WIRELESS INTERCELLULAR COMMUNICATIONS IN CARDIAC PATHOLOGY: THE ROLE OF EXOSOMES

EDITED BY: Mahmood Khan, Nazish Sayed, Prasanna Krishnamurthy and Yaoliang Tang

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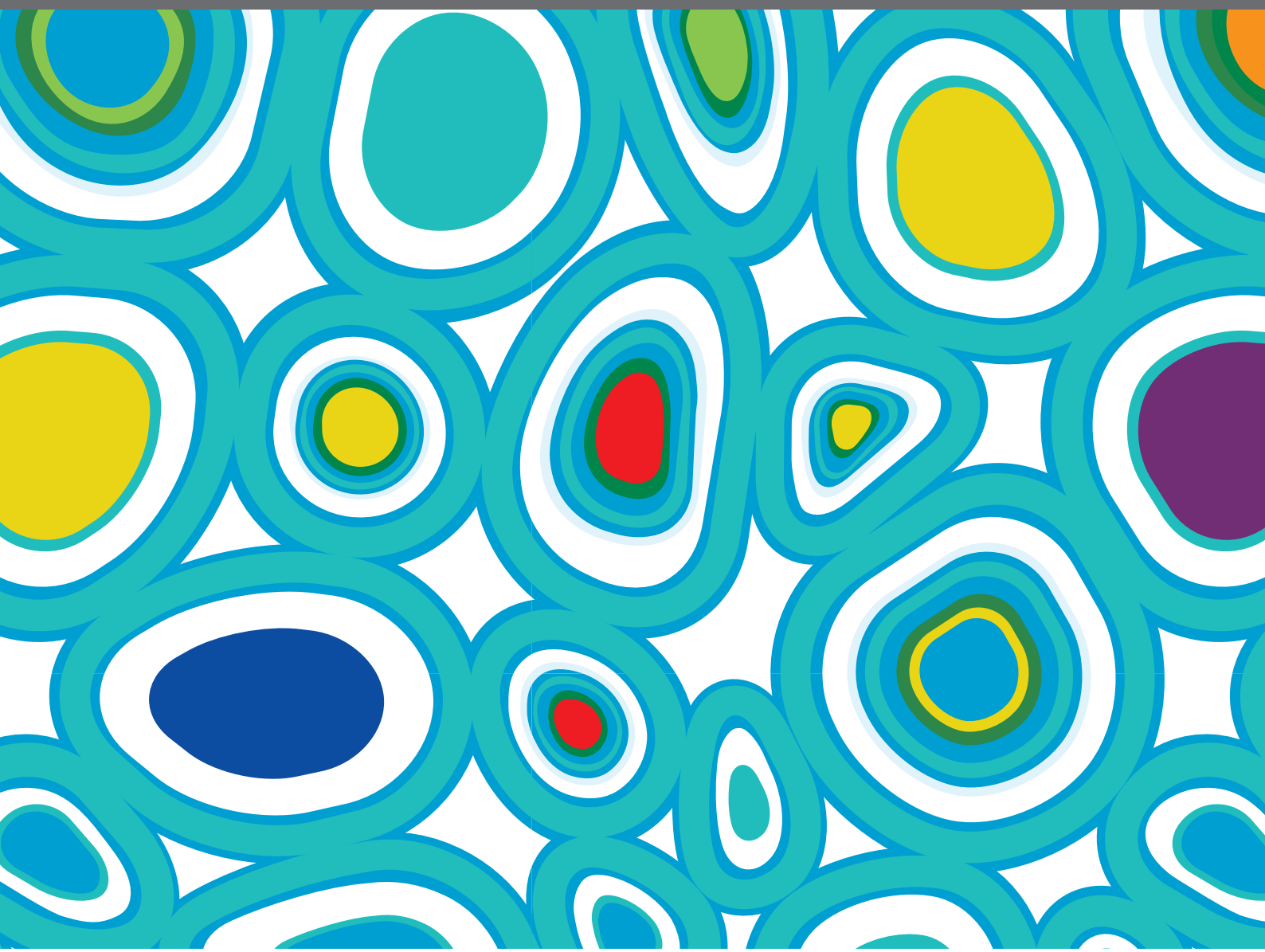

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WIRELESS INTERCELLULAR COMMUNICATIONS IN CARDIAC PATHOLOGY: THE ROLE OF EXOSOMES

Topic Editors:
Mahmood Khan, The Ohio State University, United States
Nazish Sayed, Stanford University, United States
Prasanna Krishnamurthy, University of Alabama at Birmingham, United States
Yaoliang Tang, Augusta University, United States

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REVIEW
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The Art of Intercellular Wireless Communications: Exosomes in Heart Disease and Therapy

*Mallikarjun Patil, John Henderson, Hien Luong, Divya Annamalai, Gopalkrishna Sreejit and Prasanna Krishnamurthy**

Department of Biomedical Engineering, Schools of Medicine and Engineering, University of Alabama at Birmingham, Birmingham, AL, United States

Exosomes are nanoscale membrane-bound extracellular vesicles secreted by most eukaryotic cells in the body that facilitates intercellular communication. Exosomes carry several signaling biomolecules, including miRNA, proteins, enzymes, cell surface receptors, growth factors, cytokines and lipids that can modulate target cell biology and function. Due to these capabilities, exosomes have emerged as novel intercellular signaling mediators in both homeostasis and pathophysiological conditions. Recent studies document that exosomes (both circulating or released from heart tissue) have been actively involved in cardiac remodeling in response to stressors. Also, exosomes released from progenitor/stem cells have protective effects in heart diseases and shown to have regenerative potential in the heart. In this review we discuss- the critical role played by circulating exosomes released from various tissues and from cells within the heart in cardiac health; the gap in knowledge that needs to be addressed to promote future research; and exploitation of recent advances in exosome engineering to develop novel therapy.

Keywords: exosomes, cardiac remodeling, immune modulation, diabetes, exosome engineering

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Gianandrea Pasquinelli,
University of Bologna, Italy

***Correspondence:**
Prasanna Krishnamurthy
prasanak@uab.edu

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INTRODUCTION

Exosomes are bi-layered extracellular nanoscale vesicles released by almost all cells in the body that carry proteins, lipids, cytokines, transcription factors, and nucleic acids. Since their discovery three decades ago, exosomes have been implicated in various pathophysiological conditions. These extracellular vesicles were discovered back in the 80's by two independent research groups investigating the fate of transferrin receptors in reticulocytes (Harding and Stahl, 1983; Pan and Johnstone, 1983). They observed that small vesicles were associated with transferrin receptors and were also released from reticulocytes to the extracellular fluid during recycling of the receptor. The work highlighted the mechanism by which reticulocytes lose these receptors during their maturation to erythrocytes (Harding et al., 2013). This was the first described biological phenomenon mediated by extracellular vesicles. These extracellular vesicles were later named exosomes (Pan and Johnstone, 1983). Exosomes were subsequently shown to be released from many cell types including dendritic cells (Zitvogel et al., 1998), macrophages (Ramachandra et al., 2010), B cells (Raposo et al., 1996), T cells (Blanchard et al., 2002), mast cells (Skokos et al., 2003), stem cells (Barile et al., 2014), and cancer cells (Taylor and Gercel-Taylor, 2008).

Exosomes measure between 30 and 100 nm and are secreted by cells in response to pathophysiological stimuli. They are actively synthesized by the endolysosome pathway and secreted into the intercellular space or into the systemic circulation. Exosomes were thought to be non-functional contents excreted from the cells in the form of vesicles, however, research in the recent past has shown that they function as intercellular communicators involved in cellular and organ cross talk. The cargo of exosomes contains lipids, proteins, micro-RNA, long non-coding (Lnc)-RNA, DNA and cell membrane receptors. Exosome membranes are made of a lipid bilayer and reflect the cells of origin; for example, exosomes from immune cells will have either MHC or T-cell receptor or B-cell receptor molecules whereas exosomes from cardiac progenitor cells express CD73/CD90/CD105 (Andriolo et al., 2018). However, the contents of exosomes are actively sorted and may or may not reflect the molecules from the cells of origin. For example, certain miRNA are selectively sorted into exosomes and these may potentially be absent in parent cells (Guduric-Fuchs et al., 2012; Zhang et al., 2015). Interestingly, these exosomes are taken up by the target cells through endocytosis mediated by cell adhesion molecules, specific receptors and specific membrane lipids or carbohydrates (Villarroya-Beltri et al., 2014). The contents of the exosomes can reprogram the target cells by activating specific signaling pathways via binding to specific receptors or through the delivery of enzymes and transcriptional regulators. Exosomal contents including micro-RNA have emerged as key regulators of target cell biology and function, thus exosomes are now recognized as a new class of paracrine signaling mediators in addition to classical pathways of intercellular communication by hormones, inflammatory mediators and cytokines. A detailed biology of exosomes is reviewed elsewhere (Villarroya-Beltri et al., 2014; Zhang et al., 2015; Ha et al., 2016). A present limitation of exosome research is that the tools required to identify the source and targets of exosomes in animals and humans are lacking, so cell-specific conclusions have instead been mostly been drawn from *in vitro* studies utilizing different cell types. Some of the tools that have been useful in understanding the biology of exosomes are summarized in **Table 1**.

The published data suggests that exosomes play critical roles in the patho-physiology of heart diseases; and have been increasingly investigated for their potential use in diagnosis and therapy due to their unique size, structure, and composition. In this review, we provide an overview of the role of exosomes in intercellular signaling and its effects on heart disease; the influence of various organ systems and co-morbidities including cardiometabolic syndromes on the pathogenesis of exosome-mediated heart disease; and bioengineering exosomes for efficient therapy.

TABLE 1 | Common techniques and tools for understanding the biology of exosomes.

Process	Tools
Exosome biogenesis inhibition	GW4869, Manumycin A, Tipifarnib, Netoconizole, Ketoconozole, Climbazole, Dimethyl amiloride
Exosome uptake inhibition	Heparan, Cyclochalasin, Wortmannin, Cannabidiol
Genetic tools to label extracellular vesicle membranes	EGFP and dTomato tagged to N termini of palmitoylation signal
Genetic tools to label extracellular vesicle mRNA	Palm dTomato tagging to MS2 RNA sequences (bacteriophage MS2 coat protein)
Exosome RNA labeling	Syto RNA select
Endogenous labeling	NIR_AZA1 (BF2- azadipurromethene), DiR, DiD, Rlucm, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindotricarbocyanine Iodide, PKH, cy7
Bioluminescence labeling	firefly luciferase, D-luciferin, Renilla luciferase, gaussia luciferase
Visualization	Electron microscopy, internal reflection fluorescent microscopy, single molecule localization microscope
Nanoparticle analysis and characterization	Nanosight, dynamic light scattering

EXOSOMES IN HEART DISEASE

Heart diseases are the leading cause of morbidity and mortality worldwide. According to the Center for Disease Control (CDC), 1 in 4 deaths are attributed to heart diseases in the United States and the economic burden of heart disease is estimated to be $300 billion annually (Benjamin et al., 2019). Cardiac structure and function in heart disease is regulated through communication between different cell types within the heart (fibroblasts, endothelial cells, cardiomyocytes, and Telocytes) and between the heart and peripheral tissues/organs like vasculature, kidney, bone marrow, lungs, and immune cells. Studies have shown that exosomes participate in the organ/tissue cross talk and play a critical role in the pathogenesis of heart diseases, including myocardial infarction (MI) (**Figure 1**). Extensive research has shown that exosomes can very well be utilized for diagnosis and treatment of cardiac diseases summarized in **Table 2**, and discussed below; therefore, a better understanding of the biological functions surrounding exosomes in the context of cardiac pathophysiology is necessary for the development of novel therapies.

EXOSOMES IN DIABETIC CARDIOMYOPATHY

Diabetes affects over half a billion people all around the world, cardiovascular diseases are the major cause of morbidity and mortality in diabetes, with more than half of the deaths in older diabetic patients being directly attributed to cardiovascular diseases (Donahoe et al., 2007; Matheus et al., 2013). Diabetic patients are at a higher risk of developing cardiovascular diseases compared to non-diabetic patients due to the damage caused to blood vessels, inhibition of angiogenesis, increased inflammation, metabolic changes in cardiac cells, cardiomyocyte death and defective phagocytosis, which can

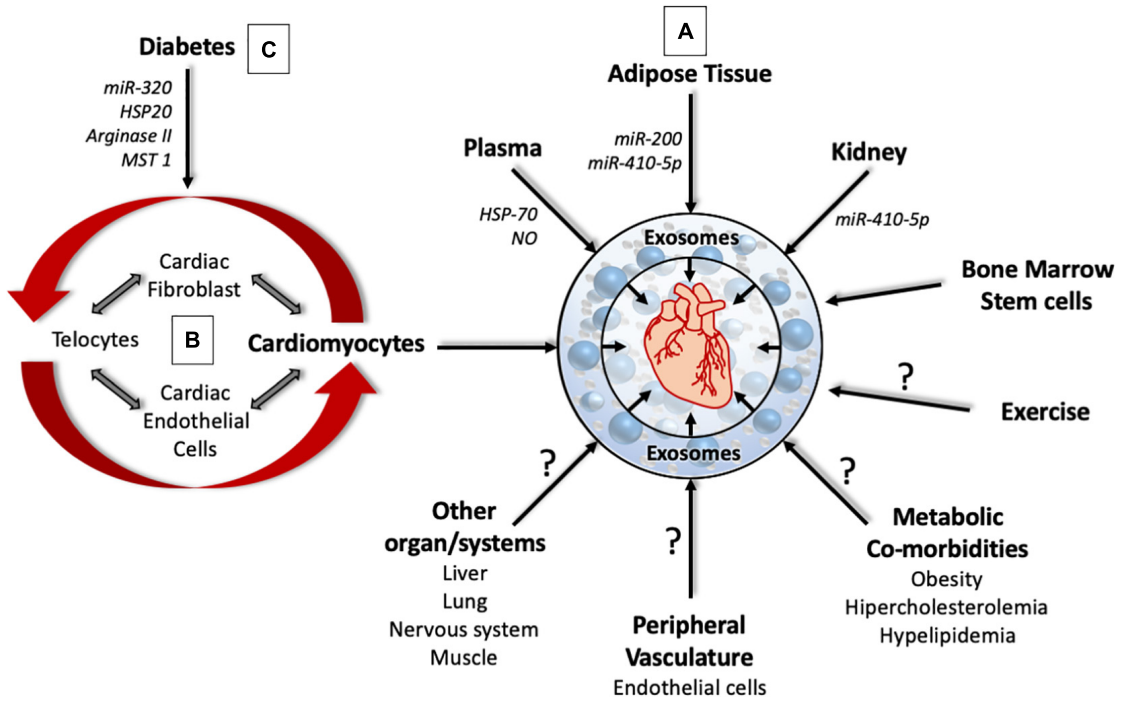

FIGURE 1 | The complex role of cardiac and non-cardiac derived exosomes in cardiac pathophysiology. **(A)** Exosomes from different organs affect the pathophysiology of the heart through the delivery of signaling molecules, micro-RNA and enzymes. However, the role of exosomes derived from the liver, lung, muscle, nervous system, metabolic co-morbidities, and exercise that can affect heart health are yet to be determined. **(B)** Exosomes from different cell types within the heart can program cells within the heart to promote cardiac remodeling that affect heart structure and function. **(C)** Under Diabetic milieu, exosomes from different cell types within the heart can bring about coronary endothelial dysfunction, cardiac hypertrophy, and cardiac fibrosis.

TABLE 2 | Studies demonstrating exosomes diagnosis and treatment of heart diseases.

Functional component	Disease	Species	References
miR-126 and miR-26	CAD	Humans	Zampetaki et al., 2010
miR-223, miR-29b RNU6-2	CV risk in smokers	Humans	Badrnya et al., 2017
miR-1, miR-21, miR-133, miR-146a, miR-208b, miR-499	Ischemic heart disease	porcine	Deddens et al., 2016
Apolipoprotein C-III, Apolipoprotein D, complement C1q subcomponent A, platelet glycoprotein 1b alpha chain, platelet basic protein	Ischemic Heart Disease	Humans	Cheow et al., 2016
miR-194, miR-34	Heart failure post-MI	Humans	Matsumoto et al., 2013
miR-146a	Peripartum cardiomyopathy	Humans	Halkein et al., 2013
miR-17, miR-197, miR-509-5p, miR-92a, miR-320a,	Metabolic syndrome	Humans	Karolina et al., 2012
miR-1, miR-133a, miR-208	Acute coronary Syndrome	Humans	Kuwabara et al., 2011
CD144 EMP	Coronary heart disease	Humans	Nozaki et al., 2009
Endothelial microparticle	Cardiovascular mortality in end stage renal failure	Humans	Amabile et al., 2010
Endothelial microparticle	Preeclampsia	Humans	González-Quintero et al., 2003
miR-126 and MiR-199a	Cardiovascular events	Humans	Jansen et al., 2014
CCL2, CCL7 and IL6	Cardiac inflammation post-MI	Mouse Humans	Loyer et al., 2018
Inflammatory and immunoglobulin class proteins	Transplant rejection	Humans	Kennel et al., 2018
Cardiac bridging integrator 1	Heart failure	Humans	Nikolova et al., 2018
miR-92b-5p	Dilated cardiomyopathy	Humans	Wu et al., 2018, p. 199
miR-223	Sepsis (microbial and non-microbial)	Mouse	Wang et al., 2015

lead to cardiac fibrosis, hypertrophy, and cardiac stiffness (Miki et al., 2013; Suresh Babu et al., 2016; Jia et al., 2018). While the interplay of diabetes and heart disease is highly complex, new evidence show that exosomes play an important role in the pathology. In diabetes, exosome cargo and structural composition are modified as the parent cells are affected

by the diabetic milieu (Kamalden et al., 2017; Bellin et al., 2019). Exosomes in diabetic milieu have been shown to inhibit angiogenesis, promote cell death, and induce due to loading of maladaptive contents that affect transcription and signaling pathways (Sahoo and Emanueli, 2016; Tao et al., 2019). Consistent with these reports, circulating exosomes in diabetics were low in proangiogenic factors like miR-126 and miR-26 (Zampetaki et al., 2010). In addition, cardiomyocyte derived exosomes under diabetic condition exhibited over expression of anti-angiogenic factor miR-320 (Wang et al., 2014). Co-culture experiments utilizing cardiomyocytes from diabetic rats and mouse cardiac endothelial cells inhibited proliferation and migration of mouse endocardial epithelial cells (MCEC). Interestingly, the endothelial cell dysfunction was abrogated by treatment with GW4869, an inhibitor of exosome formation and release, suggesting that exosomes from diabetic cardiomyocytes were responsible for myocardial endothelial cell dysfunction (Wang et al., 2014). Consistent with this finding, treatment of MCEC cells with exosomes from Goko Kakizaki rat (diabetic) cardiomyocytes inhibited endothelial cell proliferation, migration and tube formation. Mechanistically, these effects were shown to be mediated by exosome derived miR-320 from diabetic rats, which is shown to target Insulin like growth factor 1 (IGF1), HSP 20, and Ets2, important mediators of endothelial cell function. Also, transgenic mice overexpressing HSP20 in cardiomyocytes, protected mice against diabetic cardiomyopathy. In co-culture experiments, cardiomyocytes overexpressing HSP20 promote endothelial cell proliferation, migration and tube formation (Wang et al., 2016; Davidson et al., 2018a). Exosomes from HSP-Tg mice (transgenic mice overexpressing HSP20) had higher levels of phosphorylated Akt, survivin and super oxide dismutase, that are shown to protect against diabetes mediated-oxidative stress in endothelial cells and cardiomyocytes. Furthermore, cardiac pathology mediated by exosomal HSP70 in diabetes was rescued by the addition of exosomes from healthy rats. Systematically, exosomes from healthy mice activate TLR4, which in turn protects cardiomyocytes through ERK1/2 and HSP27 (Davidson et al., 2018a).

Diabetes induced endothelial dysfunction has also been known to be mediated by exosomes. In diabetes exosomes are enriched with arginase 1 and these exosomes were shown to inhibit ACE-induced endothelin dependent relaxation in aortas. This pathology was inhibited by heparin treatment, suggesting primary contributor to be exosomes (Zhang et al., 2018). It was found that the enriched Arginase1 compromised the availability of L-arginine, impairing nitric oxide (NO) production leading to defective aortic relaxation.

Exercise is known to have beneficial effects on the health of diabetic patients, especially in cardiovascular health. Several mechanisms have been proposed, including production of cardioprotective exosomes. A recent study by Chaturvedi et al. has shown that exercise enriches specific cardioprotective miRNA's such as miR-455, miR-323, miR-466, and miR-29b. Mechanistically, the cardio-protective effect was primarily due to the inhibition of MMP9, a matrix protein primarily involved in cardiac fibrosis. The expression of MMP9 was inhibited at the transcription level by miR-29b and miR-455 by the binding of its 3 prime UTR (Chaturvedi et al., 2015).

In summary, the published data suggests that exosomes under diabetic milieu carry miRNA, proteins and enzymes that are involved in the pathogenesis of cardiomyopathy. Therefore, future studies are necessary to investigate whether exosome biology/function can be re-programmed to promote cardiac health, which could be therapeutically valuable for the treatment of diabetes.

ADIPOCYTE-DERIVED EXOSOMES AFFECTING CARDIAC HEALTH

Adipose tissue was once thought to be an energy storage organ, however, adipose tissue has emerged as an endocrine organ secreting hormones, paracrine factors and inflammatory mediators. Recent studies suggest that adipocytes also secrete exosomes that play a critical role in obesity mediated metabolic disorders (Ying et al., 2017; Zhao et al., 2018). Interestingly, the majority of circulating exosomes are derived from adipose tissue and their content is determined by the health of the adipose tissue (Barberio et al., 2019). Adipose tissue biology is primarily regulated by the transcription factor PPAR-gamma and its agonists have been used for insulin sensitization in diabetic patients. However, PPAR-gamma agonists (rosiglitazone) cause heart failures that may be mediated by exosomes from adipose tissue. In co-culture experiments, rosiglitazone treated adipocytes induced cardiomyocyte hypertrophy, however, this was inhibited by treatment with GW9662 (rosiglitazone inhibitor) and GW-4869 (exosome biogenesis inhibitors), suggesting that the induction of hypertrophy was due to PPAR-gamma signaling mediated by exosomes. Molecular analysis showed that PPAR-gamma induced miR-200 expression in adipocytes and this was enriched in exosomes. miR-200 enriched exosomes induced cardiac hypertrophy by inhibiting tuberous sclerosis, the negative regulator of mTOR (Fang et al., 2016).

Adipose tissue derived exosomes have also been implicated in the development of atherosclerosis, a risk factor for heart disease. Atherosclerosis is characterized by plaque formation in the arteries that can lead to the narrowing of arteries that limits blood supply. An important aspect of atherosclerosis is the infiltration of inflammatory foam cell macrophages that take up oxidized low-density lipoproteins. Interestingly, the injection of exosomes from visceral fat explants of obese mice exacerbated atherosclerosis in mouse models of the disease. Mechanistically, exosomes from obese visceral fat explants induced the formation of M1 macrophages through the activation of NFkB pathway in RAW 264.7 cells (Xie et al., 2018). This data suggests that adipose tissue derived exosomes can reprogram macrophages into pro-inflammatory M_1 phenotype cells thus further increasing the potential for macrophage infiltration and chronic atherosclerotic inflammation.

IMMUNE CELLS-DERIVED EXOSOMES AND ITS ROLE IN CARDIAC PATHOPHYSIOLOGY

The immune system is primarily involved in the body's defense against pathogens, wound repair, inflammation and protection against non-self-antigens. Recent evidence has shown that exosomes play important role in carrying out these functions (Qazi et al., 2009; Lugini et al., 2012). Immune cells play an active role post-MI in the heart by clearing out cell debris, resolve inflammation and facilitating cardiac remodeling (Anzai et al., 2012; Hofmann et al., 2012). Consistent with this, depletion of dendritic cells resulted in sustained inflammation, inhibited endothelial cell proliferation, and thus resulting in worse outcomes (cardiac functions and cardiac remodeling), post-MI (Anzai et al., 2012). Interestingly, exosomes mediate some of these effects. For example, exosomes from cardiomyocytes exposed to ischemia or necrosis stimulate differentiation of bone marrow derived dendritic cells, which in turn secrete exosomes that program splenic $CD4^+$ T cells to secrete chemokines and inflammatory mediators; improving cardiac function post-MI (Liu et al., 2016). This suggests cardiomyocytes exposed to hypoxic conditions release exosomes that mobilize immune cells to the infarct zone and promote cardiac health. Similarly, macrophage derived exosomes enriched in miR-155 inhibited cardiac fibroblast proliferation post-MI in mice through the inhibition of son of Sevenless 1, a critical modulator of RAS activation. Macrophage derived miR-155 also promote cardiac inflammation by increasing the secretion of inflammatory cytokines IL1β, IL-6, TNF-alpha, and CCL2. Moreover, miR-155 knockout mice hearts were protected from adverse effects post-MI as shown by cardiac fibroblast proliferation and protection from cardiac rupture (Wang et al., 2017). Overall, this data suggests that immune cells orchestrate several events post-MI to promote the clearing of debris, inflammation and wound healing by distinct mechanisms that utilize exosomes.

While data on exosomes from immune cells and their effects on heart disease is evolving, studies point out to a high incidence and poor outcome of heart diseases in different metabolic settings (diabetes and obesity) in the absence of other risk factors. These differences could be explained in part by exosomes derived from immune cells, given the fact that the immune system is reprogrammed under metabolic milieu. For example, the immune system is compromised in diabetes and can lead to defective wound repair and increased inflammation. Consistent with this notion, research from our lab has shown that exposure to high glucose decreases the expression of miR-126 in macrophages and thus decreases the ability of macrophages to clear apoptotic cardiomyocytes. Moreover, overexpression of miR-126 rescued the functional deficiency of macrophages (Suresh Babu et al., 2016). In addition, Gao et al. reported that exosomes from mature dendritic cells promote atherosclerosis by inducing endothelial inflammation through exosome derived tumor necrosis factor-alpha (TNF-alpha), which activates the pro-inflammatory NF-kB pathway (Gao et al., 2016). Moreover, macrophage-derived exosomes promote the formation and mineralization of atherosclerotic plaques (Xie et al., 2018). Therefore, it is logical to speculate that the outcomes of heart diseases under metabolic conditions could be mediated by exosomes from immune cells exposed to metabolic milieu (**Figure 2**). In addition, stem cell/progenitor cell therapy can improve cardiac health post-MI through the use of exosomes (Khan et al., 2015; Kishore and Khan, 2016), however the mechanisms have yet to be fully worked out. Therefore, future research is necessary to investigate whether stem cell derived exosomes may also reprogram immune cells to improve cardiac inflammation, wound healing and resolution (**Figure 2**).

CIRCULATING EXOSOMES AND SEPSIS IN HEART DISEASE

The idea of plasma/circulating exosomes and their protective role in cardiac ischemia reperfusion injury (IR injury) arose from remote ischemia reperfusion studies. In experimental models, remote ischemia conditioning (RIC) reduced infarct size and protected against cardiomyocyte death post-MI. Molecular analysis showed that RIC increased HSP70 enriched micro vesicle release into the circulation. HSP70 protected against cardiomyocyte death via activation of ERK1/2, AKT and HSP27 through TLR4 (toll like receptor) signaling (Vicencio et al., 2015). Likewise, RIC in rats increased exosome associated miR-24 in rat plasma and protected against IR injury-induced cardiomyocyte death. Moreover, RIPC (remote ischemia pre-conditioning) exosome therapy reduced cardiomyocyte death, decrease infarct size and restored cardiac functions post-MI in

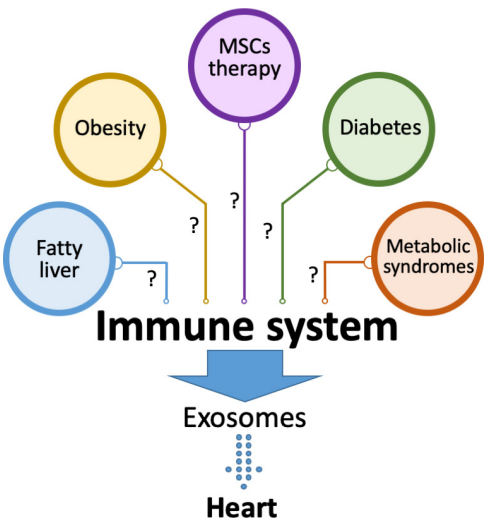

FIGURE 2 | Exosomes from the immune system affect cardiac tissue. Immune system is modulated under different metabolic settings including diabetes, obesity, fatty liver and metabolic syndromes. The role of exosomes secreted by immune cells under these conditions in heart pathology is yet to be fully described. It is also not known whether the beneficial effects of stem cell-based therapies in heart diseases are mediated by immune modulation that may lead to the release of cardio protective exosomes.

rats (Minghua et al., 2018). Contradictory results have also been reported using RIPC in the heart, so future studies are essential to elucidate the mechanisms involved.

Circulating exosomes have also been implicated in sepsis mediated heart failure. Sepsis is dysregulated body's response to infection, and is the leading cause of death in intensive care units (Raeven et al., 2018). Sepsis is primarily mediated by overactive inflammation, a process that leads to organ failure. While, a proper understanding of the molecular mechanisms surrounding sepsis is incomplete, recent studies have shown that circulating exosomes may partly explain the molecular mechanisms for sepsis induced cardiac pathology. Circulating exosomes increase with the onset of sepsis and are associated with progress of the disease (Terrasini and Lionetti, 2017); and so have been extensively investigated for diagnosis (Reviewed in detail elsewhere Raeven et al., 2018). Interestingly platelet derived exosomes in response to LPS induce vascular muscle cell death *in vitro* (Janiszewski et al., 2004). Likewise, circulating exosomes from septic patients inhibited myocardial contractility in isolated rabbit heart preparations, and these responses grew worse with prior exposure to LPS. Consistent with this, exosomes from septic patients inhibit contractions in papillary muscle preparation from rats. Interestingly, cardiac contractions were partially rescued through treatment with apocyanin, a Nox inhibitor. There was an increase in production of NO from septic exosomes that were inhibited by L-NAME, suggesting that NO could be the mediator of cardiac dysfunction in sepsis (Azevedo et al., 2007). Evidence for the involvement of exosomes in sepsis was further strengthened by an elegant study where pretreatment with GW4869 (inhibitor of exosome biosynthesis) protected against CLP (colon ligation and puncture) and LPS models of sepsis by reducing inflammation, improving cardiac function, and prolonging animal survival (Essandoh et al., 2015). Consistent with this report exosomes derived from sepsis mouse models induced disruptions of membrane podosomes, podosome cluster formation and increased vascular permeability (Mu et al., 2018). However, an interesting study by Gao et al. revealed exosomes packed with inflammatory mediators peaked 24 h after sepsis that induced proliferation of lymphocytes and differentiation of Th1, Th2 cells. Moreover, pretreatment of mice with these exosomes reduced inflammation, tissue injury, and improved survival in CLP model of sepsis (Gao et al., 2019). In light of these contradicting studies it remains to be seen whether GW4869 also inhibits synthesis of inflammatory mediators, or prevents peroxidation of membrane lipids as these are source of tissue damage. Studies have identified several microRNAs that are differentially expressed in the exosomes from septic shock patients, with very high levels of pro- inflammatory microRNA compared to exosomes from control patients. In addition, the patients that survived septic shock had high levels of microRNA involved in cell cycle regulation (Raeven et al., 2018), suggesting that exosomal microRNA has different functions at different stages of sepsis and can determine the pathogenesis and prognosis of septic patients.

These studies suggest that under healthy conditions, the body produces cardio protective exosomes that could be lost or altered under different metabolic settings or co-morbidities. Future studies would identify the source of these exosomes, characterize the cargo, and to design therapies targeting exosomal bioactive molecules.

CARDIOMYOCYTE AND CARDIAC FIBROBLASTS AFFECTING CARDIAC PHYSIOLOGY

Cardiac remodeling is a classical response to various pathophysiological stressors such as increased peripheral resistance, arterial stenosis, heart failure and myocardial infarction (MI). The classical pathways involving the neuroendocrine system are well known. However, the molecular mechanisms involving exosomes have been instrumental in understanding the pathogenesis of these diseases. Exosomes released from different cell types within the heart could serve as intercellular communicators and influence cellular functions within the heart and in peripheral organs (**Figure 1**). The composition of the exosome cargo from cardiac tissue is determined by cardiac physiology, which can convey coded messages to the target cells and reprogram their biology. For example, exosomes released from cardiomyocytes under osmotic stretches and pressure overload are enriched in angiotensin type II receptor, and these exosomes were shown to induce vascular pressure changes in heart, muscle, and intestinal vessels (Pironti et al., 2015). Interestingly, exosomes from pericardial fluid surrounding the heart were enriched in miR-let-7b-5p and were shown to induce proliferation and vascular tube formation in endothelial cells, and restore blood flow in ischemic limb models through miR-let7b-5p (Beltrami et al., 2017). This data suggests pericardial fluid exosomes promote angiogenesis. Consistent with this, exosomes from heart explants from healthy individuals and heart failure patients had opposing effects in mouse models of MI (Qiao et al., 2019). Intracardiac injections of exosomes from explants of heart failure patients had worse outcomes in terms of heart function and cardiac remodeling when compared to exosomes from healthy explants in mouse models of MI, suggesting that exosomes from healthy and failing hearts were different. Molecular analysis revealed that exosomes from healthy hearts were enriched with miR-21 that inhibited apoptosis, promoted proliferation of cardiac cells, and promoted angiogenesis. At the molecular level, miR-21-5-p inhibited PTEN, and BCL2, and activated AKT and VEGF pathways in cardiomyocytes and endothelial cells (Qiao et al., 2019).

Cardiomyocyte hypertrophy was induced in co-culture experiments using cardiac fibroblasts and cardiomyocytes (Fredj et al., 2005). In addition, conditioned media from cardiac fibroblasts induced cardiomyocyte hypertrophy, increased expression of vimentin and inhibited chronotropic contraction of cardiomyocytes *in vitro* (LaFramboise et al., 2007), suggesting that fibroblast derived factors were responsible for the phenotype. Interestingly, these factors were discovered to be exosomes derived from cardiac fibroblast (Bang et al., 2014; Lyu et al., 2015). Bang et al. have shown that these exosomes were rich in miR-21 and mediated cardiomyocyte hypertrophy through the inhibition of SORS2I and PDIM5, factors that are important

for the prevention of hypertrophy. Consistent with this report, cardiac hypertrophic patients had high amounts of miR-21* in their pericardial fluid exosomes, and injection of these exosomes in murine models induced cardiac hypertrophy which was then shown to be inhibited by antago-miR-21 treatment. In addition, in angiotensin-induced cardiac hypertrophy, Lyu et al. identified that cardiac fibroblasts released exosomes that were enriched with renin, angiotensin, angiotensin receptors I, II and inhibited the angiotensin converting enzyme (ACR). At the molecular level, cardiac hypertrophy was mediated by the activation of RAS through the MAP kinase pathway (Lyu et al., 2015). These two studies have provided mechanistic insights into how cardiac fibroblast derived exosomes trigger pressure overload-induced cardiac remodeling. Cardiac hypertrophy also involves the differentiation of cardiac fibroblasts into myofibroblasts and increased synthesis of extracellular matrix proteins including collagen and MMP's that result in cardiac fibrosis. Interestingly, studies by Datta et al. have uncovered that fibroblasts are reprogrammed by exosomes arising from cardiomyocytes. Cardiomyocyte specific HSP90 knockdown protected against fibrosis, hypertrophy, and cardiac dysfunction in renal artery ligation models of cardiac hypertrophy. In addition, conditioned media from hypertrophied cardiomyocytes induced collagen synthesis in cardiac fibroblasts that were inhibited through treatment with IL-6 neutralizing antibodies, suggesting the involvement of exosome mediated signaling (Datta et al., 2017). In addition, HSP90 increased exosome secretions that were enriched with IL-6 through p65 in cardiomyocytes that were involved in the programming of cardiac fibroblasts (Datta et al., 2017). Cardiac fibrosis and remodeling is also mediated by exosome derived miR-208 from cardiomyocytes post-MI. MiR-208 was enriched in exosomes from cardiomyocytes in response to hypoxia, angiotensin treatment and MI, that induces proliferation of cardiac fibroblasts; and inhibition of miR-208 using antagomiR protected against cardiac fibrosis and cardiac dysfunction post-MI. Exosome derived miR-208 inhibited Dyrk (dual specific tyrosine phosphorylated kinase) expression that phosphorylated NFAT to promote its nuclear export. Nuclear NFAT triggers fibrosis by inducing fibrogenic gene expression in cardiac fibroblasts. Moreover, injection of exosomes derived from the ischemic heart induced cardiac fibrosis in healthy mice (Yang et al., 2018). Likewise, miR-217 was enriched in cardiomyocyte derived exosomes in heart failure patients, thoracic aortic constriction models of heart failure and was involved in fibroblast proliferation and cardiac hypertrophy through PTEN (Nie et al., 2018). Consistent with this notion, cardiomyocyte-derived exosomes in response to isoproterenol and phenylephrine treatment were enriched with miR-300c, which inhibited angiogenesis by programing endothelial cells. In addition, treatment with antagomiR-300c protected mice against transverse aortic constriction induced cardiac hypertrophy (Ottaviani et al., 2018). Contrary to this are reports suggesting that cardiomyocyte-derived exosomes post-MI also induce angiogenesis by activating endothelial cells (Ribeiro-Rodrigues et al., 2017). Exosomes derived from H9C2 cells exposed to ischemic conditions induced endothelial cell proliferation, migration and tube formation. In addition, ischemia derived exosomes induced angiogenesis in CAM (chorio-allantoic membrane) assay using chicken embryos and Matrigel implants in rats. Furthermore, ischemic exosomes reduced infarct size, increased vascularization, and reduced fibrosis. At the molecular level, ischemic exosomes were enriched with miR-143 and miR-222, which both induce angiogenesis. In light of these contradicting reports, the speculation is that compensatory and decompensatory pathways were in action (Ribeiro-Rodrigues et al., 2017). Therefore, exosome content could be determined by the stage of cardiac pathology. In the early stages, cardiac exosomes could be enriched with cardio protective cargo and in terminal stages exosomes might be enriched with decompensatory cargo (**Figure 3**).

