrane fusion, back fusion, or lysosomal escape within recipient cells. Ideally, reporter systems should be able to indicate detailed life-cycle events of EVs, their biogenesis and diversity in donor cells, extracellular navigation, systemic distribution, as well as internalization, intracellular trafficking (between subcellular organelles), cargo delivery or escape, and fate in target cells. The advancement in EV reporter systems will likely provide more powerful and invaluable tools for the field of research in EV biology and therapeutics.

AUTHOR CONTRIBUTIONS

CH: conceptual contributions and writing the draft. GQ: conceptual contributions and editing. Both authors contributed to the article and approved the submitted version.

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REVIEW
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Insights Into Platelet-Derived MicroRNAs in Cardiovascular and Oncologic Diseases: Potential Predictor and Therapeutic Target

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Non-communicable diseases (NCDs), represented by cardiovascular diseases and cancer, have been the leading cause of death globally. Improvements in mortality from cardiovascular (CV) diseases (decrease of 14%/100,000, United States) or cancers (increase 7.5%/100,000, United States) seem unsatisfactory during the past two decades, and so the search for innovative and accurate biomarkers of early diagnosis and prevention, and novel treatment strategies is a valuable clinical and economic endeavor. Both tumors and cardiovascular system are rich in angiological systems that maintain material exchange, signal transduction and distant regulation. This pattern determines that they are strongly influenced by circulating substances, such as glycolipid metabolism, inflammatory homeostasis and cyclic non-coding RNA and so forth. Platelets, a group of small anucleated cells, inherit many mature proteins, mRNAs, and non-coding RNAs from their parent megakaryocytes during gradual formation and manifest important roles in inflammation, angiogenesis, atherosclerosis, stroke, myocardial infarction, diabetes, cancer, and many other diseases apart from its classical function in hemostasis. MicroRNAs (miRNAs) are a class of non-coding RNAs containing ∼22 nucleotides that participate in many key cellular processes by pairing with mRNAs at partially complementary binding sites for post-transcriptional regulation of gene expression. Platelets contain fully functional miRNA processors in their microvesicles and are able to transport their miRNAs to neighboring cells and regulate their gene expression. Therefore, the importance of platelet-derived miRNAs for the human health is of increasing interest. Here, we will elaborate systematically the roles of platelet-derived miRNAs in cardiovascular disease and cancer in the hope of providing clinicians with new ideas for early diagnosis and therapeutic strategies.

Keywords: platelet, MicroRNAs, cardiovascular diseases, cancer, platelet-derived microvesicle (PMV)

INTRODUCTION

The latest WHO report released in December 2020 shows that non-communicable diseases (NCDs), represented by cardiovascular and oncologic diseases, have been the leading cause of global death over the past 20 years (1). Cardiovascular diseases (CVDs), principally ischemic heart disease, heart failure and stroke, account for one-third of the annual deaths and also are the major contributors to disability (2). However, the global incidence and mortality of CVDs have continued to rise uncontrollably, with an increase of 93 and 54%, respectively, for the last three decades (1, 3). The second cause of death is cancer, with 24.5 million incident cases worldwide and 9.6 million deaths in 2017 (4, 5). With the aging of social population, the deterioration of environment, the prevalence of obesity and the deterioration of lifestyle (smoking, alcohol, drugs nightlife, and physical inactivity), the incidence of cardiovascular and oncologic diseases is bound to grow. Despite massive researches funding and government spending, the ultimate efficacy of both diseases is far from satisfactory, most likely because of limited access to timely diagnosis and standard treatment. In fact, 5-year survival rates for heart failure and lung cancer are less than 25 and 17%, respectively (6, 7).

Platelets are small anuclear cell fragments in the circulating blood. Although platelets could not transcribe the gene, the complete translational and post-transcriptional regulation machinery including mRNA, non-coding RNAs, ribosomes, and initiation/termination factors are inherited from megakaryocyte and stored in the cytoplasm and granules during thrombopoiesis (8, 9). Platelet activation dependent on specific receptors [such as glycoprotein Ib-IX-V (GPIb-IX-V), purinergic receptors (P2Y1 and P2Y12), and integrin αIIbβ3] on their surface is the key step for platelet function (10, 11). Once activated, platelets quickly secrete granules and intracellular active substances such as P-selectin, soluble CD40 ligand (sCD40L), platelet factor 4 (PF4) and interleukin-1 beta (IL-1β) *via* exocytosis pathway. These substances contribute to platelet adhesion, aggregation, platelet-leukocyte crosstalk, platelet-endothelial crosstalk and systemic inflammation states (12–14). These actions are important mechanisms by which platelets are involved in thrombosis, cardiac remodeling after myocardial infarction, atherosclerosis, diabetic microangiopathy, tumor growth, and metastasis (13, 15–18). Besides these proteins, platelets inherit a variety of nucleic acids including non-coding RNAs (miRNAs as well as lncRNAs) and messenger RNAs (mRNAs). In recent years, in-depth transcriptional analyses identified up to 532 different miRNAs and as many as 3000–6000 mRNAs in human platelets (19, 20). As we known, miRNAs are highly conserved, small endogenous non-coding RNAs negatively regulating gene expression at the post-transcriptional level by complementary sequence recognition, and are involved in many pathophysiological processes, including cardiovascular diseases and cancer. An enormous amount of research has gradually described the strong biological effects of platelet-derived miRNAs. They not only regulate the synthesis of platelet protein but also are transferred to endothelial cells, smooth muscle cells (SMCs), macrophages, and tumor cells, where they bind to host cells' mRNAs (21–23). However, a systematic review about the role and mechanism of platelet-derived miRNAs on two highly lethal diseases, cardiovascular disease and cancer, is lacking.

In this study, we explore the possibility of circulating platelet-derived miRNAs as early diagnosis and prognostic factors, and their roles in the occurrence and development of cardiovascular diseases and tumors, which may provide clinicians with new diagnostic and therapeutic targets for these diseases.

The Origin of Platelets MicroRNAs

MicroRNAs are a class of regulatory non-coding RNAs with a length of ∼22 nucleotides expressed in multicellular organisms and synthesized by an elaborate system involving numerous protein-protein and protein-RNA interactions (24, 25). Briefly, miRNA-related genes are firstly transcribed into primary miRNAs (pri-miRNAs) by RNA polymerase II (Pol II), and subsequently pri-miRNAs are processed into shorter precursor miRNAs (pre-miRNAs) by a complex formed by the RNAase-III enzyme Drosha and its interaction partner DGCR8 in the nucleus. Next, pre-miRNAs are transported out of the nucleus by exportin-5. In the cytoplasm, RNase Dicer enzyme bound to the double-stranded RNA with protein TRBP cleaves pre-miRNAs into shorter double-stranded miRNAs. Finally, double-stranded miRNAs are replicated into argonaute 2 (Ago2) and form the miRNA-induced silencing complex (RISC). One strand of the double-stranded miRNA is retained in the RISC complex, and the other strand is expelled from the complex and rapidly degraded. The RISC complex containing the miRNA single strand can function in subsequent gene regulation processes (26).

Since platelets are anucleate, mature miRNAs in platelets were previously considered remnants of megakaryocytes. However, later studies confirmed that anucleate platelets possess complete elements (Dicer, Ago2, and TRBP2) which can machine precursor miRNA (pre-miRNA) into mature miRNA in their cytoplasm (27), but lack of nuclear microprocessor components Drosha and DGCR8. The function of platelet Dicer enzyme was confirmed since miRNA-sized RNA fragments were obtained when a radioactive Dicer substrate ^{32}P-labeled pre-let-7a-3 was co-cultured with platelet extracts (27). Additionally, their research also confirmed that platelets harbor functional Ago2–miRNA complexes. The direct interaction between Ago2 with endogenous mature miR-223 was confirmed by northern blotting of platelets Ago2 immunoprecipitation and the inhibitory expression of target gene P2Y12 confirmed the regulatory function of the Ago2-miR-223 complex (27). These results suggested that platelets have the ability to produce mature miRNA by processing pre-miRNA templates.

However, evidence suggests that a large proportion of mature miRNAs contained in platelets are mainly inherited from megakaryocytes (28). Cultured megakaryocytes transcribes multiple miRNAs, which correlates well with the content of miRNAs found in platelets (28, 29). Platelets inherit RNA pool, including pre-miRNAs, from their parent megakaryocytes (30, 31). These RNAs become important sources of platelet miRNA as they may be processed into mature miRNA by functional Dicer as templates (22, 27). Besides, miRNAs can bidirectionally transfer between platelets and the surrounding environment (32).

RNA uptake from contacting cells or the circulating blood is also thought to be a contributor to miRNA diversity within platelets (30, 33). In general, platelets can not only inherit a large amount of miRNAs from megakaryocytes and take up part of miRNAs from surrounding environment, but also can process the inherited and ingested RNAs into miRNAs (**Figure 1**).

The Content of Platelets MicroRNAs

As early as in 2008, Bruchova et al. first detected abundant miR-26b in platelets in patients with polycythemia vera and essential thrombocythemia (34). This was the first study attracting our attention to platelet-derived miRNAs. Later, a large number of studies confirm that platelets contain a wide variety of miRNAs, although platelet-derived miRNAs are much less than those in nucleated blood cells (35, 36). With the development of microarray and sequencing technology, more miRNAs have been identified in platelets. Landry et al. identified 219 miRNAs in purified leukocyte-depleted platelet by locked nucleic acid (LNA) microarray profiling in 2009. Among them, miR-223, let-7c, and miR-19a were the three most abundant miRNAs according to their results (27). Subsequent studies on the most common miRNAs in platelets were inconsistent (**Table 1**). In many studies, miR-223-3p was described as the most abundant platelet-derived miRNA (37–39). Later, the first next-generation sequencing (NGS) data on platelet miRNAs was published, identifying 532 miRNAs present in platelets, with the let-7 family accounting for almost half of the total miRNA content (20). Other miRNA families highly represented in human platelets include miR-199, miR-103, miR-25, and miR-140. However, the expression of miR-223 was only ranked the tenth in this study. Overall, the top 15 miRNAs accounted for more than 90% of all miRNAs present in human platelets. More recently, Bray et al. analyzed miRNAs content in purified platelets from four healthy volunteers, expanding the number of known platelet miRNAs to approximately 750 (35). In their study, the top five miRNAs expressed most abundantly in platelets were miR-223, miR-451, miR-21, miR-23a, and miR-126. In **Table 1**, we summarized the top 20 miRNAs in platelet.

MicroRNAs and Platelet Biogenesis

Platelets are derived from megakaryocytes, and formed when the edges of mature megakaryocytes break off. Each megakaryocyte releases about 1,000–5,000 platelets (40, 41). The process of platelet production lasts approximately 7 days, involving in three main phases: megakaryocyte differentiation, megakaryocyte maturation, and platelet formation. This process is complex and regulated by multiple mechanisms including epigenetic, transcriptional as well as post-transcriptional gene

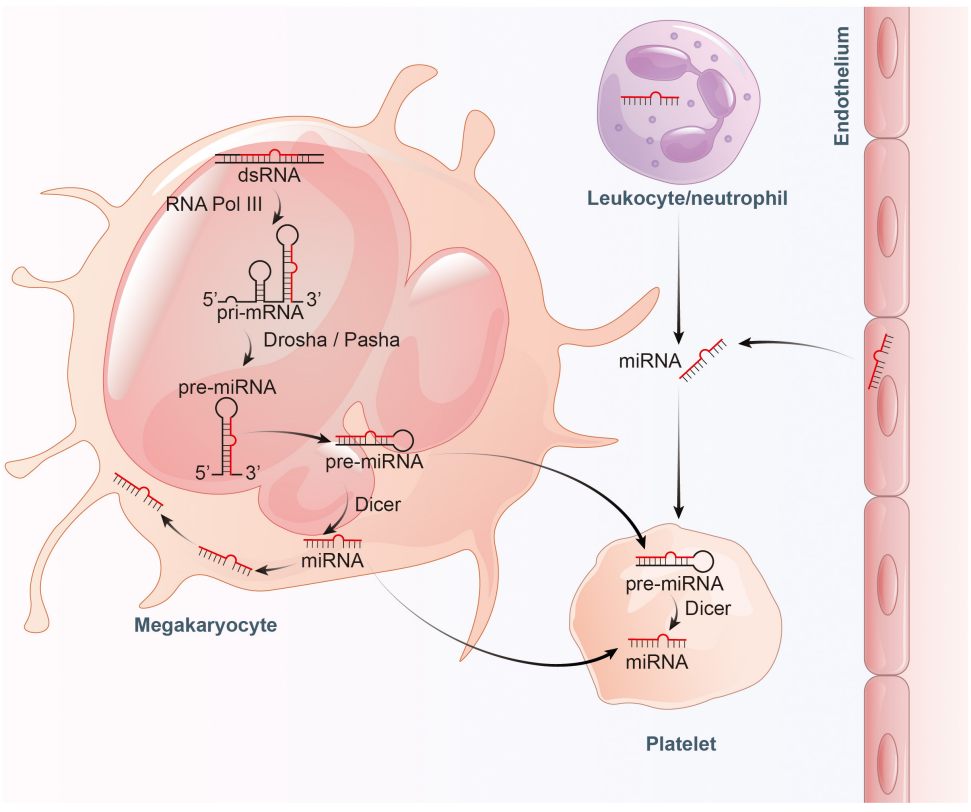

FIGURE 1 | Proposed model for the inheritance and operation of platelet miRNAs. On the one hand, platelets inherit precursor miRNA (pre-miRNA) from megakaryocytes and process them into mature miRNA by Dicer. On the other hand, platelets also inherit mature miRNA from megakaryocytes, and absorb miRNA from endothelial cells and leukocytes.

TABLE 1 | A summary of the top 20 expressed miRNAs in platelets.

Total miRNA numbers in platelets	The top 20 miRNAs in platelets	Method	References
219	miR-142-5p, miR-142-3p, miR-223, let-7a, miR-185, let-7c, let-7i, let-7b, miR-126, miR-103, miR-320, miR-30c, miR-130a, miR-26a, miR-191, miR-30b, miR-146a, miR-23b, miR-21, miR-23a	Locked nucleic acid (LNA)-based microarray profiling in human platelets	(27)
532	Let-7f-5p, let-7a-5p, miR-199ab-3p, miR-103a-3p, miR-140-3p, miR-7g-5p, miR-25-3p, let7b-5p, miR-7d-5p, miR-21-5p, miR-185-5p, miR-191-5p, miR-223-3p, miR-26b-5p, miR-423-5p, miR-221-3p, miR-107, miR-101-3p, let-7i-5p, miR-23a-3p	High-throughput sequencing in purified human platelets	(20)
750	miR-223, miR-22, miR-21, miR-126, miR-23a, miR-451, miR-17, miR-191, miR-26b, miR-15b, miR-23b, miR-484, miR-19a, let-7g, miR-221, miR-130a, miR-425, miR-142-5p, miR-185, miR-30d	RNA sequencing in highly purified, leukocyte-depleted platelet	(35)

expression control. Indeed, several studies have addressed miRNAs as well as their target proteins play important roles in megakaryocytopoiesis and platelet biogenesis (**Figure 2**; 21, 22, 29, 42).

In the megakaryopoiesis stage, hematopoietic stem cells (HSCs) differentiate and grow into megakaryocyte erythroid progenitor cells (MEPs) in bone marrow, which then develop to megakaryocytes under the influence of different factors such as thrombopoietin (TPO). Garzon et al. firstly explored the differential expression of miRNAs profiles between cultured CD34+ hematopoietic progenitor cells and megakaryocytes (43). They found a strong downregulation of 19 miRNAs during megakaryocytopoiesis suggesting that these downregulated miRNAs possibly unblock certain genes involving this process. For example, MAFB (v-maf musculoaponeurotic fibrosarcoma oncogene homolog B) and HOXA1 (Homeobox A1) gene are upregulated during megakaryocyte differentiation, as they are targets for the downregulated miR-130a and miR-10a, respectively (43). In contrast, miR-34a increases during megakaryocytic differentiation and stimulates megakaryocytopoiesis by enhancing megakaryocyte colony formation from CD34 + HSCs (44). Additionally, hematopoietic stem cells with miR-150 overexpression produced 8-fold enrichment of megakaryocyte *in vitro* and 15-fold amplification *in vivo* compared with normal controls. miR-150 was shown to maintain normal differentiation of MEPs into megakaryocyte by targeting the transcription factor c-myb (45). On the contrary, miR-28 attenuates TPO stimulating signal by downregulating the thrombopoietin receptors (TPOR, MPL), resulting in a negative effect on megakaryocyte differentiation (46). Recently, other miRNAs such as miR-10a, miR-155, and miR-125a-5p were also shown to play an important role in the formation of megakaryocytes (47–49). Interestingly, mature platelets release microvesicles (PMVs) containing many miRNAs can be internalized by bone marrow hematopoietic stem cells and regulate megakaryocytes biogenesis as a self feedback regulatory mechanism (50, 51). MiR-223, the most abundant miRNA in PMVs, enhances MK differentiation and maturation by inhibiting MYH10 and LMO2 (52). Additionally, global miR-223 knockout leads to an obstacle in the recovery of platelet production after platelet immunodepletion in mice, supporting the role of miR-223 in thrombopoiesis (53). Another miRNA, miR-1915-3p, which is highly enriched in PMV, exhibits more significant effects than miR-223 in promoting MK differentiation by suppressing Rho GTPase family member B (RHOB) expression (54).