Oxidative stress is one of the primary causes of cardiomyocyte death and heart failure, post-MI. Interestingly, cardiac-derived exosomes post-MI are responsible for the loss of the cardiac endogenous antioxidant NRF2 (Nuclear factor erythroid-2 related factor) pathway. Ventricular expression of NRF2 was inhibited whereas the expression of miR-27a, miR-28-3p, and miR-34a were upregulated, post-MI. Moreover, TNF-alpha treatment in cardiomyocytes (CM) and cardiac fibroblasts (CF) increased expression and enrichment of these microRNAs in exosomes, suggesting that CM and CF secreted these exosomes post-MI. At the molecular level exosome derived miR-27a, miR-28-3p, and miR-34-a inhibited expression of NRF2 at the transcription level (Tian et al., 2018). Exosomes also mediate progenitor cell mobilization from bone marrow that

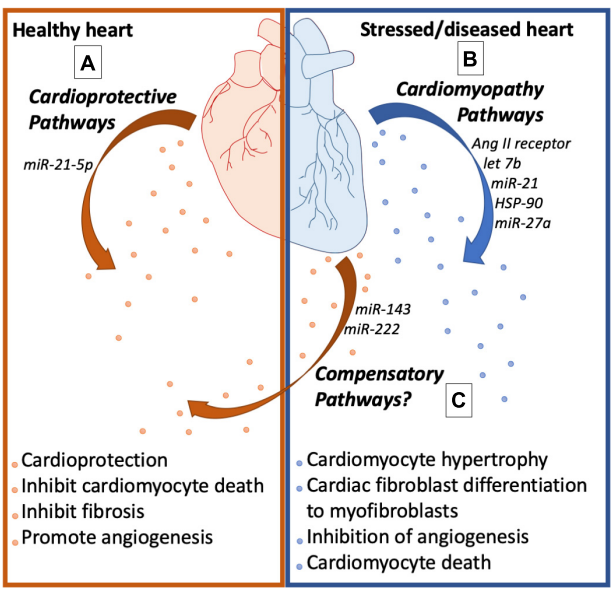

FIGURE 3 | Cardiac health affects exosome biology. **(A)** Exosomes from the healthy heart are enriched with cardioprotective molecules that promote angiogenesis, inhibit fibrosis, and inhibit cardiomyocyte cell death. **(B)** Exosomes from the stressed heart release exosomes enriched with molecules that cause cardiomyocyte hypertrophy, myofibroblast differentiation, endothelial dysfunction, and cardiomyocyte cell death. **(C)** The stressed heart also releases exosomes that activate compensatory pathways to promote cardiac health.

play critical role in cardiac inflammation and remodeling. Injection of exosomes from mice post-MI, also increased the circulation of bone marrow-derived progenitors in systemic circulation. Exosomes from MI mice were enriched with myo-miR-1a, miR-208a, miR-133a, miR-499-5p that were taken up by BM progenitor cells and inhibited CXCR4 to promote their mobilization into the systemic circulation (Cheng et al., 2019).

CARDIAC TELOCYTES-DERIVED EXOSOMES

Telocytes are interstitial, cajal like cells present in the cardiac interstitium that have long extensions (telopods) that forms supportive frame work in organs (Cretoiu et al., 2016; Yang et al., 2017). Interestingly, telocytes have been found in the niches of stem cells and are known to support their growth (Cismaşiu and Popescu, 2015). Telocytes are also involved in the horizontal transfer of macromolecules through exosomes (Mandache et al., 2007; Fertig et al., 2014; Cismaşiu and Popescu, 2015; Cretoiu et al., 2016). Exosomes from telocytes are known to induce proliferation of vascular smooth muscle cells. Additionally, telocytes induced proliferation and migration of endothelial cells in *in vitro* in co-culture experiments, suggesting molecules released from telocytes were responsible. Moreover, telocyte derived exosome treatment protected rats from adverse effects of MI leading to dysfunction as shown by echocardiography and cardiac fibrosis (Yang et al., 2017). The data suggests that telocytes are unique in regards to providing niches to stem cells, and that their exosomes have protective effects in heart diseases. Future studies are needed to investigate if telocytes can be programmed *in situ* to protect from heart diseases.

EXOSOMES FROM CARDIAC AND PERIPHERAL ENDOTHELIAL CELLS

Endothelial cells (ECs) are the innermost monolayer of vasculature that lines the entire circulatory system including the heart. The endothelium regulates the normal vascular tone and permeability, maintains homeostasis, prevents thrombogenesis, and facilitates molecular exchange between circulating blood and vessel walls (Vane et al., 1990; Rajendran et al., 2013; Cahill and Redmond, 2016). Endothelium regulate these functions though the release of mediators such as adhesion molecules, cytokines, vasodilators, and exosomes into both the local tissues and systemic circulation (Vane et al., 1990; Galley and Webster, 2004; Sandoo et al., 2010). The exosome trafficking between ECs and cardiac tissue has been gaining attention due to exosomes' ability to carry cardioprotective and proangiogenic factors to the heart. Exosomes from endothelial cells crosstalk with cardiomyocytes and other cardiac-resident cells, and are involved in many pathophysiological processes. Studies involving atherosclerosis, one of the common underlying causes of myocardial infarction, has found that communication between ECs and smooth muscle cells plays a crucial role in mediating protection against atherosclerosis development (Hergenreider et al., 2012). Hergenreider et al. showed that physiological shear stress robustly induces enrichment of atheroprotective and vasculoprotective microRNAs (such as miR-143/145) in endothelial exosomes, which when transferred to smooth muscle cells (SMCs) prevents SMC de-differentiation and induces an atheroprotective phenotype (Cordes et al., 2009; Hergenreider et al., 2012). Recently, it has been shown that exosomes derived from ECs help protect cardiomyocytes against ischemia and reperfusion conditions in AMI (Acute Myocardial infarction) by activating the ERK1/2 and MAPK signaling pathways (Davidson et al., 2018a). Another mechanism through which ECs help prevent cardiac remodeling in ischemic heart is through release of miR-126 and miR-210 enriched exosomes (Ong et al., 2014), which are well-known for activating pro-survival kinases and reducing cellular damage in recipient cells (Pan and Johnstone, 1983; Zaccagnini et al., 2017). Cardioprotective property of EC-derived exosomes were also shown in other cardiac diseases. Halkein et al. (2013) showed that in peripartum cardiomyopathy (PPCM), or pregnancy-associated heart failure, the delivery of miR-146a-enriched exosomes from ECs to CMs is increased. These miR-146a then reduce metabolic activity in CMs by decreasing the expression of N-Ras, Erbb4, Notch1, and Irak1, leading to cardiac dysfunction. Akbar et al. (2017) quantified and analyzed plasma exosomes in humans and mice after MI and found significant increases in EC-origin exosomes compared to non-AMI group (2.3–13.7-fold in human, 2.5-fold in mice) (Akbar et al., 2017). However, the author could not conclude if this increment is due to the cardiac ECs response to the MI, or the whole-body response due to activation of immune system after MI. The limitation of this paper and other studies (summarized in **Table 3**) highlights the lack of tools to determine tissue and cell specificity of exosomes in an *in vivo* setting. It is well known that ECs from different organs vary in terms of morphology, gene expression, growth factors, angiogenic potential and regulatory function; making it reasonable to believe that under a given pathophysiologic condition, the profile of exosomes from cardiac endothelial cells, the peripheral endothelium, coronary endothelium, or large vessels would differ. While exosome trafficking between cardiac ECs and cardiomyocytes is gaining attention, many of these studies have relied on HUVECs as the model instead of the targeted organ-specific ECs (such as cardiac microvascular ECs), therefore not truly mimicking the physiologic condition. Interestingly, several studies focused on non-cardiac pathology, in which cardiac EC are not the specific target, also imply the cardiac-protective effect of EC-induced exosomes. For example, study by Shyu et al. showed that under hyperbaric oxygen (HBO) therapy, human coronary artery ECs (HCAECs) can release exosomes containing long noncoding RNA MALAT1 (a proangiogenic RNA) into the circulation to reach the ECs in ischemic tissues, ultimately accelerating neovascularization and enhance wound healing process (Shyu et al., 2019). This explains the mechanism through which HBO reduce infarct size in MI *in vivo* (Sterling et al., 1993; de Jong et al., 2012; van Balkom et al., 2013). Other studies, for instance, have been carried out to investigate the role of miRNA and RNA in the

TABLE 3 | Studies demonstrating endothelial cell-derived exosomes in pathophysiology of heart diseases.

Donor cell	Target cell	Diseases	Model	Effect	References
HUVECs	Cardiomyocytes (CMs)	MI	*In vitro*	HUVEC-derived exosomes protect CMs by activating the ERK1/2 MAPK signaling pathway	Davidson et al., 2018b
HUVECs	SMCs	Atherosclerosis (cause of MI)	*In vitro*	Under shear stress, HUVECs release exosomes containing atheroprotective and vasculoprotective microRNAs	Hergenreider et al., 2012
ECs	Cardiac progenitorcells (CPC)	Ischemia	*In vitro*	Overexpression of HIF1 (stimulation of hypoxic condition) in ECs leads to releasing of miR-126/210 enriched exosomes to reduce cellular damage in recipient cells	Ong et al., 2014
HUVECs	CMs	Peripartum cardiomyopathy (PPCM)	*In vitro*	The delivery of miR-146a-enriched exosome from ECs to CMs reduces metabolic activity in CMs by decreasing the expression of Erbb4, Notch1, and Irak1	Halkein et al., 2013
ECs/HUVECs	Monocytes	MI	*In vivo/ In vitro*	After AMI, circulating EC-origin exosomes significant increased	Akbar et al., 2017
HUVECs	CMs	Diabetes/MI	*In vitro*	Under the hyperglycemic culture conditions, cardioprotective ability of HUVEC-derived exosomes is eliminated	Davidson et al., 2018a

exosome-mediated crosstalk between ECs, which can enhance angiogenesis in the injured tissue, as seen in the case of MI, hypoxia, inflammation or hyperglycemia. However, the direct role of peripheral endothelium-derived exosomes in cardiac pathophysiology remains poorly understood.

EXOSOME PROTEINS AND THEIR FUNCTION

Exosomes are actively synthesized and secreted by the endolysosome pathway, which involves active sorting of its contents including proteins. Exosomal proteomics has been instrumental in identifying the protein constituents of exosomes. Exosomes are enriched with ESCRT proteins, Tetraspanins, RNA binding proteins, selective sorting proteins, and membrane proteins that aid in sorting, cargo selection and stabilization of RNA (Xu et al., 2016). Some of these proteins- CD9, CD63, and CD81 have been extensively used as markers for exosome characterization. Exosome also carry specific proteins determined by the physiology of the donor cell that are involved in intercellular communication with specific functions. For example, several cancer cells release exosome that help in immune evasion, immunosuppression and cancer metastasis (Wortzel et al., 2019). Likewise, exosome derived proteins also participate in the pathophysiology of heart disease such as diabetic cardiomyopathy, cardiac ischemia reperfusion injury and sepsis (summarized in **Table 4**). Interestingly, several exosome-derived proteins have been targeted in diagnosis of various diseases including cancer (Cazzoli et al., 2013), myocardial infarction, sepsis, metabolic syndromes and cardiovascular outcomes (Bei et al., 2017). Due to this, active loading of specific proteins into exosomes is being used to engineer therapeutic exosomes. Several techniques including fusing of exosome sorted proteins with protein/peptide of interest, over-expression of the protein in donor cell, protein modification, *in vitro* enrichment of protein of interest in exosomes have been investigated (Liu and Su, 2019). However, therapeutic value of such exosomes is yet to be realized in clinics. More comprehensive overview of tools and technological advances in exosomal proteomics are previously described elsewhere (Barrachina et al., 2019).

STEM/CARDIAC PROGENITOR CELL-DERIVED EXOSOMES IN CARDIAC REGENERATION

An important aspect of heart disease is that cardiomyocytes have a very limited regenerative ability and any injury that leads to cardiomyocyte death results in the replacement with other cell types with limited electrophysiology function and contractile ability. Therefore, stem cell-based therapies are gaining traction for cardiac regeneration. Interestingly, the protective effect of these cells has more recently been attributed to the exosomes release (Ibrahim et al., 2014). Exosomes from stem cells can prevent cardiomyocyte death, inflammation, fibrosis, and promote angiogenesis by exporting microRNA, proteins, and signaling molecules that have regenerative capacity (summarized in **Table 5**). Consistent with this, several studies have shown that exosomes derived from cardiac progenitors and cardiosphere-derived cells have cardio protective and regenerative effects (Barile et al., 2014; Milano et al., 2019). Exosomes derived from stem cell/cardiac progenitor cell have advantages over the cells in terms of size, ease of production, problem with differentiation to undesirable non-cardiac cells and tumor formation. Therefore, exosomes from stem cells/cardiac progenitor cells can provide promising therapeutics for cardiac regeneration in the near future.

EXOSOME ENGINEERING FOR THERAPEUTICS

As previously discussed, exosomes have been shown to have a significant impact, whether positive or negative, on human

TABLE 4 | Exosome derived proteins in pathophysiology of heart diseases.

Functional component	Donor cell	Functional outcome	References
HSP60	Cardiomyocyte	Cardiomyocyte death	Kim et al., 2009
Renin, angiotensin receptor, ACE	Cardiac fibroblasts	Cardiomyocyte hypertrophy	Lyu et al., 2015
Ang type-II receptor	Cardiomyocyte	Cardiomyocyte hypertrophy	Pironti et al., 2015
HSP20	Cardiomyocyte	Endothelial cell proliferation anti-oxidant	Wang et al., 2016, p. 20
Cystasin C, Serpin F2, Serpin G1, Cd14	NA	Cardiovascular events	Kanhai et al., 2013
Microparticles (circulating and endothelial)	Endothelial cells	Prognostic marker for heart failure	Nozaki et al., 2009; Bei et al., 2017; Zamani et al., 2019
HSP70	Endothelial	Inhibit cardiomyocyte death	Davidson et al., 2018b
HSP 90	Cardiomyocyte	Cardiac fibrosis	Datta et al., 2017
Arginase 1	NA	Inhibit endothelin dependent aortic relaxation	Zhang et al., 2018
Mst1	Endothelial cells	Cardiomyocyte death in diabetes	Hu et al., 2018
Dystrophin	C2C12 cells	Restoration of dystrophin in cardiomyocytes in MDX mouse hearts	Su et al., 2018
Lamp2b	Cardiosphere derived cells	Increased retention of exosomes in the heart	Mentkowski and Lang, 2019

TABLE 5 | Stem cell/cardiac progenitor cell-derived exosomes for cardiac therapy.

Donor cells	Functional component	Functional outcome	References
Cardiac progenitor cells	miR-210, miR-132, miR-146a-3P, miR-310	Prevent cardiomyocyte death	Barile et al., 2014
Cardiosphere-derived cells	miR-146a	Promote Angiogenesis Inhibit cardiomyocyte proliferation	Ibrahim et al., 2014
Sca1 + stem cells	HSF1	Cardiomyocyte Protection against ischemic injury	Feng et al., 2014
Embryonic stem cells	miR-290-295 clusters (miR-294)	Cardiac progenitor cell proliferation	Khan et al., 2015
Cardiosphere-derived cells	Y RNA fragment	Cardioprotection against oxidative stress	Cambier et al., 2017
Cardiac progenitor cells	Pregnancy associated plasm protein, IGF	Inhibit cardiomyocyte death	Barile et al., 2018
Cardiac progenitor cells	Activation of Akt-mTOR	Inhibit cardiomyocyte death	Li et al., 2018, 2019b
Implanted cardiac progenitor cells	miR-378, miR-623, miR-941 miR-1256, miR-384, miR-525-3P, miR-315-5P, miR-1224	Improved EF Inhibit fibrosis Angiogenesis	Saha et al., 2019
Cardiac progenitor cells	miR-146a	Protection against doxorubicin induced cardiotoxicity	Milano et al., 2019
C2C12 cells	Dystrophin	Restoration of dystrophin in cardiomyocytes in MDX mouse hearts	Su et al., 2018
MSC	miR-21-5p	Cardiac contractility and expression of ca handling genes	Mayourian et al., 2018
Endothelial progenitor cells (EPC)	IL-10, miR-375	IL-10 deficiency impairs angiogenesis in ischemic heart	Yue et al., 2017

disease, and exosomes from different cell types have unique characteristics that could potentially affect these exosome-induced changes in the disease milieu. While exosomes derived from known cell types must be considered, the ability of exosome engineering to alter the disease state is of direct current interest and the focus of many studies. However, the use of naturally produced exosomes for this purpose has some limitations, as "organic" exosomes may be limited in their specificity as they are derived from the parent cell, and it may be difficult to ensure that the desired content is what is being transported (Li et al., 2019a). To address these challenges, engineered exosomes offer an opportunity to use inherent characteristics from various exosome sources, combined with novel approaches, to improve exosome specificity, content loading, and production for improved therapeutic potential (Yim et al., 2016; Luan et al., 2017).

One of the challenges in using exosomes for therapy is ensuring that the desired cells or organs receive the exosomal contents (Li et al., 2019a). The most considered idea to address this as of now is parent cell membrane modification. The most commonly used method to achieve this is through inserting a gene encoding for a target protein into the parent cell, wherein the exosomes secreted will contain the added surface protein. For example, Liang et al. employed a plasmid encoding for a CD63/ApoA1 fused gene, which was then delivered to the parent cells. This gene was then incorporated and expressed as a surface protein which allowed for specific binding to the target HepG2 cells, via the SR-B1 receptor (Liang et al., 2018). Copper-catalyzed azide alkyne cycloaddition (CLICK chemistry) has also been explored for the use of bioconjugation of surface proteins in exosomes, and it has been reported that these modifications do not impact exosome size or internalization time (Smyth et al., 2014). Along with these methods, some alternative strategies have shown promise, as seen in the work of Armstrong et al. in using noncovalent interactions

to modify the EV membrane. However, this method has challenges, as the reaction must be tightly controlled to prevent exosome loss of function due to aggregation (Qi et al., 2016; Armstrong et al., 2017).

The loading of specific exosomal content is also a popular area of research for potential exosome therapy. Passive loading usually involves incubating the drug or molecule either directly with collected exosomes, or with donor cells that secrete exosomes laden with the target molecule (Luan et al., 2017). However, passive loading has limited efficiency, and relying on donor cell secretion will incorporate nonspecific cargo along with the molecule of interest, requiring additional separation and purification steps for quality control. To combat this, active loading methods being explored to selectively incorporate a target molecule into exosomes. Sonication is one method being explored, as shown by Kim et al. (2016) in their study. In this study, macrophage derived exosomes were loaded through sonicating the exosomes to disrupt the membrane, allowing for more efficient incorporation of their molecule of interest, PTX (Kim et al., 2016). While this method allows for high loading efficiency, it can adversely compromise the exosome membrane, so care must be taken in regards to the frequency and time employed. Extrusion, the loading of the molecule of interest and exosomes through a syringe based lipid extruder, is also a method employed for active loading (Antimisiaris et al., 2018). This method shares both the high efficiency seen in sonication, as well as the drawback of potential compromised exosome membranes post loading, so care must be taken to ensure viable vesicles post loading. Electroporation is the most common method for loading siRNA or miRNAs, opening small temporary pores in the exosome membrane through electric field application to disrupt the phospholipid bilayer (Faruqu et al., 2018). However this method, while highly efficient, risks aggregation of exosomal contents, and this must be taken into consideration for study (Johnsen et al., 2016).

For clinical use, a major challenge is to increase exosome production to the necessary numbers for human patients, as current methods of exosome production and isolation are limited by low yield. The most basic way to perform this is to increase flask size for static culture, as having more cells and media would lead to greater exosome production. However, this method is generally not cost effective and can become space prohibitive with larger scale. One possible solution to increase efficiency is to increase secretion of exosomes from the cells. One of the easiest ways to achieve this is the application of stress. Several studies have shown that the application of heat, altered pH, hypoxia, or nutrient deficiency to induce cell stress all significantly increase the release of exosomes (King et al., 2012; Logozzi et al., 2018; Zou et al., 2019). However, the vesicles released under stress have been shown to have very different content profiles compared to unstressed exosome content, therefore, this must be taken into consideration for any potential use in engineered exosome therapy (Liu and Su, 2019). In addition, several drugs have been shown to regulate exosome secretion, as seen in a study by Datta et al. where they identified a number of drugs that regulate exosome biogenesis. Sitafloxacin, Forskolin, SB218795, Fenoterol, Nitrefazole and Pentetrazol were all shown to activate exosome biogenesis; while Tipifarnib and Ketoconazole were shown to be significant inhibitors of exosome release (Datta et al., 2018). Another potential approach to improve exosome secretion is to employ three-dimensional matrix cell cultures. Extracellular matrix scaffolds allow for three-dimensional signaling of the cells in culture, which can promote cell growth and vesicle release, and lead to a 20-fold increase compared to 2-D culture (Haraszti et al., 2018; Phan et al., 2018). Artificial scaffolds hold much promise, as not only do they allow for three-dimensional signaling, but also can be modified to promote vesicle release or influence vesicle content, as seen in a study by Du et al. In this study, a nitric oxide releasing polymer scaffold was used to grow mesenchymal stem cells (MSCs), and exosome release was studied. It was revealed that proangiogenic content such as VEGF and miR-126 inside the exosomes was increased due to the nitric oxide stimulation, demonstrating the efficacy of this scaffold material for potential therapeutic use (Du et al., 2017). Finally, one of the most promising technologies for large scale exosome production is the use of bioreactors. In a protocol established by Farid et al., the use of basic bioreactor flasks allowed for sequential collection of exosome containing media without passaging cells, and allowed for collection of $6.99 \pm 0.22 \times 1012$ exosomes/mL (Faruqu et al., 2018). Another study, performed by Haraszti et al., combined both bioreactor technology with 3-D scaffold culture to take advantage of the benefits from both systems. In their model, the addition of a bioreactor tangential flow system increased the exosome yield from mesenchymal stem cells sevenfold over the 3-D scaffold alone (Haraszti et al., 2018).

There are many challenges remaining for the use of engineered exosomes in therapy. There are still concerns surrounding off-target effects, difficulty with control of exosome content loading, and observation that current exosome isolation techniques present inherent limitations on scaling up exosome production (Antimisiaris et al., 2018; Phan et al., 2018; Li et al., 2019a). Despite these challenges, significant strides have been made and new solutions are being pursued, with new research exploring selective targeting and loading, while improved isolation techniques are being tested as of this writing. Exosome engineering is a very young field, showing much promise, and with further research and ingenuity solutions there is a strong possibility of their use for therapy in the near future.

CONCLUSION

Heart disease is the leading cause of morbidity and mortality in humans, and due to cardiomyocyte's limited regenerative ability, novel therapies are needed to treat heart diseases. Exosomes secreted by different cell types play critical roles in the pathophysiology of heart diseases, and their biology is determined by tissue homeostasis or disease state of the host tissue. Consistent with this, exosomes from healthy hearts were found to be cardioprotective, while exosomes from stressed hearts were involved in furthering the pathogenesis of heart

disease. Moreover, exercise was found to promote the secretion of cardioprotective exosomes into the circulation in diabetic mice, suggesting lifestyle choices may influence exosome biology to promote health. In addition, exosomes released from cardiac cells regulate the mobilization of immune cells and progenitor cells from bone marrow that are involved in the resolution of inflammation, cardiac remodeling, and wound healing. Likewise, several studies have demonstrated that exosomes can be programmed to protect and promote cardiac regeneration. Due to their unique size, content, ease of handling, non-immunogenic properties and being non-cellular, exosomes are an attractive new avenue for therapy. Therefore, further studies are needed to understand the detailed biology of exosome in health and disease, and how they may be engineered to develop novel therapies against heart disease.

AUTHOR CONTRIBUTIONS

MP and PK conceived the study. MP, HL, JH, DA, and GS collected the material and wrote the manuscript. PK provided critical feedback and helped to shape the manuscript.

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REVIEW
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Cardiac Fibroblasts and Cardiac Fibrosis: Precise Role of Exosomes

*Prabhat Ranjan[1], Rajesh Kumari[1] and Suresh Kumar Verma[1,2]**

[1] Division of Cardiovascular Disease, The University of Alabama at Birmingham, Birmingham, AL, United States,
[2] Department of Biomedical Engineering, The University of Alabama at Birmingham, Birmingham, AL, United States

Exosomes are a group of extracellular microvesicles that deliver biologically active RNAs, proteins, lipids and other signaling molecules to recipient cells. Classically, exosomes act as a vehicle by which cells or organs communicate with each other to maintain cellular/tissue homeostasis and to respond to pathological stress. Most multicellular systems, including the cardiovascular system, use exosomes for intercellular communication. In heart, endogenous exosomes from cardiac cells or stem cells aid in regulation of cell survival, cell proliferation and cell death; and thus tightly regulate cardiac biology and repair processes. Pathological stimulus in heart alters secretion and molecular composition of exosomes, thus influencing the above processes. The past decade has yielded increasing interest in the role of exosomes in the cardiovascular system and significant contribution of cardiac fibroblast (CF) and mediated cardiac fibrosis in heart failure, in this review we had overviewed the relevant literatures about fibroblast exosomes, its effect in the cardiovascular biology and its impact on cardiovascular disease (CVD). This review briefly describes the communication between fibroblasts and other cardiac cells via exosomes, the influence of such on myocardial fibrosis and remodeling, and the possibilities to use exosomes as biomarkers for acute and chronic heart diseases.

Keywords: heart failure, paracrine signaling, exosome, miR, fibrosis

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Yaoliang Tang,
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****Correspondence:***
Suresh Kumar Verma
sverma@uabmc.edu

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Abbreviations: α-SMA, α-smooth muscle actin; AP-1, activating protein-1; Azin1, antizyme inhibitor 1; CD, cluster of differentiation; CFs, cardiac fibroblasts; CM, cardiomyopathy; CVD, cardiovascular disease; DDR2, discoidin domain receptor 2; DNA, deoxyribonucleic acid; ECM, extracellular matrix; EPC, endothelial progenitor cell; EV, extracellular vesicle; EXO, exosome; Gal-3, Galectin-3; Hsp, heat-shock proteins; IGF1, insulin-like growth factor-1; IL-10, interleukin-10; IR, ischemia-reperfusion; Jnk1, c-Jun N-terminal kinases 1; KO, knock out; lncRNAs, long non-coding RNAs; MAPKs, mitogen-activated protein kinases; MI, myocardial infarction; miR, Micro RNA; MMPs, matrix metalloproteinases; NcRNAs, non-coding RNAs; NF-κB, nuclear factor κB; NFAT, nuclear factor of activated T-cell; Nm, nano meter; PDGFs, platelet derived growth factors; PDLIM5, PDZ and LIM domain 5; PPAR-α, peroxisome proliferators-activated receptor alfa; RAS, renin-angiotensin system; RNAs, ribonucleic acids; ROS, reactive oxygen species; SDF-1, stromal-derived factor-1; SORBS2, sorbin and SH3 domain-containing protein 2; TGF-β, transforming growth factor-β; TIMPs, tissue inhibitors of metalloproteinases; TNF-α, tumor necrosis factor-α.

INTRODUCTION

Intracellular communication is important in proficient and appropriate organization and function of various cells in multicellular organs. Multiple fundamental mechanisms are involved in the interactions between cells or even between different organs. For example growth factors, chemokines, adiponectin, small peptides, ECM proteins or sometimes direct cell-cell interaction are important for cellular communications (Corrado et al., 2013). However, in the last decade a considerable amount of experimental evidence has suggested that cells use a sophisticated method of communication using microvesicles called exosomes (Corrado et al., 2013; Maia et al., 2018). Exosomes are 30–120 nm size nanovesicles and have been identified in multiple cell types including stem cells for efficient intracellular communications (Mathivanan et al., 2010). Promising literature has shown that exosomes play a critical role in the shuttling of extraordinary sets of bioactive and signaling molecules which include membrane receptors, genetic materials, enzymes, cytokines and different bioactive materials in cells (Corrado et al., 2013; Cerezo-Magana et al., 2019). Thorough knowledge of a critical role for exosomes in the cardiovascular system is still developing, but establishment of novel tools and techniques in the past decade have boosted this research area significantly. Seminal work from others and our group has suggested that exosome-mediated intracellular signaling plays an important role in stem cell-mediated cardiac protection both in ischemic and hypertrophic heart failure (Sahoo et al., 2011; Mackie et al., 2012; Khan et al., 2015; Tseliou et al., 2015; Garikipati et al., 2018). Exosomes derived from stem cells provide an excellent cell-free system to improve cardiac function without significant immune response. Furthermore, cardioprotective factors such as miRs (**Figure 1**) and proteins packaged in stem cell exosomes may enhance the regenerative potential of stem cells to improve the endogenous repair process. Recently, it was shown that exosomes derived from IL-10-depleted EPCs exhibit altered exosomal content, which ultimately impairs the EPC's cardiac repair property (Garikipati et al., 2017). Interestingly, modulation of miR-375 using a miRNA antagomir in IL-10KO exosomes partially rescued endothelial cell function (Yue et al., 2017). These studies clearly indicate that the direct role of exosomes in CVDs and repair processes and alterations in exosomal contents could be beneficial in the treatment of heart disease.

Numerically, heart consists mainly of CFs (Zhou and Pu, 2016) and during ischemic/hypertrophic insults these fibroblasts become activated and involved in cardiac fibrosis and remodeling (Travers et al., 2016). Bang et al. (2014) have shown that fibroblast-derived exosomes have the ability to enhance cardiac myocytes hypertrophy in pressure-overloaded myocardium. The constituent analysis of these exosomes indicates that they are rich in passenger strands of miR such as miR-21*, a major signaling molecule which leads the hypertrophic signaling in heart. Interestingly, inhibition of miR-21 significantly reduced the cardiac hypertrophy and remodeling in this study (Bang et al., 2014). Furthermore, elevated level of miR-155 was found in macrophage-derived exosomes during heart injury (Wang et al., 2017). Intriguingly, Wang et al. (2017) has suggested that miR-155 in macrophage exosomes has potential to enhance proliferation and differentiation of resident fibroblasts and further exacerbate inflammation. These findings suggest that targeting selective molecules in cardiac fibroblast-derived (CF)-exosomes or inhibition of exosome secretion could be potential therapeutic approaches in heart failure treatment. It is also possible that exosomes from other cells such as immune cells can stimulate transition of naïve fibroblasts to activated myofibroblasts. Very limited literature is available regarding the activated fibroblasts exosomes and exosome-mediated paracrine signaling in cardiac fibrosis and remodeling. We hope that future rigorous studies on CF exosomes and mediated intercellular communications in the heart (between CFs and other cells or vice versa) will provide better understanding to develop novel therapies for CVDs. In this review article, we explore the current understanding of CFs; cardiac fibrosis; exosomes; exosomal biogenesis, structure, composition and involvement in cardiac fibrosis during heart failure. Additionally, we will discuss possibilities of exosomes as biomarkers for cardiac fibrosis and remodeling.

CARDIAC FIBROBLASTS AND CARDIAC FIBROSIS

Excessive cardiac fibrosis is a major problem in nearly all types of heart disease and significantly attributed by activation and excessive proliferation of CFs (Ali et al., 2014; Travers et al., 2016). During development, the CF population changes dramatically and regulates cardiomyocyte proliferation through multiple signaling pathways (Banerjee et al., 2007; Ieda et al., 2009). However, during disease states, excessive ECM proteins such as collagen accumulate and expand in the cardiac interstitium, which disrupts heart contractile capacity and impairs its systolic and diastolic function (Janicki and Brower, 2002; Berk et al., 2007; Kong et al., 2013). Upon cardiac insult, for example acute MI, cardiomyocytes die and a massive inflammatory and fibrogenic responses are triggered to develop a fibrotic scar as a reparative response (Frangogiannis, 2012; Humeres and Frangogiannis, 2019). Activated CFs, termed myofibroblasts, are phenotypically modified cells with differential expression of excessive ECM proteins including collagens, MMPs, and their inhibitors (Cleutjens et al., 1995; Kawaguchi et al., 2011). As compared to CFs, myofibroblasts are more contractile and express significantly more α-SMA and periostin. At an early stage of cardiac stress, these changes contribute to an adaptive repair process but eventually lead to adverse cardiac remodeling and progression toward heart failure (Tomasek et al., 2002; Hinz, 2007, 2010; Shinde et al., 2017). With or without cardiac injury, aging, chronic kidney disease, diabetes and obesity may also trigger cardiac fibrosis and remodeling (Biernacka and Frangogiannis, 2011; Cavalera et al., 2014; Hayer et al., 2018). The homeostasis of collagen turnover is tightly regulated by CFs and any imbalance in collagen metabolism leads to cardiac fibrosis (Janicki and Brower, 2002; Rathod et al., 2016). Cardiac fibrosis is mainly categorized into four types, based on the location and cause. The most prevalent two forms are the reactive

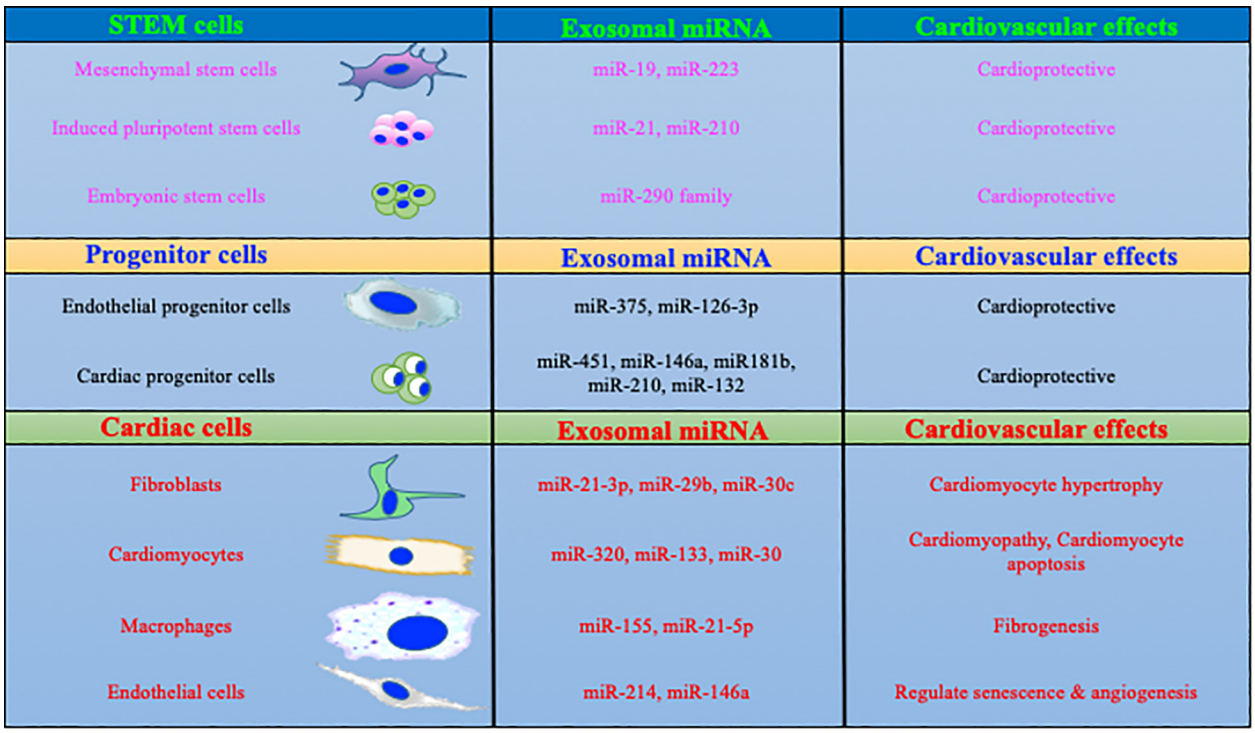

FIGURE 1 | MicroRNAs packaged in exosomes regulate cardiac biology. Exosomal content is dependent on the parent cells and its physiological status. More specifically, exosomes derived from macrophages and fibroblasts are enriched in miRs which are involved with profibrotic and inflammatory signaling. In contrast, stem/progenitor cells derived exosome contains cardio protective miRs.

interstitial and the replacement fibrosis. Interstitial fibrosis mainly describes the expansion of endomysium and perimysium, is caused by progressive deposition of extracellular proteins in the interstitial space, and leads to cardiomyocyte death. Whereas, replacement fibrosis occurs by necrosis of cardiomyocytes and is associated with systolic ventricular dysfunction, hypertrophic CM and myocarditis (Hashimura et al., 2017; Liu et al., 2017; Frangogiannis, 2019). A third category is infiltrative interstitial fibrosis, which occurs due to infiltration of inflammatory cells in right ventricles of systemic sclerosis-associated pulmonary arterial hypertension (Overbeek et al., 2010). The fourth type, termed endomyocardial fibrosis, is a primary cause of congestive heart failure in children under 2 years of age and underlying causes for this type are not well established but include infections, autoimmunity, genetic factors, and nutritional deficiencies etc. (Rohit et al., 2013; Duraes et al., 2019). The pathophysiology of cardiac fibrosis is mostly attributed to excessive synthesis and accumulation of ECM proteins by activated myofibroblasts. Even though cardiac fibrosis is involved in most forms of CVD, clinical interventions targeting cardiac fibrosis are not yet in hand. Cell population heterogeneity and lack of identification of cell-specific markers add to the complexity of designing and improving therapeutic intervention to reduce cardiac fibrosis.

Origin and Activation of Fibroblasts

Regardless of etiology, the origin of myofibroblasts remains controversial (Kong et al., 2014). Recent studies with lineage tracing strategies have suggested that cardiac fibrosis is primarily mediated by resident fibroblasts; however, other cell types (**Figure 2**) including monocytes/macrophages, endothelial cells, and hematopoietic fibroblast progenitors may also contribute to pathological fibrosis in heart (Ali et al., 2014; Kong et al., 2014; Moore-Morris et al., 2014, 2018). Endothelial cells and α-SMA-expressing mesenchymal cells have been shown to significantly contribute to fibrosis via their canonical Wnt-mediated endothelial-to-mesenchymal transition (EndMT) (Aisagbonhi et al., 2011). We have recently shown that bone marrow cells migrate to heart and transdifferentiate into myofibroblasts after myocardial damage, and thus contribute to tissue remodeling. In our study, we found that inflammatory stimulus acts as a catalyst for enhanced mobilization and homing of these bone marrow progenitor cells (Verma et al., 2017). In heart, cardiomyocytes/fibroblasts/resident macrophages secrete several chemokines such as SDF-1 which play an important role in migration of these cells to the heart (Mollmann et al., 2006; Chu et al., 2010). In addition to bone marrow cells, cardiac endothelial cells may also become myofibroblast-like cells by a process called EndMT and may be involved in pathological fibrosis during hypertrophic heart failure (Zeisberg et al., 2007).

Stimulator of Cardiac Fibrosis

Inflammatory stimulus and cardiomyocyte death are often the initial factors which stimulate a profibrotic signaling cascade in resident fibroblasts which secrete excess ECM proteins

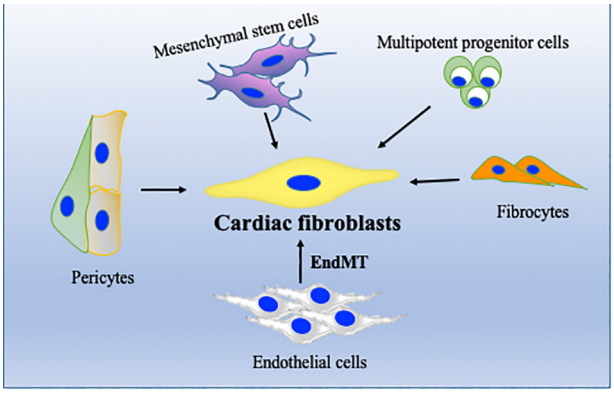

FIGURE 2 | Cellular sources of cardiac fibroblasts (CFs). In addition to resident fibroblasts, CF can originate from endothelial cells, through endothelial-to-mesenchymal transition (EndMT), from bone marrow stem/progenitor cells, from perivascular cells or from fibrocytes. Their activation and differentiation to become activated fibroblasts/myofibroblasts are highly dependent on pathological stress on the heart.

(Tomasek et al., 2002). The potential mechanism and type of profibrotic stimulation which leads this process mostly depends on the type of cardiac injury. Many different growth factors (TNFα, PDGFs, TGFβ), cytokines (IL-1, IL-10, IL-11), renin angiotensin system (RAS), and microRs are key modulators in initiation and progression of cardiac fibrosis (Saxena et al., 2013; Thum, 2014; Frangogiannis, 2015, 2019; Tao H. et al., 2016; Verma et al., 2017). RAS and TGFβ signaling are perhaps the most studied among all fibrotic pathways. Regardless of the type of cardiac injury, components of RAS are mostly produced by macrophages and resident fibroblasts and ultimately stimulate cardiac fibrosis by TGFβ signaling pathway. Upon activation, TGFβ modulates cellular functions in various cells including cardiomyocytes and fibroblasts and promotes the myofibroblast phenotype via canonical and non-canonical pathways (Kong et al., 2014). TGFβ-independent activation of human fibroblasts to myofibroblasts has also been studied and is involved in cardiac remodeling (Baranyi et al., 2019). Other than these stimulators, the roles of metalloproteinases (MMPs), TIMPs, and NF-κB are also well established in mediating cardiac fibrosis (Creemers et al., 2001; Kumar et al., 2011; George et al., 2016). As myofibroblasts are a major source of excessive ECM production, in addition to fibroblasts, macrophages also play an important role in ECM production and remodeling. Upon cardiac injury, macrophages adopt a more fibrotic M2 phenotype which has reduced expression of inflammatory cytokines like TNFα and interleukin-6 (IL-6) and increased secretion of IL-10, IGF1, TGFβ, and Gal-3 that are really key for ECM remodeling (MacKinnon et al., 2008; Suthahar et al., 2017). Non-immune cells like cardiomyocytes and CFs secrete pro-inflammatory cytokines to which myofibroblasts mostly respond and leads to excessive cardiac fibrosis (Yamauchi-Takihara et al., 1995; Porter and Turner, 2009; Aoyagi and Matsui, 2011). In addition to these inflammatory and profibrotic factors, GSK3β, β-catenin, TGFβ/SMAD-4, Wnt/β-catenin, MAPKs, and AKT signaling molecules and pathways play pivotal roles in cardiac fibrosis by regulating ECM metabolism, cardiomyocyte survival and proliferation, and maintaining wound healing after cardiac injury (Deb, 2014; Ma et al., 2018; Singh et al., 2019; Umbarkar et al., 2019; **Figure 3**). Among intracellular pathways, oxidative stress is a key factor that enhances cardiac fibrosis by triggering fibroblast proliferation and fibrotic signaling in heart. ROS generation has a dual role in cardiac fibrosis as studies reported both matrix-degradation and matrix-synthesis effects. High ROS levels increase TGFβ production and enhance CFs proliferation; paradoxically, high ROS also stimulates MMPs which facilitate ECM degradation (Siwik et al., 2001; Purnomo et al., 2013). Intriguingly, in the last decade, the role of ncRNAs, such as microRNA (miRNA), circular RNA and lncRNA, have been explored extensively in cardiovascular research and are suggested as an important trigger for various cardiovascular events including cardiac fibrosis and remodeling. Among these, microRNs are the most extensively studied NcRNA and play a critical role in regulation of fibroblast proliferation and fibrosis (Thum and Condorelli, 2015; Piccoli et al., 2016). For example, miR-21 is expressed in all heart cells and well characterized as profibrotic miRNA. It targets the expression of sprouty homology 1 (SPRY1), PTEN, and TGFβ receptor III (Huang et al., 2015). In contrast, miR-29 has been shown to reduce fibrosis by down-regulating expression of ECM genes, though in other studies miR-29 expression decreased during heart failure (van Rooij et al., 2008). Other miRNAs like miR-126, miR-15, miR-499, miR-24, miR-378, miR-1, miR-133, miR-26, miR-22, miR-199, miR-23, and miR-208 have also been studied considerably in different heart failure models (Montgomery et al., 2011; Melman et al., 2014; Tijsen et al., 2014; Suresh Babu et al., 2016; **Figure 3**).

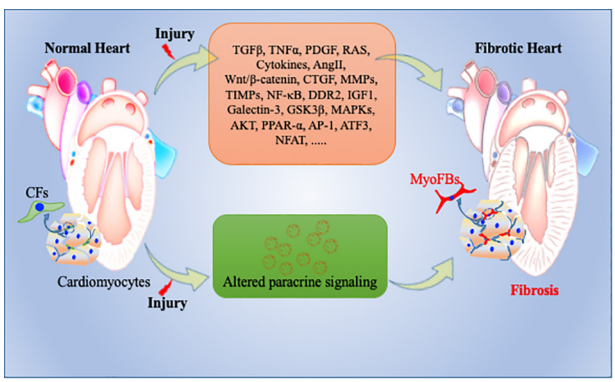

FIGURE 3 | Pathophysiology of cardiac fibrosis. During normal remodeling, adequate ECM proteins secreted by cardiac fibroblasts (CFs) are important to maintain cellular integrity in heart. However, upon cardiac injury, CFs transdifferentiate into activated myofibroblasts, secrete excessive amounts of ECM proteins, and expand the cardiac interstitium as a wound healing process. Many key molecular determents are involved in this repair process including exosomes. Altered molecular and paracrine signaling pathways can contribute to exacerbated activation and *trans*-differentiation of fibroblasts to myofibroblasts and to adverse/pathological cardiac remodeling and heart failure. CFs = Cardiac fibroblasts; myoFBs: myofibroblasts.

PARACRINE SIGNALING AND ITS ROLE IN CARDIAC FIBROSIS

Recently, enormous efforts have been made to explore the involvement of paracrine signaling in heart pathophysiology (Anthony and Shiels, 2013; Gartz and Strande, 2018). EVs, as a means of paracrine signaling, are bilayer membrane-bound cargoes and include exosomes, microvesicles and apoptotic bodies. Exosomes are extensively studied paracrine factors and can deliver various functional modulators such as proteins, lipids, DNA, mRNA, and miRNA. These transporter vesicles regulate a plethora of functions like cell differentiation, proliferation, senescence, cell death, cell–cell communication, angiogenesis, recycling of membrane lipids and proteins, and immunomodulation (Janowska-Wieczorek et al., 2005; Valadi et al., 2007; El Andaloussi et al., 2013; Xu and Tahara, 2013; De Jong et al., 2014). In the following section, we will focus on recent advancements in exosome research, especially on fibroblast biology and its role in myocardial fibrosis.

Exosomes: Structure and Function

Most living and actively functional cells secret 100–1,000 nm size micro-particles. Based on size, these microparticles are classified into three main subpopulations. The smallest particles (ranging from 30–120 nm) are termed exosomes (**Figure 4**). Exosomes were first discovered in 1946 as cellular waste and described as nanosized vesicles in 1981 (Chargaff and West, 1946; Valadi et al., 2007). Recent advances in biological science suggest that exosomes play important roles in multiple biological and pathological processes. Due to much complex biology and physiology in heart, exosome-mediated intracellular communication is an under-developed area in the cardiovascular field. Once exosomes are secreted into the extracellular system, they are stable for a relatively long period of time and can transfer cell specific signature signaling molecules to the target or recipient cells (Aryani and Denecke, 2016). Exosomes are mostly involved in cellular communication between different cell populations in multicellular organs. The main purpose of exosome-mediated intracellular signaling is to maintain cellular homeostasis and appropriate response to physiological stress. These nanovesicles contain various cellular components, such as mRNA, microRNA, DNA and membrane-bound or embedded proteins such as Alix, Tsg101 and tetraspanins (Frydrychowicz et al., 2015; Gurunathan et al., 2019). Tetraspanins are a group of transmembrane signaling proteins present in exosomes and in viable cells. The most common tetraspanins on exosomes are CD9, CD63, CD81, and CD82 and are commonly used as markers for characterization of exosomes. Exosomes may also contain Hsp (Hsp70 and Hsp90) and several intercellular adhesion molecules such as CD11a, CD11b, CD11c, CD18, CD146, CD166, and LFA-3/CD58 from their parent cells (Bobrie et al., 2011). In addition to these cellular proteins, exosomes contain a variety of genetic materials (mRNA and miRs) and are involved in angiogenesis, epigenetics, and gene regulation (Bobrie et al., 2011; Frydrychowicz et al., 2015). Although it was initially considered that exosomes are the entities whose primary function is to clear cellular waste, with increasing understanding of structural details and physiological function, study of cell-specific exosomes is currently a very hot area to explore disease pathobiology (**Figure 4**; Johnstone et al., 1987; Chang and Wang, 2019; Kelemen et al., 2019; Sole et al., 2019; Wang et al., 2019). Based on the broad array of content packaged in exosomes, we think that regulation of exosomal contents is a potential therapeutic strategy in heart disease treatment.

Role of Exosomes in Cardiac Fibrosis

Emerging evidence suggests that exosomes are secreted by most cardiac, vascular and stem cells in heart (Sahoo et al., 2011; Mackie et al., 2012; Khan et al., 2015; Tseliou et al., 2015; Garikipati et al., 2018). Thus, we surmise that all the various cells in heart use exosomes to communicate with each other viz. cardiac myocytes to endothelial cells, endothelial cells to smooth muscle cells, fibroblasts to cardiac myocytes and vice versa to regulate physiological or pathophysiological processes (Hergenreider et al., 2012; Bang et al., 2014; Wang et al., 2014). Thus, any alteration either in signaling molecules packaged in exosomes or in exosomal machinery can affect physiological homeostasis which ultimately results in heart disease. Exosomes play a central role in many cardiac diseases including MI, hypertrophy and ischemia (Xu et al., 2017). Recent evidence has shown that cardiac cell communication via exosomes is altered during fibrosis, a key mediator for heart diseases (Cosme et al., 2017). These novel findings are driving active research to study the role of fibroblast-derived exosomes and the effects of exosomes from other cells on fibroblasts to understand the pathophysiological consequences in fibrosis and heart disease.

Normally CFs contribute ∼70% of the cardiac cells and support cardiomyocytes by producing ECM and by regulating proliferation and migration of other cardiac cells (Furtado et al., 2016). Thus, fibroblasts play an important role in cardiac repair. However, in certain circumstances excessive proliferation and differentiation of fibroblasts lead to fibrosis and heart failure. Recent literature has shown that exosomes may also modify cardiac repair and fate of fibrosis via modulation of fibroblast function (Barile et al., 2017; Wang et al., 2017). Exosomes derived from cardiac progenitor cells (CPC) have potential to activate naive fibroblasts to initiate the wound healing process for myocardial repair (Barile et al., 2017). During cardiac injury, activated macrophages use exosomes enriched in miR-155 to regulate fibroblast differentiation to myofibroblasts resulting in more fibrosis. Thus, macrophage-specific inhibition of miR-155 or direct inhibition of this miRNA could be potential therapeutic approaches for regulation of cardiac injury (Barile et al., 2017). Recently, Yang et al. (2018) have found that cardiomyocyte-derived exosomes can promote cardiac fibrosis via myocyte-fibroblast cross-talk. It has been shown that injured epithelial cells secrete exosomes enriched with profibrotic factors, which can lead to fibrosis (Tomasek et al., 2002; Lee and Kalluri, 2010; Borges et al., 2013). Recently, we have shown that EPCs from IL-10KO mice secrete exosomes which are enriched with profibrotic and antiangiogenic factors and miRs. Alteration in exosomal contents significantly reduced fibrotic signaling after exosome

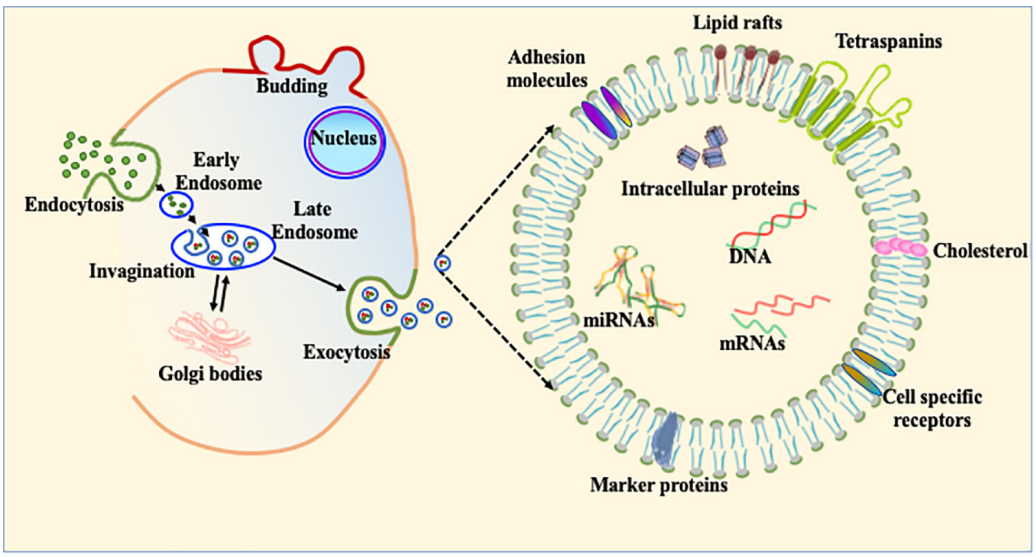

FIGURE 4 | Exosome biogenesis and its constituents. The schematic diagram of exosomal biogenesis. Exosomes are membranous vesicles 30–120 nm in diameter formed by the inward budding of the cellular membrane. This can shed off inward and fuse with early endosomes which become known as late endosomes and multivesicular bodies (MVBs). These nanovesicles carry various cellular components, such as mRNA, microRNA, DNA, and proteins within their bilayer lipid membrane such as Alix, Tsg101, and tetraspanins.

transplantation in heart post-MI (Garikipati et al., 2017; Yue et al., 2017). Thus, these publications indicate that exosomes from different cellular sources are acting as a significant determinant in regulating cardiac fibrosis. Though exosomes contain several important molecular signatures, the role of exosomal miRs and its significance in regulation of CVDs are recently highlighted in many articles (Valadi et al., 2007). We believe that modification of miRs using selective antigomir/mimic in exosomes can regulate pathological fibrosis and remodeling. To date, several miRs are identified which regulate fibroblast proliferation, differentiation and thus fibrosis (Garikipati et al., 2018; **Table 1**). Therefore, targeting those miRs in exosomes could be beneficial in reducing fibrosis and restoring heart function.

miR-Enriched Exosomes and Their Role in Cardiac Fibrosis

MicroRNAs (miRNAs, miRs) are highly conserved 21–25-nucleotide small NcRNA. They regulate target gene expression by binding to mRNAs and regulating the translation process (Thum and Condorelli, 2015; Piccoli et al., 2016). miRNA expression is altered in many CVDs including cardiac fibrosis and remodeling (Li et al., 2013; Tao L. et al., 2016). Recent advancements in technology helped to us to understand the direct role of miRs in cardiac biology and functions. For instance, during ischemic heart diseases, miRs play important roles in cardiac myocyte survival and thus improve heart function. Few miRs are exclusively expressed in muscle tissue (such as cardiac myocytes and skeletal muscles) and involved in cardiomyocyte contractility, survival and function. These miRs are termed myomiR (such as miR-1, miR-133, miR-206, miR-208, miR-486, and miR-499) (McCarthy, 2011; Chistiakov et al., 2016). As myomiRs are mainly expressed in muscle cells and play important roles in cell function, during myocardial damage, myomiR expression is altered tremendously (Gidlof et al., 2011). Interestingly, altered expression of many miRs such as miR-15, miR-21, miR-208a, miR-195, miR-29a, and miR-497 has potential to impair cardiac function post-injury (Hydbring and Badalian-Very, 2013; Porrello et al., 2013; Lin et al., 2015). Previous studies have suggested that CF activation and cardiac fibrosis are tightly regulated by sets of miRs termed fibrosis-associated miRNAs (Please see **Table 1**). Any alteration in these specific miRs can lead to exaggerated fibrosis. Interestingly, miR-433 is highly increased during myocardial ischemia and leads to cardiac fibrosis. This specific miR regulates MAPK 1 and TGF-β signaling pathways and thus enhances profibrotic signaling (Tao L. et al., 2016). Recently, Moghaddam et al. (2019) have mentioned that many miRs (such as miR-21, miR-22, and miR-24) are highly upregulated during acute ischemic injury. In addition, this group has also mentioned that miR-15, miR-34, miR-130, and miR-378 expression are noticeably reduced and are mainly responsible for the cardiac fibrosis after acute MI and IR injury models (Moghaddam et al., 2019). In summary, miRs from different cellular sources (Please see **Table 1**) can have ability to alter multiple molecular and cellular processes including cardiac fibrosis. Some sets of miRs can induce fibrosis and others can reduce it. Therefore, a balanced expression of these miRNAs is critically important during appropriate cardiac healing processes after any type of cardiac injury.

As we discussed, exosomes contain an extensive repertoire of genetic material including miRs. Recent reports have suggested that cells can also exchange miRs via exosomes, which can significantly alter the recipient cell's biology and function (Guay et al., 2015; Khalyfa et al., 2016; Qiao et al., 2019).

We have previously shown that miR-125b is an important miR in heart and plays an important role in activation of fibroblasts (Nagpal et al., 2016). Recently, Yang et al. (2018) found that exosomes derived from cardiomyocytes are enriched with miR-208a. At the molecular level, this study suggests that miR-208a enhances NFAT phosphorylation by targeting Dyrk2, preventing its entry into the nucleus in CFs, and therefore triggering fibrosis. Furthermore, Chaturvedi et al. (2015) have demonstrated that during exercise miRNA-29b and miRNA-455-enriched exosomes from cardiomyocytes can prevent fibrosis by downregulating MMP9 levels in diabetic mice. Furthermore, Bang et al. (2014) reported that in CFs, exosomal miRs are enriched with many miR passenger strands. In this study they found that fibroblast exosomes are enriched with miR-21* which has potential to induce cardiomyocyte hypertrophy by silencing expression of SORBS2 or PDLIM5 (Bang et al., 2014). Furthermore, Ang II-induced cardiac hypertrophy was effectively controlled by miR-21* inhibition in mice (Bang et al., 2014). In a very similar study, Lyu et al. (2015), showed that activated CF exosomes enhanced RAS signaling in cardiomyocytes, whereas inhibition of CF-exosome secretion by GW4869 (a potent EV inhibitor) significantly reversed Ang II-induced cardiac hypertrophy and remodeling. In addition, Wang et al. (2017) demonstrated that exosomal miR-155 inhibits both SOS and Suppressor of Cytokine Signaling 1 (SCS) expression, respectively, in fibroblasts and macrophages, thus regulating their proliferation. These studies suggest exosomes play important roles in fibroblast-mediated paracrine signaling. Furthermore, regulation of exosome biogenesis or content using pharmacological or molecular approaches could provide valuable therapeutic tools in regulation of heart failure.

Exosomes May Act as a Potential Biomarker in Cardiac Fibrosis

Recently, attempts have been made to use miRNAs or other signaling molecules in serum or plasma as diagnostic biomarkers for cancer. Researchers have found that the molecular constituents of exosomes are highly associated with parent cell phenotype and concurrent physiological/pathological condition. Thus, we can believe that exosomes are replicas of parent cells in regard to their molecular constituents. During biogenesis, exosomes receive multiple proteins via processing through endosomal pathways. These proteins are displayed on the exosome surface and include, but are not limited to, tetraspanins, heat shock proteins (HSP70) and proteins from the Rab family, Tsg101 and Alix (Bobrie et al., 2011; van der Pol et al., 2012). Therefore, proteins on the exosome surface may be utilized as diagnostic tools, as they have been proven very specific and clinically relevant (Lin et al., 2015). It has been shown that body fluids are rich in exosomes, and the specific biomolecules inside of, or on the surface of, exosomes can act as a biomedical tool to determine the disease stage or progression (Takata et al., 2008; Street et al., 2011). Careful investigation of exosomes in body fluids of patients with high risk factors for CVDs may provide us clinically useful information to diagnose these diseases at much earlier stages than previously possible. For example, cardiomyocytes secrete various muscle-specific miRNAs through

TABLE 1 | Regulatory miRNAs associated with cardiac fibrosis.

S. no.	miRNA	miRNA level	Cardiovascular disease	Target gene/pathway	Cardiovascular effects	References
1	miR-433	Overexpression	Cardiac fibrosis	AZIN1 and JNK1	Induce CF	Tao L. et al., 2016
2	miR-21-5p	Overexpression	Left ventricular hypertrophy	PPARα	Induce Hypertrophy	Chuppa et al., 2018; Moghaddam et al., 2019
3	miR-21-5p, miR-135b	Overexpression	Left ventricular hypertrophy, Cardiomyopathy	Wnt and Hippo pathway	Induce Fibrosis	Zhang H. et al., 2016; Moghaddam et al., 2019
4	miR-22	Downregulation	Cardiac fibrosis	TGFβRI	Induce CF	Jazbutyte et al., 2013; Hong et al., 2016
5	miR-29	Downregulation	Cardiac fibrosis	TGFβ/BNP	Induce CF	van Rooij et al., 2008; Chaturvedi et al., 2015
6	miR-34a	Overexpression	Cardiac fibrosis after MI and IR injury	SMAD4	Induce CF	Huang et al., 2014
7	miR-208a	Overexpression	Cardiac fibrosis	Dyrk2	Induce CF	Shyu et al., 2015; Yang et al., 2018
8	miR-132	Overexpression	Cardiac fibrosis	PTEN gene, PI3K/Akt	Inhibit CF	Zhang et al., 2018
9	miR-29a-c	Downregulation	Cardiac fibrosis	TGF-β/Smad3	Induce CF	Roncarati et al., 2014; Zhang et al., 2014; Harmanci et al., 2017
10	miR-669a	Downregulation	Cardiac fibrosis	MyoD	Induce CF	Quattrocelli et al., 2013
11	miR-455	Overexpression	Cardiac fibrosis	CTGF, LncRNA H19	Inhibit CF	Chaturvedi et al., 2015; Huang et al., 2017
12	miR-155	Overexpression	Cardiac fibrosis	TGF-β1–Smad 2	Induce CF	Zhang D. et al., 2016; Wang et al., 2017
13	miR-425, miR-744	Downregulation	Cardiac fibrosis	TGFB1 3′UTR	Induce CF	Wang et al., 2018

exosomes. During cardiac injury, elevated levels of these miRNAs can be detected in blood exosomes much earlier than detection of cardiac troponins or other markers (Xu et al., 2019). Enhanced levels of fibrosis-associated miRNAs (**Table 1**) such as miR-21, miR-425, miR-744, miR-208a, and others in plasma exosomes can also act as biomarkers in early diagnosis of hypertrophic heart diseases (Wang et al., 2018). In coronary bypass, patients' plasma exosomes are enriched with miR-1 and miR-133 and thus these miRs can be used to indicate disease progress (Emanueli et al., 2016). Matsumoto et al. (2013) has suggested that microRNAs miR-34a, miR-192, and miR-194 can be used as biomarkers to determine heart failure as well. Most studies mentioned here, and many more, have clearly indicated that miRNAs or other molecules packaged in exosomes may act as prognostic markers for heart diseases. However, rigorous investigations must be carried out in large cohorts of human patients before reaching at any final conclusion. We are optimistic that, in the near future, exosomes will be a powerful diagnostic marker to determine the progress of heart disease at early stages and will help our fellow clinicians manage this deadly disease in a more efficient manner.

CONCLUDING AND PROSPECTIVE REMARKS

Influence of exosome-mediated cardiovascular signaling and its role in CVDs have been rigorously studied and many insightful studies have been conducted and published in the recent past; however, we are still far from developing the exosome-based therapeutic for the treatment of CVDs. More in-depth research is warranted for fully understanding the biological aspects of loading, targeting, and delivery of exosomes, and for identifying the endogenous content of exosomes. Several unanswered questions remain to be addressed, such as (1) What regulates exosome biogenesis during heart failure? (2) How do cell type-specific exosomes exert their effect at the time of heart injury? (3) Does intense fibrotic response alter exosome-mediated signaling during CVD? (4) Are there qualitative and/or quantitative differences among fibroblast exosomes from various regions of the myocardium? and (5) Is it possible to alter exosomes to attenuate their detrimental effects and to enhance the benefits? It would be greatly beneficial to develop alternative strategies to engineer fibroblast (or any cell-specific) exosomes to enrich them with factors that target exosomes to the heart and appropriately repair the injury. Recent advancements in cardiovascular research indicate that exosomes may be used as a biomarker to determine heart disease at a much earlier stage than previously used biomarkers. As heart failure is a leading cause of morbidity and mortality both in developed and developing countries, developing novel biomarkers in the form of exosomes will meet a tremendous need to manage this number one lethal disease in a better manner.

AUTHOR CONTRIBUTIONS

PR generated the illustration. PR, RK, and SV wrote the manuscript. SV edited final draft of the manuscript. All authors drafted the manuscript.

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Extracellular Vesicles From Pathological Microenvironment Induce Endothelial Cell Transformation and Abnormal Angiogenesis via Modulation of TRPV4 Channels

*Brianna D. Guarino[1], Ravi K. Adapala[1,2], Anantha K. Kanugula[1], Nina M. Lenkey[1], Julie A. Dougherty[3,4], Sailaja Paruchuri[5], Mahmood Khan[3,4,6] and Charles K. Thodeti[1,2]**

[1] Department of Integrative Medical Sciences, College of Medicine, Northeast Ohio Medical University, Rootstown, OH, United States, [2] School of Biomedical Sciences, Kent State University, Kent, OH, United States, [3] Dorothy M. Davis Heart and Lung Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH, United States, [4] Department of Emergency Medicine, The Ohio State University Wexner Medical Center, Columbus, OH, United States, [5] Department of Chemistry, The University of Akron, Akron, OH, United States, [6] Department of Physiology and Cell Biology, The Ohio State University Wexner Medical Center, Columbus, OH, United States

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Karthikeyan Narayanan,
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Hirdesh Kumar,
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The University of Texas Health Science Center at Houston, United States
Fadi-Luc Jaber,
Albert Einstein College of Medicine, United States

***Correspondence:**
Charles K. Thodeti
cthodeti@neomed.edu

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The soluble and mechanical microenvironment surrounding endothelial cells influences and instructs them to form new blood vessels. The cells in the pathological tumor microenvironment release extracellular vesicles (EVs) for paracrine signaling. EVs have been shown to induce angiogenesis by communicating with endothelial cells, but the underlying molecular mechanisms are not well known. We have recently shown that the mechanosensitive ion channel transient receptor vanilloid 4 (TRPV4) expression and activity is significantly reduced in tumor endothelial cells (TEC), and that activation of TRPV4 normalized the tumor vasculature and improved cancer therapy. However, whether and how the tumor microenvironment downregulates TRPV4 and transforms the normal endothelial cell phenotype remains unknown. To explore this, we exposed normal human endothelial cells (hNEC) to human lung tumor cell conditioned media (TCM) and measured phenotypic changes and angiogenesis. We found that treatment with TCM transformed hNEC to a TEC-like phenotype (hTEC) as evidenced by increased expression of tumor endothelial cell marker 8 (TEM8) and exhibition of abnormal angiogenesis on 2D-Matrigels compared to normal hNEC. Mechanistically, expression and activity of TRPV4 was decreased in hTEC. Further, when pre-treated with exosome inhibitor GW4869, TCM failed to induce hNEC transformation to hTEC. Finally, addition of purified EVs from TCM induced

transformation of hNEC to hTEC as evidenced by abnormal angiogenesis *in vitro*. Taken together, our results suggest that the pathological (tumor) microenvironment transforms normal endothelial cells into a tumor endothelial cell-like phenotype through EVs via the downregulation of TRPV4.

Keywords: angiogenesis, endothelial cells, extracellular vesicles, tumor, TRPV4

INTRODUCTION

Angiogenesis, the formation of new blood vessels from the existing vessels, is a natural physiological process that occurs in response to tissue oxygen demand. Uncontrolled or insufficient angiogenesis can lead to ischemic heart disease, retinopathy and cancer (Folkman, 2002, 2006; Al-Latayfeh et al., 2012). In fact, solid tumors require angiogenesis for their growth and maintenance; however, tumor angiogenesis is unregulated resulting in aberrant vascular growth i.e., pathological angiogenesis (Jain, 2005a; Jain and Carmeliet, 2012). Therefore, the tumor vasculature exhibits high tortuosity, poor pericyte coverage, abnormal extracellular matrix (ECM) and hyper-permeability, causing an inefficient delivery of chemotherapies to the tumor (Siemann, 2011). Conventional anti-angiogenic therapies targeting soluble factors like VEGF have emerged as a means of overcoming the challenges posed by abnormal tumor vessels. Despite the initial results, which were promising, these anti-angiogenic therapies are often met with new challenges, such as acquired drug resistance by tumor endothelial cells (TEC) (Casanovas et al., 2005). Therefore, alternative approaches, such as restoring adequate blood flow and function of the tumor vasculature, i.e., vascular normalization is an attractive avenue of exploration (Jain, 2005b; Carmeliet and Jain, 2011).

To initiate angiogenesis, tumor cells must communicate with and recruit different cell types to form the tumor microenvironment (TME) which is made up of stromal cells such as pericytes, fibroblasts, endothelial cells (EC), ECM, and immune cells (Nyberg et al., 2008; Watnick, 2012; Samples et al., 2013; Yuan, 2016). These varying cell types release extracellular vesicles (EVs), such as exosomes and microvesicles, into the TME that are then taken up by recipient cells via endocytosis (Han et al., 2019; Maacha et al., 2019). EVs are small vesicles (30–150 nm for exosomes, 100 nm–1 μm for microvesicles) containing nucleic acids and proteins and are an important element of cell-to-cell communication. Emerging evidence has shown that EVs play a role in promoting tumor angiogenesis (Ludwig and Whiteside, 2018; Zimta et al., 2019), however, exact mechanisms have yet to be determined.

We have previously shown that transient receptor potential vanilloid 4 (TRPV4) is downregulated in TEC, and that pharmacological activation of TRPV4 channels normalizes the tumor vasculature and improves cancer therapy (Adapala et al., 2016). However, whether and how the TME induces a downregulation of TRPV4 channels in TEC remains unknown. Therefore, the main goal of this study is to show that tumor cell conditioned media (TCM) can induce downregulation of TRPV4 channels. In the present study, we investigated if a pathological microenvironment, such as TME, can transform normal ECs into a TEC-like phenotype and its underlying mechanisms.

MATERIALS AND METHODS

Tumor Cell Conditioned Media (TCM)

Adenocarcinomic human alveolar basal epithelial cells (A549) were from ATCC and cultured in normal DMEM high glucose media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. After the cells reached 80% confluence, complete media was replaced with serum free DMEM high glucose for 24 h, collected, and centrifuged at 1200 rpm. After centrifugation, TCM was filtered and stored at −80°C for future use. For the inhibition studies, A549 cells were pre-treated with the exosome inhibitor, GW4869 (10 μM), for 24 h, prior to adding serum free DMEM high glucose as described above.

Cell Culture

Human microvascular endothelial cells (HMEC-1) were purchased from ATCC (Manassas, VA, United States), cultured in MCDB-131 media supplemented with 10% (FBS), 1% penicillin streptomycin, 1% L-glutamine, 1 μg/mL hydrocortisone, and 10 ng/mL human vascular endothelial growth factor (Kanugula et al., 2019). HMEC-1 were denoted as human normal endothelial cells (hNEC). To investigate the effect of TCM on endothelial cells, hNEC were cultured in a medium containing TCM: HMEC-1 media (75:25) for five continuous passages. At the end of the five passages, these cells exhibited a tumor-endothelial like phenotype and termed as human tumor endothelial-like cells (hTEC).

Quantitative PCR (qPCR)

RNA was extracted from EC by using the RNeasy Mini Kit (Qiagen, Hilden, Germany) and quantified with a Biotech 96-well plate reader. RevertAid First Strand cDNA Synthesis Kit was used to synthesize cDNA, and Fast SYBR green master mix was used for qPCR on the Fast-Real-Time PCR system (both from Thermo Fisher Scientific). The following real-time primers were obtained from Integrated DNA Technologies (Coralville, IA, United States): β-actin (forward-5′-ACGTTGCT ATCCAGGCTGTG-3′, reverse: 5′-GAGGGCATACCCCTCGT-AGA-3′) and TEM8 (forward-5′-GCTATTATGTGTCCC GTCTCTATG, reverse: 5′-GGTGGGTGTTGGAGAGTATTG). Gene expression was normalized to β-actin, and $\Delta\Delta C_t$ values were expressed as a fold change relative to hNEC.

In vitro Angiogenesis Assay

Growth factor reduced Matrigel® (BD Biosciences) was placed in a 48-well plate and kept at 37°C for a total of 30 min (Adapala et al., 2016; Thoppil et al., 2016). Cells (1×10^5 cells/well) were plated on the Matrigel and kept at 37°C for 24 h. In some experiments, cells were pre-treated and plated together with Rho kinase inhibitor, Y27632 (10 μM) on Matrigel. Tube length was quantified using ImageJ software. For EV experiments, hNEC cultured in serum free MCDB-131 media combined with normal HMEC-1 media (75:25) were treated with 100 μg/mL of purified EVs (total EV protein) or PBS as control for 48 h before plating them on Matrigel.

Western Blot Analysis

Cells were lysed in RIPA buffer containing protease and phosphatase inhibitor cocktails (MilliporeSigma and Roche, Basel, Switzerland). Lysates were loaded into 7.5% precast polyacrylamide gels (Bio-Rad) for electrophoresis. Gels were transferred onto a PVDF membrane and blocked in 5% milk powder in tris-buffered saline (TBS) with 0.1% Tween-20. Membranes were incubated overnight at 4°C with primary antibodies: TRPV4 (1:300; Alomone Labs, Jerusalem, Israel, or 1:300; Biorbyt, San Francisco, CA, United States), and GAPDH (1:5000; Cell Signaling Technology). After incubation, membranes were washed 3× with TBS-Tween-20 for 10 min each, followed by 1 h incubation at room temperature in appropriate secondary antibody, goat anti rabbit (1:5000) conjugated with horseradish peroxidase (Cell Signaling Technology). Signals were detected with Clarity western luminol/enhancer solution and peroxide solution (Bio-Rad laboratories, Hercules CA, United States), and developed with a FluorChem M Simple Imager (Protein Simple, San Jose, CA, United States). Quantification was performed using ImageJ software.

Calcium Imaging

Endothelial cells were cultured on MatTek glass bottom dishes (MatTek, Ashland, MA, United States). Cells were loaded with Fluo-4/AM (4 μM) for 25 min and calcium influx was monitored as previously described (Adapala et al., 2011, 2016) on Olympus FluoView 300 confocal microscope (Olympus, Shinjuku, Tokyo, Japan) after stimulation with the TRPV4 agonist, GSK1016790A (100 nM).

Immunocytochemistry

Cells were cultured on glass coverslips in a 6-well plate and fixed in 4% paraformaldehyde (PFA) for 20 min. After fixing, cells were washed 3× with 1× PBS, permeabilized for 15 min with 0.25% TritonX-100 solution and blocked for 30 min in 10% FBS-containing media. Cells were incubated for 1 h at room temperature with VEGFR2 primary antibody (1:200; Cell Signaling Technology), washed 3× in 1× PBS, incubated for 1 h at room temperature with appropriate Alexa Fluor conjugated secondary antibody (1:200; Thermo Fisher Scientific). Cells were then washed 3× in 1× PBS and mounted with DAPI containing mounting medium (Vector Laboratories, Burlingame, CA, United States) on glass slides. Images were captured using an Olympus IX-71 fluorescence microscope (Olympus).

Extracellular Vesicle Isolation and Characterization

Extracellular vesicles were isolated and characterized as previously described (Dougherty et al., 2018). Briefly, $^1/5$ volume of ExoQuick-TC reagent (SBI, Mountain View, CA, United States) was added to the TCM. TCM was then incubated overnight at 4°C, followed by centrifugation at 1,500 × *g* for 30 min (4°C) to pellet EVs. Another round of centrifugation was performed to remove any residue. EVs were re-suspended in PBS and stored at −80°C for future use. Nanoparticle Tracking Analysis (NTA) was performed by diluting EVs with PBS and loading onto a Malvern Nanosight NS300 (Malvern, United Kingdom). Video was analyzed via Nanosight NS300 NTA software v3.00 (Malvern, United Kingdom). EVs were sonicated in ethanol, deposited on 400 carbon coated mesh grids and the cryo-Transmission electron microscopy (TEM) imaging was carried out in a FEI Tecnai F20 microscope operated as described previously (Gao et al., 2014).

Statistical Analysis

The data was analyzed with student's *t*-test using SPSS V. 24 software. The significance was set at $^*p \leq 0.05$; $^{****}p \leq 0.0001$. All values expressed as mean ± SEM.

RESULTS

Tumor Cell Conditioned Media Transforms Human Normal Endothelial Cells (hNEC) Into a Tumor Endothelial Cell-Like (hTEC) Phenotype

In order to mimic the TME *in vitro*, we repeatedly exposed hNEC to TCM. First, we found an increased gene expression of tumor endothelial marker 8 (TEM8) in TCM-treated hNEC (hTEC) compared to untreated cells (**Figure 1A**). Next, in *2D*- angiogenesis assays, while hNEC formed robust tubes that stabilized until 24 h, TCM-treated EC formed tubes at 6 h but were collapsed and retracted at 24 h (**Figures 1B,C**; $p \leq 0.0001$), which is reminiscent of mouse TEC (Adapala et al., 2016). Taken together, these findings suggest that TCM can transform normal EC (hNEC) into a tumor endothelial cell-like (hTEC) phenotype.

Tumor Cell Conditioned Media Induces hNEC Transformation to hTEC via Downregulation of TRPV4 and Reduction of Perinuclear VEGFR2

Next, we investigated the molecular mechanism by which TCM induces EC transformation by focusing on TRPV4 channels. We have previously shown that TRPV4 expression and activity is downregulated in mouse TEC (Adapala et al., 2016). Therefore, first, we measured the expression of TRPV4 in hNEC and hTEC. As shown in **Figures 2A,B**, western

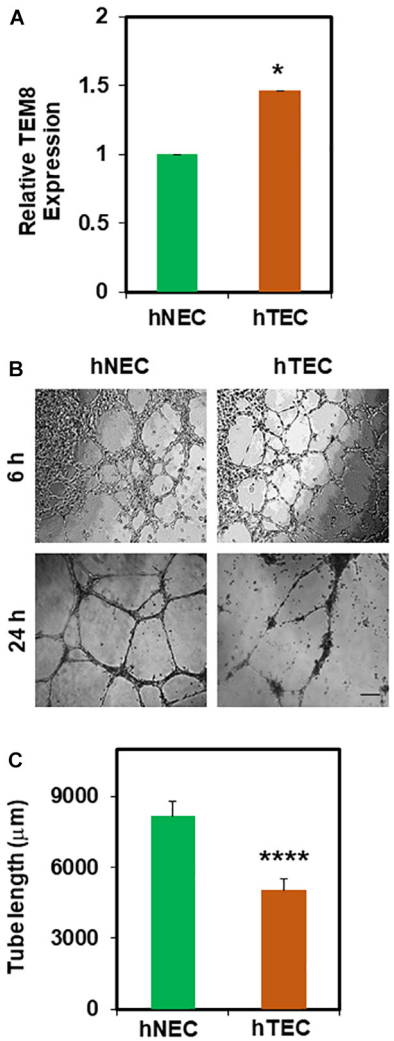

FIGURE 1 | Tumor cell conditioned media (TCM) induces transformation of hNEC to tumor endothelial-like (hTEC) phenotype. **(A)** qPCR analysis showing relative TEM8 gene expression in untreated hNEC and TCM-treated hNEC (hTEC). Note, TEM8 mRNA expression is increased significantly in hTEC ($^*p \leq 0.05$). Gene expression was normalized to β-actin and calculated as relative expression to hNEC. **(B)** Phase contrast images (4×) showing the angiogenic behavior of hNEC and hTEC when plated on 2D Matrigel at high density (100,000/well) at 6 and 24 h. Scale bar = 200 μm. **(C)** Quantitative analysis showing a significant decrease ($^{****}p \leq 0.0001$) in tube length in hTEC compared to hNEC. The results shown are a mean ± SEM from three independent experiments.