In the thrombopoiesis stage, megakaryocytes greatly enlarge their bodies with amplifying their DNA to 64-fold, filling with a high concentration of ribosomes, and synthesizing lots of platelet-specific proteins (55–57). Then, an expansive and interconnected membranous network of pools and tubules is formed, also named the demarcation membrane system (DMS) (58). DMS divides the megakaryocyte cytoplasm into small chamber where pro-platelets are split out (59, 60). TPO is widely regarded as the primary regulator of thrombopoiesis for their promoting roles on megakaryocyte endomitosis by binding to the c-Mpl receptor (61, 62). MiR-204-5p and miR-28a were reported to directly target TPO and MPL, respectively, *via* sequence-dependent 3′-UTR repression and inhibit platelet formation (46). Additionally, miR-142-3p was reported to maintain actin filament homeostasis, thereby promoting actin-dependent pro-platelet formation (63). miR-125a-5p directly targets and reduces the expression of L-plastin, an actin-bundling protein who inhibits the pro-platelet formation (49). Other miRNAs, such as miR-125b and miR-660 were found to promote platelet output from cultured megakaryocytes while miR-23a/27a/24-2 cluster blocked this process (64).

Platelet MicroRNAs and Platelet Activation

Platelets are unstable and keep hyperresponsiveness to external stimuli, such as endothelial injury, infection, and metabolic disorders. Platelet receptors, such as glycoprotein (GPIb and GPVI), adenosine receptors (P2Y12 and P2Y1), thromboxane a2 receptor (TP), and thrombin receptors (PAR1, PAR3, and PAR4), act as switches for platelet activation once binding to their ligands von Willebrand factor (vWF), collagen, ADP, TxA2, and thrombin (65, 66). MiRNAs, as an important part of post-transcriptional regulation of platelet proteins, regulate platelet activity by directly targeting several platelet proteins (**Table 2**). Landry et al. firstly reported that Ago2·miR-223 complexes negatively regulated the expression of P2Y12 receptor by targeting the 3′-UTR region (27). However, the

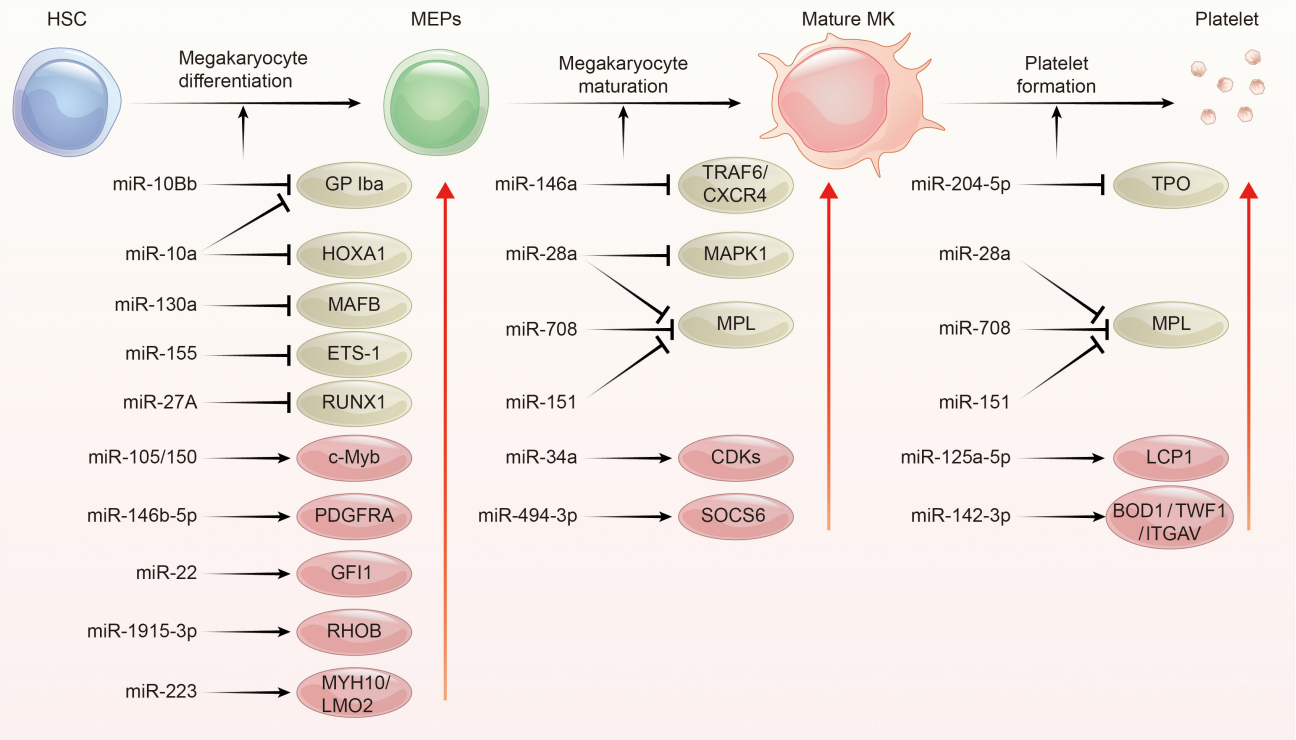

FIGURE 2 | The regulation network of miRNA during platelet biogenesis. Platelet production goes through three main stages: Firstly, hematopoietic stem cells (HSCs) were differentiated into megakaryocyte erythroid progenitor cells (MEPs), which were further differentiated and proliferated to form mature megakaryocytes, and then produced functional platelets. This process is controlled by several miRNAs through regulating the expression of their target gene. The red circle represents miRNA-mRNA pairs promoting platelet biogenesis, and the brown color represents miRNA-mRNA pairs inhibiting this process. Abbreviations: GP Iba, glycoprotein Ib platelet subunit alpha; HOXA1, homeobox A1; MAFB, V-maf musculoaponeurotic fibrosarcoma oncogene homolog B; EST-1, E26 transformation-specific sequence 1; RUNX1, runt-related transcription factor 1; PDGFRA, platelet derived growth factor receptor alpha; GFI1, growth factor independent 1; RHOB, ras homolog family member B; MYH10, myosin heavy chain 10; LMO2, LIM-only protein 2; TRAF6, tumor necrosis factor receptor associated factor 6; CXCR4, C-X-C motif chemokine receptor 4; MAPK1, mitogen-activated protein kinase 1; MPL, thrombopoietin receptor; CDKs, cyclin dependent kinases; SOCS6, suppressor of cytokine signaling 6; TPO, thrombopoietin; LCP1, L-plastin; BOD1, biorientation of chromosomes in cell division 1; TWF1, twinfilin actin binding protein 1; ITGAV, integrin subunit alpha V.

results of studies assessing the effect of miR-223 on platelet activation are contradictory. Leierseder et al. found that miR-223 did not affect platelet activation and aggregation and bleeding time while Elgheznawy et al. reported that miR-223 deletion in mice exacerbated platelet aggregation and the formation of large thrombosis (53, 67). Additionally, miR-126 transfection inhibits platelet reactivity by downregulating the expression of a disintegrin and metalloproteinase-9 (ADAM9), a protease associated with the interaction between platelet adhesion and collagen, and P2Y12 receptor expression (68, 69). In a LPS-induced sepsis model, platelet miR-26b is significantly downregulated, which contributes to elevated P-selectin (SELP) expression of MKs and platelets, and augments platelet activation (70). MiR-181a targets Ras-related protein 1B (RAP1B), an important protein participating in platelet activation and hemostasis induced by agonists, and thereby reducing platelet activation (71). Recently, high-through RNA sequencing was used to compare miRNA and mRNA profiles between hypo- and hyper-reactive platelets and helped to discover more miRNA-mRNA pairs associated with platelet activation. Nagalla et al. reported that 74 miRNAs were significantly changed in epinephrine-activated platelets compared with resting subjects. Several changed miRNAs were negatively correlated with the genes related with platelet activation, such as miR-200b: PRKAR2B, miR-495: KLHL5, and miR-107: CLOCK. The regulatory relationship was validated by miRNA-mediated inhibition of the targeted genes. Moreover, the function of these miRNA-mRNA pairs was further confirmed by reduced activation in platelets lacking PRKAR2B (72).

Platelet MicroRNAs and Platelet Secretion

Platelets contain three types of secretory organelles—alpha and dense granules and lysosomes. Alpha granules are the largest and most abundant secretory granules in platelets. More than 280 proteins are stored in alpha particles, including vWF, PF4, P-selectin, and platelet-derived growth factor (PDGF) (73, 74). Dense granules contain more than 200 small molecules, mainly including calcium, ADP/ATP, and 5-hydroxytryptamine (75).

Lysosomes only account for a small proportion in platelet and have more heterogeneous in composition and properties, containing a number of acid hydrolases, cathepsins, and lysosomal membrane proteins (76). In addition, a large number of miRNAs may also be stored in these granules, since the circulating platelet-derived miRNA increases significantly, when these particles are secreted after platelet activation (36). However, what we still don't know is their distribution and content in these particles.

The release of platelet granules is the material basis of platelet functional diversity and involves in the occurrence and development of many diseases especially cardiovascular and oncologic diseases (77). These platelet granules can fuse to the membrane of platelets *via* complex mechanisms after platelet activation and release their contents into the extracellular vascular space (78–82). The molecular mechanisms about platelet secretion mainly involve in soluble *N*-ethylmaleimidesensitive factor attachment protein receptor (SNARE) families (83). In 2010, Kondkar et al. reported that miR-96 overexpression resulted in significant inhibition of the expression of vesicle-associated membrane protein 8 (VAMP8), a SNARE protein which was elevated in hyperactive platelets and crucial for promoting platelet granules secretion (**Table 2**). In addition, miR-376c was found to inhibit the expression of phosphatidylcholine transfer protein (PCTP), an important protein contributing to secretion of dense granules by regulating PAR4-mediated platelet activation (84). Notably, the above-mentioned miRNAs who are involved in regulating platelet activation may also alter platelet particles secretion theoretically, since platelet exocytosis are initiated by platelet activation.

TABLE 2 | Overview of key platelet miRNAs and their function within platelets.

MicroRNA	Target	Role in platelets	Study samples	References
miR-223	P2y12R	Platelet activation	Mice/MEG-01	(158, 144)
	SMOC1	Platelet activation	Mice	(230)
	Factor XIII	Platelet activation	Mice	(67)
miR-126	P2y12R	Platelet activation	Mice	(146)
	PLXNB2	Platelet activation	Human	(68)
	ADAM9	Platelet aggregation	Mice	(69)
miR-30c	PAI-1	Thrombus formation	Mice	(149)
miR-148a	FcγRIIA	Thrombus formation	Mice	(231)
miR-181a	RAP1B	Platelet activation	Mice	(71)
miR-320c	RAP1	Platelet activation	Human	(232)
miR-140	SELP	Platelet activation	MEG-01 MK	(144)
miR-26b	SELP	Platelet activation	MEG-01 MK	(144)
miR-200b	PRKAR2B	Platelet reactivity	MEG-01 MK	(72)
miR-495	KLHL5	Platelet reactivity	MEG-01 MK	(72)
miR-107	CLOCK	Platelet reactivity	MEG-01 MK	(72)
miR-96	VAMP8	Platelet secretion	Human	(233)
miR-376c	PCTP	Platelet secretion	Human	(84)
miR-21	WASp	TGF-β1 secretion	Mice	(234)
miR-236	Bcl-xl/Bak1	Platelet *apoptosis*	Human	(235)

SMOC1, secreted modular calcium-binding protein 1; PLXNB2, plexin B2; ADAM9, metalloproteinase domain-containing protein 9; PAI-1, plasminogen activator inhibitor-1; RAP1B, ras-related protein 1b; SELP, selectin P; PRKAR2B, cAMP-dependent protein kinase type II-beta regulatory subunit beta; KLHL5, Kelch like family member 5; VAMP8, vesicle associated membrane protein 8; PCTP, phosphatidylcholine transfer protein; WASp: Wiskott–Aldrich syndrome protein.

Platelet Secretes and Delivers MicroRNAs

In earlier studies, researchers found that the changes of circulating miRNA were closely related to platelets activation. They identified a large number of miRNAs when comparing the miRNAs profiles in hyporeactive platelets and hyperreactive ones in response to agonist stimulation (72). However, what puzzles scientists is how they are released and maintain their stability after entering into circulation? In the past, the concept that platelets produce many membrane encapsulated extracellular vesicles (EVs) has been widely accepted (74, 85). These EVs hide miRNAs in their natural membrane barrier and isolate them from degradative components (such as nucleases) in the extracellular environment, maintaining the stability of extracellular miRNAs. Later, researchers demonstrated that platelet secreted miRNAs into circulation possibly through EVs-mediated manner (86, 87). They compared miRNAs profiles between plasma microparticles (MP) and MP-free plasma and confirmed that plasma miRNAs mainly originated from microparticles. 41–45% of circulating microparticles were of platelet, 28% of leukocyte, and 8% of endothelial origin.

Upon stimulation, platelets mainly secrete two types of EVs: exosomes (derived from exocytosis of multivesicular bodies and alpha-granules, <100 nm) and microvesicles (produced by surface shedding, 100 nm–1 μm) (74). Although exosomes from endothelial cells and tumor cells have been verified to be carriers for miRNA-based intercellular communication and a source of circulating miRNAs (88), the role of platelet-derived exosomes in miRNA transfer is still undetected. Philipp Diehl et al. firstly proposed that circulating miRNAs were mainly localized in microvesicles (MVs) derived from different tissues, especially from platelets (87). Consistently, Laffont et al. reported that platelet-derived microvesicles (PMVs) acted as intercellular transporters delivering rich miR-223 to endothelial cells and altering the gene expression (86). Thus, the transfer of platelet miRNAs is mainly mediated by PMVs (89). Indeed, platelet-derived MVs are the major source of cell-derived MVs in the circulation (90), which is consistent with the conclusion that circulating miRNAs are mainly platelets-derived (91). Then, we elaborate on the regulation of PMVs mediated miRNAs on distant cells below (**Figure 3**).

Platelet Microvesicles Transfer MicroRNAs to Endothelial Cells

Takeuchi et al. found that platelet-like particles (PLPs) derived from the megakaryoblastic cell line Meg-01 transferred labeled RNA to the endothelial recipient cells. This is the first evidence supported the phenomenon of platelet-mediated miRNA transfer (92). Later, Laffont et al. found that endothelial cells actively uptake PMVs produced by activated platelets, resulting in a significant increase of platelet miRNA (such as miRNA-223) in HUVECs when they are co-incubated. Moreover, miR-223-Ago complexes in PMVs was found to have regulatory ability,