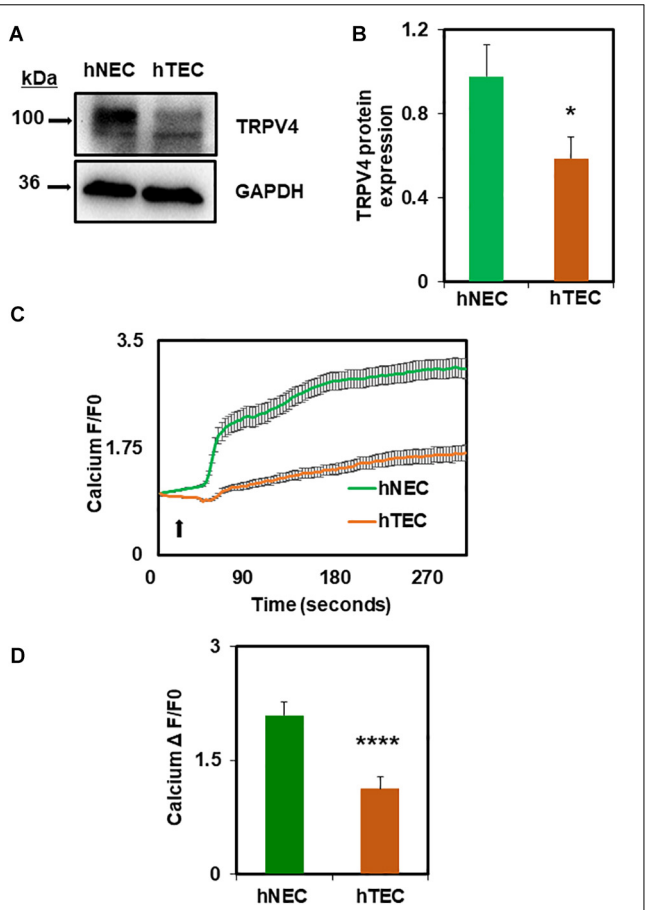

FIGURE 2 | TCM exposure downregulates functional expression of TRPV4 channels in hTEC. **(A)** Western blot analysis showing decreased expression of TRPV4 protein in hTEC when compared to hNEC. **(B)** Quantitative analysis of western blots revealed significant ($^*p \leq 0.05$) reduction of TRPV4 expression in hTEC. **(C)** Average traces showing calcium influx in response to the TRPV4 agonist, GSK1016790A (100 nM), in Fluo-4 loaded hNEC and hTEC. Arrow indicates the time of stimulation with TRPV4 agonist. **(D)** Quantitative analysis of calcium influx showed significant $^{****}p \leq 0.0001$ reduction in TRPV4-mediated calcium influx in hTEC compared to hNEC (F/F0 = ratio of normalized fluorescent intensity relative to time 0). The results shown are a mean ± SEM from three independent experiments.

blot analysis revealed that TRPV4 expression (two bands above and below 100 kDa) was significantly lower in hTEC compared to hNEC ($p \leq 0.05$). Further, calcium imaging revealed that TRPV4-mediated calcium influx in response to the specific agonist GSK1016790A, was significantly lower in hTEC ($p \leq 0.0001$) compared to hNEC (**Figures 2C,D**). We next asked if the reduction in TRPV4 altered vascular endothelial growth factor receptor 2 (VEGFR2) or Rho/Rho kinase pathways in hTEC, which we have previously shown to be activated in TRPV4 null (TRPV4KO) or TRPV4 siRNA knocked down EC (Kanugula et al., 2019). Indeed, immunostaining revealed a significant reduction of perinuclear VEGFR2 levels in hTEC compared to hNEC ($p \leq 0.0001$) (**Figures 3A,B**). Finally, we found that pre-treatment with Rho kinase inhibitor, Y-27632 (Y27), normalized abnormal angiogenesis phenotype exhibited by hTEC (**Figures 3C,D**; $p \leq 0.0001$).

Extracellular Vesicles From Tumor Cell Conditioned Media Induce Abnormal Angiogenic Phenotype in hNEC

In order to find out the active component of TCM that imparts a tumor endothelial-like phenotype, we focused on EVs from TME, which were shown to modulate angiogenesis (Kikuchi et al., 2019). To achieve this, we first treated tumor

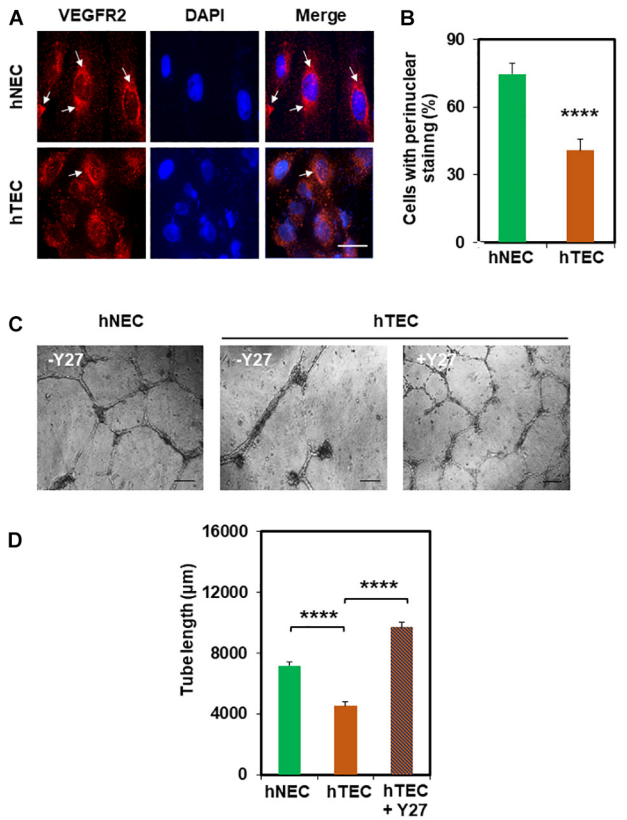

FIGURE 3 | hNEC transformation is correlated with the reduction in perinuclear VEGFR2 which is normalized by Rho kinase inhibitor Y27632. **(A)** Representative immunofluorescence images (60×) showing VEGFR2 localization in hNEC and hTEC. Cells were stained for DAPI (blue/nuclei) and total VEGFR2 (red) after exposure to TCM in 9 consecutive passages (hTEC). Scale bar = 10 μm. Note a strong perinuclear localization of VEGFR2 (arrows) in hNEC which was reduced in hTEC. **(B)** The graph represents the quantitative analysis of the percentage of cells with perinuclear localization of VEGFR2 showing a significant (****$p \leq 0.0001$) decrease in perinuclear VEGFR2 staining of hTEC. **(C)** Phase contrast micrographs (4×) showing the normalizing effects of the Rho kinase inhibitor, Y-27632 (10 μM; Y27) on hTEC angiogenic behavior, when plated on Matrigel at high density (100,000/well). Representative images were taken 24 h post plating, showing the formation of stable tubes upon inhibition of Rho kinase in hTEC. Scale bar = 200 μm. **(D)** Quantitative analysis of tube length showing a significant decrease (****$p \leq 0.0001$) in hTEC tube length compared with hNEC which was significantly increased (****$p \leq 0.0001$) after treatment with the Rho kinase inhibitor. The results shown are a mean ± SEM from three independent experiments.

cells with the exosome inhibitor, GW4869, and measured EV formation using NTA (Nanoparticle Tracking Analysis). As shown in **Figure 4A**, we found 50–200 nm sized particles in untreated TCM, the levels of which decreased in TCM-treated with GW4869 (**Figure 4A**). We then exposed hNEC to TCM untreated or treated with GW4869 for 5 consecutive passages and assessed their functional phenotype in a 2D-angiogenesis assay. We found that while cells exposed to untreated TCM formed tubes that collapsed at 24 h, GW4869-TCM exposed cells formed tubes that were stable even at 24 h (**Figures 4B,C**). Together, these data confirm the presence of EVs in TCM, and that inhibition of exosomes abolishes EC transforming ability of TCM. To further confirm the role of EVs in EC transformation, we isolated EVs from TCM and confirmed their identity with NTA and TEM. While we found 50–200 nm particles in NTA, TEM images showed round structures of 50–150 nm in EVs isolated from TCM (**Figures 5A,B**). Finally, hNECs treated with purified EVs but not PBS-treated controls, exhibited tumor EC-like tube formation with complete tube retraction at 24 h ($p \leq 0.0001$) (**Figures 5C,D**). Taken together, these data confirm that treatment with tumor derived EVs transforms normal EC to tumor endothelial cell-like EC.

DISCUSSION

The TME is a heterogenous mix of cellular and acellular components including stromal cells, extracellular matrix, soluble factors and EVs secreted by both tumor and stromal cells. Emerging evidence has shown that tumor-derived EVs play an important role in cell-to-cell communication within the TME (Han et al., 2019; Qiao et al., 2019; Tai et al., 2019). EVs have been demonstrated to carry a cargo of DNAs, RNAs, miRNAs, proteins, lipids, and cytokines that facilitate tumor growth, progression and metastasis by delivering them to stromal cells in TME. Specifically, EVs isolated from tumors/tumor cells were shown to induce angiogenesis via interaction with endothelial cells *in vitro* and *in vivo* (Becker et al., 2016; Feng et al., 2017). However, there are no reports on EV's effects on transformation of normal EC to a tumor EC-like phenotype. In the present study, we demonstrate that repeated exposure to TCM (i.e., pathological tumor microenvironment) transforms normal endothelial cells to a tumor endothelial-like phenotype as evidenced by increased TEM8 expression and abnormal angiogenesis *in vitro*. Further, we show that TCM treatment downregulated functional expression of TRPV4 in EC. Furthermore, we demonstrate that TCM treated with an exosome inhibitor significantly reduced EV production as well as transformation of hNEC. Finally, we found that EVs isolated from TCM induced a tumor endothelial cell-like phenotype.

Although tumors recruit EC from either surrounding vasculature or bone marrow for the initiation of tumor angiogenesis, over time these tumor derived EC (TEC) transform into an aberrant phenotype characterized by increased expression of TEMs, enhanced proliferation, migration and abnormal angiogenesis. However, the molecular mechanisms by which tumors impart this phenotype to TEC is not known. In the present study, we found that exposure to TCM transformed hNEC to hTEC as evidenced by increased expression of TEC marker TEM-8 and abnormal angiogenesis. Importantly, we found that the active component of TCM is EVs as evidenced by (a) their typical round structure of 50–200 nm size confirmed by TEM and NTA, (b) attenuated synthesis and abolishment of transformative effect on hNEC after the treatment with an exosome inhibitor, GW4869, and (c) transformation of hNEC to hTEC-like phenotype by isolated EVs. EVs secreted by tumors increase angiogenesis

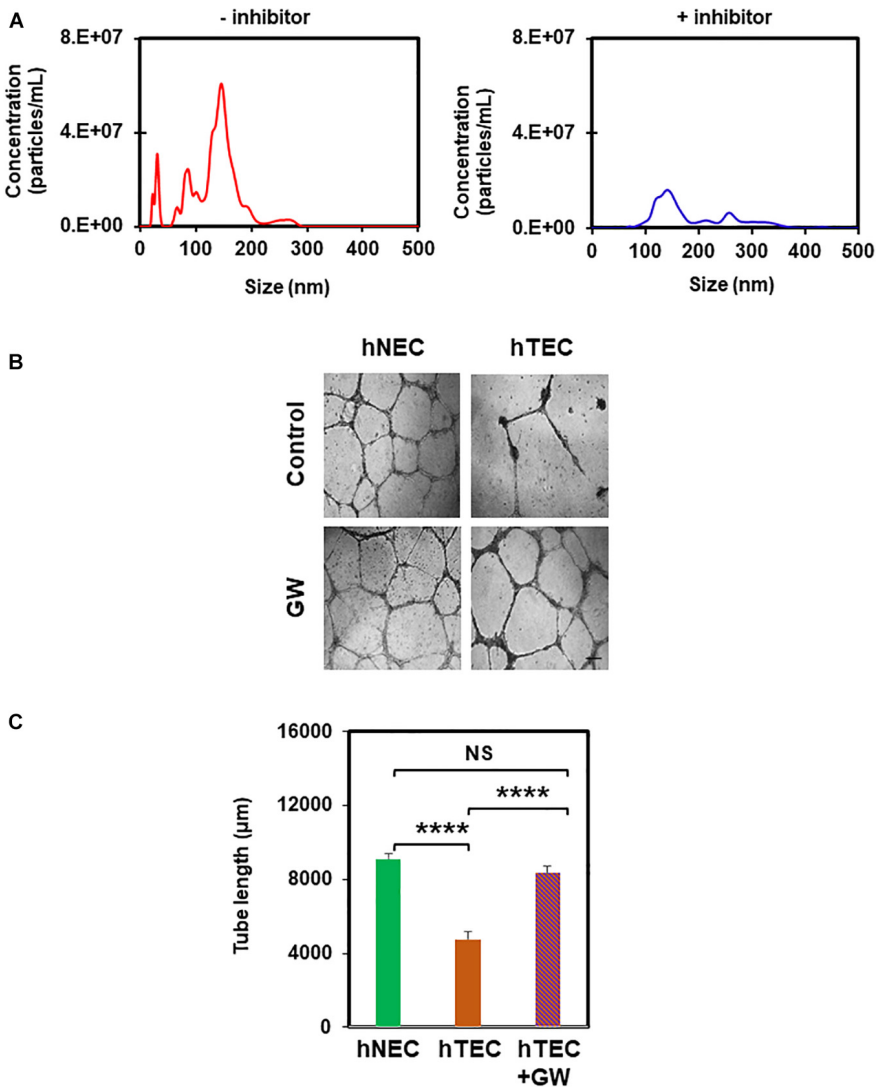

FIGURE 4 | Exosome inhibitor reduces formation of EVs and abolishes TCM-induced EC transformation. **(A)** Nanoparticle tracking analysis (NTA) showing reduction in the number of EVs from tumor cells that had been pre-treated with the exosome inhibitor GW4869 (GW). **(B)** Phase contrast micrographs (4×) showing normalized tube formation in hTEC exposed to GW4869 and plated on 2D Matrigels for 24 h compared to untreated hTEC and hNEC. Sale bar = 200 μm. **(C)** Quantitative analysis showing a significant increase (****$p \leq 0.0001$) in tube length between hTEC and hTEC + GW (GW4869) cells. Note a significant decrease in tube formation between hNEC and hTEC (****$p \leq 0.0001$), and no statistical difference (NS) between hNEC and hTEC + GW. The results shown are a mean ± SEM from three independent experiments.

by delivering pro-angiogenic growth factors or inducing pro-angiogenic genes to endothelial cells (Becker et al., 2016; Feng et al., 2017). EVs derived from glioblastoma and grown in specifically from hypoxic environment, carry number of proangiogenic cytokines and growth factors and stimulate angiogenesis via modulation of HIF, MMP 9 and LOX (King et al., 2012; Kucharzewska et al., 2013). We have previously shown that the abnormal phenotype of TEC, at least in part, comes from the functional downregulation of TRPV4 channels, as overexpression or pharmacological activation of TRPV4 normalizes TEC phenotype (Adapala et al., 2016). Here, we found that exposure to TCM significantly downregulated expression of TRPV4 in hTEC compared to hNEC. Further, TRPV4-mediated calcium influx was significantly attenuated in these cells. Downregulation of TRPV4 has been shown to activate VEGF/VEGFR2 pathway via Rho kinase/YAP (Kanugula et al., 2019), and indeed, we found that TCM treatment significantly reduced perinuclear VEGFR2 suggesting activation of VEGFR2 in hTEC (Manickam et al., 2011; Wang et al., 2017). Further, pharmacological inhibition of Rho kinase was able to normalize abnormal angiogenesis exhibited by hTEC. These findings suggest that TRPV4 downregulation by TCM/EVs transforms hNEC to hTEC via activation of VEGF/VEGFR2 pathway. Although we provide evidence for TRPV4 downregulation by EVs, the underlying molecular mechanism is not known. We speculate that EVs mediate these

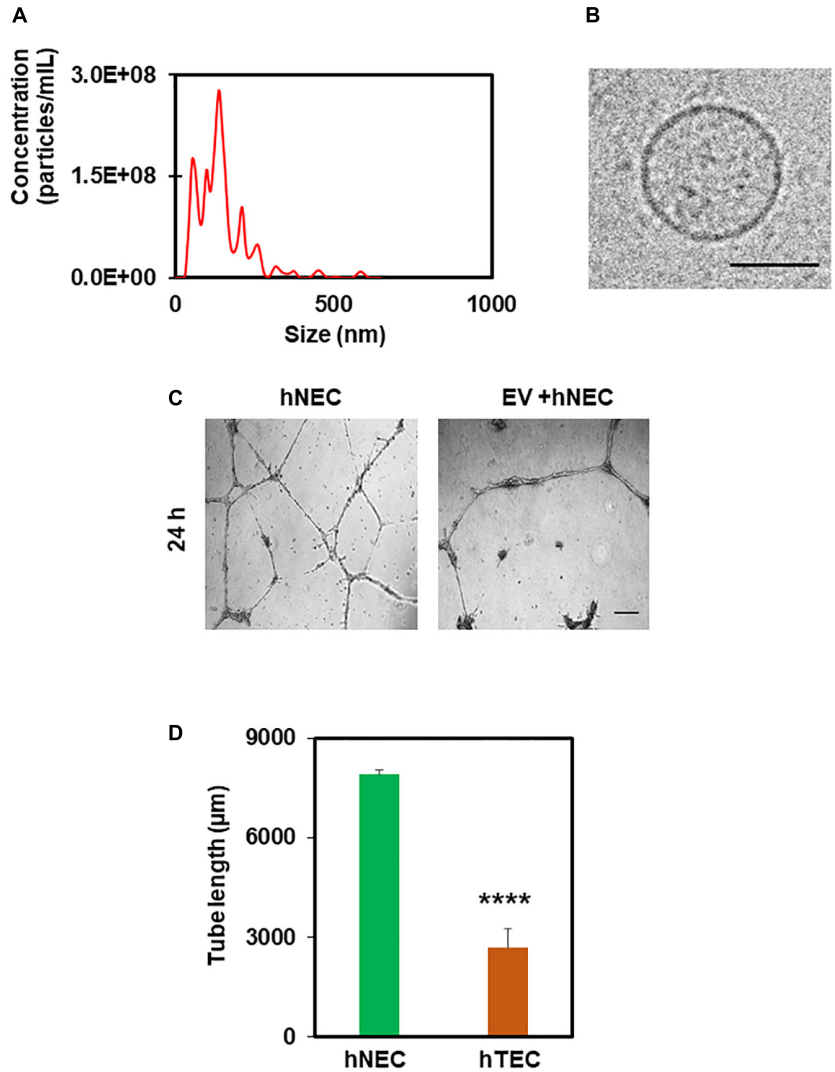

FIGURE 5 | Tumor-derived extracellular vesicles (EVs) induce hNEC transformation to hTEC as evidenced by abnormal angiogenesis. NTA analysis **(A)** and TEM images **(B)** showing 50–200 nm rounded structures confirming the isolation EVs from TCM. Scale bar = 50 nm. **(C)** Phase contrast micrographs (4×) showing tube formation of control and EV-treated cells plated on 2D Matrigel at 24 h. Scale bar = 200 μm. **(D)** Quantitative analysis showing a significant decrease (****$p \leq 0.0001$) in tube length by EC (hTEC) treated with tumor-derived EVs for 48 h. The results shown are a mean ± SEM from three independent experiments.

effects through microRNA, specifically miR-203. miR-203 is the only miRNA shown to target and downregulate TRPV4 expression in chondrocytes and hepatic stellate cells (Hu et al., 2013; Song et al., 2014). However, the role of miR-203 in EV-mediated downregulation of TRPV4 in EC needs to be determined. EVs released into TME either by tumor cells or stromal cells were shown to induce tumor angiogenesis, ECM remodeling and metastasis, albeit through activating known pro-angiogenic signaling molecules such as HIF, MMP, PI3K/AKT and LOX (King et al., 2012; Kucharzewska et al., 2013). However, the findings from our study, for the first time, show that tumor-derived EVs target a mechanosensitive calcium channel, TRPV4 and transforms normal EC to tumor EC-like phenotype, which may explain the mechanisms underlying the abnormal vasculature in the tumor.

CONCLUSION

Overall, our results demonstrate that tumor cells communicate with endothelial cells via secretion of EVs into TME, which transforms normal EC into tumor-like EC. These findings have pathophysiological significance as anti-angiogenic therapies based on VEGF have shown limited success in treating cancer (Lupo et al., 2016; Zarrin et al., 2017; Abdalla et al., 2018; Itatani et al., 2018) because tumor cells develop resistance over the time. Moreover, exposure to anti-VEGF molecules was shown to affect tumor perfusion and can impede delivery of chemotherapeutic drugs (Van Der Veldt et al., 2012). Therefore, vascular normalization approaches aimed at restoring tumor vasculature, allowing for effective delivery of chemotherapies (Jain, 2005b; Carmeliet and Jain, 2011; Goel et al., 2011)

gained much attention. Our findings show that EVs from tumor cells transform normal endothelial cells to a tumor endothelial cell-like phenotype via downregulation of TRPV4 and identifies TRPV4 as an alternative target for tumor angiogenesis and cancer therapy.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

BG, RA, AK, NL, and JD performed the research, analyzed the data, and edited the manuscript. SP edited the manuscript. MK designed and analyzed the data on exosomes and edited the manuscript. CT designed, interpreted, and analyzed the data as well as wrote the manuscript.

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REVIEW
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Exosomes in the Regulation of Vascular Endothelial Cell Regeneration

Jugajyoti Baruah[1,2] *and Kishore K. Wary*[3]*

[1] Department of Psychiatry, Harvard Medical School, Boston, MA, United States, [2] Angiogenesis and Brain Development Laboratory, Division of Basic Neuroscience, McLean Hospital, Belmont, MA, United States, [3] Department of Pharmacology, The University of Illinois at Chicago, Chicago, IL, United States

Exosomes have been described as nanoscale membranous extracellular vesicles that emerge from a variety of cells and tissues and are enriched with biologically active genomic and non-genomic biomolecules capable of transducing cell to cell communication. Exosome release, and exosome mediated signaling and cross-talks have been reported in several pathophysiological states. Therefore, exosomes have the potential to become suitable for the diagnosis, prognosis and treatment of specific diseases, including endothelial cell (EC) dysfunction and regeneration. The role of EC-derived exosomes in the mechanisms of cardiovascular tissue regenerative processes represents currently an area of intense research activity. Recent studies have described the potential of exosomes to influence the pathophysiology of immune signaling, tumor metastasis, and angiogenesis. In this review, we briefly discuss progress made in our understanding of the composition and the roles of exosomes in relation to EC regeneration as well as revascularization of ischemic tissues.

Keywords: angiogenesis, exosomes, endothelial cells, regeneration, rejuvenation

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***Correspondence:**
Kishore K. Wary
kkwary@uic.edu

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INTRODUCTION

Exosomes are nanometer (30–100 nm) sized membranous vesicles originating during the formation of multivesicular bodies (MVBs) (Dignat-George and Boulanger, 2011; Ribeiro et al., 2013; Boulanger et al., 2017; Théry et al., 2018). Biochemically, exosomes are characterized by the presence of CD63, CD14, TSG101, heat shock protein and flotillin; these exosomes can be sedimented at 120,000 × g (Théry et al., 2006; Vlassov et al., 2012; Kowal et al., 2016). Exosomes and apoptotic bodies are broadly classified as extracellular vesicles; in contrast to exosomes, apoptotic bodies are larger (500–5000 nm) (Caruso and Poon, 2018), and they sediment at 1,200–10,000 × g (Théry et al., 2006). Exosomal biogenesis occurs in the endosomal pathway and is characterized by biochemical properties (**Table 1**), whereas apoptotic bodies are characterized by membrane blebbing and their unique cell surface markers, e.g., phosphatidylserine and Annexin-V (Henson et al., 2001). Exosomes are usually released into the extracellular space when MVBs fuse with the plasma membrane, and exosomes can transport lipids, mRNAs and proteins that can alter cellular behavior in a paracrine or autocrine manner (Sahoo and Losordo, 2014; Kishore and Khan, 2016). Depending on the tissue microenvironment, and the exosome content, these vesicles mediate an array of cellular functions (Ribeiro et al., 2013). A classic example of an altered tissue microenvironment is the maintenance and repair of tissues in response to injury. Studies are

TABLE 1 | Biochemical properties of apoptosis, exosomes and MVBs.

	Size (nm)	Morphology	Sedimentation speed	Origin	Mechanism of formation	Known pathways
Apoptotic bodies	30–100	Heterogeneous	1,200–10,000 × g	Plasma membrane	Budding from the plasma membrane	Apoptotic pathway
Exosomes	30–100	Cup-shaped	100,000–120,000 × g	Multivesicular body (MVB)	Exocytosis of MVB	ESCRT-dependent, Tetraspanin, ceramide
Microvesicles	100–1000	Heterogeneous	100,000–200,000 × g	Plasma membrane	Budding from the plasma membrane	Ca^{++}-dependent, stimuli- and cell-dependent

beginning to document cell-cell signaling events that mediate restorative functions in the tissue microenvironment. In this context, the mechanisms of exosome uptake by target cells might be mediated by more than one mechanism. In cultured cells, exosome uptake can occur through: (a) clathrin-dependent endocytosis, (b) caveolae-dependent endocytosis, (c) phagocytosis, and (d) micropinocytosis (McKelvey et al., 2015). Whether exosome uptake by target cells is a physiologically regulated process remains incompletely understood.

Tissue repair mechanisms entail effective endothelial cell (EC) regeneration and reestablishment of blood flow in damaged and ischemic tissues. To accomplish this repair process, ECs that form the innermost linings of the blood vessels undergo regeneration and angiogenesis to support the restoration of tissue homeostasis (Carmeliet, 2005; Liu et al., 2019; Miyagawa et al., 2019; Williams and Wu, 2019). EC regeneration is a complex biological process that include EC migration, EC survival, rapid proliferation, tube formation, and ultimately reperfusion of injured tissues to restore homeostasis of the tissue microenvironment (Carmeliet, 2005; Bentley and Chakravartula, 2017; McDonald et al., 2018; Liu et al., 2019; Williams and Wu, 2019). Although several studies have attempted to understand the process of EC or vascular regeneration, the molecular mechanism that drives this process remains incompletely understood. Given the biological properties of exosomes and the events that they can regulate, the idea that exosomes derived from various cell types, including ECs themselves in the damaged tissue niche, can modulate EC regeneration remains an active area of research (Ibrahim et al., 2014; Li et al., 2016; Abid Hussein et al., 2017; Balbi et al., 2017; Adamiak and Sahoo, 2018; Dougherty et al., 2018; Ju et al., 2018; Bian et al., 2019; Cheng et al., 2019). In support of this notion, we describe a compendium of studies conducted over the past decade that highlight both EC- and non-EC derived exosomal molecular cargoes which drive this regenerative process. The idea and the discussions that exosomes might provide therapeutic benefit in the settings of ischemic cardiovascular diseases involving physiological injuries that might otherwise transition to disease states, should be rewarding efforts.

EC REGENERATION LIKELY INVOLVES MORE THAN ONE MECHANISM

Broadly, there are at least three major types of ECs in mammalian systems, related to arterial, venous, and lymphatic vessels (Coultas et al., 2005; Adams and Alitalo, 2007; Park et al., 2013; Qiu and Hirschi, 2019). These mature ECs are known to be arrested at the G_0-phase of the cell cycle, and they have a limited turn-over rate, cycling once every 3–5 years *in vivo*. Thus, ECs are considered terminally differentiated cells. In principle, ECs could regenerate from adult EC stem cells; however, there is conflicting evidence regarding whether adult EC-stem cells actually exist *in vivo*. Many studies suggest the existence of adult hemangioblast and or angioblast, on the basis of CD34 and Flk1 expression (and other stem cell markers, e.g., Brachyury and Er71/Etv2), and the ability of these cells to form tube-like structures (Asahara et al., 1997; Loges et al., 2004; Hirschi, 2012). On the contrary, others have argued that these cells are likely to be present in low numbers in adults *in vivo* (Rafii, 2000; Park et al., 2013; Yoder, 2018; Qiu and Hirschi, 2019). Critiques have noted that bone marrow-derived monocytes and macrophages might have been misidentified as endothelial progenitor cells, thus confusing even the experts (Medina et al., 2017). However, genetic lineage tracing experiments in mice remain inconclusive regarding the presence of EC-stem cells. Recent article summarized the proangiogenic benefit observed in preclinical and clinical studies from over 700 patients in clinical trials of CD34 + cell therapy (Sietsema et al., 2019). Nevertheless, developmental studies suggest that venous ECs can be derived from arterial ECs, whereas lymphatic ECs can be derived from venous ECs (Wang et al., 1998; Yang et al., 2012). However, depending on the type of injury or damage experienced by the ECs, more than one mechanism is likely to activate EC regeneration. Our own studies have suggested that ECs become proliferative after experimental ischemia or myocardial infarction (Kohler et al., 2014; Baruah et al., 2017). Another mechanism is dedifferentiation followed by redifferentiation of ECs in the aftermath of ischemia, a process that can also be activated by administration of low-dose small molecule inhibitors of GSK-3b called BIO (6-bromoindirubin-3-oxime) and tideglusib/NP12 (Kohler et al., 2014; Baruah et al., 2017). Yet another mechanism might be the endothelial to mesenchymal transition (EndoMT) (Dejana and Lampugnani, 2018), a biological process that occurs during the formation of cardiac valves and contributes to the emergence of several other cell lineages (Monaghan et al., 2016), and is also a response to ischemia (Manavski et al., 2018).

Thus, it is reasonable to hypothesize that exosome-mediated regeneration of ECs is likely to include at least three distinct mechanisms, but not limited to:

- Exosomes that induce EC proliferation and survival, e.g., vasculogenesis and angiogenesis.
- Exosomes that induces EC dedifferentiation/ redifferentiation (not a proven mechanism): for example, exosomes that upregulate Cyclin-D1 and

down-regulate p53, p21, and p27 mRNAs should induce EC-dedifferentiation and rapid cell cycle progression.
- Exosomes that mediate EndoMT (not a proven mechanism); in principle, exosomes containing microRNAs (miRNAs) that downregulate VE-cadherin and up-regulate Twist, Slug and Snail, and matrix metalloproteases (MMPs) could mediate EndoMT.

Thus, genomic and non-genomic cargoes in exosomes that are capable of inducing signaling to one of the above events should provide EC regenerative benefit. In addition, regeneration of ECs might be possible via exosomes that mediate transdifferentiation of somatic cells or by directly reprogramming somatic cells into ECs.

In support of this idea, a few groups have addressed the possibility of using exosome mediated reprogramming of ECs for vascular regeneration (Cheng et al., 2017; Lee et al., 2017). For example, exosomes secreted by tumor cells carry a number of potent pro-angiogenic factors such as VEGF, TGFβ, bFGF, MMP2, and MMP9, mediated angiogenic activities of ECs (Skog et al., 2008; Giusti et al., 2016; Ludwig and Whiteside, 2018). This idea is currently being explored further in several laboratories in the settings of cardiovascular regeneration and rejuvenation. However, it remains to be seen if the exosome(s) mediated reprogrammed ECs have the ability to repair effectively and reestablish blood supply productively, in aftermath of ischemic episodes.

EXOSOMES WITH NON-GENOMIC CARGOES THAT MEDIATE EC REGENERATION

Myocardial infarction represents a major cause of death among all cardiovascular diseases. Injured cardiac tissues due to myocardial infarction or ischemic insult trigger a series of adaptive response, to initiate and drive repair the injured heart. Therefore, it was surmised that in the aftermath of myocardial infarction the injured myocardium might release extracellular vesicles and exosomes that could induce a regenerative program. Cardiac extracellular vesicles or exosomes are now known to be present in both normal and infarcted heart (Chistiakov et al., 2016). Therefore, these exosomes that are secreted in an infarcted heart mediate various cell to cell communication events, including exosome biogenesis which provide cardiovascular regenerative benefits, improved cardiac function, and normalize tissue homeostasis (Barile et al., 2012; Waldenström et al., 2012; Wang et al., 2016). In a study, human pediatric cardiac progenitor cell (CPCs) prepared from the right atrial appendages from children of different ages undergoing cardiac surgery for congenital heart defects were isolated and cultured under hypoxic or normoxic conditions. In their, study, the authors found that CPC exosomes derived from neonates improved cardiac function, mediated angiogenesis, and reduced fibrosis, independent of culture oxygen levels (Agarwal et al., 2017). However, there are many open questions that need to be addressed (Bollini et al., 2018). A detailed overview of exosomes and their regenerative potential in infarcted heart can be found elsewhere (Bollini et al., 2018; Shanmuganathan et al., 2018).

At the cellular level, EC proliferation and survival represent two key events in the process of EC regeneration (Park et al., 2013; Qiu and Hirschi, 2019). These cells must proliferate rapidly and survive to make up for the loss of cells or to replace damaged and non-functional cells. Inadequate proliferation or enhanced cellular death might initiate or augment pathological event. Therefore, well-coordinated cellular proliferation and survival events are quintessential to normalizing damaged tissues (McDonald et al., 2018).

A study on exosome cargo and EC interaction has been conducted by Nazarenko et al. (2010) in a tumor microenvironment. This study has described a role of Tetraspanin (Tspan8) containing exosomes, which efficiently induce angiogenesis in tumors and tumor-free tissues. The authors have found that Tspan8 contributes to selective recruitment of proteins and mRNAs into exosomes; these markers include CD106 and CD49d, which have been implicated in exosome-EC binding and EC internalization. Exosome uptake induces vascular endothelial growth factor (VEGF)–independent regulation of several angiogenesis-related genes, including *von Willebrand factor, Tspan8*, the chemokines *CXCL5*, and *MIF*, the chemokine receptor *CCR1* and, together with VEGF, *VEGF receptor 2* (Nazarenko et al., 2010). EC uptake of Tspan8-CD49d complex–containing exosomes is accompanied by enhanced angiogenic activities of EC, such as proliferation, migration, and sprouting. Several studies subsequently exploited the potential of exosome cargoes in a tumor-free environment. Accordingly, one elegant investigation by Sahoo et al. (2011) has shown that exosomes derived from human $CD34^+$ stem cells mediate EC proliferation and survival, thereby stimulating the angiogenic activities of ECs. As expected, exosomes purified from human induced pluripotent stem cells have been found to induce angiogenesis and improve recovery in a mouse model of hind limb ischemia (Hu et al., 2015). Bian et al. (2013) have examined the effects of mesenchymal stem cell (MSC) derived extracellular vesicles which also included exosomes, and found that exosomes mediated efficient regeneration of ECs in a rat model of acute myocardial infarction. Although this study did not conclusively identify the types of molecules involved in this process, it highlighted the potential role of exosomes in mediating angiogenic processes in an injured tissue microenvironment (Shabbir et al., 2015; Teng et al., 2015). The Wnt/b-catenin signaling pathway is crucial in regulating both developmental and therapeutic angiogenesis (Dejana and Kühl, 2010). Interestingly, MSC exosomes express Wnt4, which induces translocation of (β-catenin into the nuclei of recipient ECs, thereby promoting angiogenic events in a rat skin burn model (Zhang et al., 2015). Similar studies have demonstrated that the Sonic hedgehog signaling pathway, the presence of platelet derived growth factor receptor in the extracellular vesicles or PKA signaling might contribute to the proangiogenic activity (Benameur et al., 2010; Ma et al., 2017; Xue et al., 2018). In a study, cardiomyocyte derived exosomes containing heat

shock protein (Hsp20) showed increased EC proliferation by interacting with VEGF receptor-2 (Zhang et al., 2012). This finding highlighted the key role of exosomes in tissue restorative processes. Notch-Dll4 signaling has been extensively studied in relation to angiogenesis, whereby the expression of Dll4 ligand in tip cells regulates the sprouting of ECs (Gerhardt et al., 2003; Kangsamaksin et al., 2014; Pitulescu et al., 2017). In a 3D matrix microenvironment, exosomes containing Dll4 freely moved to target ECs and mediated efficient Notch activation upon interaction with the recipient ECs (Sharghi-Namini et al., 2014). In addition, Dll4-containing exosomes increased EC motility while decreased proliferation. Dll4 is known to be present during tissue reparative processes, and targeting Dll4 will be critical to mediating efficient angiogenic recovery in injured tissues. Angiogenesis is also regulated by the activities of MMPs, which mediate cell-matrix or cell-cell interaction during the migratory phase. In this context, MMP14 containing exosomes have been shown to cleave VEGFR1 and promote VEGF-A induced migration and proliferation of ECs (Han et al., 2019).

Ding et al. (2019) have studied the effects of exosomes derived from bone marrow MSCs and found that they have superior angiogenic properties and enhance cell proliferation. The authors additionally found that deferoxamine conditioned exosomes activate the PI3/AKT pathway, thereby enhancing cell proliferation and decreasing wound lesions (Ding et al., 2019). Interestingly, exosomes derived from MSCs released high levels of the proangiogenic molecule stromal cell derived factor 1 (SDF1), which not only prevented apoptotic cell death of myocardial cells but also induced cardiac EC regeneration in a mouse model of myocardial infarction (Gong et al., 2019). Hypoxia inducible factor-1α (HIF-1α) is an important mediator of angiogenic activity during ischemic insult. Exosomes prepared from human umbilical cord MSCs have been found to enhance fracture repair and angiogenesis in a rat model of stabilized fracture through HIF1α (Zhang et al., 2019). Beyond studies of heart and skin injury models, the ability of exosome mediated regeneration has also been tested in a mouse model of traumatic brain injury (Gao et al., 2018). Here, the authors addressed the role of exosomes derived from endothelial colony forming cells in their ability to restore the blood brain barrier continuity (Gao et al., 2018). However, whether exosomes can also mediate EC regeneration via EndoMT in addition to the above mentioned mechanisms remain incompletely understood. **Figure 1** and **Table 2** summarize some of the key genomic and non-genomic cargoes implicated in the regeneration of ECs and angiogenesis.

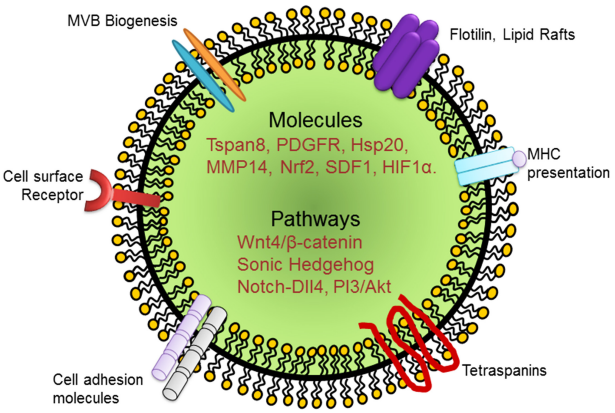

FIGURE 1 | Schematic representation of an exosome and its cargo carrying growth factors, cytokines, and signaling molecules that have been implicated in EC regeneration. PDGFR, platelet derived growth factor receptor; Hsp, heat shock protein; MMP, matrix metalloprotease; SDF, stromal derived factor; HIF, hypoxia inducible factor.

TABLE 2 | Non-genomic and genomic exosomal constituents and their known endothelial cell activities.

Cellular activity	Cargo	References
EC proliferation and survival	**Non-genomic**	
	• Tetraspanin (Tspan8)	Nazarenko et al., 2010
	• Wnt4	Zhang et al., 2015, 4
	• PDGFR	Ma et al., 2017
	• Sonic hedgehog pathway	Benameur et al., 2010
	• Protein kinase A signaling	Xue et al., 2018
	• pathway	Zhang et al., 2012
	• Heat shock protein (Hsp20)	Sharghi-Namini et al., 2014
	• Notch-Dll4	Han et al., 2019
	• MMP14	Ma et al., 2017
	• Nrf2	Gong et al., 2019
	• SDF1	Zhang et al., 2019, 1
	• HIF1α	
EC proliferation and survival	**Genomic**	
	• miRNA-146a	Ibrahim et al., 2014
	• miRNA-294	Khan et al., 2015
	• miR-21-3p	Hu et al., 2018
	• miR-939	Li et al., 2018
	• miR-423-5p	Xu et al., 2019
	• miR-210	Ma et al., 2018
	• miR-199-5p	Ye et al., 2019)

EC, endothelial cells.