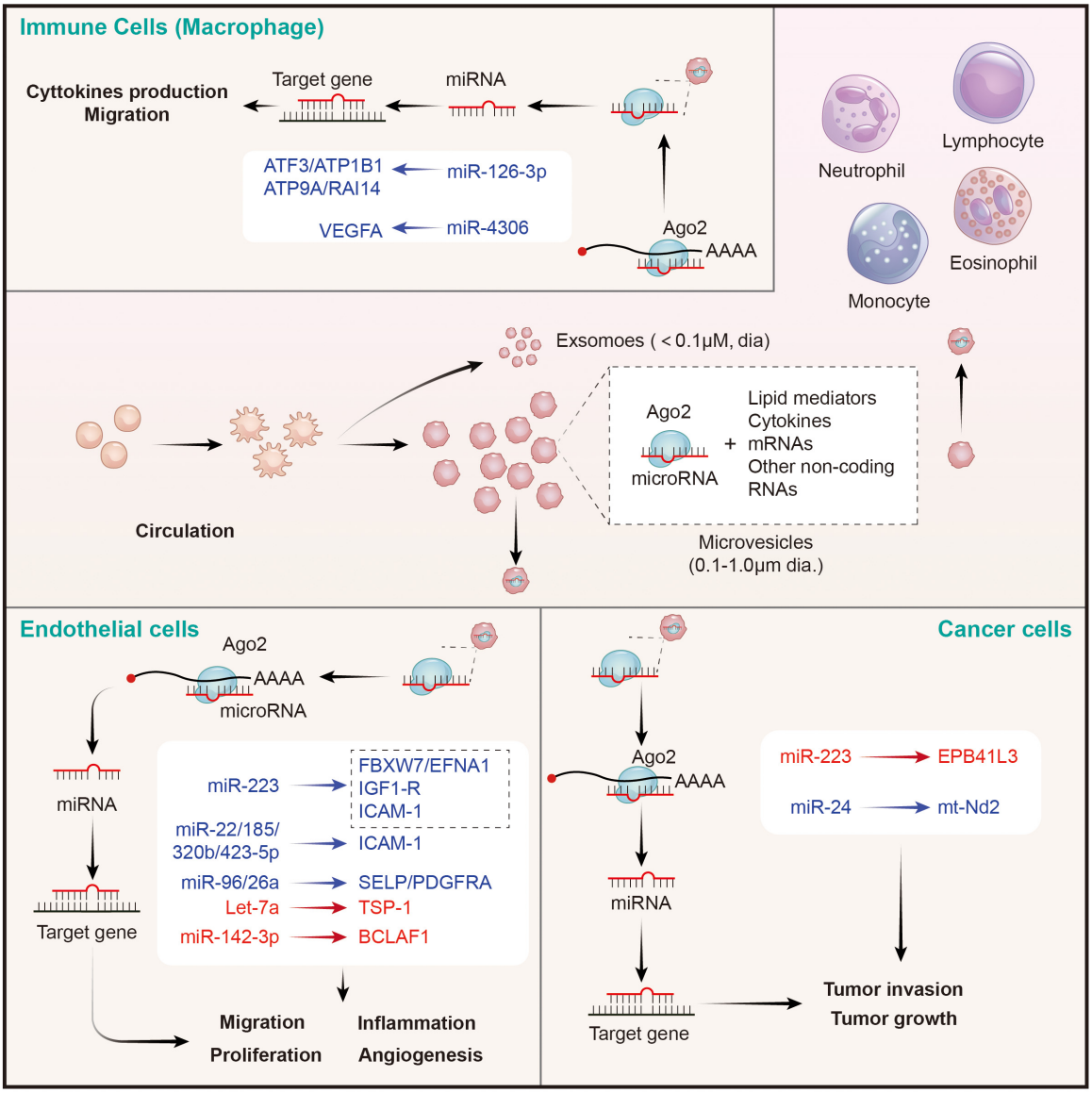

FIGURE 3 | Platelet MPs mediate the transfer of intercellular miRNAs to other cells in the circulatory system, and participate in the regulation of gene expression of recipient cells. MPs released by activated platelets are rich in bioactive lipid mediators, cytokines, mRNAs and a wide variety of non-coding RNAs (including miRNAs). Platelets derived miRNAs can be efficiently transferred into endothelial cells, immune cells, and cancer cells through MPs-mediated manner. Platelet MPs form a tent to protect miRNAs from extracellular nucleases degradation and act as intercellular transporters to deliver functional Ago2·microRNA complexes, through which they modulate the genes of recipient cells in the circulatory system and perform a wide range of biological functions. The red represents miRNA-mRNA pairs that produce promoting functions and the blue represents miRNA-mRNA pairs that produce inhibitory functions. Abbreviations: Ago2, argonaute 2; ATF3, activating transcription factor 3; ATP1B1, sodium/potassium-transporting ATPase subunit beta-1; ATP9A, ATPase phospholipid transporting 9A; RAI14, retinoic acid induced 14; VEGFA, vascular endothelial growth factor A; FBXW7, F-box and WD-40 domain protein 7; EFNA1, ephrin A1; IGF1-R, insulin like growth factor 1 receptor; ICAM-1, intercellular adhesion molecule 1; SELP, selectin P; PDGFRA, platelet derived growth factor receptor alpha; TSP-1, thrombospondin 1; BCLAF1, BCL2 associated transcription factor 1; EPB41L3, erythrocyte membrane protein band 4.1 like 3; mt-Nd2, mitochondrially encoded NADH: ubiquinone oxidoreductase core subunit 2.

resulting in significant downregulation of the expression of two endogenous target genes FBXW7 and EFNA1 (86). Consistently, increased miR-223 in thrombopoietin-induced platelets leads to decreased IGF1-R expression in cultured endothelial cells, and exacerbates their apoptosis (93). Additionally, platelet-derived miR-223 also was predicted to target the ICAM-1 gene and proved to inhibit its expression in HUVECs during inflammation process *via* blocking the NF-κB and MAPK pathways (94). Besides miR-223, co-culture of fluorescent labeling platelets with miR-Scr-FITC and HMEC-1 endothelial cells confirms that 4 platelet-derived miRNAs (miR-22, −185, −320b, and −423-5p) are uptake by endothelial cells and restrains the expression of ICAM-1 (23). Moreover, miR-96 and −26a were also found to be transferred from PMVs to HUVECs, and inhibited the migration

and tube formation of HUVECs (95). In conclusion, platelet-derived miRNAs are taken up by endothelial cells through microvesicles, inhibit endothelial inflammation, migration and tubule formation, and promote apoptosis. However, the opposite results also exist. Let-7a highly expressed in PMVs was found to significantly promote endothelial cell angiogenesis by targeting the anti-angiogenic molecule thrombospondin-1 (TSP-1) (96). Moreover, miR-142-3p from PMVs was also found to be delivered into endothelial cells and enhanced their proliferation *via* inhibiting the expression of Bcl-2-associated transcription factor (BCLAF)1 (97). Therefore, the effects of platelet-derived miRNAs on endothelial cells depend on the type of miRNA ingested. However, it is still unclear whether the uptake of platelet-derived miRNA types by endothelial cells is selective in different states, or only depends on the content of miRNAs in PMVs. It is worth noting that SMCs are identified as other recipients on vascular for PMV-mediated miRNAs. Thrombin-stimulated platelets produced numerous PMVs containing miR-223, miR-339, and miR-21, which were transferred into SMCs and inhibited their proliferation by downregulating the expression of platelet-derived growth factor receptor beta (PDGFRb) (98).

Platelet Microvesicles Transfer MicroRNAs to Immune Cells

Circulating leukocytes are a kind of cells that can be directly coupled with activated platelets through surface receptors, and subsequently modifying their phenotypes. In this study, we focused on leukocytes as recipient cells that receives miRNAs from PMVs and the biological effects of the miRNAs on the former. Laffont et al. found that fluorescently labeled PMVs were internalized by primary human macrophages when they were co-incubated, subsequently causing the aggregation of miR-126-3p in the recipient macrophage (99). Further analysis using transcriptome-wide microarray, 34 miRNAs were identified to be significantly elevated in macrophages upon incubation with PMVs. Correspondingly, 367 mRNAs, including important cytokines and chemokines such as CCL4, CSF1, and TNF-α, acting as potential targets for these changed miRNAs, were confirmed to be significantly downregulated. Co-incubation of macrophages with PMVs enhanced their ability of phagocytosis, pointing toward a potential role of PMVs-mediated miRNAs in shaping macrophage functions (99). However, it didn't identify which miRNAs besides miR-126-3p are involved in the cellular reprogramming of macrophages. A later study from Yang et al. suggested that miRNA-4306 mainly from PMVs was effectively delivered into human monocyte-derived macrophages, and inhibited their migration *in vitro* and reduced macrophage infiltration in myocardial infarction tissues. The inhibitory effect of miR-4306 on macrophages was possibly mediated by restraining the ERK/NF-κB signaling (100). Besides macrophages, PMVs also interact with natural killer (NK) cells and shift their function *via* transferring platelet-derived miR-183 and suppressing the expression of NK activation adapter DAP12 (101). However, the mechanisms of PMV-miRNAs transfer and mediated gene regulation on other immune cells require further exploration.

Platelet Microvesicles Transfer MicroRNAs to Cancer Cells

Although the role of PMVs in tumor regulation has been largely confirmed, there are few studies on whether the miRNAs carried by PMVs have direct effects on tumor cells (102). In 2015, Liang et al. found that PMVs-derived miR-223 was rapidly delivered into human lung cancer A549 cells once they were nurtured together. Subsequently, the invasion ability of A549 cell was increased. This effect may be explained by the inhibition of tumor suppressor-associated gene EPB41L3 by platelet miR-223 in A549 cells (103). By contrary, Michael et al. reported that platelet-derived RNAs, including miRNAs, were transferred into tumor cells, leading to tumor cell apoptosis as their vector PMVs left the circulation and entered the tumor microenvironment. MiR-24 was a major species in this transfer (104). These findings provide novel insights of horizontal miRNA transfer from PMVs to tumor cells and their roles on cancer progression. However, the mechanisms of PMVs infiltration and miRNA transfer, as well as the types of transferred miRNAs and global effects on tumor gene expression, remain to be further investigated.

Functions of Platelet-Derived MicroRNAs in Cardiovascular Diseases and Cancer

For the past few decades, platelet hyperactivation had been elaborated to play important roles in the development and progression of cardiovascular diseases and cancer (105–107). Activated platelets release a variety of vasoactive substances, cytokines and growth factors, promoting platelet-leukocytes crosstalk, mediating the migration of leukocytes, and inducing smooth muscle cell migration and proliferation, further aggravating the damage of vascular-related diseases (108). Platelet secretion also plays multiple roles in cancer fate, including promoting proliferation, resisting cell death, inducing angiogenesis, accelerating invasion and metastasis, and evading immunodetection (109). Activated platelets release and deliver abundant miRNAs as above mentioned; however, few studies have systematic revealed their association and value with clinical diseases. Here, we will explore the possible association of platelet-derived miRNAs with cardiovascular disease and cancer (**Tables 3**, **4** and **Figures 4**, **5**).

Platelet-Derived MicroRNAs and Atherosclerosis

Atherosclerosis (AS) is a chronic cardiovascular disease that underlies the pathology of cerebral infarction and coronary heart disease (110). The occurrence and development of AS involve in a series of pathological and physiological processes, mainly including vascular endothelial damage, inflammatory cell and lipid infiltration, platelet activation, and intimal thickening (111–113). Platelets are considered to be important contributors to atherosclerosis for their ability to induce inflammatory cascades. Activated platelets lead to vascular damage through the expression and release of inflammatory mediators and promote the activation and degeneration of endothelial cells to form atherosclerosis and vascular thrombotic lesions (114). They also promote intercellular communication and adhesion between blood cells and the vessel wall, proliferation of SMCs and chemotaxis of foam cells (115, 116).

TABLE 3 | The potential roles of platelet miRNAs on cardiovascular diseases and diabetes.

Diseases	MicroRNA	Target	Associated phenotypes	References
Atherosclerosis	miR-21	MAP3K10	Inhibit macrophage inflammation and atherosclerosis progression	(119)
	miR-223	ICAM-1, NFAT5	Inhibited endothelial inflammation and intra-arterial thrombosis in atherosclerosis; inhibited proliferation and migration of SMCs and atherosclerosis progress	(94) (122)
	miR-34a	Sirt1	Aggravated atherosclerotic plaque development	(121)
	miR-25-3p	Adam 10	Inhibited ox-LDL-induced vascular endothelial inflammation, lipid deposition, and atherosclerosis	(123)
	miR-22-3p	HMGB1	Suppressed the proliferation and migration of SMCs, and neointimal hyperplasia during atherosclerosis	(236)
	Let-7g	PDGF/MEKK1	Decreased atherosclerotic plaques	(237)
	Let-7	IL-6/TGFβR1	Reduced diabetes-associated carotid atherosclerosis	(155)
Myocardial infarction	miR-22-3p	PTAFR	Suppressed the fibrogenesis and collagen deposition of post-MI	(238)
	miR-4306	VEGFA	Reduced macrophage infiltration in cardiac tissue in myocardial infarction mice	(100)
	miR-320b	ICAM-1	Alleviated endothelial inflammation in myocardial infarction mice	(23)
	miR-223-3p	KLF15, PARP-1 RASA1	Protected cardiomyocyte from hypoxia-induced apoptosis and oxidative stress; promoted the proliferation, migration, and differentiation, and aggravated myocardial fibrosis	(239) (128) (127)
	miR-126	HIF-1α VEGFA	Promote angiogenesis in AMI patients	(129)
Hypertension	miR-142-3p	BCLAF1	Promoted endothelial cell proliferation in hypertension	(97)
	miRNA-126	PI3KR2	Prevented microvascular abnormalities in hypertension	(134)
	miR-140-5p	Nrf2/Sirt2	Worsen hypertension and oxidative stress	(240)
	miR-21-3p	HDAC8 ADRA2B	Lowered blood pressure and weakened reduced organ damages in hypertension	(241) (242)
Diabetes	miR-223	P2y12R SMOC1 NLRP3 FOXO1/SOX6 GLUT4	Restrained platelet hyperreactivity associated with diabetes; inhibited endothelial injury induced by high-glucose and high-fat; promoted β-cell proliferation and improved β-cell function in diabetes; Increased glucose metabolism of cardiomyocytes and improved diabetic cardiomyopathy	(158) (230) (154) (243) (244)
	miR-126	ADAM9	Rescued diabetes-induced impairment in efferocytosis of apoptotic cardiomyocytes	(245)
	miR-140	FOXK2	Improved angiogenic dysfunction in diabetes mellitus	(246)
	miR-143	ORP8	Inhibited insulin-stimulated AKT activation and impaired glucose metabolism	(247)
	miR-103b	SFRP4	Early diagnosed DM2	(148)

ICAM-1, intercellular adhesion molecule 1; NFAT5, nuclear factor of activated T cells 5; Adam 10, a disintegrin and metalloprotease 10; HMGB1, high mobility group box 1; PDGF, platelet derived growth factor; MEKK1, mitogen-activated protein kinase kinase kinase 1; TGFβR1, transforming growth factor beta receptor 1; PTAFR, platelet-activating factor receptor; VEGFA, vascular endothelial growth factor A; KLF15, kruppel like factor 15; PARP-1, poly (ADP-ribose) polymerase 1; RASA1, RAS p21 protein activator 1; BCLAF1, BCL2 associated transcription factor 1; PI3KR2, phosphoinositide-3-kinase regulatory subunit 2; Nrf2, nuclear factor erythroid 2-related factor 2; Sirt2, sirtuin 2; HDAC8, histone deacetylase 8; ADRA2B, adrenoceptor alpha 2b; SMOC1, secreted modular calcium-binding protein 1; NLRP3, nod-like receptor protein 3; Foxo1, forkhead box O1; SOX6, SRY-box transcription factor 6; GLUT4, glucose transporter 4; FOXK2, forkhead box K2; ORP8, oxysterol-binding protein-related protein 8; SFRP4, secreted frizzled related protein 4.

As mentioned above, platelet-derived miRNAs not only regulate platelet function, but also participate in endothelial cell function and SMC proliferation, suggesting that they may play a significant role in the occurrence and development of atherosclerosis. Several studies have found platelet-derived miRNAs changed in the circulation of atherosclerotic patients. In study performed by Sondermeijer group, circulating miR-624 and miR-340 are found to be significantly elevated in patients with CAD as compared to healthy controls (117). However, the expression of miR-126 and miR-223 in platelets were reduced in AS patients. Among them, miR-126 expression level was proven to have a negative correlation with plaque morphology and coronary stenosis (118). The mechanism may involve the targeted regulation of MAP3K10 by miR-21 to inhibit macrophage inflammation and atherosclerosis progression (119). In a atherosclerosis animal model, more miRNAs such as miR-19a, −21, −126, −26b, −92a, −155, −204, −210, −221, −222, and −34a are reported to be delivered by PMVs and contribute to the richness of circulating miRNAs (120). Elevated miR-34a aggravated atherosclerotic plaque development by inhibiting Sirt1 signaling in an atherosclerosis mouse model (121). Other demonstrated the roles of platelet-derived miRNAs in many key links of atherosclerosis development. MiR-223 released by thrombin-activated PMVs inhibits ICAM-1 expression by regulating NF-κB and MAPK pathways and is protective against atherosclerosis and endothelial inflammation (94). MiR-223 also inhibits the platelet-derived growth factor-BB (PDGF-BB)-induced proliferation and motility of human aortic smooth muscle cells by targeting NFAT5 and exhibits potential therapeutic effects for atherosclerosis (122). Platelet-derived

TABLE 4 | The potential roles of platelet miRNAs on cancer.

MicroRNA	Target	Associated phenotypes	References
miR-223	Mef2c RhoB EPB41L3	Promoted lung cancer cell invasion	(168) (169) (103)
miR-126	VEGF-A ZEB1 ADAM9 CCR1	Inhibit the progression of ovarian cancer, cervical cancer, prostate cancer, and NSCLC	(172) (173) (174) (175)
miR-24	mt-Nd2	Inhibited growth of both lung and colon carcinoma ectopic tumors	(104)
miR-939	E-cadherin	Induced epithelial-mesenchymal transition and promoted progression of epithelial ovarian cancer	(178)
miR-22	ACLY SIRT1/FGFR1	Inhibited the growth and metastasis in breast cancer, inhibits the angiogenic activities of endothelial cells and consequently NSCLC growth	(180) (187)
Let-7d	HIF1α	Inhibited breast cancer metastasis to the brain	(248)
miR-27b	THBS-1	Enhanced the pro-angiogenic activities	(168)
miR-21	KRIT1 PTEN	Leading to tumor progression	(182) (183)
miR-142	TGF-β	Leading to decreased growth and metastasis of hepatocellular carcinoma by antagonizing angiogenesis	(186)

HIF1α, hypoxia inducible factor 1 subunit alpha; VEGF-A, vascular endothelial growth factor A.

exosomes overexpressing miR-25-3p attenuate coronary vascular endothelial cell inflammation induced by oxidized low density lipoprotein *via* targeting NF-κB/Adam10 Pathway in ApoE-/- mouse models of atherosclerosis (123). However, whether other differentially expressed platelet miRNAs are also involved in the occurrence and development of atherosclerosis remains unclear.

Platelet-Derived MicroRNAs and Myocardial Infarction

The rupture of unstable plaque leading to rapid activation and aggregation of circulating platelets, and following thrombosis, is the main mechanism of myocardial infarction. Several selected platelet miRNAs were found to significantly change in acute myocardial infarction (AMI) and become indicators of thrombosis. Candidate platelet miR-21 and miR-126 were significantly descended in patients with AMI compared with the controls while the results of platelet miR-150 and miR-223 were opposite. Hromadka et al. found that miR-126 and miR-223 were potential independent predictors of thrombotic events and recommended for ischemic risk stratification after AMI (124). The expression levels of platelet miR-587 were relatively higher in AMI patients than unstable angina (UA) and control groups and showed positive association with the degree of coronary stenosis (125). These results suggest that platelet-derived miRNAs may induce atherosclerotic plaque instability or thrombosis besides platelet function. Elgheznawy et al. found that in a mouse FeCl3-induced arterial thrombosis model, miR-223 deficiency increased thrombus size after FeCl3 carotid treatment, and increased embolization after laser-induced vascular injury of the small dorsal skin (67). MiR-223 directly targets kindlin3, an integrin-binding protein, and FXIII-A, both of which contribute to thrombus formation. Therefore, platelets unexpectedly antagonize the formation of thrombi by releasing specific miRNAs, such as miR-223, when they are abnormally activated in the early stage of MI, through above mechanisms. Additionally, miR-223 has also been found to exhibit contradictory direct cardiac effects in myocardial infarction, including protecting cardiomyocytes from ischemic injury, promoting fibroblast proliferation and collagen formation (126–128). Of course, platelet miRNAs also influence platelet activity by targeting its surface receptors, such as miR-223/P2Y12 and miR-126/ADAM9, accounting for their potential behaviors during thrombosis (27, 68). Besides, inflammatory response accelerating myocardial fibrosis after myocardial infarction is closely related to the deterioration of cardiac function and long-term prognosis. MiR4306 from PMVs noticeably inhibited macrophage infiltration in cardiac tissue in myocardial infarction mice, which may also restrain the progression of post-infarction remodeling (100). Endothelial cells expressing ICAM-1 triggering the adhesion and migration of inflammatory cells to the damaged myocardium, has been found to be regulated by platelets releasing miR-320b (23) and miR-223 (122). Additionally, exosomes containing miR-126 extracted from AMI patients promote angiogenesis by increasing HIF-1α and VEGFA expression (129).

Platelet-Derived MicroRNAs and Hypertension

Hypertension is a complex, multifactorial disease, and its occurrence and development have definite relationships with miRNA regulatory network (130). Marketou et al. assessed platelet-derived miRNAs in 82 patients with essential hypertension and 28 healthy individuals and found that miR-22 and −223 levels are significantly decreased in hypertension and negatively correlated with SBP levels (131). Additionally, let-7 is reported to have a positive correlation with carotid intima-media thickness in patients with essential hypertension (132) while miR-21 has a negative association with arterial stiffness (133). These results suggest that platelets are involved in the formation and progress of hypertension by delivering miRNAs, although the mechanisms seem not very clear. Abnormal proliferation of endothelial cells (EC) induced by pathologic factors contributes to vascular remodeling in hypertensive conditions. MiR-142-3p from PMVs enhanced EC proliferation by downregulating the expression of BCLAF1 (97). In a spontaneously hypertensive rat (SHR) model, miRNA-21 levels were increased by 36% levels while miRNA-126 levels were reduced by 29%. Both of them are abundant in platelets. Aerobic exercise training reversed their changes and prevented microvascular abnormalities in hypertension *via* increasing the expression of anti-apoptotic protein Bcl-2 and inhibiting the expression of anti-angiogenic regulator PI3KR2 (134). Thus, regulating endothelial cell proliferation in hypertensive vascular remodeling is one of the mechanisms to explain the potential

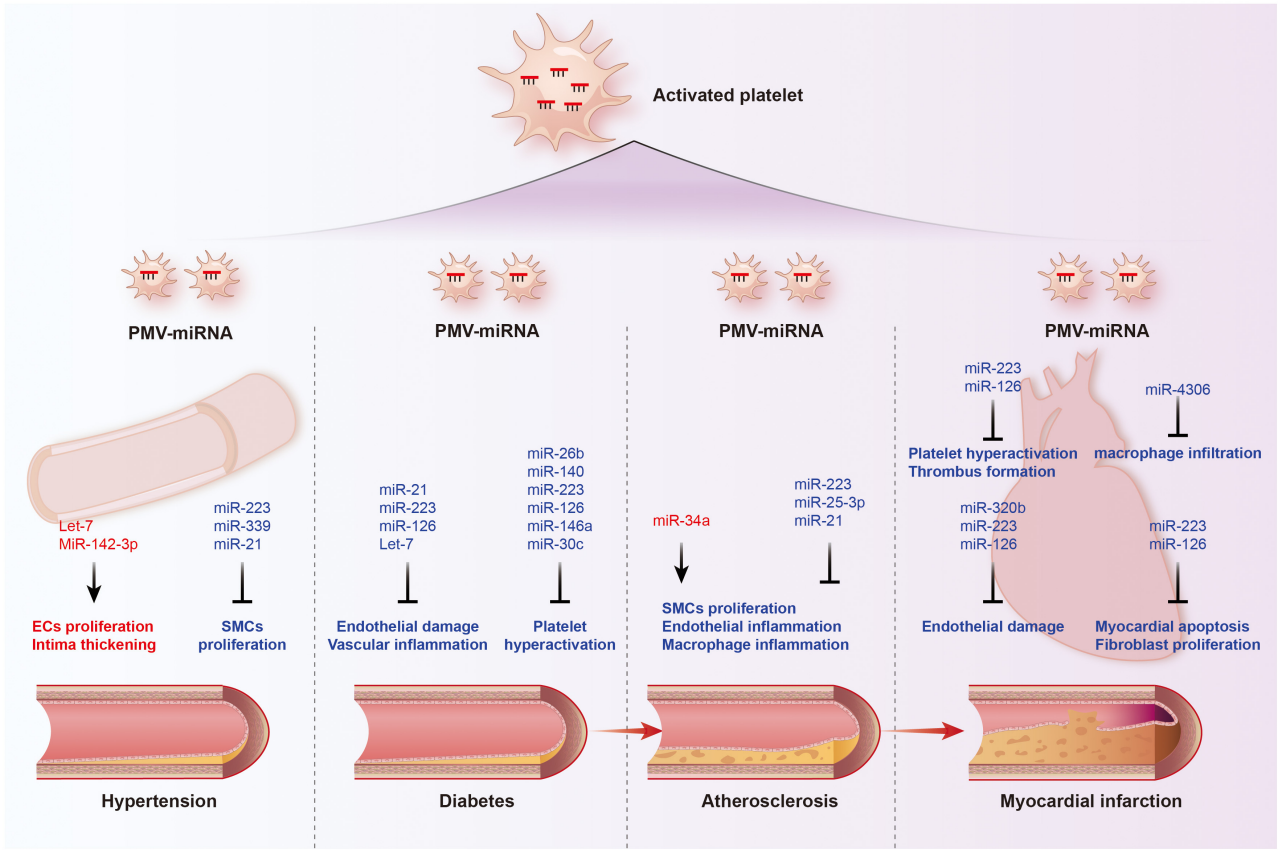

FIGURE 4 | Functions of platelet-derived miRNAs in cardiovascular diseases. The transport of miRNAs from platelet microvesicle (PMV) to the cardiovascular system participate in the occurrence and development of hypertension, diabetes, coronary heart disease, and myocardial infarction, involving multiple mechanisms such as endothelial homeostasis, smooth muscle cell proliferation, inflammatory cell infiltration, and cardiomyocyte apoptosis. Red labeled miRNAs promoted their downstream processes, while blue labeled miRNAs inhibited the downstream processes.

role of platelet-derived miRNAs. Additionally, PMVs from thrombin-stimulated platelets transferring miR-223, −339, and −21 into SMCs were shown to inhibit their proliferation by downregulating the expression of PDGFRb (98). However, whether their proliferation effects on SMCs is enough to change the phenotype of hypertension deserves further study.

Platelet-Derived MicroRNAs and Diabetes

Diabetes mellitus is a serious threat to human lifespan worldwide, resulting in a 2–4 times increase in mortality compared with non-diabetic subjects (135, 136). Cardiovascular complications are the leading cause of death from diabetes, accounting for 50–75% of deaths (137, 138). It has been widely accepted that diabetes are prone to pro-thrombic condition by increasing coagulation activity, impaired fibrinolysis, endothelial dysfunction, and platelet hyper-reactivity. Although the detail mechanism of hypercoagulable state in diabetes remain unclear, hyperactivation, and endothelial dysfunction observed in diabetes contribute to this pathological process (139). In 1993, Nomura et al. reported that platelets were activated in diabetic patients and the microparticles released from them also increased in plasma (140, 141). Activated platelets are able to modulate the function of ECs and SMCs, contributing to both the initiation and progression of atherosclerosis, and even the ensuing atherothrombotic sequelae (142, 143). Recently, it has been reported that platelets have been found to be a major source of circulating miRNAs and specific miRNAs from platelets changed in diabetes, suggesting that platelets-derived miRNAs may be potential predictive markers and therapeutic targets.

Fejes et al. reported that hyperglycemia suppresses miRNAs expression in platelets. They found the expression of miR-223, miR-26b, miR-140, and miR-126 in mature platelets had been significantly inhibited in T2DM subjects (144), which at least partly because hyperglycemia decreased platelet Dicer activity (145). Additionally, miR-26b and miR-140 showed direct target on the gene SELP and miR-223 and miR-126 inhibited the expression of P2Y12 in platelets (144, 146). Similarly, Elgheznawy et al. found human subjects and mice with diabetes showed decreased levels of platelet miR-142, miR-143, miR-155, and miR-223, which possibly related to the inhibition of Dicer enzyme. Using calpain inhibitors to prevent the loss of platelet Dicer in diabetic mice can save the decrease of platelet-miRNAs and increase their target genes (67). Besides miR-223, the expression of miR-146a was also downregulated

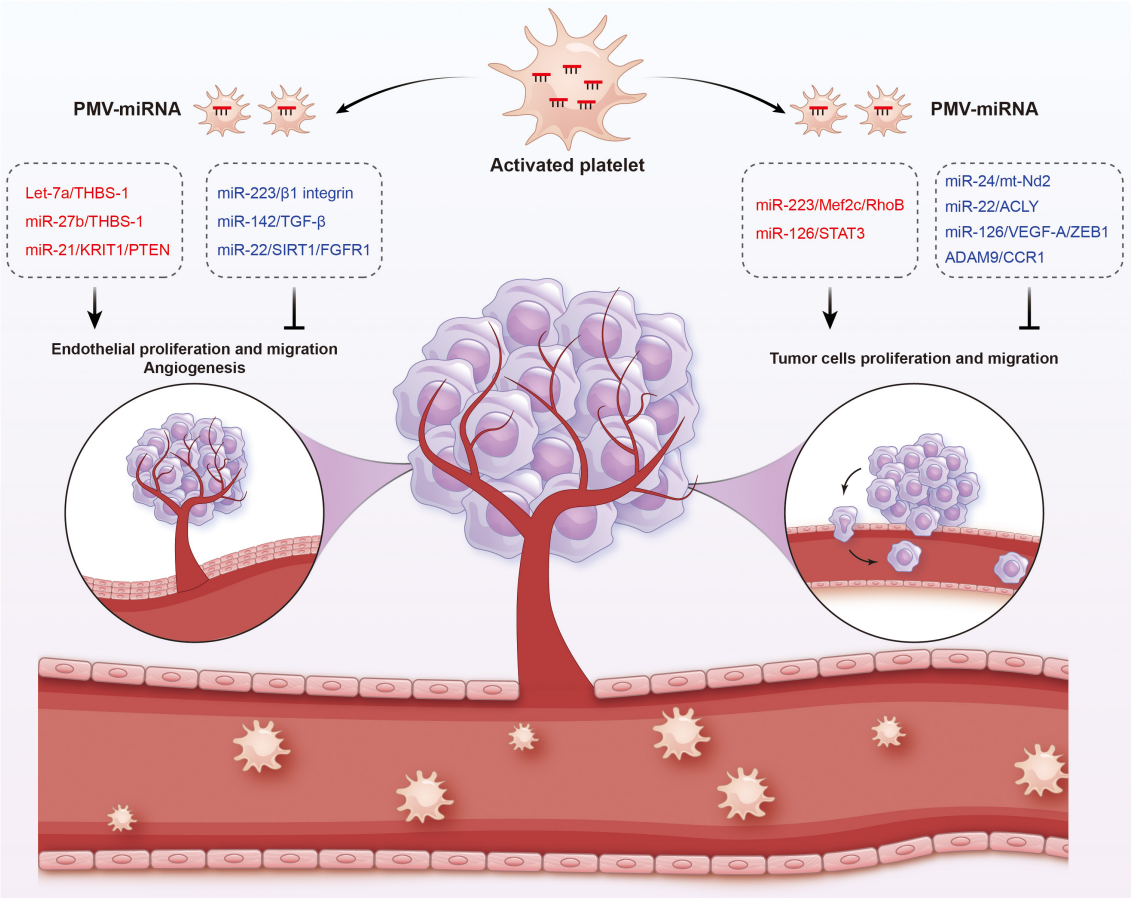

FIGURE 5 | Functions of platelet-derived miRNAs in cancer. Platelet-derived miRNAs change tumor fates in two ways: on the one hand, PMVs transfer platelet miRNAs to vascular endothelial cells and are associated with enhanced tumor metastasis and cancer progression; on the other hand, PMVs can penetrate the blood vessels and enter the tumor microenvironment to directly transfer platelet miRNAs into tumor cells, thus regulating gene expression in tumor cells and tumor progression. The red represents miRNA-mRNA pairs that produce promoting functions and the blue represents miRNA-mRNA pairs that produce inhibitory functions. Abbreviations: THBS-1, anti-angiogenic protein thrombospondin-1; KRIT1, krev interaction trapped protein 1; PTEN, phosphatase and tensin homolog deleted on chromosome ten; TGF-β, transforming growth factor β; SIRT1, sirtuin 1; FGFR1, fibroblast growth factor receptor 1; Mef2c, myocyte enhancer factor 2C; STAT3, signal transducer and activator of transcription 3; ACLY, proto-oncogene ATP citrate lyase; VEGFR-A, vascular endothelial growth factor receptor A; ZEB1, zinc finger E-box binding homeobox 1; CCR1, chemokine (C-C motif) receptor 1.

by hyperglycemia intervention, resulting in subsequent platelet activation in patients with diabetes. Moreover, low level of miR-223 and miR-146a in patients plasma with diabetes increases the risk of ischemic stroke (147). Platelet-derived miR-103b is significantly downregulated and also suggested as a novel biomarker for the early diagnosis of T2DM (148). Additionally, decreased miR-30c level induced by hyperglycemia promotes thrombus formation in T2DM by increasing the expression of plasminogen activator inhibitor-1 (PAI-1) (149).

The change of platelet-derived miRNAs contributed to diabetes-associated vascular lesion. Endothelial dysfunction in patients with T2DM is well recognized, resulting in the vascular system susceptible to thrombotic and atherosclerotic effects (150). Recent plasma miRNA analysis confirmed that the downregulation of miR-126 in a group of diabetic patients are partially responsible for vascular damage in diabetic patients (151, 152). Elevated plasma miR-21 in diabetic patients was found to decrease the production of reactive oxygen species and inflammatory cytokines in vascular endothelial cells and reduce the area of atherosclerotic plaque by targeting the degradation of ADAM10 mRNA (153). Additionally, miR-223-3p improves the injury of cardiac microvascular endothelial cells from diabetic mice by targeting the expression of NLRP3 (154). Let-7, an abundant miRNA in PMVs, is decreased in human and mice carotid plaques with diabetes, and promotes the inflammatory phenotypes of SMCs including proliferation, migration, monocyte adhesion, and NF-κB activation (155).

Targeting platelet-derived miRNAs appears to confer some protective effects in diabetes. For example, aerobic training improves platelet function in type 2 diabetic patients *via* increasing miRNA-130a and decreasing the target gene GPIIb (156). Long-term moderate-intensity aerobic training increased miRNA-223 expression, leading to decreased expression of P2Y12 receptor and platelet activity, which may be one of internal

mechanisms for reducing the occurrence of atherothrombotic events in T2DM patients (157, 158). Additionally, inhibiting platelet activation by aspirin reduced levels of circulating miR-126 (159), which may protect endothelial from inflammation.

Platelet-Derived MicroRNAs and Cancer

Cancer is the leading cause of death following CVDs and shows close associations with CVDs. Tumors are often accompanied by overactive platelets and hypercoagulable state, as well as endothelial proliferation and angiogenesis in the tumor microenvironment, overlapping some pathogenesis with many cardiovascular diseases (160, 161). Long-term platelet inhibition in cardiovascular disease is considered to regulate tumor fate by alleviating chronic inflammation and endothelial angiogenesis (162, 163). Additionally, tumor therapy often leads to cardiovascular disease, which is well-known as cardio-oncology (164). Therefore, platelet function and its derived miRNAs may be the common markers for diagnosis and common targets for treatment about these two diseases.

In fact, platelets are active participants in all steps of tumorigenesis, including tumor growth, angiogenesis, and metastasis. On the one hand, PMVs transfer platelet miRNAs to vascular endothelial cells and are associated with enhanced tumor metastasis and cancer progression. On the other hand, due to the high permeability of the vascular system of solid tumors, PMVs can penetrate the blood vessels and enter the tumor microenvironment to directly transfer platelet miRNAs into tumor cells, thus regulating gene expression in tumor cells and tumor progression (165, 166). Subcutaneously implanted tumors with platelet miRNA knockout in mice aggravated sarcomatoid growth and progress, verified the tumor-promoting effects of platelet miRNAs (167).