EXOSOMES WITH GENOMIC CARGOES THAT MEDIATE EC REGENERATION

In addition to transporting growth factors and receptors, exosomes possess the unique ability to transfer miRNAs to recipient cells. miRNAs regulate downstream signaling events through base pairing of their seed sequence with complementary mRNA (Lu and Clark, 2012). MSCs, as well as ECs, contain different regulatory miRNAs, which alter cellular function in target cells (Hromada et al., 2017; Ferguson et al., 2018). In this context, miRNA-146a enriched exosomes secreted from cardiosphere-derived cells have been shown to enhance angiogenesis while simultaneously stimulating proliferation and inhibiting apoptosis of cardiomyocytes (Ibrahim et al., 2014). Exosomes derived from embryonic stem cells have also been exploited in this regard. Accordingly, mouse ESC-derived exosomes have been shown to provide beneficial effects in regeneration after myocardial injury via miR-294 (Khan et al., 2015). Different miRNAs have been implicated in this reparative

process. In a more recent study, miR-21-3p enriched exosomes secreted by human umbilical cord blood cells have been shown to accelerate cutaneous wound healing and promote angiogenic events (Hu et al., 2018). Yet another interesting study conducted in a patient population with myocardial ischemia has reported that coronary serum exosomes regulated angiogenesis through miR-939 in this sample group (Li et al., 2018). Human adipose derived stem cell exosomes also exert similar proangiogenic effects via miR-423-5p and Sufu (Xu et al., 2019). In addition, exosomes loaded with miR-210 exert beneficial effects favoring EC function and reoxygenation (Ma et al., 2018). Thus, exosomes can transport different combinations of miRNAs depending on the tissue environment and cell type (Kim et al., 2012; Ferguson et al., 2018). We have listed a select group of miRNA cargoes transported by exosomes known to regulate EC regeneration and angiogenesis in **Figure 2** and **Tables 2**, **3**. Nevertheless, continued analyses of the miRNA compositions of various exosomes should be useful in designing custom exosomes for the induction of potent EC regeneration in relation to angiogenesis and revascularization of ischemic cardiovascular tissues. A complete understanding of the role of exosomes in regenerative process in the aftermath of myocardial infarction could bridge an important gap in knowledge of the repair mechanism after myocardial injury.

FUTURE PERSPECTIVES

The capacity of the exosomes to induce EC regeneration should benefit organ repair and survival after injury. EC regeneration and the ways in which therapeutic exosomes contribute to this process have the potential in treating ischemic cardiovascular diseases. Thus, substantial progress has been made in the field of exosome research, providing insights into exosome composition and function. Ongoing methodological and technical innovations are beginning to help further synthesize new knowledge, functional understanding and potential applications. However, detail studies are needed to address the possible heterogeneity of exosomes and how this new knowledge could benefit the understanding of EC regeneration and EC pathology. For example, are there specific stimuli that induce the release of "exosomes" that mediate angiogenic activities of ECs, but do not alter the behavior of any other cell type? Are there exosomes that induce rapid proliferation of ECs, but not non-ECs? Are there specific exosomes that inhibit fibrosis, but induce productive wound healing in the aftermath of acute myocardial infarction? These are some of the few questions that come to mind as we ponder the future of exosomes in applications in EC regeneration and re-establishing blood flow to the ischemic cardiovascular organs. Studies have attempted to determine the regenerative ability of exosomes primarily in inbred (e.g., C57BL/6) mouse strains, in experiments such as hind limb ischemia and myocardial infarction. Usually, C57BL/6 mice show robust EC regenerative activities. The question remains whether exosomes provide potent EC regenerative responses in a strain-specific manner. Experiments are also needed in clinically relevant models, for example, mice with defective revascularization potential, such as diabetes. Unraveling the molecular and functional attributes of exosomes and how they may be harnessed should contribute meaningfully to the pursuit of controlling the biology of ECs for regenerative therapy. The answers to these questions and concerns should arrive soon, as new technological innovations such as organoids, data science, computational modeling and artificial intelligence are being incorporated into cardiovascular research (Garikipati et al., 2018; Trac et al., 2019).

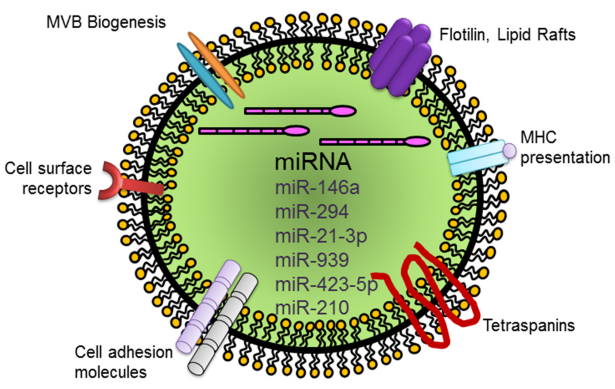

FIGURE 2 | Schematic representation of a generic exosome and its cargo carrying miRNAs that mediate EC regeneration. Abbreviation: miRNA, microRNA.

TABLE 3 | Exosomes in endothelial cell proliferation and angiogenesis.

Key findings	References
Improved angiogenesis in rat hind limb ischemia model	Johnson et al., 2019
Promoted EC repair in a rat model of balloon –induced carotid artery injury	Hu et al., 2019
Enhanced repair effect in a rat model of myocardial infarction	Ni et al., 2019
Promoted postnatal angiogenesis in mice bearing ischemic limbs	Ye et al., 2019
Human induced-pluripotent stem cell-derived cardiomyocytes promoted angiogenesis	Dougherty et al., 2018
Exosomes derived from ischemia subjected cardiomyocytes promoted cardiac angiogenesis	Ribeiro-Rodrigues et al., 2017
Human pericardial fluid derived exosome promoted therapeutic angiogenesis	Beltrami et al., 2017
Enhanced the density of new functional capillary and blood flow recovery in rat myocardial infarction model	Teng et al., 2015

CONCLUSION

In principle, more than one mechanism is likely to be involved in regulating EC repair and regeneration, and reestablishing flow

of blood to the ischemic organs. However, there are technical challenges that must be addressed before exosomes could be used as therapeutic biologics from bench to bedside. In this review, we have attempted to summarize how the cargo composition of exosomes derived from several human and non-human sources might benefit EC repair and regeneration. It would be a "giant leap" to be able to reprogram autologous somatic cells directly to ECs by using exosomes, thereby eliminating the use of viral vectors. However, continued research will be required before this interesting idea can be translated into therapy through EC regeneration and restoration of cardiovascular function.

AUTHOR CONTRIBUTIONS

Both authors wrote, corrected, and reviewed the final manuscript. KW submitted the manuscript online.

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frontiers in Cell and Developmental Biology

REVIEW
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Utilization of Human Induced Pluripotent Stem Cells for Cardiac Repair

Chengming Fan[1,2], Eric Zhang[1], Jyotsna Joshi[3], Jinfu Yang[2], Jianyi Zhang[1] and Wuqiang Zhu[3]**

[1] Department of Biomedical Engineering, School of Medicine and School of Engineering, University of Alabama at Birmingham, Birmingham, AL, United States, [2] Department of Cardiovascular Surgery, The Second Xiangya Hospital, Central South University, Changsha, China, [3] Department of Cardiovascular Medicine, Physiology and Biomedical Engineering, Mayo Clinic, Scottsdale, AZ, United States

The paracrine effect, mediated by chemical signals that induce a physiological response on neighboring cells in the same tissue, is an important regenerative mechanism for stem cell-based therapy. Exosomes are cell-secreted nanovesicles (50–120 nm) of endosomal origin, and have been demonstrated to be a major contributor to the observed stem cell-mediated paracrine effect in the cardiac repair process. Following cardiac injury, exosomes deriving from exogenous stem cells have been shown to regulate cell apoptosis, proliferation, angiogenesis, and fibrosis in the infarcted heart. Exosomes also play a crucial role in the intercellular communication between donor and recipient cells. Human induced pluripotent stem cells (hiPSCs) are promising cell sources for autologous cell therapy in regenerative medicine. Here, we review recent advances in the field of progenitor-cell derived, exosome-based cardiac repair, with special emphasis on exosomes derived from hiPSCs.

Keywords: exosomes, induced pluripotent stem cells, cardiac, repair, regeneration

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****Correspondence:***
Jianyi Zhang
jayzhang@uab.edu
Wuqiang Zhu
Zhu.Wuqiang@mayo.edu

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INTRODUCTION

Cardiovascular disease caused by coronary obstruction accounts for up to 80% of all cardiovascular-related deaths (Lloyd-Jones et al., 2010). Cell-based therapies centered around the transplantation of stem cells and/or their derivatives into the infarcted heart, has been tested in preclinical and clinical studies over the past decade and demonstrated to be a promising strategy for the treatment of cardiovascular diseases (Barile et al., 2017b). Since the development of induced pluripotent stem cell (iPSC) technology in 2006 (Takahashi and Yamanaka, 2006), iPSCs have emerged as one of the most promising stem cell sources for therapeutic applications in cardiovascular field (Zhu et al., 2018). As patient-specific iPSCs are derived from patients themselves, they possess extensive self-renewal and differentiation potential. Despite encouraging advances in iPSCs technology, challenges remain in administration of iPSCs in the preclinic studies, including low rate of engraftment and the potential risk of tumorigenesis, which still impede clinical application of iPSCs and iPSCs-based derivatives (Jung et al., 2017; Taheri et al., 2019).

While the primary goal of stem cell-based therapy is to generate new cardiac muscle, recent data from both clinical and preclinical studies have indicated that transplanted stem cells may exert their functional beneficial effects largely through their secretome, by which the paracrine activity

of the transplanted cells is mediated in part through their secreted vesicles (Merino-Gonzalez et al., 2016). Significant preclinical developments of exosome-based regeneration medicine have been achieved thus far through recognizing that it is the paracrine cues from transplanted iPSCs and/or its derivatives, that impart the major beneficial effects of regeneration within the injured tissues, rather than the direct effect of surviving transplanted cells (Wang et al., 2015; Dougherty et al., 2017, 2018; Ye et al., 2019). Furthermore, recent studies establish that exosomes derived by donor cells play a critical role in this paracrine regenerative mechanism (Kim et al., 2015; Merino-Gonzalez et al., 2016; Ju et al., 2018; Youn et al., 2019). In addition, significant functional improvement after intravenous cell therapy in patients with heart failure are most likely caused by the release of exosomal vesicles from the transplanted cells which are accumulated in organs such as the lung (Bartolucci et al., 2017). The application of stem cell−derived exosome-based technology as a cell-free strategy of preclinical application, has been identified in the progress of several fields, such as molecular diagnostic markers, drug delivery systems and potential target therapeutic agents (Joladarashi et al., 2015; Joladarashi and Krishnamurthy, 2017; Jung et al., 2017). In the cardiac regenerative medicine, stem cell-derived exosomes have been recognized as one of the key therapeutic factors, including the stimulation of cardiac repair of the injured heart tissues (Taheri et al., 2019). Exosomes are powerful mediators and functional regulators of cardiac cells, including cardiomyocytes and endothelial cells, which improve heart function and stimulate angiogenesis by enhancing regeneration of both blood vessels and injured myocardium in the peri-infarcted area; In general, the biologically active molecular cargoes include lipids, proteins and nucleic acids, such as DNA, mRNA, miRNA, and lncRNA. Thus, the potential advances of exosomes-based therapy include their role in the promotion of angiogenic, anti-apoptotic, anti-immunogenic, proliferative, or anti-fibrotic effects (Chaput and Thery, 2011; Wang et al., 2015; Yang, 2018; Ye et al., 2019). Therefore, exosomes indicate a huge therapeutic potential in the prevention and treatment of ischemic heart disease (Moghaddam et al., 2019).

In this review, we summarize the current advances on the utilizing exosomes to treat ischemic heart disease and conclude with a discussion of current challenges and future prospects in this field.

Formation and Secretion of Exosomes

Extracellular vesicles (EVs) include exosomes (diameter range: 30–150 nm), microvesicles (diameter range: 50–1000 nm), and apoptosomes (diameter range: 50–5000 nm) (Barile et al., 2017b). Exosomes are secreted by most cell types (Kishore et al., 2016; Gao et al., 2017) and contain a variety of proteins and nucleotides (van der Pol et al., 2012). The secretion of exosomes is an ATP-dependent, multi-step process requiring transporter molecules (Colombo et al., 2014). In general, along with the inward budding of endosomal membrane, the primary exosomes are formed and then gradually mature with releasing into a structure known as multi-vesicular bodies (MVBs) (Taheri et al., 2019). After maturation, MVBs are either guided to destructive pathways or are secreted extracellularly by the cells (H Rashed et al., 2017). Cells use exosomes to deliver bioactive components for intercellular communication. For example, exosomes can enter into the target cells and deliver their cargo through a variety of endocytic pathways, including endocytosis, and clathrin-independent pathways, such as phagocytosis, macropinocytosis, caveolin-mediated uptake, and lipid raft mediated internalization (Mulcahy et al., 2014). Surface proteins on the exosome surface, including integrins, CD9, CD63 and CD81, are readily internalized by specific ligands in target cells and message delivery is mediated between cells via release of exosome cargo into the cytoplasm or nucleus of the recipient cells (Rana et al., 2012).

Exosomes From Stem Cells

Exosomes equip a unique and powerful carrier for cells to deliver a variety of bioactive components and promote intercellular communication. Recently, exosomes from many stem cells, including mesenchymal stem cells (MSCs) (Lai et al., 2010), embryonic stem cells (ESCs) (Khan et al., 2015), cardiac progenitor cells (CPCs) (Barile et al., 2018), induced pluripotent stem cells (iPSCs) (Adamiak et al., 2018), and adipose-derived stem cells (ADSCs) (Xu et al., 2019) have been well investigated for their cardiac repair potency in several types of cardiovascular diseases, such as myocardial ischemia/reperfusion injury or myocardial infarction. Among these cell types, iPSCs is the most promising candidate for therapeutic applications because they can be generated from the patient's own somatic cells, possess extensive cell-renewal capability and potentially provide a variety of cell types that can be re-administered to the patient (Zhu et al., 2018). On the other hand, contrary to the stem cell therapy, exosomes confer minimal tumorigenicity (Lai et al., 2015) and immune response (Bradley et al., 2002) as they are readily recognized and endocytosed by recipient cells or are metabolically eliminated through the blood and urine. Unlike iPSCs, the very limited number of endogenous MSCs, and the steadily dropping number of isolatable MSCs in patients, particularly in aging patients, make the therapeutic application of endogenous MSC-derived exosomes particularly challenging (Phinney and Pittenger, 2017). Resident CPCs in adult heart and their exosomes may be well suited to treat cardiac pathologies (Xiao et al., 2016). However, these exosomes are difficult to isolate in patients due to low availabilities. ADSCs are redundant and can be harvested from adipose tissues (Hong et al., 2019). ADSCs and their exosomes represent new approaches for myocardial repair (Perea-Gil et al., 2018; Bai et al., 2019; Yang et al., 2019). Cardiosphere-derived cells (CDCs) are cardiac progenitor cells with anti-inflammatory, anti-oxidant, anti-fibrotic, and cardiomyogenic properties (Gallet et al., 2017; Marban, 2018). ESC-derived exosomes may be obtained indefinitely from ESCs and large-scale of production is not a problem (Chen et al., 2019). However, the ethical issues about using ESCs in regenerative medicine remain as major challenges, in particular, human embryos are destroyed during the derivation of human embryonic stem cells (Pessina and Gribaldo, 2006). hiPSCs emerged as a promising alternative to human ESCs and have no ethic issues. hiPSCs-derived exosomes may be produced

in large quantity, and are stable during cryostorage without loss of function. hiPSC are good cell resources for generating reliable "off-the-shelf" product and are ideal cell models for personalized medicine (Menasche, 2018). The advantages and disadvantages of exosomes from iPSCs and other stem cells were summarized in **Table 1**.

Preclinical studies have shown that exosomes can be used as an effective therapeutic strategy for a variety of diseases as they regulate a broad range of cellular behaviors and promote intercellular communications. Secreted exosomes may be collected from the cell culture medium via well-established protocols (Momen-Heravi et al., 2013; Witwer et al., 2013; Greening et al., 2015). Pilot studies from Sahoo et al. (2011) have shown that exosomes derived from human CD34 positive bone marrow-derived cells promote the survival, proliferation and angiogenic activity of endothelial cells. Studies have also shown that almost all exosomes have potential to increase the survival and proliferation of cardiac cells, attenuate ischemic injury, promote angiogenesis, and improve heart function in both small and large animal models (Khan et al., 2015; Gallet et al., 2017; Gao et al., 2017). Khan et al. (2015) reported that ESCs-derived exosomes, after being directly injected to infarcted mouse hearts, promote cardiac pro-angiogenesis and cardiomyocyte survival, improve cardiac function and reduce fibrosis after myocardial infarction. More recent papers confirmed the therapeutic effects of exosome in other types of myocardial injuries (Chen et al., 2019; Singla et al., 2019) and explored the potential mechanism underlying the beneficial effects (Lee et al., 2017). The therapeutic delivery of exosomes through the circulation system by using miRNAs mimics or synthetic exosomes as cargos and vehicles have been widely studied. Exosomes from CPCs (CPC-EXOs), ADSCs (ADSC-EXOs), MSCs (MSC-EXOs) or other stem cells have indicated positive results in the field of ischemic heart repair, including stimulation of angiogenesis and suppression of apoptosis (Xu et al., 2019). Because of the specific miRNA and cytokines in the cargo of CPC-EXOs, the therapeutic effect of CPC-EXOs is reported to be better than that of CPCs and MSC-EXOs (Barile et al., 2018; Xu et al., 2019). Hypoxia pretreatment of ADSC-EXOs and regulation of miRNAs in ADSC-EXOs including miR126-Spred1-ERK1/2-MAPK signaling pathway in angiogenesis, miR93-5p/TLR4 and Wnt/β-catenin signaling pathways in alleviated apoptosis both enhanced the therapeutic efficacy of acute myocardial infarction, suggesting the effectiveness of engineered exosomes as an alternative therapy for ischemic heart disease and regenerative medicine (Xu et al., 2019). As such, it appears that exosomes secreted from different cell types and under different conditions carry vast different varieties of bioactive molecular cargoes and subsequently provide different biomolecular effects (Li et al., 2013). The recent *in vivo* cardiac protective effect of the exosomes released by iPSCs and their derivatives is summarized in **Table 2**. However, further endeavors are warranted to investigate the

TABLE 1 | Advantages and disadvantages of exosomes from different stem cells.

Origin of exosomes	Advantages	Disadvantages	References
MSC	Most studied, well isolated and purified	Low number of endogenous MSCs, and the constantly diminishing number of isolatable MSCs found in the aging individual	Phinney and Pittenger, 2017
CPC	CPCs are specialized to function in the heart, CPC derived exosomes may be particularly well suited to treat cardiac pathologies	Impractical to obtain a sufficient amount of CPCs from the limited amount of available heart tissue	Xiao et al., 2016
ADSC	Readily accessible by routine liposuction, higher number of stem cells can be harvested from adipose tissue	A consensus in the doses of exosomes has not been reached, and the related studies are inadequate and limited. The organ diseases that can be effectively treated by ADSC-Exos are limited.	Hong et al., 2019
CDC	Acellular and non-replicating, facilitating the development of a stable and reliable "off-the-shelf" product, less immunogenic	Low number of endogenous CDCs, need to isolated from the human heart	Gallet et al., 2017; Marban, 2018
ESC	Qualified exosomes can be obtained infinitely from ESCs; Capable of instigating cell analogous response in target cells.	Ethical issue: human embryos are destroyed during the process of harvesting embryonic cells, this makes the research unpopular with those that believe human life begins at conception and that this life is being destroyed	Pessina and Gribaldo, 2006; Chen et al., 2019
iPSC	iPSCs have emerged as a promising alternative to ESCs; readily accessible, possibilities of large-scale production, stability after cryostorage without loss of function and can be applied to personalized medicine.	Laborious and inefficient isolation techniques same as the isolation from other stem cells	Menasche, 2018
iCMs/iECs/ iMSCs/iPgs	Generated from patient-specific iPSC-derivatives and can be used for an autologous therapy by activating endogenous repair.	Inefficient purity of iPSC-derivatives and exosomes isolation	Bian et al., 2019

ADSC, adipose-derived stem cells; CDC, cardiosphere-derived cells; CPC, cardiac progenitor cells; ESC, embryonic stem cells; iPSC, induced pluripotent stem cell; iCMs, iPSC-derived cardiomyocytes; iECs, iPSC-derived endothelial cells; iMSCs, iPSC-derived mesenchymal stem cells; iPgs, iPSC-derived cardiovascular progenitors; MSC, mesenchymal stem cells.

TABLE 2 | Cardioprotective effects of exosomes secreted by iPSC and its derivatives.

Releasing source	Cargo	Administration route	Animal model	Biological effect	References
iPSC	A set of miRNAs and proteins	Intramyocardial, 48 h after reperfusion	Mouse, I/R	Improve LV function, promote angiogenesis, ameliorate apoptosis and hypertrophy; no effect on infarct size.	Adamiak et al., 2018
iPSC	miR-21 and miR-210	Intramyocardial, before reperfusion	Mouse, I/R	Ameliorate apoptosis through suppression of caspase 3/7 activation	Wang et al., 2015
iPSC-CM	A set of miRNAs and lncRNAs	Intramyocardial, cell injection	Rat, MI	Improve cardiac function	Lee et al., 2017
iPSC-CM	RNAs, peptides, and small molecules	–	–	Salvage the injured cardiomyocytes in the peri-infarct region against apoptosis, necrosis, inflammation, remodeling, and fibrosis	Yang, 2018
iPSC-Pg	Enriched genes for tissue reparative pathways	Intramyocardial, 3 weeks after MI	Mouse, MI	Improve cardiac function, no effect on infarct size, hypertrophy and vascular density.	El Harane et al., 2018
iPSC-CM, iPSC-MSC, iPSC-EC	–	Intramyocardial after MI	Mouse, MI	Improve LVEF and restore the function of the injured myocardium	Vaskova et al., 2018

I/R, ischemic reperfusion injury; iPSC, induced pluripotent stem cell; iPSC-CM, iPSC-derived cardiomyocytes; iPSC-EC, iPSC-derived endothelial cells; iPSC-MSC, iPSC-derived mesenchymal stem cells iPSC-Pg, iPSC-derived cardiovascular progenitors; LVEF, Left ventricular ejection fraction; MI, Myocardial infarction.

candidate cell types as potent sources of exosomes for therapeutic approaches (Rezaie et al., 2019).

Anti-apoptotic Effects of iPSC Exosomes

Transplanted iPSCs produce paracrine factors that enhance survival of native cells in the ischemic heart, however, the mechanisms remain largely unknown. It has been reported that exosomes, a cell-free component secreted from iPSCs, contain some specific miRNAs and cytokines that can provide robust cardiac protection and promotion of myocardial regeneration (Moghaddam et al., 2019). Another study has reported that exosomes secreted by murine cardiac fibroblasts-derived iPSCs exhibit enhanced cytoprotection against acute ischemia/reperfusion-induced cardiomyocyte apoptosis by reducing the activity of caspase 3/7 proteins in the ischemic myocardium (Wang et al., 2015). The anti-apoptotic effects may be explained by cardioprotective miRNAs inside the iPSCs-derived exosomes, such as miR-21 and miR-210 (Wang et al., 2015). Another miRNA carried by exosomes, miR-92a, was also shown to have cardioprotective effects via inhibition of apoptosis, promotion of angiogenesis, and reduction of fibrosis in injured hearts (Lee et al., 2017). Although detailed mechanisms of their anti-apoptotic effects are not fully deciphered, exosomes from iPSC-CMs has been shown to possess a powerful cardiac protective effect by preserving the mitochondrial membrane potential, decreasing the translocation of Bax to the mitochondria, delaying the mPTP opening time, and inhibiting caspase 3/7 protein activity under (hypoxic/ischemic) conditions [need citations]. These cardiac protective effects depend on the ERK1/2 and p38-MAPK signal pathway (Gartz et al., 2018). It has been found that programmed cell death 6 interacting protein, also known as Apoptosis-linked gene 2–interacting protein X (ALIX), is an endosomal sorting complex required for transport complex-associated protein. ALIX plays direct role in exosome biogenesis such as packing the cargos, aiding in their entry into vesicles, and regulating the formation of vesicles (Baietti et al., 2012; Hurley and Odorizzi, 2012) and promotion of cell degeneration, aging, and apoptosis (Chatellard-Causse et al., 2002). In addition, a recent *in vitro* and in aortic rings *ex vivo* study reported that exosomes from ALIX-overexpressing and ALIX-knockout hiPSCs provide stronger and weaker therapeutic benefits respectively, against cisplatin and oxidative damage in epithelial, epidermal, and endothelial cells (Sun et al., 2019). Furthermore, exosomes released by iPSC-derived MSCs alleviate hepatic ischemia reperfusion injury (I/R) possibly by decreasing oxidative stress, reducing inflammatory responses and inhibiting apoptosis. In addition, exosomes secreted by iPSC-derived MSCs promote the growth, proliferation, and migration of human dermal fibroblast by stimulating ERK1/2 (Kim et al., 2018). A recent study reported that after 7 weeks of peri-infarct injections, the best preservation of left ventricle function was found in the exosome (released by iPSC-derived cardiovascular progenitors) injected hearts compared to those injected with iPSC-CMs, iPSC-derived cardiovascular progenitors or PBS. The authors found that the exosomes were enriched with signaling cues crucial for pathways beneficial to chronic heart failure, such as enhanced metabolism, growth, survival, proliferation, angiogenesis, vasculogenesis, and reduced organismal morbidity and mortality (El Harane et al., 2018).

Pro-angiogenic Activities of iPSC Exosomes

Angiogenesis is the formation of new blood vessels that helps to establish and support the normal structure and function of the cardiac tissues. Angiogenesis is defined as the migration, development and differentiation of endothelial cells to form new blood vessels (Kubis and Levy, 2003). Exosomes secreted by various cell types have been demonstrated to

possess proangiogenic effects. For instance, exosomes isolated from MSCs and CPCs promote migration of endothelial cells (Vrijsen et al., 2010), while exosomes derived from human pericardial fluid have been shown to stimulate the proliferation of endothelial cells (Beltrami et al., 2017). Furthermore, exosomes secreted from CDCs have shown stimulation of angiogenesis in tube formation assays and have also shown enhancement of vessel density when locally delivered to chronic infarcted mouse hearts (Ibrahim et al., 2014). A very recent study demonstrated that exosomes released by immune response-free monkey autologous iPSCs provided enhanced wound healing through promotion of angiogenesis and cell viability of injured endothelial cells in the wounded regions (Lu et al., 2019). On the contrary, a study has reported that the effects of hiPSC-derived exosomes on normal human umbilical vascular endothelial cells (HUVECs) were minimal (Ding et al., 2018). However, under high glucose conditions, these exosomes were able to reduce senescence of endothelial cells, promote cell proliferation and enhance the formation of capillary-like structures (Ding et al., 2018). Vaskova et al. (2018) compared the reparative capacities of the exosomes secreted by iPSC-derived cardiomyocytes (iCMs), endothelial cells (iECs), and MSCs (iMSCs) and they found that iCM, iEC, and iMSC-exosomes possess the pleiotropic ability to generate a capillary network and improve the function of the damaged myocardium. A recent study has demonstrated that hiPSC-CMs-derived exosomes stimulate *in vitro* angiogenesis in several facets of tube formation, accompanying with increased expression of growth factors such as PDGFA, VEGF2A, and FGF2 in endothelial cells (Dougherty et al., 2018). Investigations have demonstrated that miRNA-199b play key role in iECs differentiation by modulating VEGF expression via targeting Notch signaling (Chen et al., 2015; Du et al., 2016). Another study indicated that exosomes derived by hiPS-ECs is enriched with miR-199b-5p that significantly promotes neovascularization via transcriptional upregulation of VEGFR2, regulated through Jagged1/Notch1 signaling pathway (Ye et al., 2019). It is documented that exosomes derived from iPS-MSCs significantly enhance angiogenesis (Qi et al., 2016), and promote the proliferation, migration and tube-forming abilities of endothelial cells *in vitro* (Hu et al., 2015; Zhang et al., 2015), via the activation of PI3K/Akt signaling pathway (Liu et al., 2017).

Pro-cell Cycle Effects of iPSC Exosomes

Recent studies have demonstrated the beneficial effects of exosomes in enhancing the cell cycle activity in animal models of MI. For instance, exosomes secreted by CDCs were found to promote the proliferation of cardiomyocyte in mouse MI hearts (Ibrahim et al., 2014). Similarly, exosomes derived by iMSCs promoted the proliferation of human fibroblasts *in vitro* in a dose-dependent manner (Zhang et al., 2015) while iMSCs-derived exosomes enhanced the viability and cell cycle progression in human keratinocytes and human dermal fibroblasts (Kim et al., 2018). Ye et al. (2019) in one of their studies treated bovine aortic endothelial cells with 100 μg/ml of hiPSC-CM-derived exosomes and found a significant increase in cell proliferation when compared to control (no exosomes). Khan et al. (2015) reported that mouse hearts treated with ESC-derived exosomes promote myocyte proliferation by enhancing cardiomyocyte cycling, as evidenced by both BrdU+ (S-phase) and phosphorylated histone H3 (PH3+; M-phase) cardiomyocytes, at 28 days of infarction when compared to those treated with embryonic fibroblasts-derived exosomes orsaline. Similarly, ESC-derived exosomes also stimulated mRNA expression of cyclins A2, D1, D2, and E1 but suppressed the expression of cyclin inhibitors p16, p19, p21, and p53 at day 5 following myocardial infarction (Khan et al., 2015), indicating that iPSC-derived exosomes may activate cell cycle activity and promote cell proliferation.

Teratogenic Potential of iPSC Exosomes

Teratoma is a benign tumor formed by cells from all three germ layers. Although iPSCs and their derivatives have been demonstrated to be effective for myocardial repair, the teratogenic risk of these cells remains a concern and limits the routine clinical uses of these cells (Ben-David and Benvenisty, 2011; Zhao et al., 2011). Several studies had shown that iPSCs indeed form teratoma in mice and non-human primates (Zhao et al., 2011; Hong et al., 2016; Lu et al., 2019). iPSC-derived exosomes may possess unique advantage in this regard. Although exosomes secreted from iPSCs and its derivatives were reported to enhance angiogenesis and cell cycle of cells in the host animal hearts (Khan et al., 2015; Adamiak et al., 2018), exosomes are non-proliferative and chromosome-free. Therefore, exosomes are not expected to form teratoma-like tumor masses cells (Lu et al., 2019). iPSC-derived exosomes represent a special cell-free system to regenerate the injured myocardium which will have significant applications in cardiac regenerative medicine (Ailawadi et al., 2015; Das and Halushka, 2015; Singla, 2016).

Anti-fibrotic Effects of iPSC-Exosomes

Although the beneficial components in the exosomes secreted from iPSCs are not fully identified yet, studies have shown that iPSC culture medium have the capability to improve alveolar epithelial wound-healing and reduce lung fibrosis in a lung epithelial wound healing model (Gazdhar et al., 2014). Furthermore, it is reported that exosomes derived from macrophage contain miR-155 which reduces the proliferation and stimulates inflammation of fibroblast during cardiac injury (Wang et al., 2017). However, other previous studies have shown that exosomes secreted from iMSCs promote proliferation and migration of human fibroblasts and also increase their secretion of collagens and elastin (Zhang et al., 2015). Secretions of type I and type III collagens, elastin and their associated mRNA transcripts in the fibroblasts were increased in response to the treatment of exosomes from hiPSC-MSCs in a dose-dependent manner. These discrepancies may be explained by the different bioactive components in exosomes derived by different cell types or even from the same cell type but under different conditions. Further studies need to be done to address these issues.

It is noteworthy to mention that in some cases, depending on cell- type as well as genotypic and functional status of the cells, specific components in the exosomes may not be beneficial but rather, harmful to the cardiovascular system (Barile et al., 2017a). For example, the progression of heart failure may alter

the miRNA cargos in cardiac-derived exosomes and suppress their regenerative activities (Qiao et al., 2019). As an individual miRNA transferred via exosomes from different cell types or in different status may elicit divergent biological responses, exosomes extracted from the serum of dilated cardiomyopathy patients were reported not only to cause dramatic pathological changes in gene expression in both neonatal rat cardiomyocytes and hiPSC-CMs *in vitro* but were also associated with the accelerated heart failure progression in patients (Jiang et al., 2017). Exosome content may be manipulated via genetic modification of the cells. It is reported that exosomes secreted from GATA-4 overexpressing stem cells highly expressed several anti-apoptotic miRNAs and displayed better myocardial repair potency in ischemic heart diseases (Yu et al., 2015). Yi et al. (2019) reported that exosomes from osteocalcin-overexpressed EPCs showed a beneficial effect by stimulating the endothelial OCN-GPRC6A signaling. An et al. (2019) reported that exosomes from ADSCs overexpressing miR-21 promote vascularization. Despite these beneficial effects, it is worthy to note that these genetic modifications of donor cells may also cause unwanted changes in exosomes. Accordingly, the effects and safety of modified exosomes should be evaluated sufficiently before their clinical applications (Yamashita et al., 2018). Application of hiPSCs with a mutant genotype should be very cautious unless they are first corrected into wild-type genotype. Thus, a thorough investigation of iPSC-derived exosomes to identify the beneficial ("good guys") and potential harmful components ("bad guys") should be performed prior to the routine clinical application of exosome-based therapy (Jung et al., 2017).

Targeted Delivery of Exosomes to Injured Heart and Future Perspectives

Three different ways had been used to deliver exosomes to the ischemic heart tissue which include intravenous, intramyocardial and intracoronary injections (**Figure 1**). Exosomes signal cardiomyocytes and other supporting cells, including endothelial cells, smooth muscle cells and fibroblasts, and modulate their response to ischemic damage. Unfortunately, it is reported that majority of delivered exosomes are rapidly trapped in the liver, spleen, and lung when administrated intravenously, which may resulting in undesirable side effects and reduction of their beneficial pro-regenerative effects (Lai et al., 2014; Morishita et al., 2015; Smyth et al., 2015). Interestingly, when exosomes delivered 30 min after coronary occlusion and subsequent reperfusion, via an intramyocardial route led to significantly decreased infarct size in mini-pigs, and showed no effect through intracoronary delivery (Gallet et al., 2017). It is likely that exosomes injected via intracoronary route are prone to be flushed and drained through the coronary vein and subsequent uptake by macrophages, thereby resulting in poor retention in the heart (Barile et al., 2017b). As open chest surgery for intramyocardial injection of exosomes in MI patients is unlikely a viable approach from a translational perspective, there remains a need to maximize the therapeutic effects of exosome-based therapy. Targeted delivery of exosomes to the damaged cardiac tissue has emerged as an important goal in this field (Huang et al., 2019). Different attempts have been investigated to aid the targeted delivery of exosomes to the injured myocardium, such as fusion of biocompatible materials onto the stem cell surface membranes or modification of cell membrane via expression of cardiac or endothelial cell-specific surfaces markers (Li et al., 2018; Tang et al., 2018; Huang et al., 2019; Shen et al., 2019). In this perspective, Liu et al. (2018) generated an engineered hydrogel patch for prolonged release of extracellular vesicles secreted by hiPSC-CMs. They demonstrated that the delivery of this engineered hydrogel reduces infarct size, cardiomyocyte apoptosis and hypertrophy, and improve heart function in a rat MI model. Likewise, Vandergriff and colleagues chemically marked and ameliorated exosomes with a cardiac homing peptide

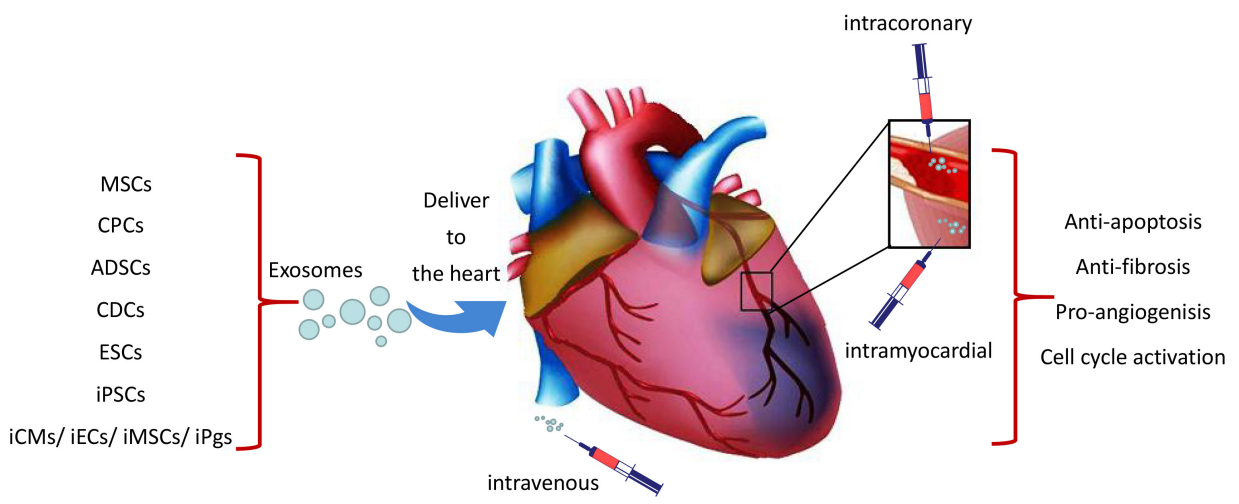

FIGURE 1 | Schematic representation of the cardiac delivery of exosomes from iPSCs and other stem cells. Exosomes were delivered to the ischemic heart tissue through intravenous, intracoronary or intramyocardial which may lead to cardio-protection through the effects of anti-apoptosis, angiogenesis, anti-fibrosis and cell cycle activation.

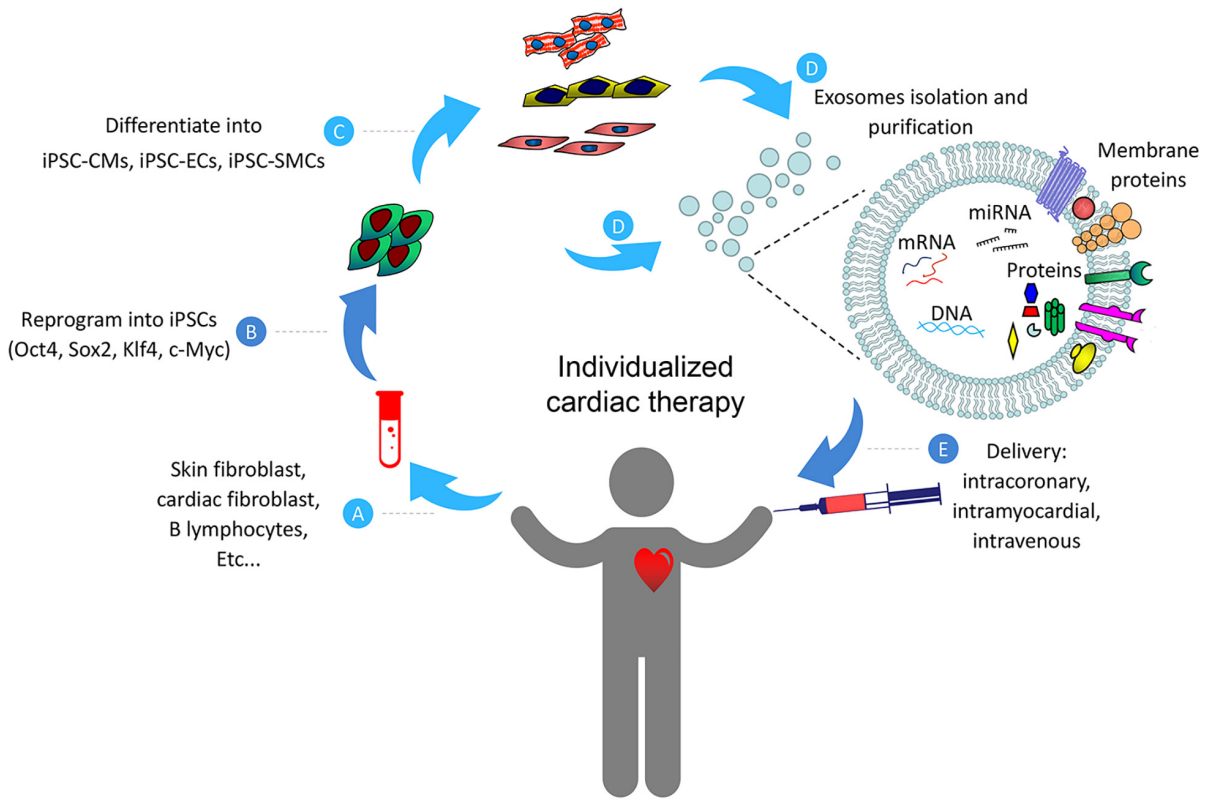

FIGURE 2 | Schematic representation of the individualized cardiac therapy with exosomes generated from iPSCs and its derivatives. Patient-specific Cells **(A)** were isolated and reprogrammed into iPSCs **(B)**, then differentiated into its derivatives including iPSC-CMs, iPSC-ECs and iPSC-SMCs **(C)**. Exosomes **(D)** generated from iPSCs and its derivatives were then used as a platform for personalized cardiac therapy by simulating endogenous repair through intracoronary, intramyocardial or intravenous delivery **(E)**.