In different cancer cells, PMVs derived miRNAs target different oncogenes and tumor suppressor genes. It has been reported that miR-223 enhances breast cancer invasion by inhibiting the expression of myocyte enhancer factor 2C (Mef2c) and increases the progression of gastric cancer by specifically targeting RhoB (168, 169). It has also been reported that miR-223 directly targets the 3′-UTR of the tumor suppressor EPB41L3, which is the most upregulated gene in recurrent tumors (170). Increased miR-223 was observed in platelets and PMVs from NSCLC patients and effectively delivered to human lung cancer cell A549 *via* PMVs (103), thus facilitating the invasion of A549. Physiological delivery of platelets miR-223 and miR-126 altered the phenotypes of breast cancer cells, including cell cycle arrest, migration inhibition, and increased responsiveness to cisplatin (171). MiR-126 can inhibit the progression of some cancers (including ovarian cancer, cervical cancer, prostate cancer, and NSCLC) *via* negative control of numerous validated targets such as VEGF-A, ZEB1, ADAM9, and CCR1 (172–175). In some instances, however, miR-126 supports cancer progression *via* inhibiting STAT3-mediated tumor apoptosis (176, 177). MiR-24 in PMVs inhibits the growth of ectopic tumors of lung and colon cancer by targeting mitochondrial protein mt-Nd2. Blocking miR-24 in tumor cells accelerates their growth *in vivo* and eliminates the inhibitory effect of PMVs on tumor growth (104). In ovarian cancer cells, miR-939 delivered by PMVs induces mesenchymal transformation of epithelial cells and cancer progression by inhibiting the expression of e-calponin expression (178). In small-cell lung cancer (SCLC), overexpression of miR-24-3p blocked the autophagy process by targeting autophagy-associated gene 4A (ATG4A), finally enhancing the sensitiveness of SCLC cells to combined chemotherapy (etoposide and cisplatin) (179). In breast cancer, miR-22 inhibited the growth and metastasis by downregulating the expression of the proto-oncogene ATP citrate lyase (ACLY) (180). In solid tumors, PMVs interact with tumor cells by directly transferring platelet-derived miRNAs and inhibit the growth of ectopic tumors in colon and lung cancers by downregulating TC genes and inducing tumor cell apoptosis (104).

Platelet miRNAs also transfer to vascular endothelial cells and regulate tumor development and drug resistance by enhancing or inhibiting angiogenesis. When PMVs were co-cultured with HUVECs on extracellular matrix gels, PMVs-derived let-7a was adopted to endothelial cells and induced robust capillary like structure formation by promoting the release of pro-angiogenic regulators and reducing the expression of anti-angiogenic protein thrombospondin-1 (THBS-1) (96). In another *in vitro* experiment, THBS-1 expression was inhibited by transfection with elevated platelet miR-27b, which was subsequently enhanced the pro-angiogenic activities of platelet (181). Exosomal miR-21 in the tumor microenvironment had been widely known as a strong proangiogenic factor *via* targeting krev interaction trapped protein 1 (KRIT1) and PTEN (182, 183), finally leading to tumor progression. However, miR-223 is identified as an antiangiogenic miRNA by targeting β1 integrin (184), thereby promoting resistance to cetuximab in head and neck squamous cell carcinoma (185). Moreover, miR-142 was found to directly target and inhibit transforming growth factor β (TGF-β), leading to decreased growth and metastasis of hepatocellular carcinoma by antagonizing angiogenesis (186). MiR-22 also acts as a potent angiogenesis inhibitor that inhibits the angiogenic activities of endothelial cells and consequently NSCLC growth through targeting SIRT1 and FGFR1 (187).

Platelet Inhibitors and Platelet-Derived MicroRNAs

Antiplatelet drugs are considered as the cornerstone for the prevention and treatment of atherothrombotic diseases, and have saved numerous lives since inception. However, a considerable number of patients receiving standard antiplatelet medication still exhibit high levels of platelet activation, increasing their risk of progression and recurrence of cardiovascular events (188, 189). Several possible mechanisms were proposed to explain the phenomenon, such as genetic polymorphisms, drug–drug interactions, or high on-treatment platelet reactivity (HTPR) (190, 191). However, identifying patients who has inadequate response to state-of-the-art antiplatelet treatment remains a challenge. Thus, real-time monitoring of platelet activity seems to be more accessible and important for patients with atherothrombotic diseases and can ideally guide personalized antiplatelet treatment. Current platelet function tests are measured *ex vivo* and susceptible to interference by many

confounding factors, bringing obvious limitations for guiding treatment decisions. As mentioned above, platelet activation leads to the production of PMVs carrying abundant miRNAs, which in turn change platelet function and affect protein expression in other cells upon internalization. Antiplatelet drugs including COX inhibitors and P2Y12 receptor inhibitors were found to significantly change the expression of platelet-derived miRNAs (36, 192, 193). Circulating platelet miRNAs are relatively stable and convenient for detection *in vivo*, making them potential and reliable markers for monitoring platelet activity and antiplatelet response.

Willeit et al. firstly examined the responsiveness of platelet-derived miRNAs to platelet inhibition. They introduced that plasma levels of platelet miRNAs, including miR-126, miR-150, miR-191, and miR-223, are significantly reduced by aspirin and prasugrel treatment (91). Another recent study found that the levels of plasma miR-223 and miR-197 from platelets are significantly downregulated in subjects treated with clopidogrel or ticagrelor when compared with health controls (194). Acute coronary syndrome patients treated with clopidogrel alone resulted in 2-fold reduction in miR-223, 1.8-fold reduction in miR-130, and 4.1-fold reduction in miR-126 (195). Apparently, inconsistent miRNA alterations in different researches may be attributed to the kind of antiplatelet reagents, which results in varied degree of platelet inhibition. A recent study reported that plasma platelet miRNAs (such as miR-126, miR-150, and miR-223) were significantly reduced in ACS patients who completed the replacing treatment from clopidogrel to another more potent antiplatelet agent ticagrelor, in proportion to the degree of platelet inhibition (196). Additionally, monotherapy with potent P2Y12R inhibition prasugrel in T2DM reduced the levels of miR-24, miR-191, miR-197, and miR-223 when compared with aspirin treatment (197). Therefore, consensus on which miRNA is the best biomarker for the response to antiplatelet therapy has not yet been reached. Circulating miR-223 and miR-126 are expected to be the options since they reached similar conclusion in multiple studies (198–201).

Plasma level of platelet-derived miRNAs can also be used as a marker of antiplatelet insensitivity or resistance, also named high on-treatment platelet reactivity (HTPR). Kok et al. proposed that miR-19b-1-5p is a suitable marker of platelets insensitive to aspirin (202). MiR-365-3p is found to be positively correlated with HTPR in coronary artery disease patients (203). Additionally, platelets in ACS patients with HTPR exhibit upregulation of miR-204-5p after dual antiplatelet therapy (204). Lower expression of miR-126, miR-130, and miR-223 is also been observed in the ACS patients with high platelet reactivity (HPR) to clopidogrel than those with low platelet reactivity (LPR) (195). A more recent study also reported that increased expression of miR-24-3p, miR-142-3p, and miR-411-3p was positively correlated with clopidogrel resistance (CR) in CAD patients (205). Similarly, miR-29, miR-34, miR-126, miR-142, and miR-223 are also reported to be novel biomarkers for P2Y12 inhibitor resistance prediction (206). Although the mechanisms of antiplatelet resistance are complex, the relationship between plasma miRNAs and platelet resistance may be explained by their regulation on platelet surface proteins. Liu et al. found that miR-34b-3p overexpression inhibited the expression of thromboxane A synthase 1 (TBXAS1), leading to the enhanced antiplatelet efficiency of aspirin (207). The downregulated miR-107 and miR-223 in the HPR group are negatively correlated with P2Y12 expression, indicating that platelet miR-107 and miR-223 possibly mediated CR by inhibiting P2Y12 expression (208, 209). A more recent study also found that platelet miR-15b promoted platelet insensitivity in patients undergoing PCI because it suppressed Bcl-2 protein expression and enhanced platelet apoptosis (210).

In general, on the one hand, platelet-derived miRNAs are significantly altered by antiplatelet drugs and become potential indicators of platelet activity level. On the other hand, changes in platelet miRNA levels may in turn affect their response to antiplatelet drugs by altering the expression of platelet-activated receptors.

Clinical Significances of Platelet-Derived MicroRNAs in Cardiovascular and Neoplastic Diseases

As mentioned above, platelets are excessively activated in various vascular diseases, diabetes and tumors, and secrete many cell-specific miRNAs through PMVs. These miRNAs remain stable in peripheral blood, allowing a convenient detection, bringing them potential perspectives of early clinical diagnosis for platelet-related diseases (**Table 5**). Additionally, platelet miRNAs control and regulate the biological functions of themselves and other neighboring cells, participating in the occurrence and development of cardiovascular diseases and tumors, becoming novel potential targets for treatment.

Platelet-Derived MicroRNAs as Potential Markers of Cardiovascular Diseases

Considering the high mortality and morbidity of cardiovascular diseases and the lack of timely diagnosis, the discovery of novel predictive biomarkers is necessary. However, current diagnostic techniques based on electrocardiogram and troponin, are severely limited because they may be non-specifically altered in certain diseases, such as myocarditis and secondary myocardial damage. Platelet hyperactivation is another important clinical feature during acute thrombotic events (211), yet no corresponding test accurately reflects its states. Platelet-derived miRNAs have been reported to be biomarkers for platelet activation and are expected to be diagnostic and/or prognostic biomarkers for cardiovascular disease.

MiR-1, one of platelet-rich miRNAs, was found to increase rapidly and peak within 2 h after the onset of cardiac infraction and positively correlated with serum creatine kinase MB (CK-MB) levels (212). Another study showed that both increased miR-1 and miR-29b were associated with the decreased parameters of cardiac function (such as LVEDV and LVEF) in patients suffered AMI, indicating their potential predictive roles for adverse ventricular remodeling (213). MiR-126 and miR-223 were the most frequently investigated platelet miRNAs and it is well established that they are significantly reduced in CVD patients, making them an indication for the presence of cardiovascular

TABLE 5 | Changes of platelet miRNAs in cardiovascular disease and cancer.

Changed microRNAs	Disease	References
miR-340↑, miR615-5p↑, miR-545:9.1↑, miR-451↑, miR-454↑, miR-624↑, miR-624↑, miR-12801↓	Premature coronary artery disease	(117)
miR-150↑, miR-223↑, miR-21↓, miR-126↓	STEMI patients	(215)
miR-142-3p↑, miR-107↑, miR-338-3p↑, miR-223-3p↑, miR-21-5p↑, miR-130b-3p↑, miR-301a-3p↑, miR-221-3p↑	ACS patients	(219)
miR186-5p↓, miR185-5p↓, miR20a-5p↓, miR942↓, miR127-3p↑, miR221-3p↑, miR483-5p↑, miR146a-5p↑	Acute coronary syndrome	(249)
miR-150↑	Heart failure with atrial fibrillation	(250)
miR-144↑, miR-146a↓, miR-223↓	Diabetes mellitus type 2 patients with ischemic stroke	(221) (147)
miR-223↑	NSCLC patients	(103)
miR-34c-3p↑, miR-18a-5p↑	Nasopharyngeal carcinoma patients	(226)

diseases (131, 214). Five candidate platelet miRNAs, including miR-1, miR-21, miR-126, miR-199, and miR-233, were compared in patients with ST segment elevation myocardial infarction (STEMI) and healthy volunteers. Among them, only miR-126 exhibited correlation with plasma cTnI and was expected to be a potential novel biomarker for STEMI (215). Moreover, miR-126 also is proved as a strong and independent predictor of long-term all-cause mortality among patients with T2DM (216) and patients with venous thromboembolism (217). In a large patient cohort with CAD, Serum miR-223 as well as miR-197 levels were found to be predictors for cardiovascular death (218). Additionally, the predictive accuracy for one-year comprehensive ischemic endpoint was significantly increased when miR-223 and miR-126/miR-223 ratios were served as predictors and added into the model calculating the ischemic risk (124). However, another study assessed potential biomarkers of ACS based on the miRNA profiles of platelets and found that eight platelet miRNAs were markedly elevated in ACS patients and associated with platelet reactivity and functionality. Among them, miR-142-3p is the only potential biomarker confirmed to accurately predict the risk of ACS (219).

Circulating platelet miRNAs may also be sensitive and specific biomarkers for ischemic stroke. In T2DM patients with ischemic stroke, the miR-144 level in platelets increased significantly (220). However, the expression of platelet miR-223 was significantly reduced in these subjects when compared with T2DM patients in without thromboembolic complications (221). Similarly, Duan et al. found that the expressions of platelet miR-223 and miR-146a was obviously lower in diabetic and ischemic stroke patients than in healthy donors (147). Additionally, the expression level of these two miRNAs was correlated with blood platelet activation rates.

Platelet-Derived MicroRNAs as Potential Markers of Cancer

Currently, the diagnosis of cancer mainly depends on clinical manifestations, radiological and biochemical tests and pathological analysis. Although biopsies are the current gold standard for cancer diagnosis, the information obtained from individual biopsies provides a limited snapshot of tumors in space and time, and is not suitable for repeated sampling. Therefore, the liquid biopsy is considered a promising tool for early detection and subsequent monitoring of cancer (222, 223). Platelets are an important component of blood, and their ability to store and release numerous miRNAs to the environment, which enables them to reflect different disease states, early diagnose tumors, predict prognosis, monitor response to treatment, and detect disease recurrence and metastasis (224).

Platelet miRNAs were significantly altered in tumor patients compared with healthy donors (225). For example, platelet derived miR-223 was specifically overexpressed in NSCLC patients than healthy subjects (103). Additionally, tumor-educated platelet was reported to express higher levels of receiving miR-34c-3p and miR-18a-5p from nasopharyngeal carcinoma (NPC) cells, making them potential makers for NPC diagnosis (226). The positive rates for NPC diagnosis based on platelet miR-34c-3p and miR-18a-5p were 93.8 and 87.5%, respectively, significantly higher than these based on Epstein-Barr virus DNA (66.7%) (226). Moreover, a large prospective trial by Best et al. in 2015 showed that the RNA profile of tumor-affected platelets was different from that of healthy individuals, and identified the location of the primary tumor with 96% accuracy and 71% accuracy (227). Therefore, platelets and their derived miRNAs may be a potential source for the development of oncology blood biomarkers.

Platelet miRNAs can also be used to evaluate efficacy and patient outcomes. miR-21, miR-25, miR-19b, and miR-146a and in patients with NSCLC may be potential indicators to predict response to platinum-based treatments (228). Gasperi et al. reported that ω6/ω3-PUFA supplementation enhanced platelet antitumor activities by promoting PMVs derived miRNA (miR-223 and miR-126) delivery into breast cancer. These two miRNAs inhibited the cancer proliferation and metastasis and increased the sensitivity to cisplatin once internalized by breast cancer cells in a dose-dependent manner (171). Additionally, cardiovascular damage caused by tumor treatment contributes to the increasing mortality of cancer (229). Platelet-derived miRNAs may also become potential markers in the field of cardio-oncology. The levels of miR-223-3p decreased in radiation-induced heart in a time-dependent manner and exhibits potential protection against radiation-induced cardiac toxicity (126).

However, we still face many difficulties in trying to apply platelet miRNAs to the clinic. The primary problem is how to solve the potential infection of plasma miRNA background

on platelet miRNA. To avoid this problem, limiting miRNA measurements to PMVs has been recommended, but this would imply a greater workload and a more complex workflow. Future studies will need to explore convenient, economical and accurate detection methods.

Treatment Prospect

Alterations in tissue-specific or cell-specific miRNA expression and their regulation of pathogenic genes under different disease conditions provide a theoretical basis for the use of miRNA technologies to treat diseases. In the past few years, chemically modified oligonucleotides called antagomirs have been developed to silence specific endogenous miRNAs *in vivo* and *in vitro*. Intravenous systemic administration of antagomirs has been widely demonstrated to effectively and specifically recognize and inhibit the activity of target miRNAs in a sequence complementary binding manner in many cells. Several miRNA-targeted therapies have entered the stage of clinical development. For example, a mimetic tumor suppressor miR-34 has reached phase I clinical trials for the treatment of cancer. Additionally, a biological bead targeting miR-122 has entered the phase II trials for the treatment of hepatitis. However, drug delivery issues remain a barrier to their therapeutic use, particularly for targeting miRNAs in cardiovascular disease states. The properties of platelet endocytosis and transport of circulating RNAs have given us new solutions. Vesicle storage coupled with the relatively long half-life of miRNAs could allow miRNAs to persist in circulation for a long time, thus effectively silencing targets in various organ systems.

CONCLUSION

Overall, platelets are involved in several normal physiological processes such as hemostasis, inflammation, vascular repair, and generation, and play a role in diseases such as atherosclerosis, diabetes and cancer. Several studies have determined that activated platelets release miRNA-rich PMVs to regulate other cellular functions. With the intensive study of platelet-derived miRNAs, we realize that platelet miRNAs have the potential to be excellent diagnostic tools covering multiple pathological mechanisms simultaneously. Studies have shown that platelet miRNAs can directly or indirectly reflect platelet activity, thus indicating the emergence of pathological states at an early stage or assessing efficacy after treatment. We can also exploit the organ specificity of some miRNAs within platelets to enhance the effect of existing drugs or to find new therapeutic targets. In conclusion, studying platelet-derived miRNAs can be of great benefit to patients by helping to modify the use of existing drugs and finding new drug targets, as well as for assessing treatment efficacy and patient prognosis. Although there are still questions to be answered, platelet-associated miRNAs are promising biomarker candidates.

AUTHOR CONTRIBUTIONS

QL and LW wrote the manuscript, prepared the tables, and drew the figures. JD helped to process the figures. LW and YW designed the experiments, guided this study, and revised the manuscript. MD, LL, QF, and DW checked this manuscript. All authors contributed to this article and approved the submitted manuscript.

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TGF-β-Containing Small Extracellular Vesicles From $PM_{2.5}$-Activated Macrophages Induces Cardiotoxicity

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Numerous epidemiological and experimental studies have demonstrated that the exposure to fine particulate matter (aerodynamic diameter <2.5 μm, $PM_{2.5}$) was closely associated with cardiovascular morbidity and mortality. Our previous studies revealed that $PM_{2.5}$ exposure induced cardiac dysfunction and fibrosis. However, the corresponding underlying mechanism remains largely unaddressed. Here, $PM_{2.5}$-induced cardiotoxicity is presented to directly promote collagen deposition in cardiomyocytes through the transforming growth factor-β (TGF-β)-containing small extracellular vesicles (sEV). The sEV transition may play an important role in $PM_{2.5}$-induced cardiac fibrosis. Firstly, long-term $PM_{2.5}$ exposure can directly induce cardiac fibrosis and increase the level of serum sEV. Secondly, $PM_{2.5}$ can directly activate macrophages and increase the release of tumor necrosis factor α (TNF-α), interleukin-6 (IL-6), and TGF-β-containing sEV. Thirdly, TGF-β-containing sEV increases the expression of α-smooth muscle actin (α-SMA), collagen I, and collagen III in mouse cardiac muscle HL-1 cells. Finally, TGF-β-containing sEV released from $PM_{2.5}$-treated macrophages can increase collagen through the activation of the TGF-β-Smad2/3 signaling pathway in HL-1 cells from which some fibroblasts involved in cardiac fibrosis are thought to originate. These findings suggest that TGF-β-containing sEV from $PM_{2.5}$-activated macrophages play a critical role in the process of increasing cardiac collagen content *via* activating the TGF-β-Smad2/3 signaling pathway.

Keywords: $PM_{2.5}$, macrophage, small extracellular vesicles (sEV), TGF-β, cardiotoxicity

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HIGHLIGHTS

- $PM_{2.5}$ exposure increased the level of sEV, which can promote collagen deposition in cardiomyocytes.
- TGF-β-containing sEV from $PM_{2.5}$-induced macrophages activated the TGF-β-Smad2/3 signaling pathway and caused cardiotoxicity.

INTRODUCTION

Exposure to particulate matter (PM) with an aerodynamic diameter <2.5 μm ($PM_{2.5}$) causes certain health risks and toxic effects on various tissues and systems (1). Epidemiological and

experimental studies have demonstrated that long-term or high-concentration $PM_{2.5}$ exposure can increase the morbidity and mortality of cardiovascular diseases (CVD) (2–4). PM induces many pathological processes, such as systemic inflammation and oxidative stress, and causes acute arterial vasoconstriction, endothelial dysfunction, arrhythmia, and procoagulation/thrombosis, which could aggravate the occurrence and development of CVD (2–4). Existing studies have indicated that $PM_{2.5}$ exposure can induce ECG changes such as abnormality of heart rhythm, tachycardia, and T-wave reduction, and increase the level of inflammatory cell infiltration and fibrosis of cardiac tissue in mice (5, 6). Our previous research also found that $PM_{2.5}$ induced cardiac dysfunction and fibrosis (7). However, the relevant molecular mechanisms of $PM_{2.5}$-induced cardiac injury need to be elucidated.

Recently, small extracellular vesicles (sEV) have received extensive attention in intercellular communication and signaling (8). sEV are a group of extracellular vesicles (40–160 nm) containing nuclear acids, proteins, lipids, and metabolites (9), which are selectively loaded to regulate the biological functions in receptor cells (10). Multiple studies have found that sEV are involved in the regulation of pathological processes of cardiovascular diseases, including atherosclerosis, hypertension, and myocardial infarction (11–13). These findings indicate that sEV may play a key role in cardiac fibrosis after $PM_{2.5}$ exposure.

To explore the mechanism of sEV regulating cardiac fibrosis after $PM_{2.5}$ exposure, we firstly assessed cardiac injury in $PM_{2.5}$ exposed mice (14). Then we confirmed the increase of fibrosis-related proteins in cardiomyocytes cocultured with serum sEV after $PM_{2.5}$ exposure. To investigate whether sEV from macrophages and lung epithelial cells regulate the progression of cardiac fibrosis through body circulation, we examined the levels of key fibrosis-inducing mediators TNF-α, IL-6, and TGF-β in sEV with different exposure times. Our study indicated that TGF-β-enriched sEV, which were released from macrophages after $PM_{2.5}$ exposure, could promote the process of cardiac fibrosis. The findings provide a reference for the cardiovascular-related diseases induced by ambient particulate matter exposure.

MATERIALS AND METHODS

Experimental Animals

The 7-week-old C57BL/6J mice were purchased from Beijing Huafukang Bio-Technology Co., Ltd. After being adaptively fed for 1 week, mice were randomly assigned to the filtered air (FA) group and $PM_{2.5}$ group. The $PM_{2.5}$ concentration in the FA group ranges from 0 to 5 $\mu g/m^{-3}$. During the whole exposure period, all the mice were fed with commercial mouse chow and distilled water, and the exposed warehouse was maintained under temperature (22 ± 2 °C) and relative humidity (40–60%) conditions with a 12 h light/dark cycle. After 4 months (July-October 2017) of exposure, blood samples and heart tissues were obtained for the following experiments. The whole animal studies were performed in accordance with the principles of laboratory animal care (NIH publication no. 85–23, revised 1985) and with approval from the University of Chinese Academy of Sciences Animal Care and Use Committee.

$PM_{2.5}$ Sampling and Preparation

The $PM_{2.5}$ samples were collected from September to November 2016 by a medium-volume air particle sampler (TH-150D-I, Wuhan Tianhong, China) located on Zhongguancun East Road, Beijing. The $PM_{2.5}$ particles were collected by Teflon-coated filters (diameter = 90 mm, Whatman, St. Louis, MO) and stored at −20 °C until use. To extract the $PM_{2.5}$ sample from Teflon filters, a sonicator (Catalog No. KQ-700 V, Shumei, China) was applied for 30 min. The extracted $PM_{2.5}$ was diluted to a final concentration of 10 mg/ml and stored at −80 °C prior to the study.

Cell Culture and $PM_{2.5}$ Exposure

RAW264.7, MLE-12, and HL-1 cells were grown in high-glucose Dulbecco's modified Eagle medium with 10% fetal bovine serum (FSP500, ExCell Bio, China) and 1% penicillin and streptomycin. The cells were kept in a humidified incubator at 37 °C with 5% CO_2. RAW264.7 and MLE-12 cells were seeded into 6-well plates and exposed to 50 μg/ml $PM_{2.5}$ for 1, 12, 24, and 48 h for total RNA extracted. After a 24-h-treatment with 50 μg/ml $PM_{2.5}$, a conditioned medium of RAW264.7 and MLE12 cells were harvested to isolate sEV.

Coculture

The coculture of RAW264.7 and HL-1 cells was applied in a transwell model (Corning, USA), which separates two chambers by a membrane with 0.4 μm pores. RAW264.7 cells were incubated in the upper chamber, while the HL-1 cells were cultured in the bottom chamber. RAW264.7 and HL-1 cells were cultured separately in the upper and bottom chambers until cells were grown to 80% confluence. GW4869 (Sigma-Aldrich, USA, 10 μM) was added to pre-treat RAW264.7 cells for 2 h to block sEV release. After 2 h of treatment, the upper chamber medium was removed. Subsequently, the upper chamber was inserted into the bottom chamber and exposed to 50 μg/ml of $PM_{2.5}$ for 24 h. We extracted the cardiomyocyte protein for Western blot.

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from tissues and cell lysates by using TRIzol Reagent (Invitrogen, Carlsbad, CA), and reverse transcription was performed with PrimeScript™ RT Master Mix (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. Real-time PCR was performed using SYBR Premix Ex TaqII (Takara Bio, Japan) on a QuantStudio 7 Flex system (Thermo Fisher Scientific, USA). The primers used in this study are listed in **Table 1**. To determine the relative expression of mRNA in response to various stimuli, 18 s was used as the internal reference. The gene expression was quantified using $2^{-\Delta\Delta Ct}$ method.

Western Blot Assay

Total proteins from cardiac tissue, myocardial cell, and sEV were lysed with RIPA buffer (Beyotime, China) with 1% PMSF and protease and phosphatase inhibitor cocktails (Bimake, USA). Protein concentration was measured by enhanced BCA protein

TABLE 1 | Primers used for real-time PCR.

Primer	Forward primer (5′-3′)	Reverse primer (5′-3′)
α-sma	GTCCCAGACATCAGGGAGTAA	TCGGATACTTCAGCGTCAGGA
Mmp9	CTGGACAGCCAGACACTAAAG	CTCGCGGCAAGTCTTCAGAG
Col1α1	GCTCCTCTTAGGGGCCACT	CCACGTCTCACCATTGGGG
Col1α2	GTAACTTCGTGCCTAGCAACA	CCTTTGTCAGAATACTGAGCAGC
Col3α1	CTGTAACATGGAAACTGGGGAAA	CCATAGCTGAACTGAAAACCACC
TGF-β	CTCCCGTGGCTTCTAGTGC	GCCTTAGTTTGGACAGGATCTG
18s	TTCTGGCCAACGGTCTAGACAAC	CCAGTGGTCTTGGTGTGCTGA

assay kit (Beyotime, China). Protein samples were analyzed on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with color prestained protein marker (LABLEAD, China) and transferred to PVDF membranes (Millipore, USA). The PVDF membranes were blocked with 5% non-fat milk for 1 h at room temperature. The membranes were incubated with primary antibodies overnight at 4 °C and then incubated with diluted secondary antibody at room temperature for 1 h. Immunoreactive bands were detected with ECL reagents (Bio-Rad, CA). β-Tubulin was used as a protein-loading control. Primary antibodies F4/80 (#70076T) and E-cadherin (#3195S) were purchased from Cell Signaling Technology (CST, USA), CD63 (#ab217345), CD9 (#ab92726), α-SMA (#ab124964), and Collagen I (#ab260043) were purchased from Abcam (UK); TGF-β (#orb11468) and Collagen III (#orb371960) were purchased from Biorbyt (UK); Alix (#sc-53540) was purchased from Santa Cruz (USA); TSG101 (#28283-1-AP) was purchased from Proteintech (China); smad2 (#ET1604-22), p-smad2 (#ET1702-34), smad3 (#ET1607-41), p-smad3 (#ET1609-41) were purchased from HUABIO (China); and tubulin (#AF1216) was purchased from Beyotime (China). Secondary HRP-conjugated antibodies (#S0101, #S0100) were purchased from Beyotime (China).

Cell Viability Assay

RAW264.7 and MLE-12 were seeded in a 96-well plate at a density of 1×10^4 cells/well. The cells were exposed to 12.5, 25, 50, 100, or 200 μg/ml of $PM_{2.5}$ for 1, 12, 24, or 48 h. Cell viability was measured by using MTT (Sigma, Sigma Aldrich, St. Louis, MO) based on the protocol used in the previous study (15). Briefly, MTT (final concentration of 500 μg/ml) was incubated for 2 h at 37°C and then treated with 100 μl DMSO. Next, the absorbance was read in the microplate spectrophotometer (Synergy H1, BioTek, USA) at a wavelength of 492 nm. Data were expressed as the percentage of untreated cells.

Isolation of sEV and TEM Observation

The sEV were isolated from conditioned medium or serum by ultracentrifugation according to the standard methods. Briefly, conditioned medium or serum was centrifuged at 300 g for 30 min to remove cells; 3,000 g for 30 min to eliminate cell debris, 10,000 g for 30 min to remove large particles, and then 120,000 g for 2 h to isolate sEV with a Type 70 Ti rotor (Beckman, Germany). The new supernatant was removed and the pellet sEV was resuspended in 100 μl sterile PBS. The sEV were stored at −80 °C before use. The transmission electron microscopy (Tecnai G2 F20 TWIN TMP 200 kV, FEI, USA) was applied to observe the ultrastructue of sEV.

Nanoparticle Tracking Analysis

The isolated sEV was analyzed by nanoparticle tracking analysis (NTA) to determine the concentration and the size. sEV isolated from 1 ml of plasma or 10 ml cell supernatant were resuspended in 1 ml of PBS and then analyzed by NanoSight NS300 (Marvel, UK).

sEV Labeling and Uptake

The sEV were labeled with PKH26 Fluorescent Cell Linker Kits for General Cell Membrane Labeling (Sigma, St. Louis, MO) based on the manufacturer's protocol. The labeled exosomes were incubated with HL-1 cells for 24 h. Cells were fixed with 4% paraformaldehyde (PFA) for 10 min, permeabilized with 0.1% Triton X-100 for 10 min, and then blocked by 3% BSA for 1 h at room temperature. The cell samples were incubated overnight with anti-tubulin primary antibody at 4°C, followed by incubation with secondary antibody labeled with Alexa Fluor 488 (#ZF-0512, ZSGB-BIO, China) for 1 h at room temperature. Nuclei were labeled with 4,6-diamido-2-phenylindole dihydrochloride (DAPI) (Beyotime, China). A Zeiss LSM 880 confocal microscope system was applied to capture images. A Zeiss LSM 880 confocal microscope system was applied to capture images.

ELISA

The level of TGF-β, TNF-α, and IL-6 in sEV were analyzed with ELISA kits (CSB-E04726m, CSB-E04741m, CSB-E04639m, CUSABIO, China) based on the manufacturer's instructions. The data of signals were determined by a microplate spectrophotometer (Synergy H1, BioTek, USA). The concentration of cytokines was calculated according to the standard curve and OD value.

Immunohistochemistry

Histopathology was performed as previously described. After perfusion with cold PBS, the heart sample was fixed with 4% paraformaldehyde (PFA) for 48 h, embedded with paraffin, and then cut into a heart section (5 μm). The tissue sections were stained with hematoxylin and eosin (H&E) stain and Masson's trichrome stain kit according to the standard techniques to evaluate the cardiac injury and collagen distribution of heart tissue. ImageJ software was used to determine the percentage of fibrotic area.

Statistical Analysis

All data were expressed as means ± standard error of the means (SEM), and were analyzed with GraphPad Prism 9 software. If the data of two groups conformed to be normal distribution and homogeneity of variance, they were analyzed by a two-tailed *t*-test. Otherwise, they were analyzed by ANOVA (three or more groups) followed by Bonferroni's correction. For *in vitro* experiments, all the results were based on at least 3 independent experiments. A $p < 0.05$ was defined as statistical significance.

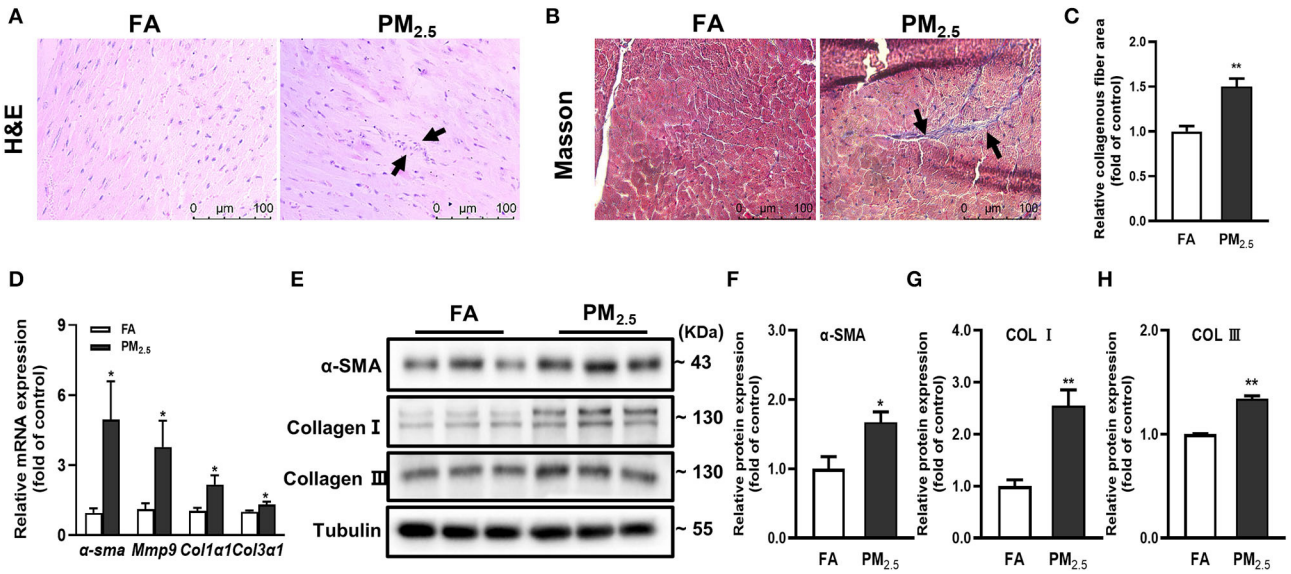

FIGURE 1 | $PM_{2.5}$ induced cardiac inflammation and fibrosis. **(A)** Representative images of hematoxylin-eosin (H&E) staining in heart issue, scale bars: 100 μm. The black arrow indicated the infiltration of inflammatory cells. **(B)** Representative images of Masson staining in heart issue, scale bars: 100 μm. **(C)** Quantitation of cardiac fibrosis area. **(D)** Relative mRNA expressions of α-SMA, mmp9, Col1α1, and Col3α1 in the hearts of mice were analyzed by qPCR. **(E–H)** Relative protein expressions of α-SMA, Col I and Col III in the hearts of mice were analyzed by Western blot. All the data were presented as mean ± SEM (*t*-test), $n = 5$, *indicates $p < 0.05$, **indicates $p < 0.01$.