(CHP; CSTSMLKAC) in order to augment their accumulation in the peri-infarct site after intravenous administration. Such targeted delivery has been shown to increase the myocardial reparative capacity of exosomes (Vandergriff et al., 2018) and has established the intravenous administration of exosomes as a feasible approach for myocardial repair.

Although detailed mechanisms on exosome-based myocardial repair have not been fully understood, here we would like to summarize the potential beneficial effects of such therapy from current studies discussed in this review (**Figure 2**). Collectively, data from these studies indicate that naïve exosomes from iPSCs and/or their engineered vesicles (cell-free) may be effective for the treating heart diseases. However, despite the encouraging advances in this field at present, there are still number of challenges that need to be addressed before clinical promotion of exosome-based therapy. First, since exosomes from various types of stem cells may confer completely different response in target cells (Xiao et al., 2016), detailed investigations are necessary to identify the optimal cell types and determine the optimal functional status of these cells, and examine the molecular contents responsible for any observed beneficial effects of these exosomes. Second, the mechanism of exosome biogenesis, metabolic kinetics, curative effect, and *in vivo* biodistribution need to be carefully investigated (Taheri et al., 2019). Third, the number of exosomes stay in the cardiac area is very limited after being administered intravenously. This limitation has been shown to attenuate the therapeutic efficiency of the exosomes (Ni et al., 2019). Exosome-based cardiac therapies may be modified with the utilization of engineered biomaterials which provide platforms for durable retention and prolonged release of cargos in the injured myocardium.

AUTHOR CONTRIBUTIONS

CF, JZ, and WZ wrote the manuscript. EZ, JJ, and JY made the revision.

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Extracellular Vesicles From Notch Activated Cardiac Mesenchymal Stem Cells Promote Myocyte Proliferation and Neovasculogenesis

*Wanling Xuan[1], Mahmood Khan[2] and Muhammad Ashraf[1]**

[1] *Vascular Biology Center, Department of Medicine, Medical College of Georgia, Augusta University, Augusta, GA, United States,* [2] *Department of Emergency Medicine, Wexner Medical Center, The Ohio State University, Columbus, OH, United States*

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***Correspondence:**
Muhammad Ashraf
mashraf@augusta.edu

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Cardiac mesenchymal stem cells (C-MSCs) are a novel mesenchymal stem cell (MSC) subpopulation derived from cardiac tissue, which are reported to be responsible for cardiac regeneration. Notch signaling is believed to aid in cardiac repair following myocardial injury. In this study, we have investigated the role of extracellular vesicles (EVs) from Notch1 engineered C-MSCs on angiogenesis and cardiomyocyte (CM) proliferation in ischemic myocardium. C-MSCs were isolated from Notch1^{flox} mice (C-MSC^{Notch1FF}). Notch1 gene deletion was accomplished by adenoviral vector-mediated Cre recombination, and Notch1 overexpression was achieved by overexpression of Notch1 intracellular domain (N1ICD). EVs were isolated by using the size exclusion column method. Proteomic composition of EV was carried out by mass spectrometry. A mouse myocardial infarction (MI) model was generated by permanent left anterior descending (LAD) coronary artery ligation. Intramyocardial transplantation of Notch1 knockout C-MSCs (C-MSCs^{Notch1KO}) did not have any effect on cardiac function and scar size. On the other hand, transplantation of N1ICD-overexpressing C-MSCs (C-MSCs^{N1ICD}) showed significant improvement in cardiac function and attenuation of fibrosis as compared to the control (PBS) group and non-modified C-MSC groups. C-MSCs^{N1ICD} differentiated into smooth muscle cells and formed new vessels. Proteomics profiling identified several proteins, such as lysyl oxidase homolog-2 and biglycan, as highly enriched proteins in EV-C-MSCs^{N1ICD}. Go term analysis indicated that EV-C-MSCs^{N1ICD} were enriched with bioactive factors, potent pro-repair proteins responsible for cell migration and proliferation. EV-C-MSCs^{Notch1FF} and EV-C-MSCs^{N1ICD} were strongly proangiogenic under both *in vitro* and *in vivo* conditions. EV-C-MSCs^{N1ICD} caused dense tube formation *in vitro* and increased neovasculogenesis in the peri-infarct area *in vivo*. Furthermore, EV-C-MSCs^{N1ICD} attenuated endothelial cell (EC) and CM apoptosis under oxidative stress and ischemic injury. Similarly, EV-C-MSC^{Notch1FF} and EV-C-MSC^{N1ICD} treatment improved cardiac

function and decreased fibrosis in mice post-MI. EV-C-MSCs^{N1ICD} were very effective in improving cardiac function and decreasing fibrosis. Notch1 signaling is a strong stimulus for cardiac regeneration by C-MSCs. EVs secreted by Notch1-overexpressing C-MSCs were highly effective in preventing cell death, promoting angiogenesis and CM proliferation, and restoring cardiac function post-MI. Overall, these results suggest that Notch1 overexpression may further enhance the effectiveness of EVs secreted by C-MSCs in cell-free therapy.

Keywords: cardiac mesenchymal stem cells, extracellular vesicles, Notch1, angiogenesis, proliferation, myocardial infarction

INTRODUCTION

Cardiac mesenchymal stem cells (C-MSCs) are a novel mesenchymal stem cell (MSC) subpopulation arising from cardiac tissue, which are predominantly Sca-1$^+$ cells and express mesenchymal surface antigens. Compared with bone marrow-derived MSCs (BM-MSCs), C-MSCs expressed GATA4 (an early cardiac transcription factor marker) (Chen et al., 2012). C-MSCs have the advantage of being preconditioned by the cardiac micro-environment and epigenetic profile, which showed enhanced levels of histone acetylation at the promoter regions of the cardiac-specific genes (Wang et al., 2013). Although these stem cells are capable of transforming into new cardiac cells, their contribution toward functional improvement of the heart under ischemic conditions remains controversial.

Cardiac mesenchymal stem cells play an important role in angiogenesis of the ischemic heart. A lineage tracing study demonstrated that cardiac-resident Sca-1$^+$ cells abundantly contributed to the cardiac vasculature in mice during physiological growth and during cardiac remodeling after myocardial infarction (MI) (Vagnozzi et al., 2018). Thus, C-MSCs might serve as a potential candidate for cell therapy. However, differentiation of C-MSCs is believed to depend on close cell-to-cell interaction via Notch1 signaling as trypsinization causes a loss of cell–cell contact and impairs C-MSC differentiation during *in vivo* transplantation (Li et al., 2006).

Notch signaling is involved in mammalian cardiogenesis, including cell fate decisions, differentiation and proliferation, formation of heart tissues, and angiogenesis (del Monte et al., 2011; MacGrogan et al., 2014; Zhou and Liu, 2014). Key signaling pathways responsible for cardiac morphogenesis become transiently reactivated in the damaged heart. Multiple studies reported that Notch signaling was reactivated during myocardial injury and initiated cardiac repair following myocardial injury (Li et al., 2010; Nistri et al., 2017). Notch signaling determines both the extent of myocardial damage and pathological left ventricular remodeling involving regeneration, cardiomyocyte (CM) survival, fibrotic response, and angiogenesis (Rizzo et al., 2014). For instance, activation of Notch1 in neonatal rat CMs and intact mouse myocardium elevated phospho-Akt^{S473} levels as well as proliferation of myocytes in the infarcted heart (Gude et al., 2008). The cardioprotective effect of Notch1 against ischemic damage was reported to be mediated by AMPK signaling via an interaction with upstream liver kinase beta 1 (LKB1) (Yang et al., 2016). Moreover, both systemic and BM-MSC-specific ablation of Notch1 led to impaired cardiac repair following MI (Li et al., 2011). However, the role of Notch1 signaling in C-MSCs remains unclear.

A previous study reported that overexpression of Notch1 intracellular domain (N1ICD), the active form of Notch1, promoted cardiosphere derived cells (CDCs) toward vascular smooth muscle cell (VSMC) differentiation both *in vitro* and *in vivo* (Chen et al., 2012). In this regard, Notch1 activation in C-MSCs might potentially stimulate vascular repair by angiogenesis. It has been demonstrated that C-MSC administration improved cardiac function in animal models of heart failure (Moore et al., 2017). Nevertheless, whether Notch1 overexpression could further enhance the regenerative capability of C-MSCs and improve cardiac function remains unclear and requires further investigation. More recently, extracellular vesicles (EVs) secreted by stem cells have been reported as final effectors of protection against ischemic injury. These EVs carry miRNAs and proteins which facilitate cell–cell communication in addition to other cellular effects (Mathieu et al., 2019). Bioactive molecules in EVs secreted by C-MSCs presumably contributed to the afforded benefits (Wysoczynski et al., 2019). Therefore, the present study was designed to investigate whether Notch1 overexpression in C-MSCs could render their EVs more effective in cardiac repair following MI.

MATERIALS AND METHODS

C-MSC Isolation and Culture

Mouse C-MSCs were obtained from Dr. Yaoliang Tang at Augusta University, which were isolated from the hearts of 2- to 3-month-old Notch1^{flox} mice (The Jackson Laboratory, stock number: 006951) according to the procedure as previously described (Ju et al., 2018). The isolated cells were purified using a mouse hematopoietic lineage depletion cocktail kit (Stemcell Technologies) and Sca-1 magnetic beads (MiltenyiBiotec Inc.) with magnetic activated cell sorting (MACS). These cells expressed the mesenchymal cell surface makers CD105, CD44, and CD140 by flow cytometric analyses (Ju et al., 2018). Cells were cultured in high-glucose DMEM medium supplemented with 10% fetal bovine serum (FBS), 200 mM L-glutamine, 55 nM β-mercaptoethanol, and 1% MEM non-essential amino acid. Before EV collection, culture medium was switched to

medium supplemented with exosome-deleted FBS (Gibco) for 48 h. C-MSCs isolated from Notch1^{flox} mice were designated as C-MSCs$^{Notch1\ FF}$.

Generation of Notch1 Knockout C-MSCs (C-MSCs^{Notch1KO}) and N1ICD Overexpression in C-MSCs (C-MSCs^{N1ICD})

Notch1 knockout C-MSCs were generated by deletion of Notch1 genes in C-MSCs isolated from Notch1^{flox} mice via adenoviral vector–mediated Cre recombination. N1ICD-overexpressing C-MSCs were generated via adenoviral vector-mediated transient overexpression of N1ICD. N1ICD recombinant adenovirus was generated as described in a previous study (Chen et al., 2012). The expression of N1ICD in C-MSCs was determined by western blot.

Mouse Aortic Endothelial Cell Isolation and Culture

Primary mouse aortic endothelial cells (ECs) were isolated as described previously (Wang et al., 2016). Briefly, the mouse thoracic aorta was quickly removed using micro-dissection forceps and gently flushed with ice-cold PBS to remove the blood. Then the aorta was cut into 1 mm rings. These aortic segments were cultured on matrix in EC growth medium for EC sprouting. After 2–3 days, the proliferating ECs were harvested and passaged. The cells were characterized by CD31 staining.

Human EC Culture

Human cardiac microvascular ECs (CMVECs, CC-7030) and human aortic ECs (HAECs, CC-2535) were obtained from Lonza Company. Cells were maintained in EC growth medium V-2 (213-500, CELL APPLICATIONS, Inc.). Cells at passage 2–6 were used for experiments.

Isolation of EVs

Extracellular vesicles were isolated using the size exclusion column method. Briefly, conditioned media were collected, and EVs were isolated by centrifugation at 3000 r/min for 30 min to remove cells and debris, followed by filtration through a 0.22 μm filter to remove the remaining debris. Then the medium was further concentrated using Amicon Ultra-15 100 kDa centrifugal filter units (Millipore). Isolation of EVs in the concentrated medium was carried out through qEV size exclusion columns (Izon Science). EV fractions were collected and concentrated by an Amicon Ultra-4 10 kDa centrifugal filter (Millipore). The purified EVs were stored at -80°C and subsequently characterized by particle size, electron microscopy, and proteomic profile.

Concentration and Particle Size Measurement With Tunable Resistive Pulse Sensing

Particle size and concentration were analyzed using the tunable resistive pulse sensing (TRPS) technique with a qNano instrument (Izon Science) as described in a previous study (Vogel et al., 2016). Briefly, the number of particles was counted (at least 600–1000 events) at 20 mbar pressure. Beads of CPC200 (200 nm) were used for calibration. Data were analyzed using Izon Control Suite software.

Transmission Electron Microscopy

Tissue samples were processed for transmission electron microscopy (TEM) by the Electron Microscopy and Histology Core Laboratory at Augusta University. Briefly, EV suspension was fixed with an equal volume of 8% paraformaldehyde (PFA) to preserve ultrastructure. Ten microliters of suspended/fixed exosomes was applied to a carbon/formvar-coated 200 mesh copper grid and allowed to stand for 30–60 s. The excess was absorbed by Whatman filter paper. Ten microliters of 2% aqueous uranyl acetate was added and treated for 30 s. Grids were allowed to air-dry before being examined in a JEM 1230 transmission electron microscope (JEOL USA Inc., Peabody, MA, United States) at 110 kV and imaged with an UltraScan 4000 CCD camera and First Light Digital Camera Controller (Gatan Inc., Pleasanton, CA, United States).

EV Internalization

Purified EVs were labeled with PKH26 (Sigma–Aldrich), according to the manufacturer's protocol. Briefly, 300 μL of EVs was suspended into 100 μL of Diluent C, which was mixed with 1.4 μL of PKH26 dye. The labeling reaction was stopped by adding an equal volume of exosome-free FBS. Exosome Spin Columns (Thermo Fisher Scientific) were used to remove unincorporated PKH26. The CMVECs were incubated with labeled EVs for 3 h. After incubation, cells were stained with WGA conjugated with Alexa Fluor 488 (Thermo Fisher Scientific). Cells were fixed with 2% formaldehyde for 5 min and mounted with DAPI mounting media (Southern Biotech). Images were taken with a fluorescent microscope (Olympus, Japan).

Tube Formation Assay

HAECs (1×10^5 cells/well) were seeded on Matrigel (Corning) in a 24-well plate and treated with or without 1 μg EVs from different groups of C-MSCs in EGM-2V basal medium (Lonza). Complete EGM-2V medium was used as positive control. After 16 h, cells in Matrigel were stained with Calcein AM, and images were taken by fluorescent microscope. Tube formation was analyzed by Wimasis Image Analysis Platform.

Induction of Myocardial Infarction

Animal experiments were carried out according to experimental protocols approved by the Augusta University Animal Care and Use Committee. An MI model was generated as previously described. Briefly, MI was induced in 8-week-old C57/B6 mice weighing 22–25 g (The Jackson Laboratory), which were anesthetized with 2% isoflurane (isoflurane USP, Henry Schien). The mice were incubated with a 24-gauge tube and ventilated using a Harvard Rodent Ventilator (MiniVent Type 845, Holliston, MA, United States). Before incision, mice were treated with buprenorphine SR (1.0 mg/kg). The left anterior descending (LAD) coronary artery was permanently ligated with a prolene #8-0 suture. Ten minutes after LAD ligation, 20 μL EVs (particle

concentration: 1×10^{12}/mL) from different groups of C-MSCs or 5×10^5 C-MSCs suspended in 20 μL PBS were injected into the myocardium bordering infarction zone. Before transplantation, C-MSCs were labeled with LuminiCell Tracker 540 (Millipore) according to the manufacturer's protocol. The same volume of PBS was injected in the control group.

Echocardiography

Echocardiography was performed in mice anesthetized mildly with inhaled isoflurane (0.5%) using a Vevo 2100 imaging system (VisualSonics Inc.). Hearts were imaged in 2D parasternal short-axis view at the level of the mid-papillary muscle. The M-mode images were used to measure left ventricular end diastolic diameter (LVDd) and left ventricular end systolic diameter (LVDs). Left ventricular ejection fraction (EF) and left ventricular fractional shortening (FS) were analyzed using LV trace for three consecutive cardiac cycles.

Immunostaining

Hearts were fixed with 4% PFA for 1 h at room temperature and then immersed in 30% sucrose overnight at 4°C. At day 2, hearts were cryopreserved in an optical cutting temperature (OCT) compound (Tissue Tek) at -80°C. Hearts were sliced into 5-μm-thick frozen sections and incubated with primary antibodies including α-sarcomeric actinin (A7811, Sigma, 1:200), ki67 (ab16667, abcam, 1:500), and SMA (ab5694, abcam, 1:300). Slides were incubated with anti-rabbit/mouse secondary antibodies conjugated to Alexa Fluor 594, Alexa Fluor 647, or Alexa Fluor 488 (Life Technologies). Images were taken using a confocal microscope (FV1000, Olympus, Japan). Ki67-positive CMs were analyzed in 22 animals ($n = 5$ in PBS and C-MSC$^{\text{Notch1KO}}$ groups; $n = 6$ in EV-C-MSC and EV-C-MSC$^{\text{N1ICD}}$ groups) at 7 days after MI and 12 animals ($n = 3$) at 1 month post-MI. The proliferating CMs were blindly counted in 132 sections (six sections cut at 400 μm intervals from apex to base per heart) at 7 days post-MI and 72 sections at 1 month post-MI. Vessel density was assessed in 20 animals ($n = 5$ in each group) 1 month post-MI. The number of vessels was blindly counted in 60 sections (three sections per heart, 15 sections per group) in the infarct and border areas of all mice after staining with an antibody α-SMA using a fluorescence microscope at a magnification of 400. Vascular density was determined by counting α-SMA-positive vascular structures. The number of vessels in each section was averaged and expressed as the number of vessels per field (0.2 mm^2). For cells, C-MSCs were fixed with 4% PFA and blocked with 10% FBS, followed by incubation of anti-N1ICD antibody (sc-376403, Santa Cruz) and secondary antibody conjugated to Alexa Fluor 594 (Life Technologies).

TUNEL Staining

Twenty-four hours after LAD ligation, mice were sacrificed, and heart tissue was embedded and sectioned for TUNEL staining. TUNEL staining was performed using a kit (Thermo Fisher Scientific) according to the manufacturer's instruction. CMs were identified by α-sarcomeric actinin staining. Cells were counterstained with DAPI to visualize nuclei. Total number of α-sarcomeric actinin and TUNEL double-positive cells were determined in five fields (20 ×) from the border area in each heart ($n = 3$). PBS-treated mice served as control groups.

Trichrome Masson Staining

Hearts were embedded in paraffin and cut into 5-μm-thick sections. Masson trichrome staining was carried out for scar tissue measurement according to the manufacturer's protocol (HT-15, Sigma). The size of the left ventricle (LV) area and scar area were measured using the ImageJ software. Six sections with 400 μm intervals from apex to basal were analyzed per heart. The fibrosis area was expressed as the ratio of scar area to LV area.

Western Blotting

Extracellular vesicles or C-MSCs were lyzed with radio immunoprecipitation assay (RIPA) buffer supplemented with Complete Protease Inhibitor Mixture tablets (Roche Diagnostics). The cell lysate was sonicated in ice using a sonication device (Sonic dismembrator Model100, Fisher Scientific). Five micrograms of protein of EVs or 10 μg protein of cells was separated by SDS/PAGE and transferred to PVDF membrane (BioRad). Membranes were incubated with rabbit anti-CD63 antibody (EXOAB-CD63A-1, SBI System Biosciences), mouse anti-Tsg101 antibody (sc-7964, Santa Cruz), mouse anti-calnexin antibody (sc-23954, Santa Cruz), anti-N1ICD antibody (sc-376403, Santa Cruz), or anti-GADPH antibody (sc-32233, Santa Cruz) overnight at 4°C followed by incubation with an anti-mouse or anti-rabbit goat peroxidase conjugated secondary antibody. Immunoreactive bands were visualized by the enhanced chemiluminescence method (Bio-Rad) with a western blotting detection system (Fluorchem E, ProteinSimple, United States).

Proteomic Profile by Mass Spectrometry

Sample preparation and analyses were performed at the Proteomics and Mass Spectrometry Core Laboratory at Augusta University. Briefly, EV proteins were extracted and solubilized with acid-labile surfactant, followed by trypsin digestion and peptide cleanup using a C18 spin column. Peptide digests were analyzed on an Orbitrap Fusion tribrid mass spectrometer (Thermo Scientific) coupled with an Ultimate 3000 nano-UPLC system (Thermo Scientific). Two microliters of reconstituted peptide was first trapped and washed on a Pepmap100 C18 trap (5 μm, 0.3 × 5 mm) at 20 μL/min using 2% acetonitrile in water (with 0.1% formic acid) for 10 min and then separated on a Pepman 100 RSLC C18 column (2.0 μm, 75 μm × 150 mm) using a gradient of 2–40% acetonitrile with 0.1% formic acid over 40 min at a flow rate of 300 nL/min and a column temperature of 40°C.

Samples were analyzed by data-dependent acquisition in positive mode using an Orbitrap MS analyzer for a precursor scan at 120,000 FWHM from 300 to 1500 m/z and an ion-trap MS analyzer for MS/MS scans at top speed mode (3 s cycle time). Collision-induced dissociation (CID) was used as a fragmentation method. Raw data were processed using Proteome Discoverer (v1.4, Thermo Scientific) and submitted for SequestHT search against the Uniprot human database. The fixed value PSM validator algorithm was used for peptide

spectrum matching validation. SequestHT search parameters were 10 ppm precursor and 0.6 Da product ion tolerance, with dynamic carbidomethylation (+57.021 Da). To compare the relative expression difference across different sample groups, the number of PSM was used for each sample as an expression of its relative abundance. The number of PSM was normalized by the sum of PSM in each sample ($n = 3$), which represented the overall sample loading amount on the column. Go terms for enriched protein were analyzed using QuickGO (Binns et al., 2009).

Statistical Analysis

Data were expressed as mean ± SD. After a test for normality, statistical analysis of differences among different groups was compared by ANOVA with Bonferroni's correction for multiple comparisons. Differences were considered statistically significant at $P < 0.05$. Statistical analyses were performed using Graphpad Prism 6.0 (Chicago, IL, United States).

RESULTS

In vivo Effect of C-MSC Transplantation on Ischemic Injury in an MI Model

First, we verified Notch1 deletion or N1ICD overexpression in C-MSCs by western blot (**Supplementary Figure S1A**). The transfection efficiency of Ad-GFP-N1ICD was more than 95% as visualized by GFP fluorescence (**Supplementary Figure S1B**). By immunostaining, N1ICD expression was localized in C-MSC nuclei (**Supplementary Figure S1C**). Next we determined the role of Notch1 in the effectiveness of C-MSCs on cardiac injury. Transplantation of C-MSCs^{Notch1FF} improved cardiac function compared with the PBS control group (EF: 53.21 ± 2.68% vs. 39.04 ± 9.69%; FS: 27.00 ± 1.78% vs. 18.92 ± 5.21%, $P < 0.05$) (**Figure 1A**) and decreased infarct size 1 month post-MI (**Figure 1B**). Transplantation of Notch1 knockout C-MSCs did not have any effect on cardiac function (EF: 42.32 ± 6.67%; FS: 20.68 ± 3.66%, $P > 0.05$) and scar size (**Figure 1**). On the other hand, C-MSCs^{N1ICD} had a significant protective effect on EF and FS (EF: 62.47 ± 6.70%; FS: 33.37 ± 4.79%, $P < 0.05$) and fibrosis (**Figure 1**) compared to all other groups ($P < 0.01$). For fibrosis assessment, $n = 3$ was used in each group. Besides their beneficial effect on cardiac function, these cells were colocalized with smooth muscle cells and blood vessels (**Supplementary Figure S2**). A profound effect of N1ICD-overexpressing C-MSCs was observed on cardiac function and vascularization.

Characterization of EVs From C-MSCs

We isolated and characterized EVs from different C-MSCs. The appearance and size of EV from C-MSCs^{Notch1FF}, C-MSCs^{Notch1KO}, and C-MSCs^{N1ICD} under TEM and TRPS were similar (**Figures 2A,B**). EVs from all C-MSCs expressed exosome specific markers Tsg101 and CD63, while they did not express calnexin (**Figure 2C**).

Internalization of EVs From C-MSCs by ECs

The therapeutic efficacy of EVs depends on their internalization by recipient cells where they release their contents (Camussi et al., 2010; Feng et al., 2014). Upon incubation with CMVEC, these were internalized by CMVEC and observed in the perinuclear region (**Figure 2D**). However, in order to rule out the possibility of excess dye remaining, we used an equal concentration of dye in equal volume as used in EV labeling and incubated it with ECs after filtration. After filtration, no red color was observed in the fluid after filtration, suggesting no retention of the dye (**Supplementary Figure S3A**). In addition, no red fluorescence signal was detected when incubated with EC (**Supplementary Figure S3B**).

Proteomic Profile in EVs Derived From N1ICD-Overexpressing C-MSCs

Next, we explored the protein cargo in EVs derived from N1ICD-overexpressing C-MSCs using mass spectrometry. **Figure 3A** shows a list of enriched proteins in EV-C-MSCs^{N1ICD} relative to EV-C-MSC^{Notch1FF}. Differentially expressed proteins of EVs were annotated on the basis of the GO terms. Considering the EV data set, we found GO terms significantly enriched in the "biological process" category. Proteomics profiling identified several proteins, such as Lysyl oxidase homolog-2 and biglycan, as highly enriched proteins in EV-C-MSCs^{N1ICD} vs. EV-C-MSCs^{Notch1FF}. The enriched proteins in EVs derived from N1ICD-overexpressing C-MSCs were related to blood vessel development, cell proliferation, angiogenesis, EC proliferation and migration, heart development, and response to hypoxia (**Figure 3B**). Proteomic raw data are available in ProteomeXchange with identifier PXD016578.

Effect of EVs From N1ICD-Overexpressing C-MSCs on Apoptosis and Tube Formation *in vitro*

We wanted to determine whether EVs derived from N1ICD-overexpressing C-MSCs had an effect on apoptosis and tube formation. We successfully isolated mouse aortic EC which expressed CD31 (**Supplementary Figure S4**). Under oxidant stress, pretreatment of EC with EVs from C-MSCs attenuated apoptosis as detected by TUNEL staining (**Figures 4A,B**). Interestingly, EVs from Notch1 knockout C-MSCs abrogated such effects. Moreover, apoptosis was significantly lower in EV treatment from C-MSCs^{N1ICD} than control or Notch1 knockout C-MSCs (**Figures 4A,B**). Given the role of Notch1 signaling activation in postnatal angiogenesis, we also determined the effects of EV on tube formation. Using the HAECs' tube formation assay, we found that EV-C-MSCs^{N1ICD} dramatically promoted tube formation *in vitro* compared to PBS control or EVs from wild-type C-MSCs or Notch1 knockout C-MSCs (**Figure 4C**). Covered area, total tube length, total branching points, and total loops were all significantly increased with treatment of EV-C-MSCs^{N1ICD} when compared to EVs from other C-MSCs and control groups (**Figure 4D**).

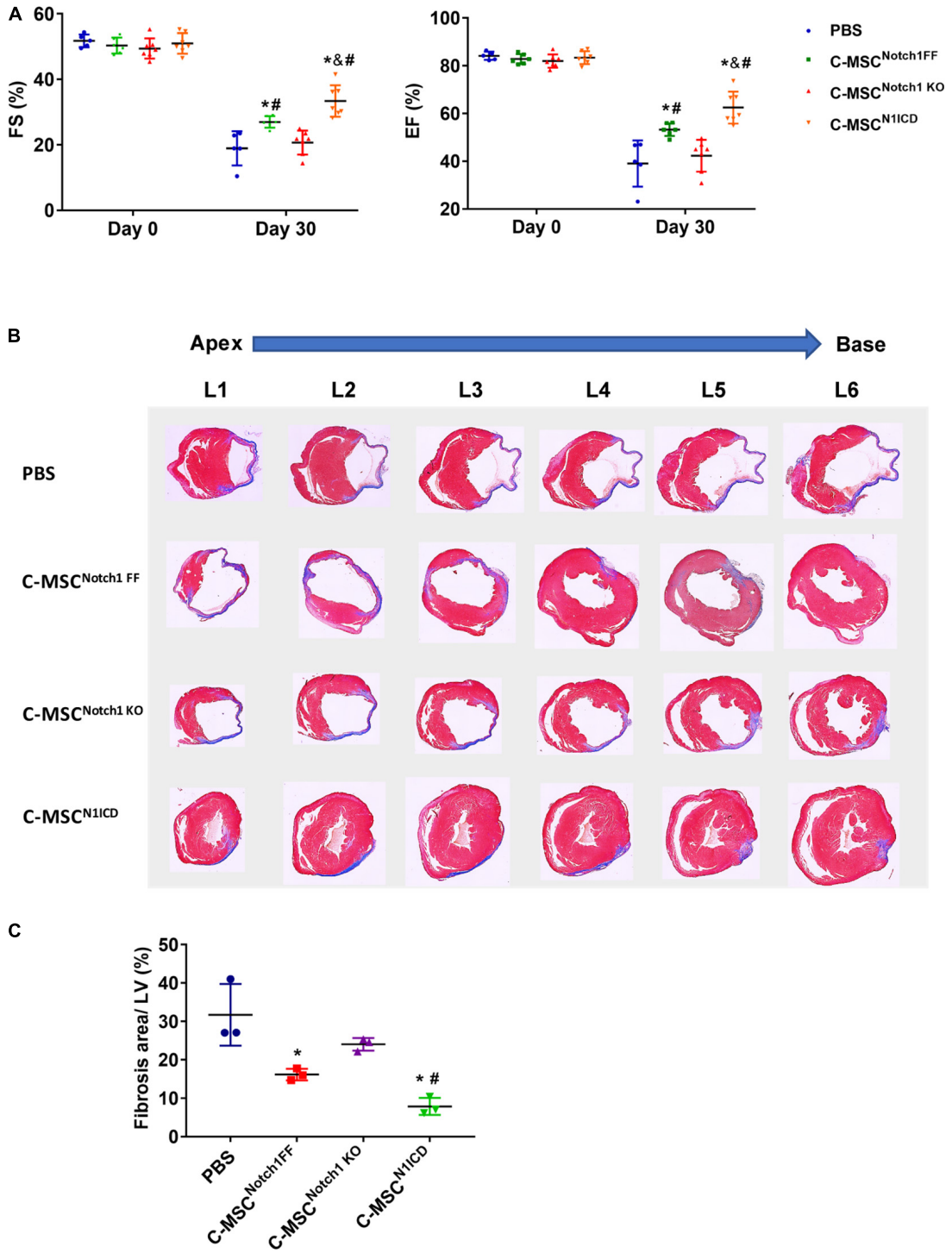

FIGURE 1 | Notch1 intracellular domain (N1ICD)-overexpressing cardiac mesenchymal stem cells (C-MSC) (C-MSC^{N1ICD}) transplantation improved cardiac function and attenuated cardiac fibrosis after myocardial infarction (MI). **(A)** Intramyocardium transplantation of both C-MSCs^{Notch1FF} and C-MSCs^{N1ICD} improved fractional shortening (FS) and ejection fraction (EF) compared with PBS-treated mice ($P < 0.001$). $n = 5$ in PBS, $n = 7$ C-MSC^{Notch1KO} and Exo-C-MSC^{N1ICD} groups; $n = 6$ in C-MSC^{Notch1FF} group. **(B)** Representative Masson trichrome-stained sections of hearts from mice treated with C-MSC^{Notch1FF}, C-MSC^{Notch1KO}, and C-MSC^{N1ICD} 1 month after MI. **(C)** Quantitative analysis of fibrosis size from different treated mice 1 month after MI C-MSCs^{N1ICD} significantly decreased fibrosis size compared with other groups, respectively ($n = 3$). * vs. PBS group, # vs. C-MSC^{Notch1KO} group, & vs. C-MSC^{Notch1FF}, $P < 0.01$.

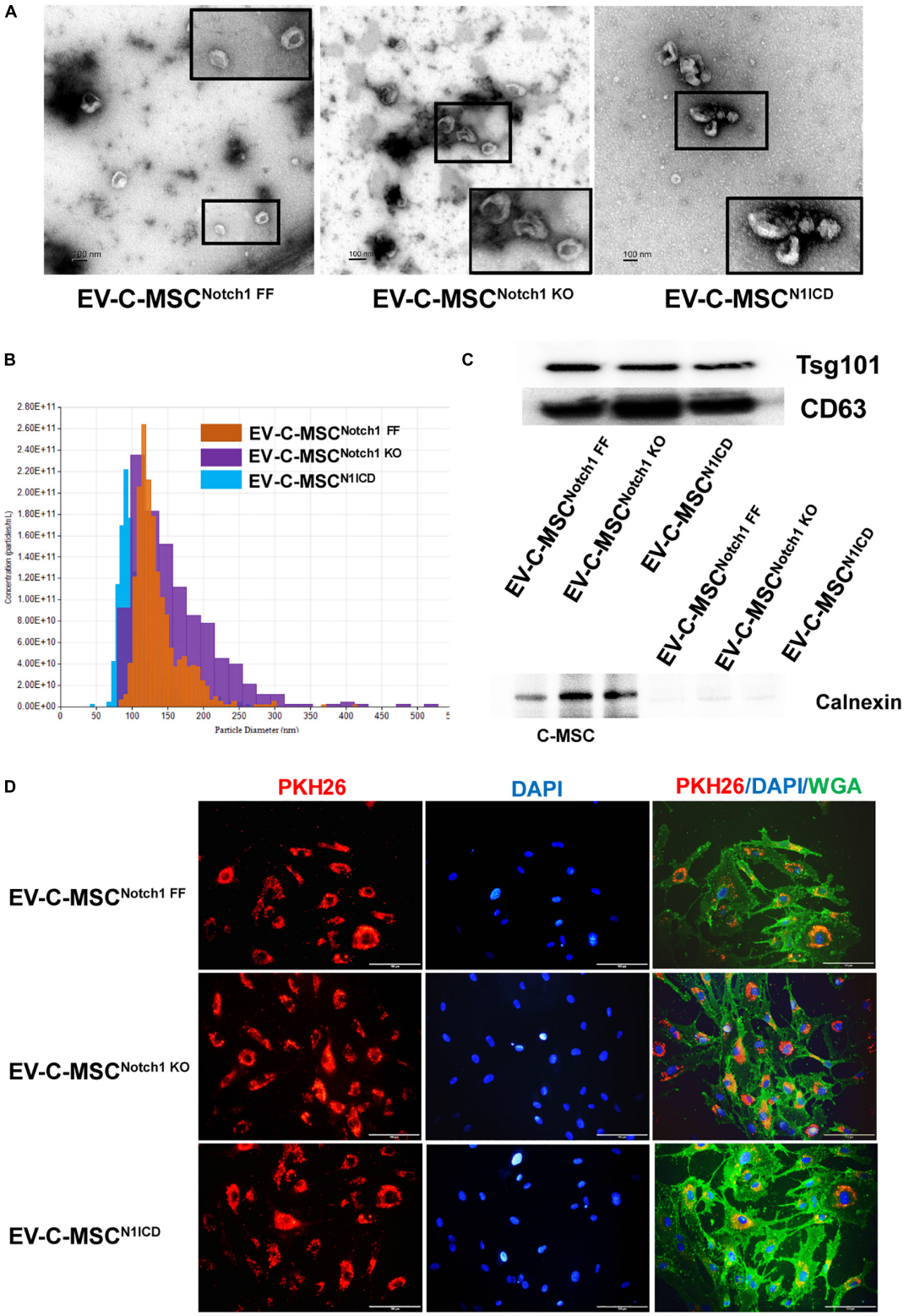

FIGURE 2 | Characterization and internalization of extracellular vesicles from C-MSCs. **(A)** Extracellular vesicles (EVs) secretion from the different C-MSCs as imaged by transmission electron microscopy (TEM). Inset shows higher magnification of EVs. Bar = 100 nm. **(B)** Representative graph of size distribution of EVs from C-MSCs^{Notch1FF}, C-MSCs^{Notch1KO}, and C-MSCs^{N1ICD} detected by tunable resistive pulse sensing (TRPS). **(C)** Representative images of western blot showed that EVs from C-MSCs^{Notch1FF}, C-MSCs^{Notch1KO}, and C-MSCs^{N1ICD} were enriched in EV-specific marker CD63 and Tsg101. Negative marker calnexin was not expressed in EVs. **(D)** EV internalization in cardiac microvascular endothelial cells (CMVECs). PKH26-labeled EVs (red) from different groups of C-MSCs were observed inside the CMVECs (green, WGA), mostly located at the perinuclear region. Bar indicates 100 μm.

A

Protein	Fold change
Collagen alpha-1(I) chain	1.610704137
Collagen alpha-2(I) chain	1.594698541
Nidogen-2	1.390402746
Fibronectin	1.40751941
Collagen alpha-1(III) chain	1.781245834
Biglycan	1.964302774
Nidogen-1	1.610110582
Tenascin	1.349265543
Procollagen C-endopeptidase enhancer	1.589373009
Basement membrane-specific heparan sulfate proteoglycan core	1.315790801
Fibulin-2	1.210750398
Laminin subunit gamma-1	1.260847085
Collagen alpha-2(V) chain	5.288386814
Lysyl oxidase homolog 2	2.523245362
Tubulin beta-4B	1.210750398
Laminin subunit beta-1	1.703988677
Fibrillin-1	3.032592876
Inter-alpha-trypsin inhibitor heavy chain H2	1.584901631
Collagen alpha-2(VI) chain	18.98345885
Collagen alpha-1(XV) chain	4.399499169

B

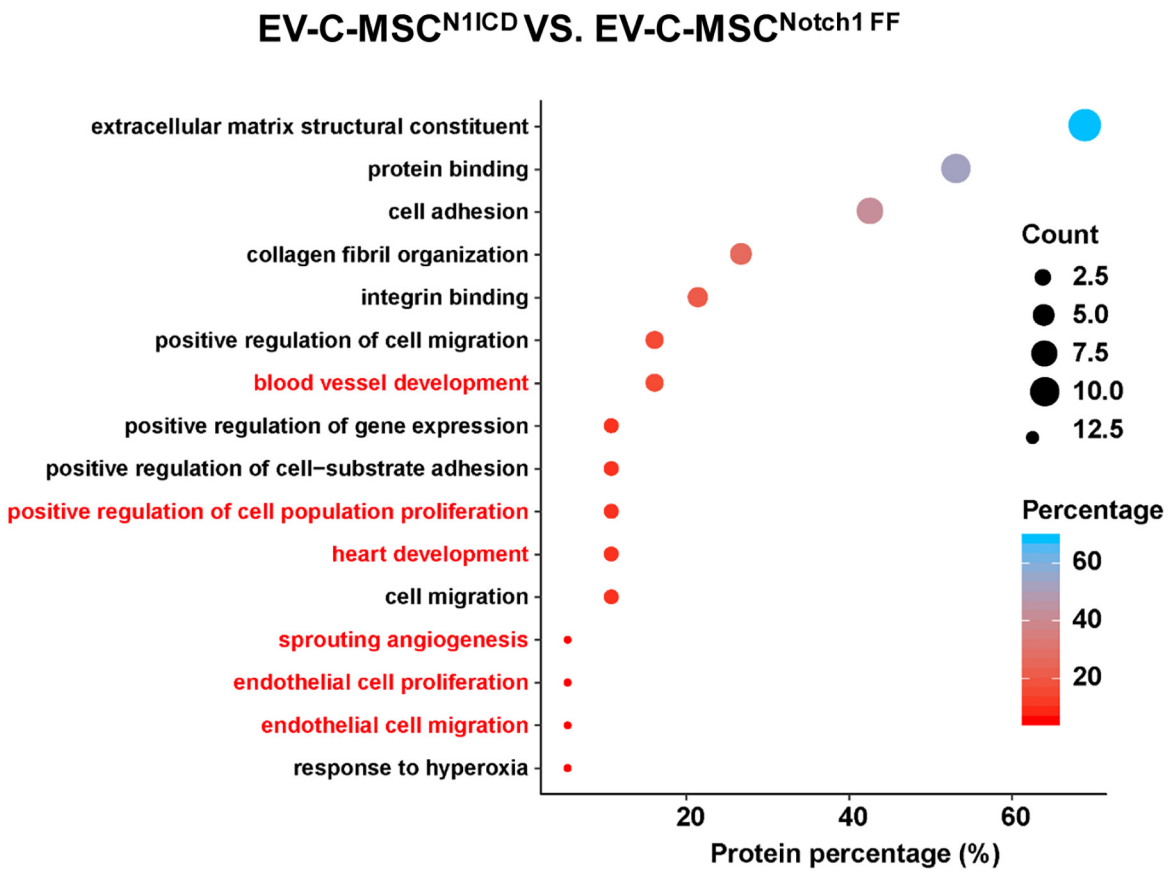

FIGURE 3 | Proteomic profile in EVs derived from N1ICD-overexpressing C-MSCs. **(A)** List of enriched proteins in EV-C-MSCs^{N1ICD} relative to EV-C-MSCs^{Notch1FF}. **(B)** GO protein annotation and pathway enrichment analysis. **A**, Pathway enrichment analysis on differentially expressed proteins in EVs. As expected, many terms referring to biological processes related to various functions of C-MSCs, including extracellular matrix, collagen, proliferation, apoptosis, cell cycle, differentiation, migration, heart development, and angiogenesis were pointed out.

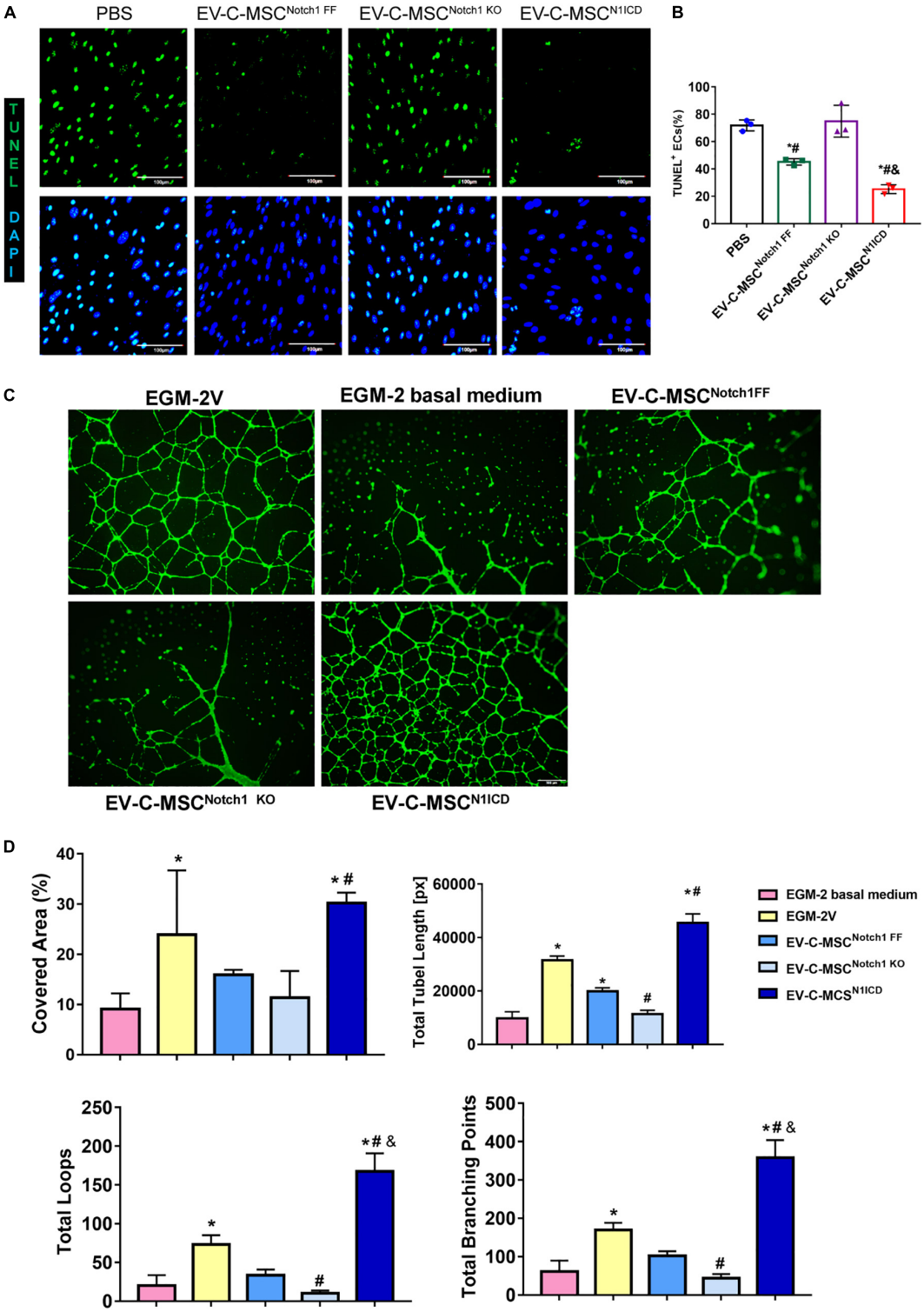

FIGURE 4 | *In vitro* cardioprotective effects of EV-C-MSCs^{N1ICD}. **(A)** Representative images of TUNEL staining in mouse ECs which were pretreated with EVs from C-MSCs^{Notch1FF}, C-MSCs^{Notch1KO}, and C-MSCs^{N1ICD} (1 μg/well, 12-well plate) for 24 h and then were subjected to 8 h H_2O_2 (300 μM) treatment. Bar = 100 μm. **(B)** Quantitative analysis for TUNEL staining in mouse ECs. * vs. PBS group, # vs. EV-C-MSC^{Notch1KO} group, & vs. EV-C-MSC^{Notch1FF} group, $P < 0.05$. **(C)** Representative images of tube formation in human aortic endothelial cells (HAECs) with EV treatment from C-MSCs^{Notch1FF}, C-MSCs^{Notch1KO}, and C-MSCs^{N1ICD} (1 μg/well, 24-well plate). EGM-2V medium and EGM-2V basal medium (without VEGF) served as controls. HAECs were labeled with Calcein AM (Green). Bar = 500 μm. **(D)** Quantitative evaluation for tube formation assay. Covered area, total tube length, total branching points, and total loops were analyzed from three biological repeated experiments. Bar indicates 500 μm. * vs. EGM-2 basal medium group, # vs. EV-C-MSC^{Notch1FF} group, & vs. EGM-2V group, $P < 0.05$.

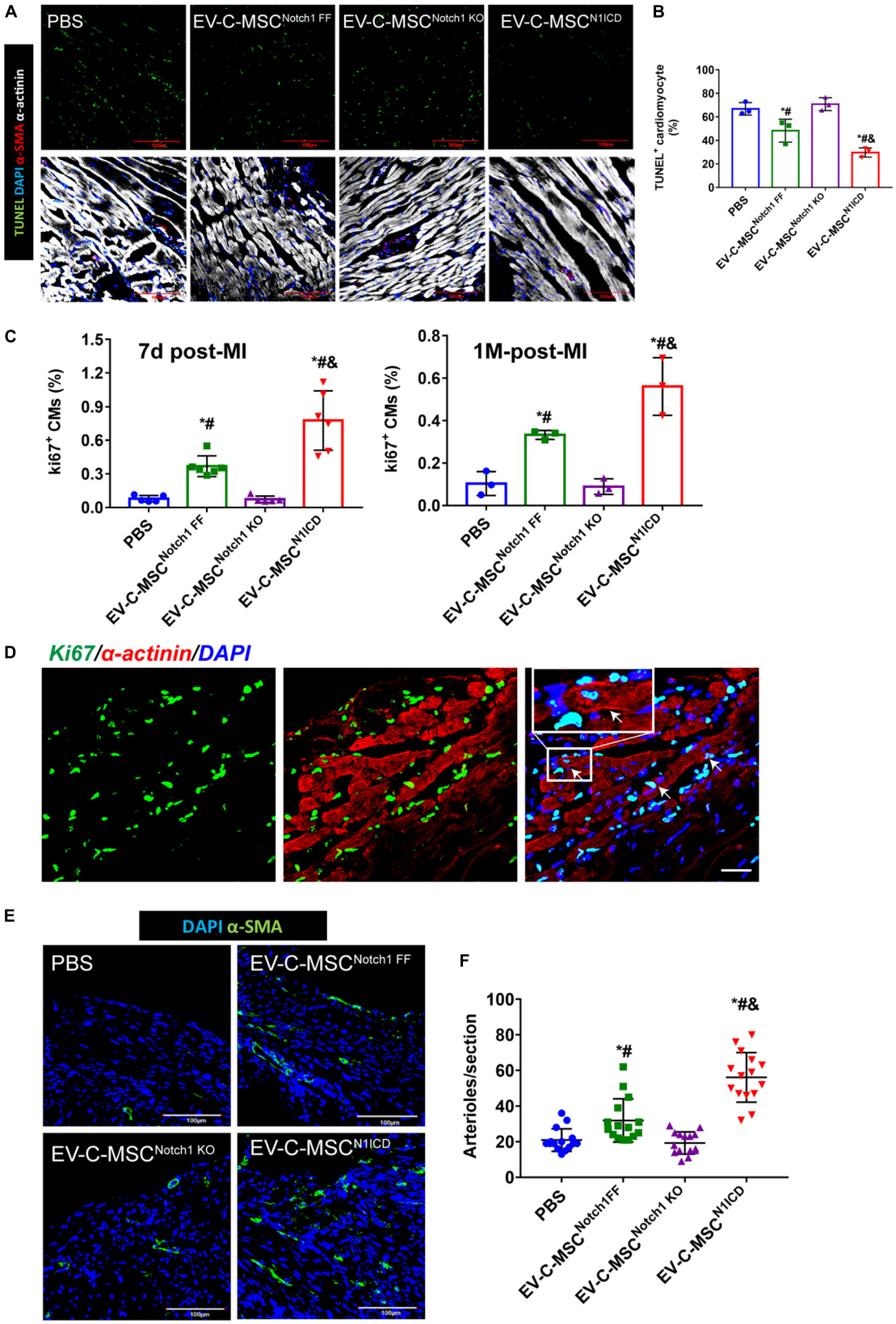

FIGURE 5 | Continued

FIGURE 5 | *In vivo* cardioprotective effects of EV-C-MSCs^{N1ICD}. **(A)** Representative images of TUNEL staining in infarcted mouse hearts with EV treatment from C-MSCs^{Notch1FF}, C-MSCs^{Notch1KO}, and C-MSCs^{N1ICD} 24 h after MI. **(B)** Quantitative analysis for TUNEL staining in infarcted mouse hearts. * vs. PBS group, # vs. EV-C-MSC^{Notch1KO} group, & vs. EV-C-MSC^{Notch1FF} group, $P < 0.05$. **(C)** Quantitative analysis for ki67-positive cardiomyocytes (CMs) in mice with EV treatment from C-MSCs^{Notch1FF}, C-MSCs^{Notch1KO}, and C-MSCs^{N1ICD} at 7 days and 1 month after MI. * vs. PBS group, # vs. EV-C-MSC^{Notch1KO} group, & vs. EV-C-MSC^{Notch1FF} group, $P < 0.001$. $n = 5$ in PBS and C-MSC^{Notch1KO} groups; $n = 6$ in EV-C-MSC and EV-C-MSC^{N1ICD} groups at 7 days after MI; and $n = 3$ at 1 month post-MI. **(D)** Representative image of ki67-positive CMs (α-actinin-positive) in EV-C-MSC^{N1ICD}-treated mouse 7 days after MI. Inset shows higher magnification, and white arrows indicate ki67-positive CMs. Bar = 50 μm. **(E)** Representative images of arteriole density in peri-infarct area from mice 1 month after MI. Arterioles were identified by α-SMA positive-staining (green) in the vessels. Bar = 100 μm. **(F)** Quantitative analysis of arteriole density from MI mice with different EV treatment. Arteriole density was markedly increased in EV-C-MSC^{N1ICD}-treated hearts in the peri-infarct area compared with other treatments respectively. * vs. PBS group, # vs. EV-C-MSC^{Notch1KO} group, & vs. EV-C-MSC^{Notch1FF} group, $P < 0.05$. $n = 15$ sections from five mice in each group.

In vivo Effect of EVs Derived From C-MSCs on Ischemic Injury in Mouse MI Model

Next we investigated whether EVs derived from N1ICD-overexpressing C-MSCs prevent CM apoptosis, stimulate CM proliferation, and promote neovascularization after MI. Twenty-four hours after MI, we observed both that EV-C-MSCs^{Notch1FF} and EV-C-MSCs^{N1ICD} decreased CM apoptosis compared with the PBS control group (**Figures 5A,B**). EV-C-MSCs^{N1ICD} further increased CM proliferation at a higher rate than with EV-C-MSCs^{Notch1FF} (**Figure 5C**) at both 7 days and 1 month post-MI. **Figure 5D** shows representative images of Ki67- and α-actinin-positive CMs in the peri-infarct region of EV-C-MSC^{N1ICD}-treated mouse hearts 7 days post-MI. In agreement with our *in vitro data*, the vessel density as identified by α-SMA staining and tube-like structures (**Figures 5E,F**) in the infarcted region was also increased by treatment with both EV-C-MSCs^{Notch1FF} and EV-C-MSCs^{N1ICD} but significantly higher in the EV-C-MSC^{N1ICD} group than all other groups (**Figures 5E,F**). In line with a previous study (Ju et al., 2018), EVs from C-MSCs had, in general, a significant effect on cardiac function compared with PBS control (EF 55.30 ± 6.83% vs. 40.98 ± 9.77%; FS: 28.74 ± 4.57% vs. 20.31 ± 5.38%, $P < 0.05$.) (**Figures 6A,B**). However, EV-C-MSCs^{N1ICD} had a profound effect on functional parameters (EF: 65.54 ± 4.77% vs. 55.30 ± 6.83%; FS: 35.84 ± 3.54 vs. 28.74 ± 4.57%, $P < 0.05$.) (**Figures 6A,B**). EVs from Notch1 knockout C-MSCs had the opposite effect on cardiac function and reversed the beneficial effect of EV-C-MSCs^{N1ICD} (**Figures 6A,B**). Histological evidence of a larger amount of collagenous mass by the latter treatment further supports the negative role of EVs from Notch1 knockout C-MSCs ($n = 6$) (**Figures 6C,D**).

DISCUSSION

In the adult heart, C-MSCs participate in stromal cardiac tissue renewal by differentiating into SMCs and ECs and releasing a variety of paracrine factors responsible for trophic, angiogenic, and anti-inflammatory effects (Martini et al., 2019). Here we report that transplantation of C-MSCs after N1ICD overexpression has a superior and significant effect on cardiac function improvement and attenuation of cardiac fibrosis compared with C-MSCs. Second, EVs secreted by these modified C-MSCs also played a significant role in cardiac rejuvenation and healing compared to EVs from simple C-MSCs. In contrast, Notch1 deletion resulted in loss of regenerative capabilities of C-MSCs and their EVs as well.

Proangiogenic therapy appeared to be a promising strategy for MI. Neovasculogenesis has the potential to salvage ischemic myocardium at early stages post-MI. Notch1 signaling plays a critical role in postnatal angiogenesis including cardiac angiogenesis during ischemia (Yoshida et al., 2014; Zhou et al., 2018), thus enhancing survival of cardiac cells. Notch1 activation promotes VSMC differentiation of CDC through an RBPJ-dependent signaling pathway (Chen et al., 2012). Consistent with these findings, we discovered that N1ICD-overexpressing C-MSCs differentiated into VSMCs in the infarcted heart after transplantation and led to significant functional improvement and attenuation of fibrosis compared to C-MSCs. Despite low expression of N1ICD, C-MSC transplantation had some therapeutic effect (Moore et al., 2017). It appears that Notch1 activation boosts the function of C-MSCs in the regenerative process. However, Notch1 is altered in aging (Rizzo et al., 2018), which also compromises the function of C-MSCs (Martini et al., 2019). How Notch signaling is affected in the aging heart has not been extensively investigated yet. Cardiac progenitor cells (CPCs) lose their reparative potential as they age (Trac et al., 2019). Spherical aggregation rescued the reparative potential of CPCs from older donors due to increased Notch1 signaling (Trac et al., 2019). Our study clearly demonstrates the importance of Notch1 signaling in C-MSC activation. Although the molecular mechanisms of action by Notch1 in aged C-MSCs remain to be characterized, our study emphasizes its importance not only in MI but also for aging heart diseases.

Cardiac mesenchymal stem cells have the potential to differentiate into cardiac lineage cells in the ischemic myocardium, but significant improvement in cardiac function does not correspond to regeneration against scar area by stem cells (Mirotsou et al., 2011). Transdifferentiation and paracrine signaling are suggested to underlie their cardiac reparative effects. More recently, the EVs secreted by stem cells have drawn more attention as final effectors of protection against ischemic injury. Majority of recent studies support the notion that MSCs mediate their effect by paracrine factors (Timmers et al., 2011; Ju et al., 2018). This paracrine signaling is now believed to occur through the release of small vesicles. The therapeutic efficacy of EVs is dependent not only on the synergy of a select permutation of individual EV components but also on the amount of EVs, their internalization by recipient

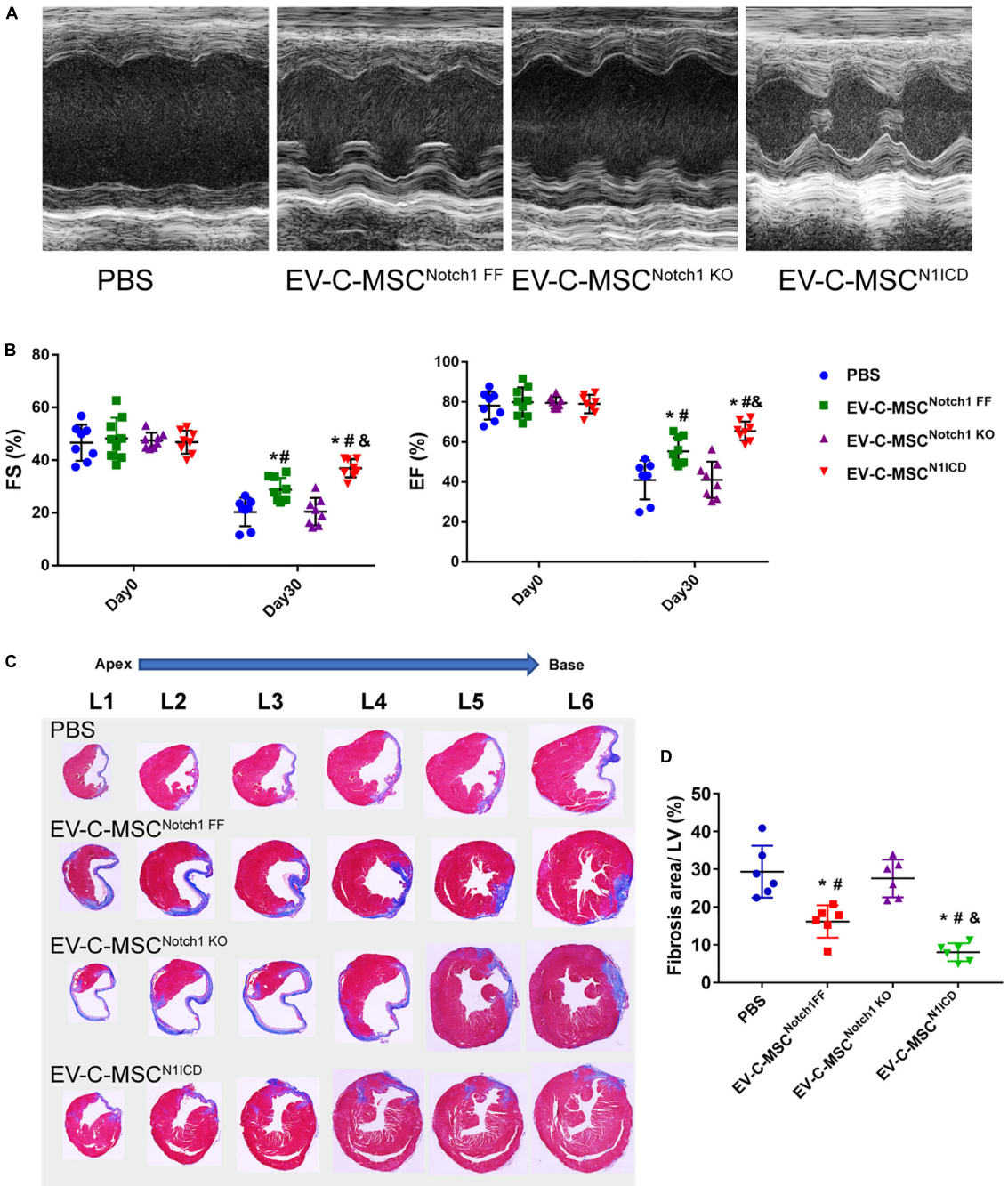

FIGURE 6 | EV-C-MSCs^{N1ICD} improved cardiac function and attenuated cardiac fibrosis after MI. **(A)** Representative M-mode echocardiography images from different EVs treated. **(B)** Intramyocardium injection of both EV-C-MSCs^{Notch1FF} and EV-C-MSCs^{N1ICD} improved fraction shortening (FS) and ejection fraction (EF) compared with PBS-treated mice ($P < 0.001$). $n = 8$ in PBS, EV-C-MSC^{Notch1KO}, and EV-C-MSC^{N1ICD} groups; $n = 9$ in EV-C-MSC^{Notch1FF} group. **(C)** Representative Masson trichrome-stained sections of hearts from mice treated with EV-C-MSC^{Notch1FF}, EV-C-MSC^{Notch1KO}, and EV-C-MSC^{N1ICD} 1 month after MI. **(D)** Quantitative analysis of fibrosis size from different treated mice 1 month after MI. EV-C-MSCs^{N1ICD} significantly decreased fibrosis size compared with other groups, respectively. * vs. PBS group, # vs. EV-C-MSC^{Notch1KO} group, & vs. EV-C-MSC^{Notch1FF} group, $P < 0.01$. $n = 6$.

cells, and release of their contents (Camussi et al., 2010; Feng et al., 2014). Manipulation of Notch activity for increased cellular survival and proliferation has recently been promoted as a potential approach for regenerative medicine (Androutsellis-Theotokis et al., 2006). Our data support the notion that EVs from Notch1 engineered C-MSCs have a superior and significant effects on angiogenesis and myocyte proliferation in the ischemic heart following coronary artery

ligation. EVs secreted by C-MSCs with Notch1 deletion were not effective in preventing apoptosis and myocyte proliferation and angiogenesis. It appears from the proteomic profile that EVs from N1ICD-overexpressing C-MSCs are enriched with bioactive factors, potent pro-repair proteins responsible for cell migration and proliferation and CM protection. For example, biglycan, one of the enriched proteins in EVs from N1ICD-overexpressing C-MSCs, has been demonstrated as an angiogenic factor (Yamamoto et al., 2012; Xing et al., 2015; Myren et al., 2016). Biglycan enhanced promoter activity of hypoxia-inducible factor-1α (HIF-1α), resulting in increased HIF-1α mRNA levels, as well as augmented HIF-1 activity, leading to increased VEGF expression (Hu et al., 2016). Lysyl oxidase-like protein-2 (LOXL-2), a highly enriched protein, was also related to regulation of sprouting angiogenesis and played an essential role in developmental angiogenesis (Zaffryar-Eilot et al., 2013). It is expressed in neovessels as a hypoxia target and accumulated in the endothelial extracellular matrix (ECM) (Bignon et al., 2011). A gain-and-loss-of-function experiment demonstrated that LOXL-2 overexpression increased capillary formation and LOXL-2 knockdown dramatically reduced EC migration and proliferation, resulting in decreased tubulogenesis (Bignon et al., 2011; de Jong et al., 2019). In addition, biglycan overexpression in transgenic mice has been shown to induce cardioprotective genes [nitric oxide (NO) synthases] in the heart (Bereczki et al., 2007). Biglycan protected CMs against hypoxia/reoxygenation injury in an NO-dependent mechanism (Csont et al., 2010). Consistent with these studies, EVs from N1ICD-overexpressing C-MSCs prevented apoptosis of ECs and CMs exposed to oxidative stress and ischemic injury and promoted cardiac angiogenesis. It is likely that biglycan and LOXL-2 contributed to such protective effects. Sprouting angiogenesis is related to extensive ECM remodeling (Neve et al., 2014; Crosby and Zoldan, 2019). Multiple levels of cell–ECM interactions are potentially involved in capillary formation (Edgar et al., 2014; Rauff et al., 2019). We noticed that some laminin subunits and collagen alpha chains were upregulated in EVs from C-MSCs^{N1ICD}. Thus, the molecular mechanisms of action by these proteins in EVs remain to be determined.

CONCLUSION

Notch signaling is important in cardiac repair following myocardial injury. In this study, we have investigated the role of EVs from Notch1 engineered C-MSCs in angiogenesis and CM proliferation in ischemic myocardium. EV-C-MSCs^{N1ICD} were very effective in improving cardiac function and decreasing fibrosis. Notch1 signaling is a strong stimulus for cardiac regeneration by C-MSCs. EVs secreted by Notch1-overexpressing C-MSCs were highly effective in preventing cell death, promoting angiogenesis and CM proliferation, and restoring cardiac function post-MI. In conclusion, proangiogenic factors from EVs of N1ICD-overexpressing C-MSCs might be a novel strategy for boosting angiogenesis in ischemic hearts.

DATA AVAILABILITY STATEMENT

The proteomic raw data generated in this study has been deposited in ProteomeXchange with identifier PXD016578.

ETHICS STATEMENT

All study protocols were approved by the Institutional Animal Care and Use Committee and carried out consistent with the recommendations of the American Veterinary Medical Association guidelines.

AUTHOR CONTRIBUTIONS

WX participated in experimental design, acquisition, and analysis of experimental data, and drafted the manuscript. MK helped in experimental design, manuscript writing, and proofreading. MA conceived the idea, helped in experimental design, finalized the manuscript, and financially supported the study through NIH 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/fcell.2020.00011/full#supplementary-material

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ORIGINAL RESEARCH
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Human Cardiac Progenitor Cells Enhance Exosome Release and Promote Angiogenesis Under Physoxia

*Julie A. Dougherty[1,2†], Nil Patel[1†], Naresh Kumar[1], Shubha Gururaja Rao[3], Mark G. Angelos[1], Harpreet Singh[2,3], Chuanxi Cai[4] and Mahmood Khan[1,2,3]**

[1] Department of Emergency Medicine, The Ohio State University Wexner Medical Center, Columbus, OH, United States, [2] Dorothy M. Davis Heart Lung and Research Institute, The Ohio State University Wexner Medical Center, Columbus, OH, United States, [3] Department of Physiology and Cell Biology, The Ohio State University Wexner Medical Center, Columbus, OH, United States, [4] Division of Cardiac Surgery, Department of Surgery, The Ohio State University Wexner Medical Center, Columbus, OH, United States

Studies on cardiac progenitor cells (CPCs) and their derived exosomes therapeutic potential have demonstrated only modest improvements in cardiac function. Therefore, there is an unmet need to improve the therapeutic efficacy of CPCs and their exosomes to attain clinically relevant improvement in cardiac function. The hypothesis of this project is to assess the therapeutic potential of exosomes derived from human CPCs (hCPCs) cultured under normoxia (21% O_2), physoxia (5% O_2) and hypoxia (1% O_2) conditions. hCPCs were characterized by immunostaining of CPC-specific markers (NKX-2.5, GATA-4, and c-kit). Cell proliferation and cell death assay was not altered under physoxia. A gene expression qPCR array (84 genes) was performed to assess the modulation of hypoxic genes under three different oxygen conditions as mentioned above. Our results demonstrated that very few hypoxia-related genes were modulated under physoxia (5 genes upregulated, 4 genes down regulated). However, several genes were modulated under hypoxia (23 genes upregulated, 9 genes downregulated). Furthermore, nanoparticle tracking analysis of the exosomes isolated from hCPCs under physoxia had a 1.6-fold increase in exosome yield when compared to normoxia and hypoxia conditions. Furthermore, tube formation assay for angiogenesis indicated that exosomes derived from hCPCs cultured under physoxia significantly increased tube formation as compared to no-exosome control, 21% O_2, and 1% O_2 groups. Overall, our study demonstrated the therapeutic potential of physoxic oxygen microenvironment cultured hCPCs and their derived exosomes for myocardial repair.

Keywords: cardiac progenitor cells, stem cells, hypoxia, extracellular vesicles, angiogenesis, cardiac repair

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Sveva Bollini,
University of Genoa, Italy

Reviewed by:
Lucio Barile,
University of Zurich, Switzerland
Marta Tapparo,
University of Turin, Italy

****Correspondence:***
Mahmood Khan
mahmood.khan@osumc.edu

† These authors have contributed equally to this work

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INTRODUCTION

Myocardial infarction (MI) is responsible for the death of one American every 40 s (Benjamin et al., 2017). MI is caused by a limited supply of blood and oxygen to the heart, which leads to cardiac dysfunction, fibrosis, and, ultimately, heart failure. Post-MI, there is a permanent loss of cardiomyocytes and scar tissue formation that results in irreversible damage and maladaptation,

which affects cardiac function (Prabhu and Frangogiannis, 2016). Existing therapies have been used to prevent additional damage to the heart muscle and reduce the risk of future MI, however, they only slow the progression to heart failure. Therefore, effective cardiac repair is essential to restore function of the heart following MI. Currently, cell-based therapies to aid in cardiac tissue regeneration, such as the application of stem cells, serve to be the most promising therapeutic option today (Krishna et al., 2011; Choi et al., 2015). Nevertheless, optimal cell type and conditions have not yet been identified for clinically relevant repair.

Stem cells have been previously used as a cell-based therapy to restore cardiac function. Embryonic stem cells (ESCs) hold promise as a cellular therapy because they are pluripotent; however, the clinical use of this cell line raises several ethical issues and political controversies. Today, adult stem cells are the only cells utilized as a cell-based therapy to treat MI in the United States (Le and Chong, 2016). Cardiac progenitor cells (CPCs) have been of particular interest for stem cell therapy in the treatment of MI since they have the ability to differentiate into three cardiac lineages: cardiomyocytes, smooth muscle cells, and endothelial cells. However, cell survival post-transplantation is very poor. Interestingly, despite this, moderate improvements in cardiac function are observed (den Haan et al., 2012; Noort et al., 2012; van der Spoel et al., 2012; Zuo et al., 2012; Bao et al., 2017; Wu et al., 2017), which points toward a paracrine mechanism of repair.

Extracellular vesicles, including exosomes and microparticles, have recently become of particular interest as they have been identified to be key players in paracrine signaling (Hergenreider et al., 2012; Raposo and Stoorvogel, 2013; Maas et al., 2017; Sullivan et al., 2017) and have become a major focus of research in this area. Exosomes are 50–150 nm (Yanez-Mo et al., 2015) vesicles formed by inward budding of endosomal membranes (Thery, 2011). Microvesicles are larger, 100–1000 nm (Cocucci and Meldolesi, 2015), and directly bud from the cell membrane (Colombo et al., 2014). Despite their differences, practical isolation of the two types of particles is difficult and we will refer to exosomes and microvesicles collectively as EVs. These EVs are nanoparticles that contain lipids (Record et al., 2014), proteins (Choi et al., 2015), and nucleic acids (Gezer et al., 2014; Ahadi et al., 2016; Ohno and Kuroda, 2016), which are specifically packaged depending on their source cell type and microenvironment (Xiao et al., 2016; Dougherty et al., 2017). EVs have already demonstrated therapeutic potential in treating the heart post-MI. In 2010, a study demonstrated that exosomes secreted by MSCs reduce myocardial ischemia/reperfusion injury via a mouse Langendorff heart model. In this study, exosomes were administered prior to reperfusion and results showed decreased infarct size (Lai et al., 2010). Another study demonstrated proangiogenic activities of atrial appendage CPC-derived EVs both *in vitro* and *in vivo* (Barile et al., 2014). Specifically, this study showed that these EVs inhibited cardiomyocyte apoptosis and enhanced angiogenesis, as they were enriched in miRNAs with anti-apoptotic and proangiogenic activities (Barile et al., 2014). Oxygen concentration used for culture were not reported for either study, thus, one then assumes cells were cultured at standard laboratory cell culture conditions of 21% O_2.

The role of oxygen is severely critical in the survival of any type of cell line including stem cells. Oxygen controls the cellular microenvironment, serving as both a metabolic substrate and a signaling molecule (Abdollahi et al., 2011). Standard cell culturing protocols utilize 21% O_2 for culturing and maintaining the cells. These conditions are considered normoxia, as it is the atmospheric level of oxygen. On the contrary, in an *in vivo* scenario, the oxygen microenvironments for cells are much lower than 21% O_2. The relative oxygen concentration of arterial blood is approximately 12% and most tissue is around 3.4 to 6.8% with concentration varying based on location (reviewed in Abdollahi et al., 2011; McKeown, 2014). McKeown proposes that 5% O_2 be termed "physoxia" as it is a better estimate of tissue oxygenation (McKeown, 2014). Conversely, hypoxic culture of cells affects their functional behavior and can have therapeutic applications. In two different studies, hypoxic culture (1% O_2) of adipose stromal cells enhanced cytokine production and increased their angiogenic properties (Rehman et al., 2004; Thangarajah et al., 2009). Also, hypoxic culture (2% O_2) of stem cells has demonstrated various benefits including a 30-fold increase in the expansion of cells compared to normoxic conditions in a study utilizing human bone marrow-derived mesenchymal stem cells (BM-MSCs) (Grayson et al., 2007). Another study demonstrated that hypoxic preconditioning (1% O_2 hypoxia for 6 h) enhanced CPC function by demonstrating increased invasion ability and pro-survival pathway activation (Hernandez et al., 2018). Thus, culturing cells *in vitro* at physoxic and hypoxic conditions mimics the *in vivo* microenvironment and that of the ischemic heart post-MI. Additionally, previous studies have reported that short-term hypoxic culture resulted in enhanced exosome release from mouse CPCs and altered their molecular contents (Gray et al., 2015; Barile et al., 2017). Therefore, the focus of this paper was to investigate whether low-oxygen culturing (5 or 1% O_2) of hCPCs modulates hypoxia signaling genes and their derived exosomes for cardiac repair post-MI.

MATERIALS AND METHODS

Culture of Cardiac Progenitor Cells

Human cardiac progenitor cells (hCPCs) were isolated from the right atrial appendage and sorted for expression of c-kit cell surface marker, as described previously (Zhang et al., 2017). Cells were used at passage 7–10 for these studies. Cells were initially cultured for 48 h at normoxic conditions (37°C, 21% O_2) then placed in medium with exosome-depleted FBS (SBI, Palo Alto, CA, United States) and continuously cultured at normoxic condition of 21% O_2 physoxic condition of 5% O_2 or hypoxic condition of 1% O_2 using a controlled C-chamber incubator (ProOx P110 O_2 Controllers, BioSperix, Parish, NY, United States). Media was refreshed every other day, retaining 20% of conditioned media. Phase-contrast images

were captured using a DM IL LED microscope and MC170 HD digital camera (Leica Microsystems, Inc., Buffalo Grove, IL, United States).

Immunofluorescent Staining

Cells were seeded on glass cover-slips coated with 0.5% gelatin and cultured at 21, 5, and 1% O_2 for 48 h. Cells were fixed with 4% paraformaldehyde in PBS for 10 min at room temperature, permeabilized with 0.25% Triton-X-100 in PBS for 20 min at 4°C, and incubated overnight at 4°C with antibodies in antibody dilution buffer (1% w/v BSA, 0.3% Triton-X-100 in PBS): NKX-2.5 (1:25, PA5-49431, Invitrogen, Carlsbad, CA, United States), GATA-4 (1:300, PA1-102, Invitrogen, Carlsbad, CA, United States), and c-kit (1:25, MA5-12944, Invitrogen, Carlsbad, CA, United States). Cells were washed three times for 5 min with PBS, treated with secondary antibody (1:500 anti-rabbit IgG Alexa Fluor 488 ab150077 or anti-mouse IgG Alexa Fluor 488 ab 150113, Abcam, Cambridge, MA, United States), and incubated in the dark for 2 h at room temperature. Cover-slips were washed three times for 5 min with PBS. F-actin was stained using ActinRed™ 555 ReadyProbes™ reagent (R37112, Invitrogen, Eugene, OR, United States) and nuclei were stained with NucBlue™ Live Cell Stain ReadyProbes™ reagent (R37605, Invitrogen, Eugene, OR, United States) for 30 min at room temperature. Cover-slips were washed a final time, mounted with ProLong™ Glass Antifade Mountant (Invitrogen, Eugene, OR, United States) and allowed to cure for 24 h. Imaged were acquired with a FluoView 1000 Filter Confocal Microscope (Olympus, Center Valley, PA, United States). Images were median-filtered (Singh et al., 2012, 2013) and analyzed by FIJI software.

Cell Proliferation Assay

The proliferation of hCPCs was performed using XTT reagent (Cayman Chemical, Ann Arbor, MI, United States) for cells cultured at 21, 5, and 1% O_2. Briefly, 6,000 cells were seeded in replicate wells of 96-well plates for analysis at time 0 and after 24 h. XTT reagent was prepared per the manufacturer's instructions, mixed with cells and media blanks, and incubated at 37°C at their respective oxygen concentrations for 2 h then read with a spectrophotometer at 450 nm for 1 s (Victor300, Perkin Elmer, Waltham, MA, United States). Absorbance values were blank-corrected and proliferation was calculated relative to their time 0 value. Data are plotted as fold increase relative to time 0, mean ± SD, $n = 4$.