RESULTS

$PM_{2.5}$ Induced Cardiac Inflammation and Fibrosis

To best mimic human exposure, mice were exposed to ambient airborne $PM_{2.5}$ from Beijing Zhongguancun District for 4 months; more details were described in our previous study (14). To evaluate the effects of $PM_{2.5}$ exposure on cardiac inflammation and fibrosis, we used hematoxylin and eosin (H&E) and Masson staining in heart tissues of mice. The results showed the obvious inflammatory cell infiltration (**Figure 1A**) and significantly increased myocardial collagen fibers (**Figures 1B,C**) in $PM_{2.5}$ exposed mice. Besides, the mRNA and protein expressions of fibrosis markers, including α-smooth muscle actin (α-SMA), collagen I (Col I), and collagen III (Col III) were increased in the heart tissue of mice after $PM_{2.5}$ exposure (**Figures 1D–H**). All the data proved that long-term $PM_{2.5}$ exposure could induce cardiac inflammation and fibrosis.

Serum sEV From $PM_{2.5}$-Exposed Mice Increased the Level of Fibrosis-Related Proteins in Cardiomyocytes

Our previous study has demonstrated that $PM_{2.5}$ played a key role in overall heart failure progression by regulating lung oxidative stress, inflammation, and remodeling (16). To better understand how lung injury regulates cardiac inflammation and fibrosis, we isolated serum sEV from FA mice and $PM_{2.5}$ exposed mice. Transmission electron microscopy (TEM) showed typical cup or saucer-like particles in two groups (**Figure 2A**). Nanoparticle tracking analysis (NTA) showed an obvious increase of concentration and no significant difference between FA-sEV and $PM_{2.5}$-sEV (**Figures 2B–D**). Western blot confirmed that the isolated sEV were enriched with sEV marker proteins, including Alix, CD9, and CD63 (**Figure 2E**). However, we also detected two major lung cells surface markers, macrophage (F4/80) and epithelial cell (E-cadherin), in serum sEV. The notable increase of F4/80 implied that macrophage-sEV may be the critical factor of $PM_{2.5}$-induced cardiac fibrosis.

To investigate whether sEV could regulate the progression of cardiac fibrosis, sEV were co-incubated with mouse cardiac muscle HL-1 cells for 24 h. $PM_{2.5}$-sEV, but not FA-sEV, promoted the protein levels of α-SMA, Col I, and Col III in HL-1 cells (**Figures 2F–I**).

sEV Released From $PM_{2.5}$-Treated Macrophage Increased Collagen Expressions in Cardiomyocytes

Due to the difficulties in distinguishing the major origin of sEV after $PM_{2.5}$ exposure, a transwell co-culture system (0.4 μm)was used to confirm the crosstalk between $PM_{2.5}$-exposure macrophages or epithelial cells and cardiomyocytes *via* sEV (**Figure 3A**). GW4869, a confirmed sEV secretion inhibitor, was added into the upper culture media for a 2-h-pretreatment in mouse macrophage cells (RAW264.7) and alveolar epithelial cells (MLE-12) (17). Then, we treated them with or without $PM_{2.5}$. The results showed the obvious increase of α-SMA, Col I, and Col III in HL-1 cells cocultured with RAW264.7 and the tendency to decrease after pretreatment of GW4869

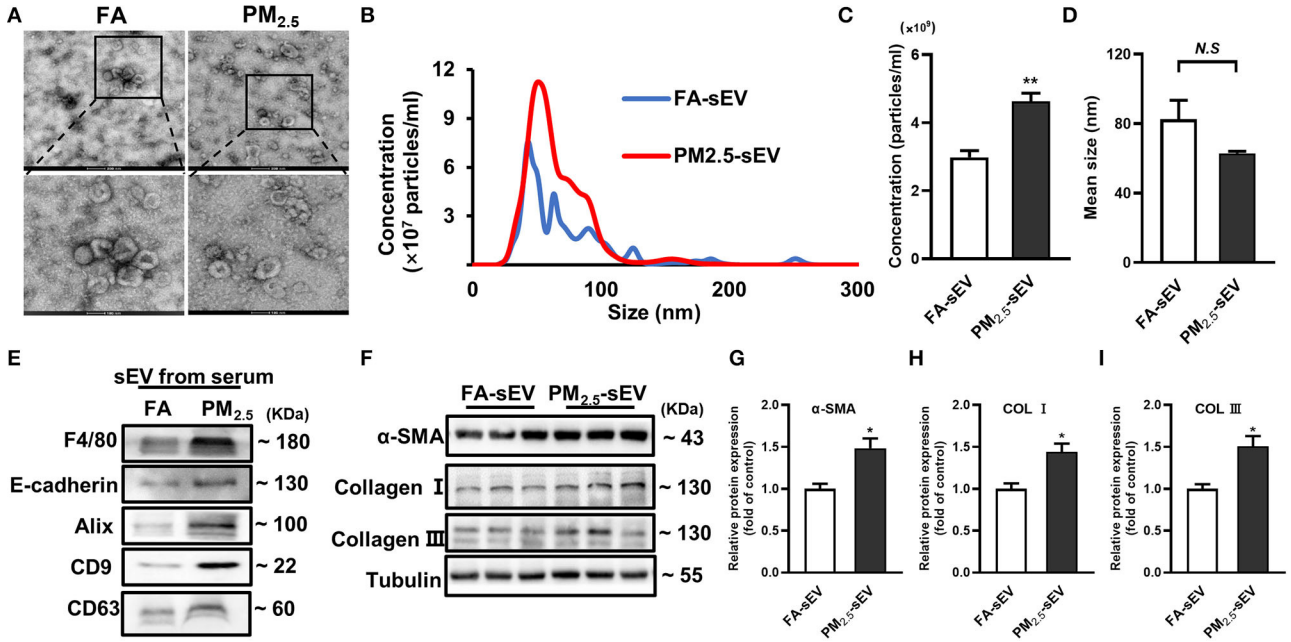

FIGURE 2 | Serum sEV from $PM_{2.5}$-exposed mice increased the level of fibrosis-related proteins in cardiomyocytes. **(A)** Representative TEM images of isolated serum sEV, scale bars: 500 nm, 100 nm. **(B)** Representative results of NTA demonstrated the concentration and distribution in FA-sEV and $PM_{2.5}$-sEV. **(C)** The average concentration in FA-sEV and $PM_{2.5}$-sEV. **(D)** Mean size in FA-sEV and $PM_{2.5}$-sEV. **(E)** Representative images of Western blot in macrophage marker (F4/80), epithelial cell marker (E-cadherin), and sEV marker (Alix, CD9, and CD63) in FA-sEV and $PM_{2.5}$-sEV. **(F–I)** Relative protein expressions of α-SMA, Col I, and Col III in HL-1 cells treated with sEV analyzed by Western blot ($n = 4$). All the data were presented as mean ± SEM (*t*-test), $n = 5$, *indicates $p < 0.05$, **indicates $p < 0.01$. N.S indicates no significance.

(**Figures 3B–E**). However, there was no significant change in HL-1 cocultured with MLE-12 (**Supplementary Figure S1**). All the data indicated that macrophage-derived sEV played a critical role in the regulation of cardiac fibrosis.

Separation and Characterization of sEV From RAW264.7 and MLE-12 After $PM_{2.5}$ Exposure

To better explore the function of sEV from different cells in lung-mediated cardiac fibrosis induced by $PM_{2.5}$ exposure, we isolated sEV from mouse macrophage cells (RAW264.7) and alveolar epithelial cells (MLE-12) with or without $PM_{2.5}$-treated. We firstly applied MTT viability assay to test the effects of $PM_{2.5}$ on RAW264.7 cells and MLE-12 cells. The cells were exposed to $PM_{2.5}$ for 1, 12, 24 and 48 h at concentrations of 0 to 200 μg/mL and showed a concentration-dependent decrease (**Figures 4A,B**). A total of 50 μg/mL is chosen as our dosing concentration, of which 80–90% of the cells were viable compared with controls. NTA, TEM, and western blotting analyses were performed to identify the purity of sEV derived from RAW264.7 and MLE-12. NTA reflected the increase of sEV in both two types of cells after $PM_{2.5}$ exposure (**Figure 4C**). TEM showed the representative images of sEV in two groups (**Figure 4D**). Western blotting showed the presence of sEV surface markers, including Alix, CD9, and CD63 (**Figure 4E**). All these data manifested that our purified nanoparticles were sEV.

Release of Cytokines in sEV by RAW264.7 and MLE-12 After $PM_{2.5}$ Exposure

Cytokines, including TNF-α, TGF-β, and IL-6, play remarkable roles in cardiac fibrosis, which combined with fibroblast surface receptors to activate fibrosis-related signal pathways (18, 19). We examined the release pattern of these cytokines in sEV from different cells after 1, 12, 24, and 48 h of $PM_{2.5}$ exposure. We found that TNF-α increased rapidly in RAW264.7-derived sEV in the initial 1 h of $PM_{2.5}$ exposure (**Figure 5A**). TNF-α from RAW264.7-sEV increased over exposure time and became steady till 48 h (**Figure 5A**). Besides, we observed a continuous growth of TGF-β content in RAW264.7-sEV (**Figure 5B**) and an increased IL-6 covering after $PM_{2.5}$ exposure (**Figure 5C**). However, there was no significant difference in MLE-12-derived sEV after $PM_{2.5}$ exposure, and the levels of cytokines in MLE-12-sEV were lower than RAW264.7-sEV (**Figures 5D–F**). Hence, $PM_{2.5}$ exposure mainly altered TNF-α and TGF-β in macrophage-sEV, which may mediate the levels of fibrosis-associated proteins in cardiomyocytes.

TGF-β-Containing sEV Induced Production of Collagen Through TGF-β-Smad2/3 Signaling Pathway in Cardiomyocytes

To determine the regulation of TGF-β-containing sEV from macrophages on myocardial fibrosis process, firstly, we labeled sEV with red fluorescence PKH-26 and then cocultured

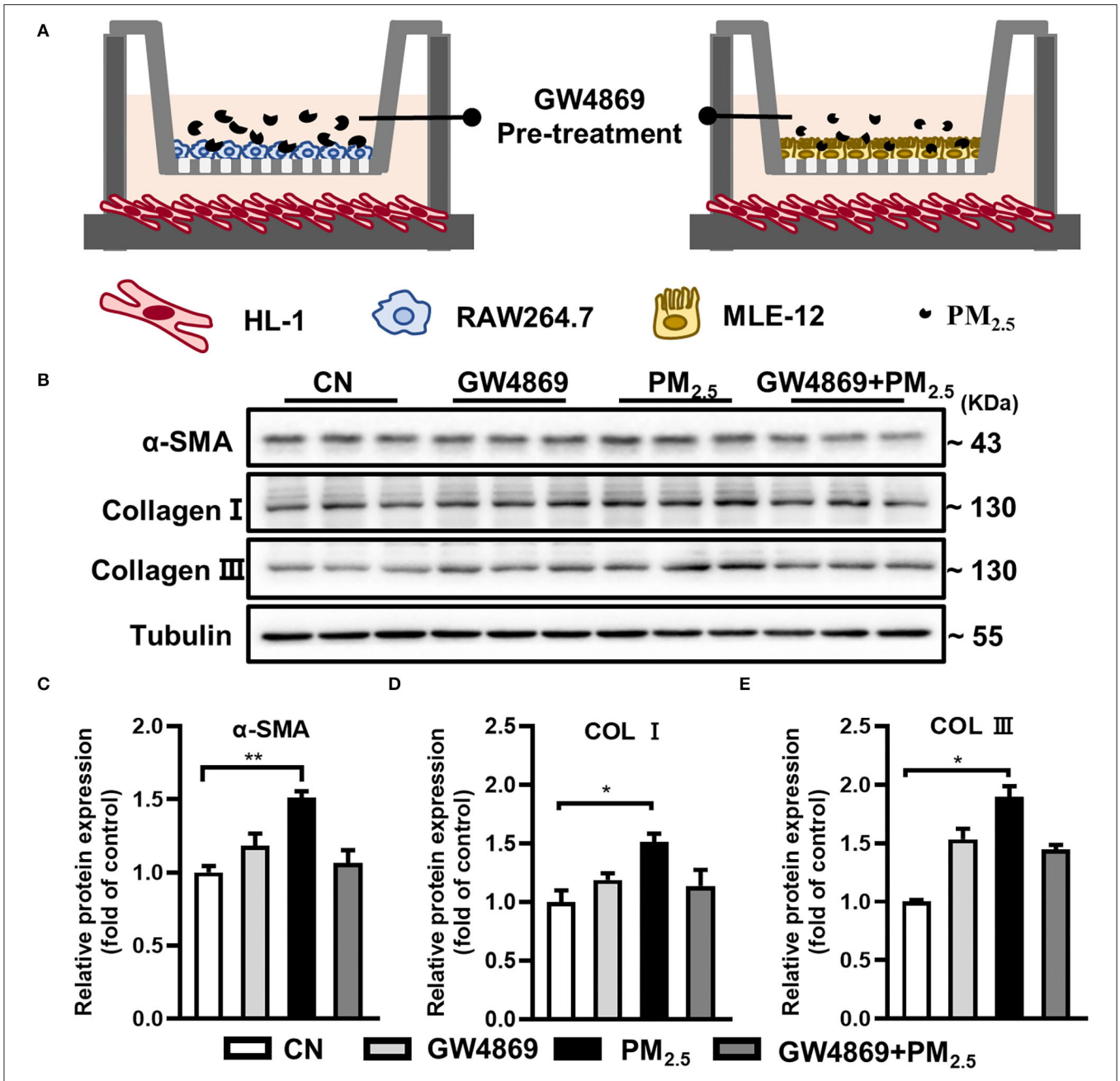

FIGURE 3 | $PM_{2.5}$-treated macrophage-sEV increased collagen expressions in cardiomyocytes. **(A)** HL-1 cells cocultured with RAW264.7 and MLE-12 in the presence or absence of $PM_{2.5}$ and GW4869 in a transwell (0.4 μm) plate. **(B–E)** Cocultured with RAW264.7 and $PM_{2.5}$ promoted the protein levels of α-SMA, Col I, and Col III in HL-1 cells through transwell system. All the data were presented as mean ± SEM (*t*-test, one-way ANOVA), *indicates $p < 0.05$, **indicates $p < 0.01$.

them with HL-1 cells for 24 h. The intracellular uptake of labeled sEV was observed under a laser scanning confocal microscope (**Figure 6A**). Then, we detected the expression of the mRNA and protein expressions of TGF-β, α-SMA, Col I, and Col III in HL-1 cells. The results showed that $PM_{2.5}$-induced RAW264.7-sEV could promote the mRNA and protein expressions of α-SMA, Col I and Col III (**Figures 6B–G**). Phosphorylation of Smad2/3 and total Smad2/3 in HL-1 cells were then analyzed. RAW264.7-sEV co-culture increased the protein expression of p-Smad2/Smad2 and p-Smad3/Smad3 (**Figures 6H–J**). Our data preliminary indicated that $PM_{2.5}$ increased the expression TGF-β in macrophage–sEV, which activated the TGF-β-Smad2/3 signaling pathway in cardiomyocytes.

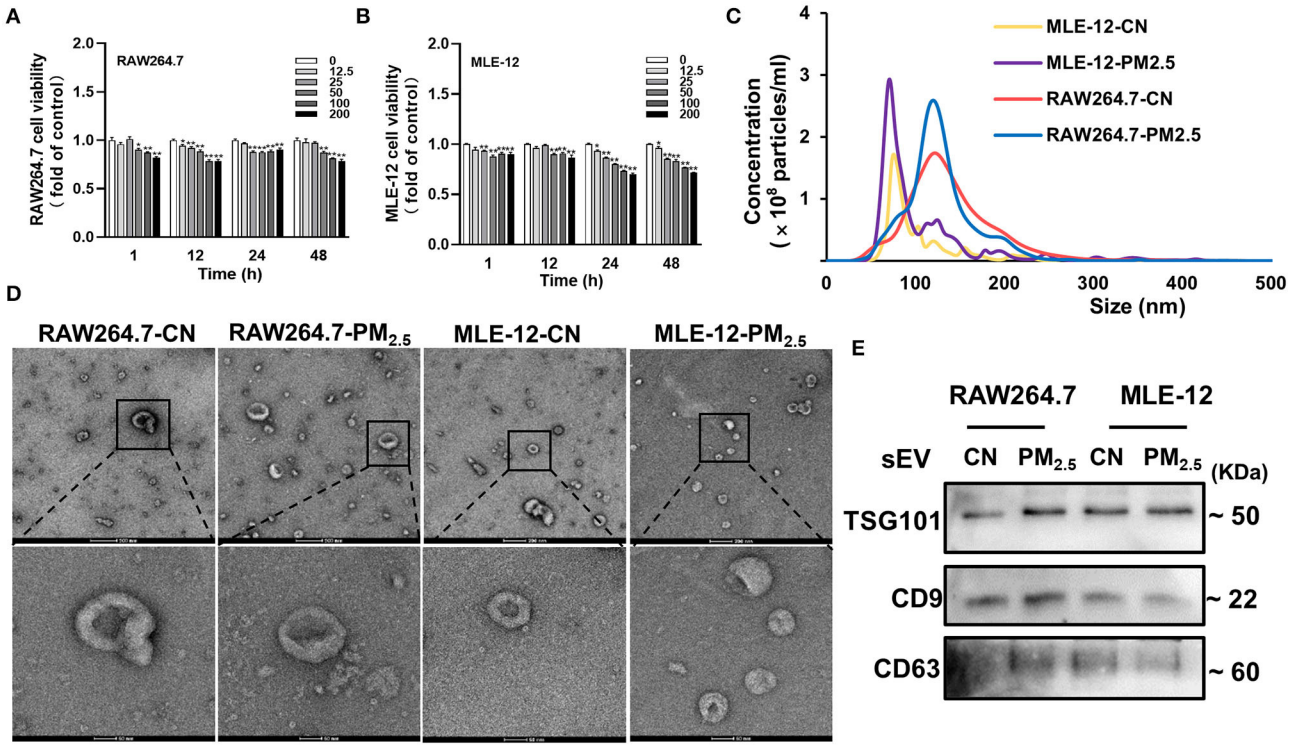

FIGURE 4 | Separation and characterization of sEV from RAW264.7 and MLE-12 after $PM_{2.5}$ exposure. **(A,B)** Cell viability analyzed by MTT assay. The RAW264.7 and MLE-12 were treated with various concentrations of $PM_{2.5}$ for 1, 12, 24, and 48 h ($n = 5$). **(C)** The results of NTA demonstrated the concentration and distribution in CN-sEV and $PM_{2.5}$-sEV. **(D)** Representative TEM images of isolated sEV, scale bars: 200 nm, 50 nm. **(E)** Representative images of Western blot in sEV marker Alix, CD9 and CD63 in CN-sEV and $PM_{2.5}$-sEV. All the data were presented as mean ± SEM (*t*-test), $n = 3$–4, *indicates $p < 0.05$, **indicates $p < 0.01$.

DISCUSSION

Our study showed that $PM_{2.5}$ exposure modulated the intercellular communication between macrophages and cardiomyocytes, increasing TGF-β in macrophage sEV, which upregulated the levels of cardiomyocyte fibrosis-related proteins and aggravated myocardial fibrosis.

It is well accepted that air pollution is a significant cause of non-communicable diseases worldwide, and particulate matter is one of the main air pollutants (20). Although previous studies have demonstrated that particulate matter exposure activated the ROS/TGF-β1/Smad3 signaling pathway to induce myocardial fibrosis (21), our study explained that $PM_{2.5}$ exposure not only increased cardiomyocyte and fibrosis levels by TGF-β, but also promotes this outcome through macrophage–sEV secretions.

Some studies focused on the role of sEV-induced chronic diseases by respiratory exposure, and it has been shown that exposure of the respiratory tract to ambient particulate matter promoted the secretions of sEV from different cellular sources and alter their composition (22). These sEV may remain in the lungs, or regulate systemic inflammatory responses through blood circulation. Plasma EV levels were significantly increased in short-term PM exposure populations and steel plant workers exposed to occupational PM, and the significantly altered miRNAs in sEV could regulate coagulation function, inflammatory response, and fibrin levels (23, 24). To expound whether $PM_{2.5}$ exposure induced cardiac fibrosis through sEV, serum sEV were isolated from 4-month-exposure mice and cocultured with cardiomyocytes. The results reflected that serum sEV could induced myocardial fibrosis after $PM_{2.5}$ exposure.

However, there are still some uncertainties about the potential sources of increased levels of serum sEV after exposure. Additional studies have reported that macrophage-derived sEV containing angiotensin II type 1 receptor played an important role in BLM-induced pulmonary fibrosis (25), and macrophage-derived sEV also activated the fibroblast in an endoplasmic reticulum stress-dependent manner to mediate silica-induced pulmonary fibrosis (26). In addition, sEV from alveolar epithelial cells activated alveolar macrophage in sepsis-induced acute lung injury (27), and cigarette smoke extract-treated lung epithelial Beas-2B-derived sEV could promote macrophage polarization (28). All these studies revealed the critical role of sEV from macrophages and lung epithelial cells in the regulation of lung microenvironment homeostasis. To investigate whether and how sEV from lung cells mediated myocardial fibrosis through blood circulation after $PM_{2.5}$ exposure, we established sEV models of lung epithelial cells and macrophages *in vitro* to explore the regulation of sEV from different cells on cardiomyocyte fibrosis. We firstly used GW4869, a specifically selective inhibitor of N-SMase to reduce sEV release, which can successfully blocked sEV by pre-treatment of cells. The results reflected that $PM_{2.5}$-induced RAW264.7-sEV increased α-SMA and collagen levels in

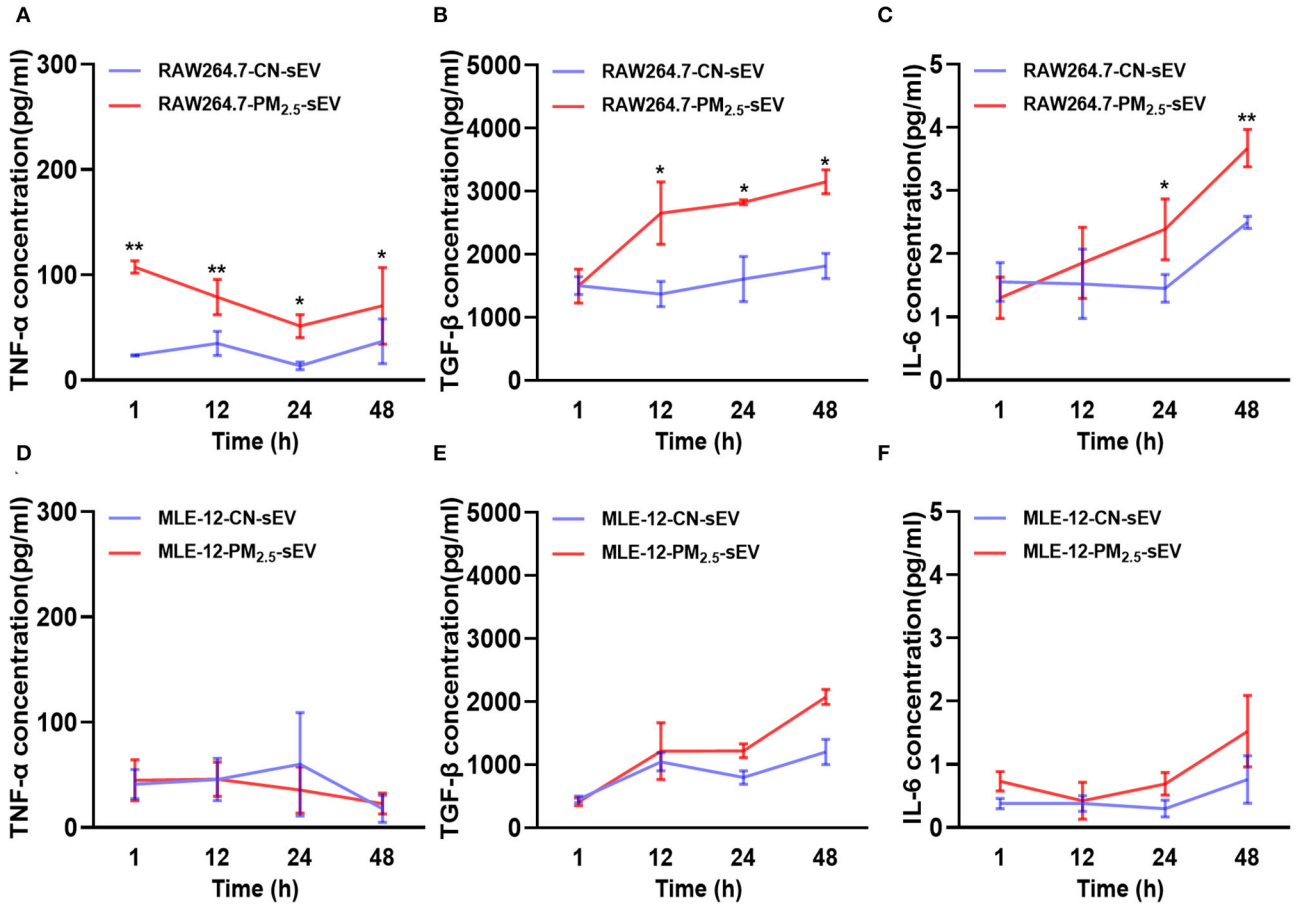

FIGURE 5 | Release of key cytokines in sEV by RAW264.7 and MLE-12 after 1, 12, 24, 48 h of $PM_{2.5}$ exposure. **(A–C)** ELISA for TNFα, TGF-β, and IL-6 in sEV from RAW264.7 cells after 1, 12, 24, and 48 h of $PM_{2.5}$ exposure. **(D–F)** ELISA for TNFα, TGF-β, and IL-6 in sEV from MLE-12 cells at 1, 12, 24, and 48 h after $PM_{2.5}$ exposure. All the data were presented as mean ± SEM (two-way ANOVA), $n = 3$. *indicates $p < 0.05$, **indicates $p < 0.01$.

HL-1 cells, while the similar results were not observed in MLE-12-sEV.

Afterward, we isolated sEV from RAW264.7 cells and MLE-12 cells. It is found that $PM_{2.5}$ increased the level of sEV in two cells. A large amount studies have established that macrophages released a large amount of pro-fibrotic growth factors such as TGF-β, platelet-derived growth factors (PDGFs), and fibroblast growth factors (FGF) (29). Meanwhile, the pro-inflammatory cytokines IL-1β, IL-6, and TNF-α induced the transcription of IL-10, PDGF, or TGF-β to promote the fibrotic macrophage (M2c) phenotype (29). The growing evidences reflected that sEV transferred the cytokines to modulate the functions of recipient cells (30–32). According to previous studies, $PM_{2.5}$ increased the levels of TGF-β1 in mice lung tissue and bronchoalveolar lavage fluid, suggesting that $PM_{2.5}$ exposure may increase TGF-β1 and cause myocardial fibrosis (33). Thus, we detected the levels of TNF-α, TGF-β, and IL-6 from RAW264.7 cells and MLE-12 cells. Various cell exposure models at different times were designed to track the changes of cytokines in sEV. The rapid response of TNF-α in RAW264.7-derived sEV may promote macrophage polarization and pro-fibrotic factors transcriptions. However, with the increase of $PM_{2.5}$ exposure time, the expression of TGF-β increased, which may be a major factor in the fibrosis process. In addition, the levels of cytokines in sEV from RAW264.7 cells significantly increased as compared with sEV from MLE-12 cells.

Finally, we found that macrophage-derived sEV upregulated the levels of TGF-β and fibrosis-associated proteins after $PM_{2.5}$ exposure. TGF-β in cardiac tissue caused Smad2/Smad3 phosphorylation, which would affect various profibrotic gene expressions and stimulate cardiac fibrosis development (34). To gain a comprehensive understanding of collagen increase induced by macrophage-derived sEV, we measured the phosphorylation of Smad2/3 and total Smad2/3 in HL-1 cells. Our data revealed that $PM_{2.5}$-induced TGF-β-containing sEV caused collagen deposition by activating TGF-β-Smad2/3 signaling pathway in cardiomyocytes.

To summarize, our study suggested that $PM_{2.5}$-treated cell-to-cell communication between macrophages/lung epithelial cells and cardiomyocytes promotes cardiac fibrosis. More importantly and practically, this critical communication through sEV, which connects macrophages and cardiomyocytes, may provide new ideas for preventive treatment and facilitate the development

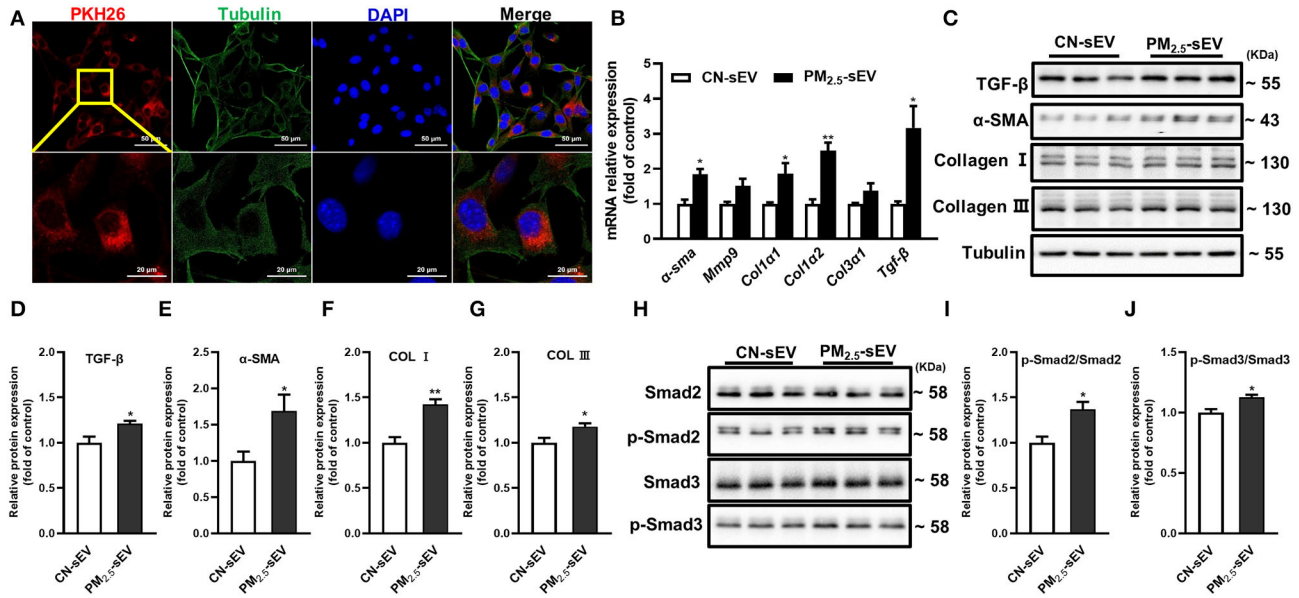

FIGURE 6 | TGF-β-containing sEV-induced production of collagen through TGF-β-Smad2/3 signaling pathway in cardiomyocytes. **(A)** Representative images of HL-1 cells that were incubated with the RAW264.7-sEV of PKH26-labeled sEV (red), cytoskeleton was stained with tubulin (green) and nuclei with DAPI (blue). Scale bar: 50 μm, 20 μm. **(B)** Relative mRNA expressions of α-SMA, mmp9, Col1α1, Col1α2, Col3α1, and TGF-β in HL-1 after being treated with RAW264.7-sEV analyzed by qRT-PCR. **(C–G)** Relative protein expressions of GF-β, α-SMA, Col I, and Col III in HL-1 were analyzed by Western blot. **(H–J)** Quantification of normalized p-Smad2/Smad2, p-Smad3/Smad3 in HL-1. All the data were presented as mean ± SEM (*t*-test), $n = 3–4$, *indicates $p < 0.05$, **indicates $p < 0.01$.

of diagnosis and treatment for patients with $PM_{2.5}$-associated CVD. However, there are still some deficiencies in our study, including that the regulatory role of other important components in sEV, such as miRNAs or proteins, on cardiac fibrosis has not been determined. In addition, the sEV inhibitor GW4869 can be used in mice to better confirm the regulation of sEV after $PM_{2.5}$ exposure. These unanswered questions will provide a more comprehensive understanding of cardiovascular disease caused by $PM_{2.5}$ exposure.

CONCLUSION

In summary, our study put forward for the first time that the $PM_{2.5}$ induced cardiac fibrosis by regulating the TGF-β-Smad3/2 signaling pathway *via* macrophage-derived sEV. This study provides a novel insight into the mechanism underlying $PM_{2.5}$-induced cardiovascular diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The animal study was reviewed and approved by University of Chinese Academy of Sciences Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

WD, FZ, and XH conceived, designed the research, and revised the manuscript. XH, MC, XC, and XY performed the experiments. XH analyzed the experiment results and edited the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcvm.2022.917719/full#supplementary-material

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