Apoptosis Flow Cytometry

Cells were cultured at 21, 5, and 1% O_2 for 48 h then harvested for staining with the ApoDETECT AnnexinV-FITC kit (Invitrogen, Thermo Scientific, Waltham, MA, United States) to assess apoptosis without any additional stress. Cells were trypsinized, collected, and spun down 10 min at 800 *g*. The cell pellet was resuspended in 1 ml ice-cold PBS and transferred to a 1.5 ml microcentrifuge tube and spun down 1 min at 3000 rpm. The cell pellet was resuspended in 1X binding buffer and counted, volume was adjusted so cell density was $2–5 \times 10^5$ cells/ml. 190 μl cell suspension was combined with 10 μl of Annexin V-FITC, mixed gently, and incubated at room temperature for 10 min. Cells were washed 1X with binding buffer, spun down 1 min at 3000 rpm, and resuspended in 190 μl binding buffer. 10 μl of 20 μg/ml propidium iodide stock solution was added to cells and incubated at room temperature for 10 min. Cells were spun down 1 min at 3000 rpm and resuspended in 200 μl of PBS. Cells were analyzed by flow cytometry using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, United States), with single dye controls determining gate parameters. Apoptotic cells are positive for Annexin V-FITC and negative for PI, dead cells are dual positive, and live cells show little to no fluorescence.

Analysis of Gene Expression

RNA Isolation

Human CPCs were cultured at the three distinct oxygen concentrations for 48 h. Cells were then lysed with TRIzol (Invitrogen, Carlsbad, CA, United States) for 3 min at room temp. Total RNA was then isolated using the Direct-zol RNA miniprep kit (Zymo Research, Irvine, CA, United States) per manufacturer's instructions with on-column DNA digestion and analyzed with a spectrophotometer (NanoDrop2000, Thermo Fisher, Pleasanton, CA, United States) for quantity and purity.

cDNA Synthesis

200 ng of total RNA was used to prepare cDNA using the RT^2 First Strand Kit (330404, Qiagen, Germantown, MD, United States) per manufacturer's protocol with synthesis performed for 1 h at 37°C. Reactions were prepared from biological triplicates at the same time, with the same master mix.

qPCR Analysis

cDNA reactions were analyzed with RT^2 SYBR Green ROX qPCR Mastermix (330523, Qiagen, Germantown, MD, United States) with the human hypoxia signaling pathway array (PAHS-032Z, Qiagen, Germantown, MD, United States) according to manufacturer's instructions. Sample master mixes were thoroughly mixed and 25 μl was added to each well of the array plate using an electronic pipette. The reaction was performed on a QuantStudio 3 thermocycler and Ct values were determined by the QuantStudio Design & Analysis Software, version 1.4.1 (Thermo Fisher, Waltham, MA, United States). Relative quantification was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with normalization to five stably expressed housekeeping genes (ATR, CTSA, HIF1AN, LGALS3, RBPJ) and fold-change calculated relative to 21% O_2 cells. Data are shown mean ± SD, $n = 3$.

Isolation of EVs

Human CPC-derived EVs were derived from the cell-conditioned medium collected at each oxygen concentration after culturing for 48 h in media supplemented with exosome-depleted FBS. Conditioned media was clarified with a 0.22 μm syringe filter and concentrated with an Amicon-Ultra 100 kD centricon. EVs were precipitated using ExoQuick-TC®(System Biosciences,PaloAlto, CA, United States) according to the manufacturer's protocol. EVs were then suspended in PBS and stored at −80°C.

Nanoparticle Tracking Analysis (NTA) of EVs

An equal number of cells were seeded to 10 cm dishes and EVs were isolated from equal volumes of conditioned media from normoxic and low-oxygen hCPCs. Isolated EVs were suspended in equal volumes of PBS and analyzed for size, concentration, and distribution with a NanoSight NS300 (Malvern Panalytical, Malvern, United Kingdom). Camera setting and detection threshold were kept constant for all samples for direct comparison, samples were analyzed in triplicate, and a total of at least 1000 validated tracks per sample was measured (NTA v3.3, Malvern Panalytical, Malvern, United Kingdom). Curves represent mean of triplicate measurements.

Cryo-TEM of Isolated EVs

EVs isolated from 21, 5, and 1% O_2 culture were sent for Cryo-TEM processing and imaging, as described in Gao et al. (2014). Briefly, a FEI Vitrobot Mark IV plunge freezer (Thermo Scientific, Waltham, MA, United States), set at room temperature and ∼95% humidity, was used to prepare vitrified cryo-TEM specimens from the aqueous samples. About 2.5 μL of the solution was applied to a TEM grid coated with lacey carbon film. After blotting using two filter papers, the grid was plunge-frozen in liquid ethane (Cavalier et al., 2009). The vitrified specimen was mounted onto a Gatan 626.DH cryo-TEM holder and transferred into a FEI Tecnai F20 TEM equipped with a Gatan twin blade retractable anti-contaminator. The cryo-TEM observation was carried out at ∼ −174°C.

Exosome Antibody Array of Isolated EVs

Isolated EVs from 21, 5, and 1% O_2 cultured hCPCs were analyzed for expression of known exosome markers using the Exo-Check Exosome Antibody Array (System Biosciences, Palo Alto, CA, United States) per the manufacturer's instructions. Briefly, 50 μg of EVs (by total EV protein) were added to 10X lysis buffer and vortexed. Labeling reagent was added, sample was vortexed, and incubated at room temperature with orbital shaking for 30 min. Excess labeling reagent was removed with provided columns and lysate was added to Blocking Buffer and mixed by inversion. The array membrane was incubated in distilled water at room temperature for 2 min, the water was then decanted, the lysate/blocking buffer mixture was added to the membrane, and it was incubated overnight at 4°C on a rocker. The lysate/blocking mixture was decanted, the membrane was washed at room temperature twice for 5 min with rocking. Detection Buffer was added to the membrane and incubated for 30 min at room temperature with rocking. Detection buffer was decanted and the membrane was washed three times for 5 min at room temperature. SuperSignal™ West Femto Maximum Sensitivity Substrate (Thermo Scientific, Waltham, MA, United States) was used to develop the membrane by mixing reagents 1:1 and incubating on the membrane for 5 min. Imaging was performed with an Azure c600 Imaging System (Azure Biosystems, Dublin, CA, United States).

Wound Healing (Scratch Assay) for Cell Migration

Triplicate wells of a 24-well plate were seeded with bovine aortic endothelial cells (BAECs) cultured at 37°C, 5% CO_2, 21% O_2, and grown to confluence. A scratch was made down the center of each well with a 200 μl standard pipet tip. Human CPC-derived EVs were added to cells at 100 μg/mL and imaged at 0 and 8 h time points. An EVOS FL Auto 2 (Thermo Scientific, Waltham, MA, United States) with a programmable stage was used to take images of the same fields over time. The wound area was analyzed for four fields per well with ImageJ by a blinded operator. The area of closure was calculated as A = (1- (*n* h area/0 h area)) x 100% per frame then averaged per well.

Tube Formation Assay for Angiogenesis

Bovine aortic endothelial cells cultured at 37°C, 21% O_2 were seeded in quadruplicate in Geltrex-coated wells on a 24-well plate. Human CPC-derived EVs from normoxic 21% O_2, physoxic (5% O_2), hypoxic (1% O_2) conditions or an equal volume of PBS (No-EVs Control) were added to BAECs at 100 μg/mL. Plates were incubated at normoxic conditions (37°C, 5% CO_2, 21% O_2) for 16 h. Wells were gently washed with PBS and fixed with 4% paraformaldehyde in PBS for 20 min at room temperature. Images were taken with a 4X objective on a Leica DM IL LED microscope (Leica Microsystems, Inc., Deerfield, IL, United States). Tube formation was analyzed with the Angiogenesis Analyzer plug-in on ImageJ. Six images per well were analyzed, with four wells per group. Well totals were calculated and normalized to the No-EVs Control.

Statistical Analysis

Data were analyzed with a one-way ANOVA and Holm-Sidak post-test, when meeting the assumptions of normality and equal variance. A p-value <0.05 was considered statistically significant. All values are expressed as mean ± SD.

RESULTS

Cell Morphology and Cardiac Marker Expression of hCPCs

Human CPCs were cultured at each oxygen concentration for 48 h with hypoxia which was achieved in a regulated hypoxia chamber at 37°C and 5% CO_2, with humidity. Phase microscopy images were taken and revealed that hCPCs showed no changes in cell morphology compared to normoxic 21% O_2 when subjected to low-oxygen (**Figure 1**). Immunofluorescent staining for cardiac markers NKX-2.5 and GATA-4 was preformed to confirm their cardiac lineage (**Figure 1** and **Supplementary Figures 1, 2**), which confirmed that low-oxygen culture conditions did not alter their cardiac lineage. Similarly, immunostaining for c-kit was also performed and results showed that cells maintained c-kit expression under low-oxygen conditions as well (**Figure 1** and **Supplementary**

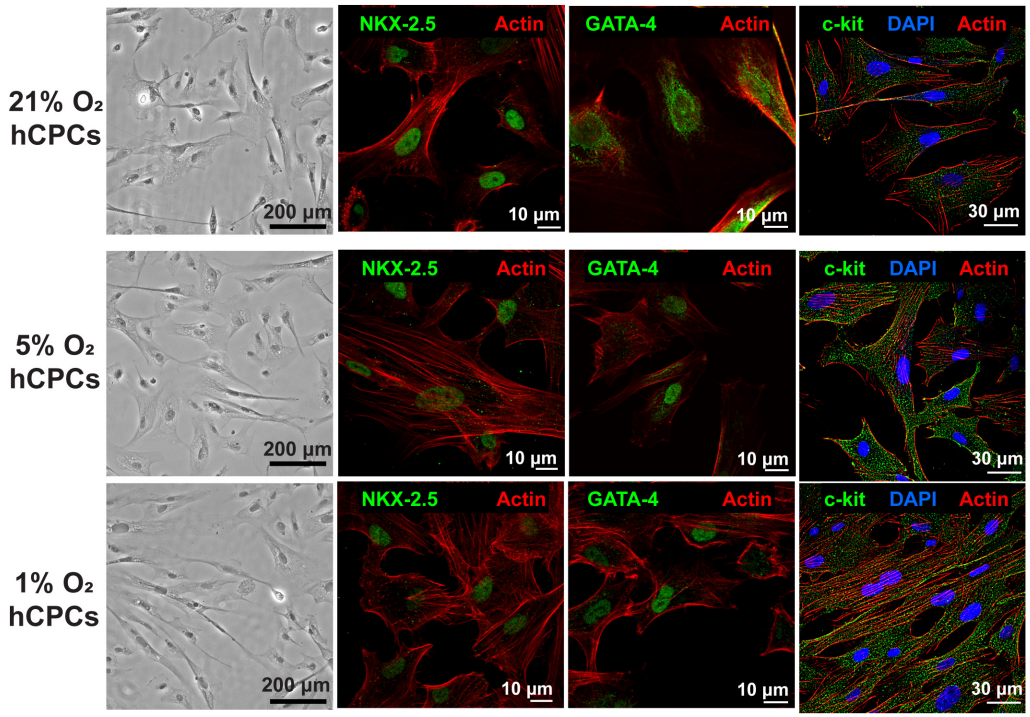

FIGURE 1 | hCPCs morphology and cardiac gene expression under normoxic and hypoxic microenvironments. Human CPCs were cultured under 21, 5, and 1% O_2 for 48 h. DIC imaging shows the typical morphology of cells, which is unchanged under hypoxia. Immunofluorescent staining for cardiac lineage markers NKX-2.5 (green, nuclear), GATA-4 (green, nuclear), and c-kit (green) showed their expression was maintained under all oxygen conditions. Nuclei are stained blue and F-actin is stained red.

Figure 3). Therefore, low-oxygen culture did not affect CPC morphology or identity.

Analysis of Cellular Health Under Hypoxic Culturing Conditions

Hypoxia is known to negatively affect cellular health due to oxidative stress. To test whether 5% O_2 and 1% O_2 negatively affected cellular health we analyzed cell proliferation and cell death for all conditions. Cells were analyzed by XTT assay at time 0 and after 24 h. Results demonstrated that cell proliferation was unchanged under either low-oxygen condition as compared to 21% O_2 (**Figure 2A**), thus the cells were dividing as normal. Furthermore, cells were analyzed for apoptosis and cell death to determine whether culturing under low-oxygen conditions was stressful and induced apoptosis or necrosis. We incubated the cells under the three oxygen concentrations for 48 h then harvested and stained the cells with Annexin V-FITC and propidium iodide (PI) for analysis by flow cytometry. Results showed that the percentage of healthy cells (dual negative) is similar for all oxygen conditions (**Figure 2B**). Thus, culturing cells at 5 and 1% O_2 for 48 h did not induce apoptosis or necrosis.

Changes in Cellular Gene Expression Under Varying O_2 Environments

A qPCR array for hypoxia-related genes was performed to assess the changes in gene expression of hCPCs under normoxic, physoxic, and hypoxic culture. Cellular gene expression was analyzed for all three oxygen conditions after 48 h of culture using an array with 84 genes involved in the hypoxia signaling pathway including: HIF1α and its co-transcription factors and other interactors; hypoxic responsive genes involved in: angiogenesis, coagulation, DNA damage and repair, metabolism, regulation of apoptosis, regulation of cell proliferation, transcription factors, transporters, channels, receptors, and others. Data were normalized to five housekeeping genes and fold change was calculated relative to the normoxic cellular expression level. Culturing under 5% O_2 induced a significant ($p < 0.05$, $n = 3$) increase in the expression of five genes (IGFBP3, EDN1, CA9, MMP9, VEGFA) and a significant ($p < 0.05$, $n = 3$) decrease in the expression of four genes (NAMPT, PLAU, ODC1, EGR1) (**Figure 3A**). Culturing at 1% O_2 caused a significant ($p < 0.05$, $n = 3$) increase in the expression of 23 genes (IGFBP3, BDRG1, CA9, ANGPLT4, MMP9, ADM, DDIT4, PGF, PFKFB3, SLC2A3, SLC2A1, ANKRD37, VEGFA, HK2, GPI, PDK1, ANXA2, BLM, LOX, TXNIP, PFKFB4, ERO1A, GBE1) and a significant ($p < 0.05$, $n = 3$) decrease in the expression of eight genes (P4HA1, NAMPT, ADORA2B, TFRC, HIF1A, HMOX1, FOS, EGR1) (**Figure 3B**). While the upregulated genes show some commonality, increased expression of EDN1 was unique to 5% O_2 culture (**Figure 3C**). Entire array data can be found in **Supplementary Table 1**. These results demonstrate that culturing hCPCs under 5% O_2 modulated

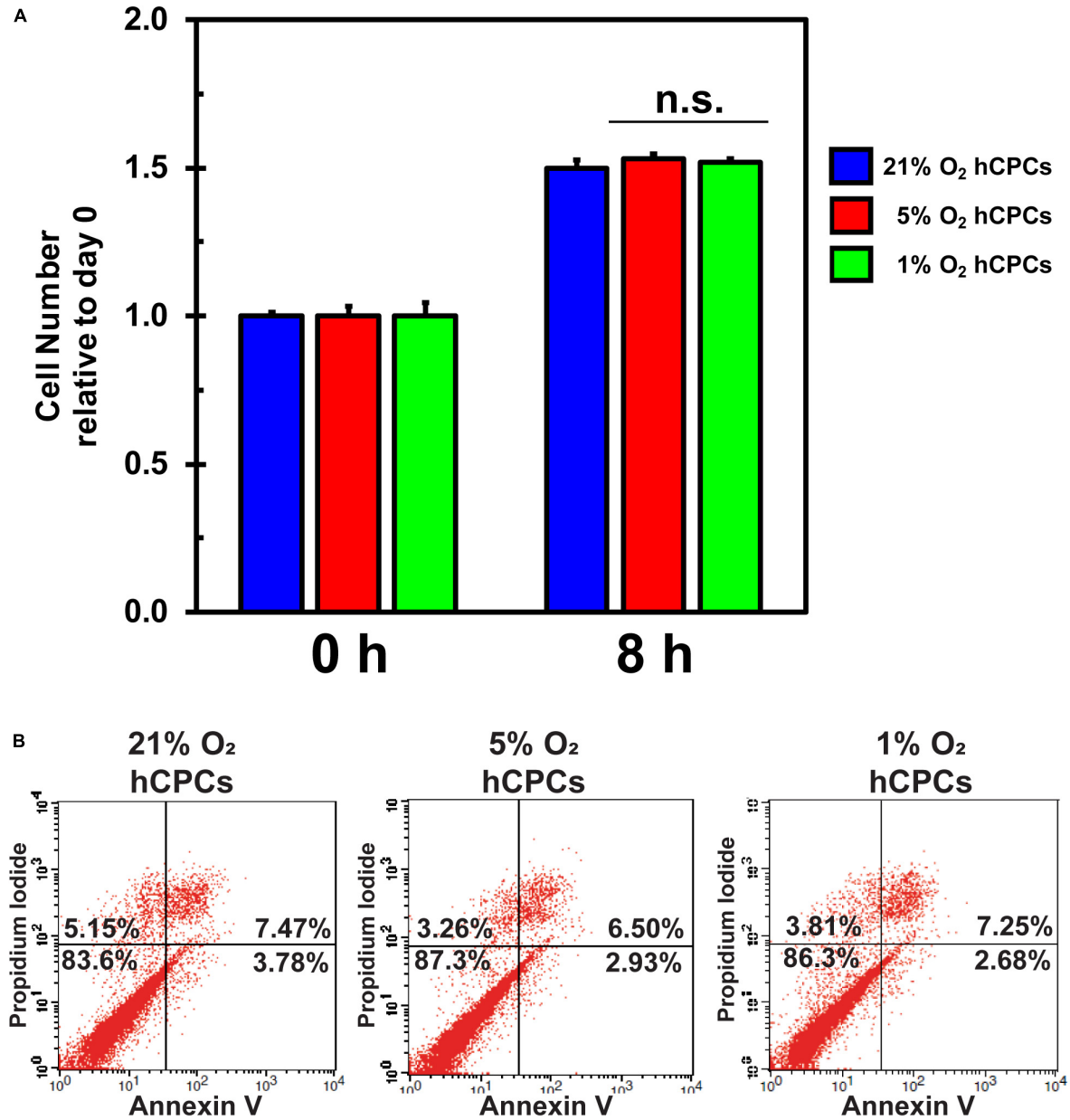

FIGURE 2 | hCPCs maintain normal function under hypoxia. hCPCs were cultured under 21, 5, and 1% O_2. **(A)** XTT assay for cell proliferation shows that cells maintained a similar level of proliferation over 24 h. Data represents mean ± SD, $n = 4$. **(B)** Flow cytometry for Annexin V/PI show that cells are equally healthy under the various oxygen conditions as the number of non-apoptotic/non-necrotic cells remains similar after 48 h of culture.

few hypoxia-related genes and culturing hCPCs under 1% O_2 modulated numerous hypoxic genes.

Isolation and Characterization of hCPC-Derived EVs

EVs were successfully isolated from hCPCs under all three O_2 conditions and analyzed via Cryo-TEM to preserve their shape and structure. The lipid bilayer is easily identified in representative TEM images (**Figure 4A**, yellow arrows). EVs were further analyzed for common exosomal markers with an antibody array. To verify exosome identity, isolated particles were tested to comply with international standards (Thery et al., 2018) demonstrating at least three positive markers and one negative marker. Results show that these EVs expressed the exosomal transmembrane or lipid-bound markers ICAM, CD81, CD63, ANXA5, and cytosolic markers ALIX, FLOT-1 and TSG101 (**Figure 4B**) (de Gassart et al., 2003; Lotvall et al., 2014). Detection of the negative marker GM130 (Lotvall et al., 2014; Keerthikumar et al., 2015; Samaeekia et al., 2018), a Golgi-associated protein, was not seen, thus confirming their identity as exosomes. Detection patterns were similar for the EVs isolated under all

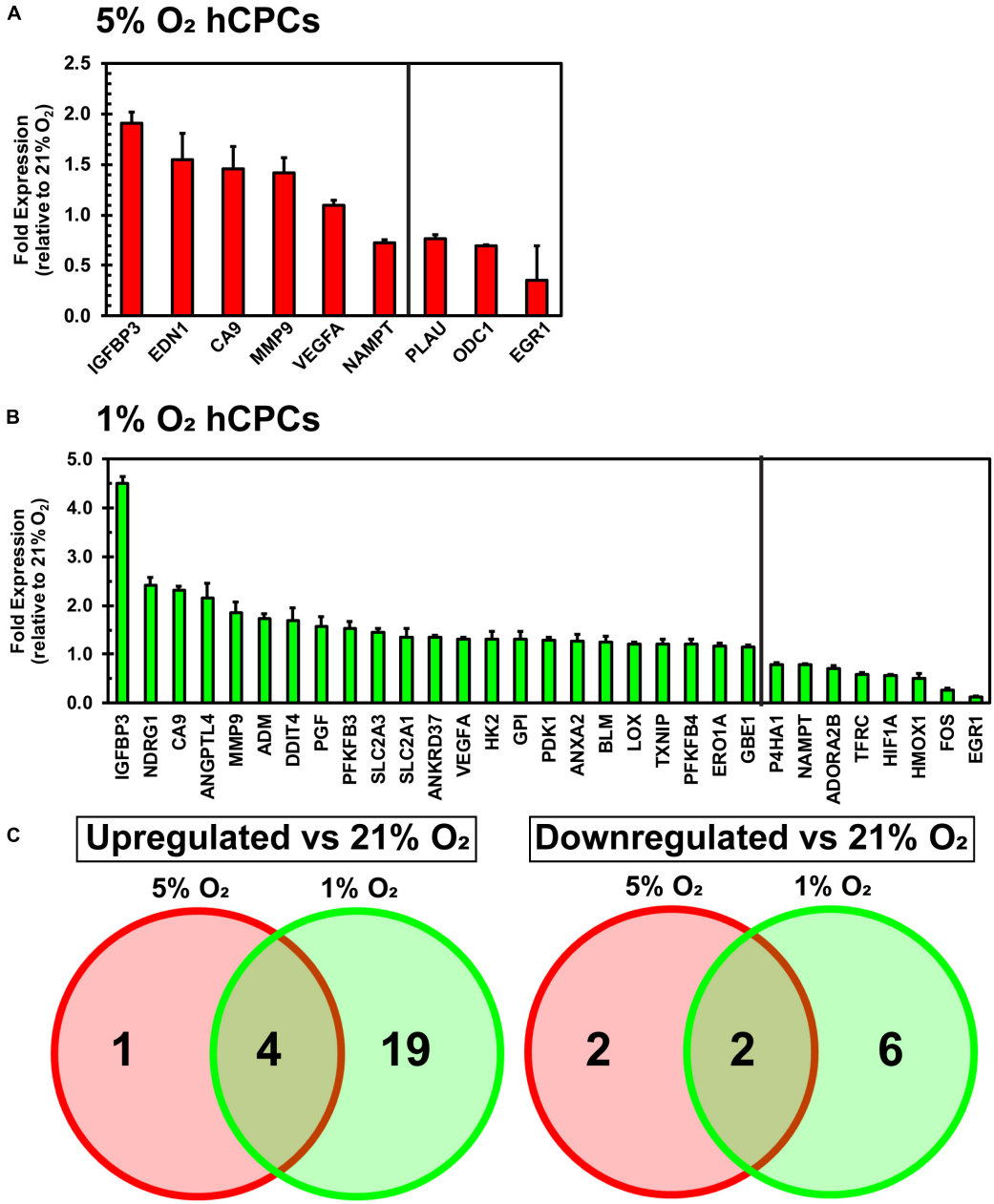

FIGURE 3 | Culturing hCPCs under hypoxia modulates hypoxic gene expression. hCPCs were cultured under 21, 5, and 1% O_2 for 48 h then total RNA was harvested and gene expression analyzed via real-time PCR. Gene expression was normalized to five housekeeping genes and calculated as a fold change relative to levels at 21% O_2. **(A)** 5% O_2 culturing of hCPCs significantly increased expression of five genes, and significantly decreased expression of four genes. Data represented as mean ± SD, $n = 3$, all have $p < 0.05$ as compared to 21% O_2. **(B)** 1% O_2 culture altered expression of numerous hypoxia-related genes. 23 genes were significantly increased in expression and 8 genes were significantly decreased in expression, as compared to 21% O_2. Data represented as mean ± SD, $n = 3$, all have $p < 0.05$ as compared to 21% O_2. **(C)** Venn diagram illustrating similarly and differentially regulated genes under 5 and 1% O_2, as compared to 21% O_2.

three oxygen conditions. Nanoparticle tracking analysis (NTA) revealed the size and concentration distribution for EVs derived from the three oxygen conditions (**Figure 4C**). Their sizes are consistent with those of exosomes (50–150 nm) (Yanez-Mo et al., 2015) and small microvesicles (100–1000 nm) (Cocucci and Meldolesi, 2015). The mean and mode sizes of the particles isolated from the differing oxygen concentrations were similar (**Figure 4D**). Interestingly, concentration, and thus yield, of EVs was 1.6-fold greater under 5% O_2 conditions (**Figure 4E**). Since EVs were isolated from identically seeded plates with equal volumes of media and were resuspended in identical volumes of PBS the concentration of EVs is congruent to their yield. Furthermore, this data showed that 5% O_2 EVs had a unique size distribution. Collectively, these data verify the identity of the

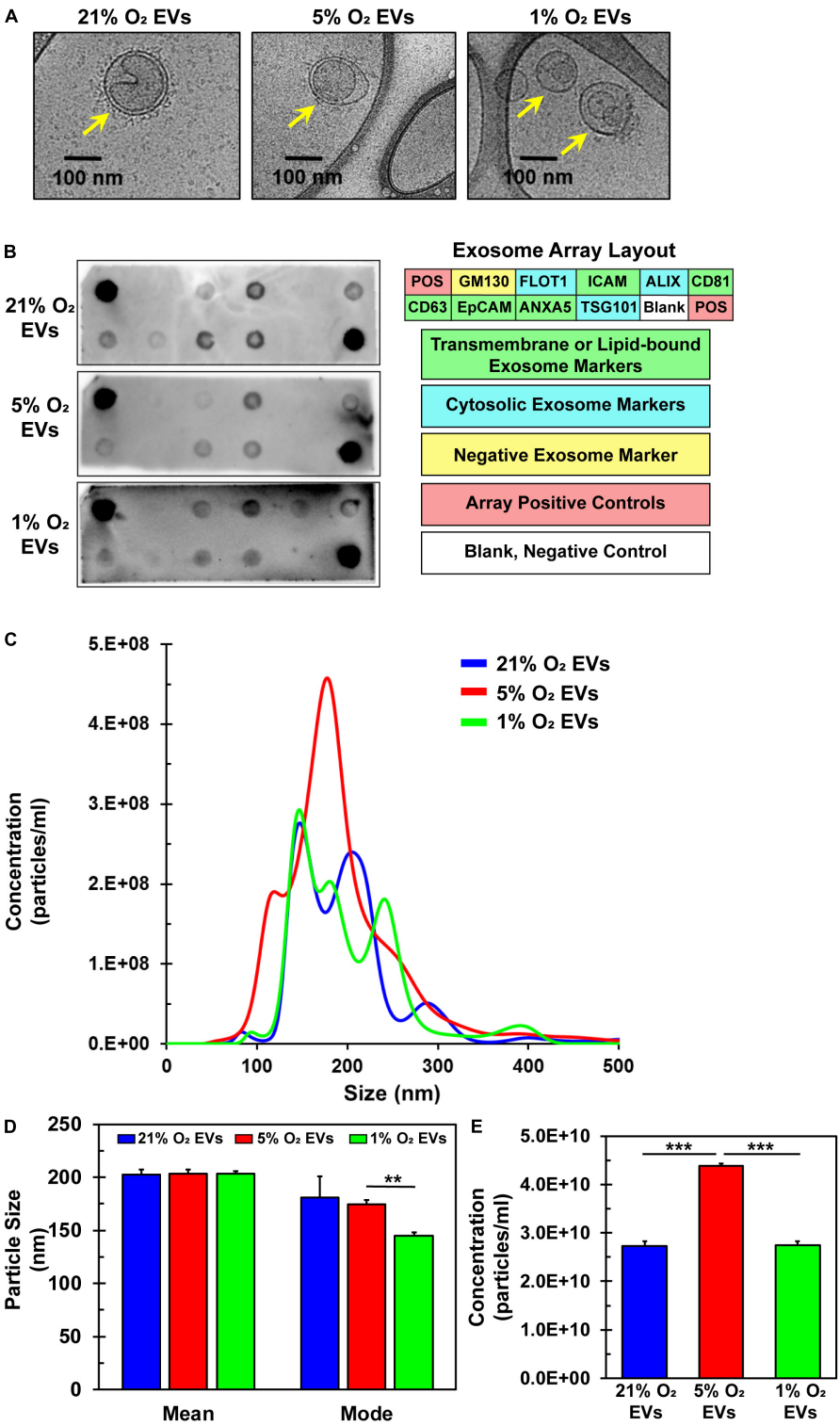

FIGURE 4 | Characterization of EVs derived from hCPCs under varying oxygen microenvironment. hCPCs were cultured under three different oxygen condition (21, 5, and 1% O_2) to generate EVs for analysis. **(A)** Cryo-TEM of isolated EVs shows their characteristic morphology of a round shape with a lipid bilayer (yellow arrows), which was identified under all three conditions. **(B)** EVs derived from 21, 5, and 1% O_2 hCPCs were analyzed with an exosome antibody array for known exosome markers and a negative exosome marker. The isolated EVs from all three O_2 conditions met the ISEV minimum requirements for identification. **(C)** NTA of triplicate samples shows the size and concentration distribution of EVs. **(D)** Mean and mode sizes of isolated EVs were similar and within the accepted range for extracellular vesicles, data is mean ± SD, $n = 3$. **(E)** The concentration of EVs isolated from hCPCs cultured under 5% O_2 microenvironment was higher than 21 and 1% O_2 groups, data is mean ± SD, $n = 3$. $^{**}p < 0.01$, $^{***}p < 0.001$.

isolated particles to include small microvesicles and exosomes. Varying oxygen concentrations generated EVs that were similar in mean and mode size, however, their secretion was increased under 5% O_2 and also had a unique size distribution pattern as compared to 21 and 1% O_2 culture.

Angiogenic Potential of hCPC-Derived EVs

A key tenet of angiogenesis is cell migration as cells must migrate toward the formation site of the new vessel (Adair and Montani, 2010). The wound-healing/scratch assay is a well-established assay for 2-D cell migration (Rodriguez et al., 2005). BAECs were grown to confluence in 24-well plates, wounded with a pipet tip, and treated with hCPC EVs from 21, 5, and 1% O_2 culture at 100 μg/ml EVs (total EV protein) at time 0. Results demonstrate that all groups of EVs significantly increased BAEC migration after 8 h as compared to No-EVs control (**Figure 5A**). The 5% O_2-derived EVs had the greatest percentage wound closure, however, this was not statistically significant compared to 21 or 1% O_2 (**Figure 5B**). These results indicate that hCPC-derived EVs increase endothelial cell migration, a crucial component of angiogenesis, but this was not enriched by generation of EVs under hypoxic conditions.

Additionally, endothelial cells that are to form new blood vessels must reorganize into 3D tubules to allow for subsequent blood flow (Adair and Montani, 2010). The tube formation assay using ECs is a well-characterized model system for *in vitro* angiogenesis (Arnaoutova et al., 2009), which we used to assess the hCPC-derived EVs. BAECs were seeded with media containing 100 μg/ml EVs (total EV protein) from 21, 5, and 1% O_2 cultured hCPCs onto basement membrane-coated wells and allowed to form tubules for 16 h (**Figure 6A**). Results demonstrate that treatment with 5% O_2-derived EVs has shown the greatest enhancement of tube formation. These EVs significantly ($p < 0.05$, $n = 3$) increased the number of master junctions, total master segment length, and total mesh area as compared to No-EV control (**Figure 6B**). Impressively, 5% O_2 hCPC-derived EVs also significantly ($p < 0.05$, $n = 3$) enhanced tube formation by all measurements as compared to both 21 and 1% O_2 EV treatment (**Figure 6B**). Thus, culturing hCPCs at 5% O_2 generated EVs with the greatest angiogenic potential in terms of tube formation.

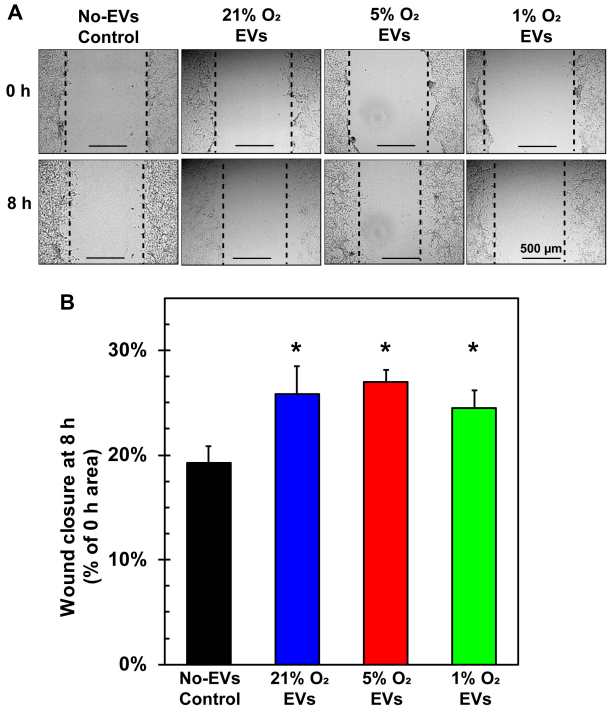

FIGURE 5 | Effect of hCPC-derived EVs on endothelial cell migration. BAECs were grown to confluence, wounded with a pipet tip, and treated with 100 μg/ml of total EV protein from each condition. **(A)** Representative images from 0 and 8 h show the extent of cell migration, with dotted lines approximating the cell front. **(B)** Wound healing assay demonstrates that treating ECs with hCPC-derived EVs increased cell migration significantly when compared to No-EVs group. However, no significant differences were observed between 5 and 1% O_2 derived EVs. Data are shown as mean ± SD, $n = 4$, $^*p < 0.05$ vs. No-EVs control.

DISCUSSION

Overall, this study demonstrated the therapeutic potential of human CPC-derived EVs for cardiac repair. Our study elucidated the optimal oxygen concentration required for culturing hCPCs and showed that the EV-cargo released under 5% O_2 is very potent for promoting angiogenesis. Furthermore, only a few hypoxic genes were modulated under 5% O_2 conditions, when compared to numerous genes altered under 1% O_2 culturing. Our results clearly demonstrate that physoxic (5% O_2) culturing of hCPCs may play a crucial role in myocardial repair applications, especially during cell transplantation studies to prevent hypoxic shock for transplanted cells in the ischemic heart. Furthermore, physoxic culturing also influences the EV-cargo released by hCPCs as a source for cell-free therapeutics.

In this study, we have analyzed hCPCs and the functionality of their derived exosomes cultured under three different micro-environments (21, 5, and 1% O_2). Our results denote that hCPCs cultured under a low-oxygen, physiological microenvironment (a.k.a physoxic, 5% O_2) were able to maintain normal cell morphology and expression of cardiac markers, thereby demonstrating their robustness under physoxic oxygen conditions. NTA analysis indicated our ability to successfully isolate exosomes from cells under all oxygen conditions. Cells cultured at 5% O_2 exhibited increased exosome secretion with a unique size and concentration profile as compared to normoxia and 1% O_2 culturing. Functional assays from this study signify our novel hypothesis that physoxic oxygen concentration affects the therapeutic potential of hCPC-derived EVs. Additionally, gene expression profiling of the hCPCs revealed a possible molecular mechanism underlying the increased potency of 5% O_2-derived EVs. On the other hand, EDN1 was uniquely and significantly upregulated in 5% O_2 cultured hCPCs when compared

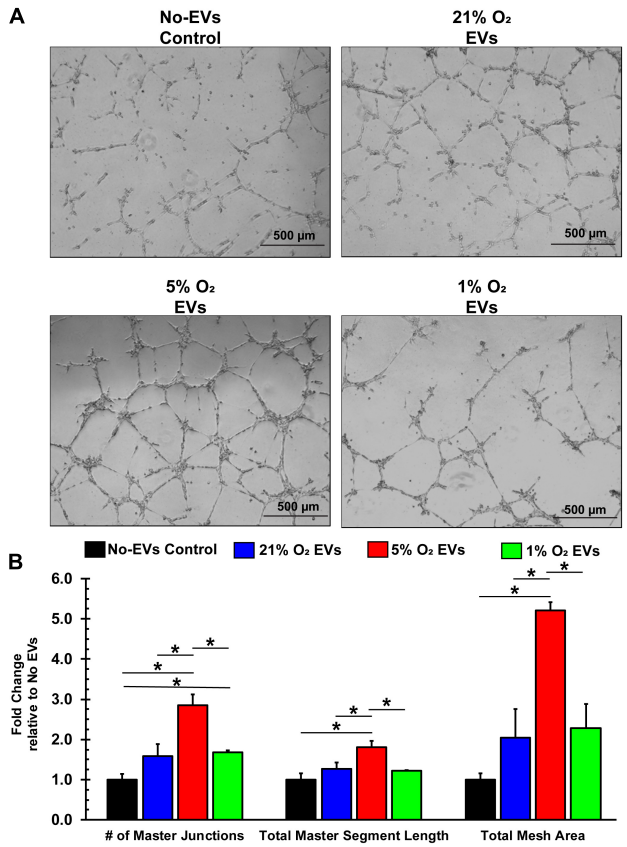

FIGURE 6 | EVs derived from hCPCs under physoxia enhances angiogenesis *in vitro*. BAECs were seeded onto Matrigel-coated wells with 100 µg/ml of total EV protein from each condition and incubated for 16 h. **(A)** Representative images illustrate the degree of tube formation for each group. **(B)** 1% O_2-derived EVs significantly increased tube formation in terms of the number of master junctions as compared to No-EVs control. However, 5% O_2-derived EVs significantly increased tube formation as compared to all groups in terms of the number of master junctions, the total master segment length, and the total mesh area. Data shown represents mean ± SD, $n = 3$, $^*p < 0.05$ for comparison.

to normoxia or 1% O_2 cultured hCPCs. *EDN1* encodes endothelin 1 (ET-1), a secreted peptide that acts as a paracrine signaling factor mediating growth (Soh et al., 2016), survival (Del Bufalo et al., 2002; Nelson et al., 2005), and angiogenesis (Salani et al., 2000; Wulfing et al., 2004; Wu et al., 2014). Future proteomic analysis of exosomes derived from 5% O_2 cultured hCPCs would reveal if EDN1 is delivered to target cells via exosomes and play a crucial role promoting angiogenesis.

Our NTA analysis results showed an increased EV release under 5% O_2 condition, but not under at 1% O_2. Other studies have observed increased secretion of EVs under hypoxia (King et al., 2012; Bian et al., 2014; Zhu J. et al., 2018; Patton et al., 2019; Zhang et al., 2019), however these studies were not performed with hCPCs. Multiple studies have demonstrated enhanced functional effects of hypoxia-derived versus normoxia-derived EVs. A study of MSC-EVs observed increased proliferation, migration, and tube formation with hypoxia-derived EVs (1% O_2 for 72 h) as compared to those from normoxic conditions (Bian et al., 2014). Another study on glioma cells observed significantly increased tube formation by endothelial progenitor cells when treated with hypoxia (<0.5% O_2 for undisclosed time) versus normoxia-derived EVs; however, the treatments equally protected glioma cells from oxidative stress (Kore et al., 2018). Similarly, human cardiosphere-derived EVs isolated under 18 and 1% O_2 showed increased tube formation as compared to PBS control. However, the two conditions were either not analyzed to each other or their difference was not statistically significant (Namazi et al., 2018). Zhu L.P. et al. (2018), showed that intramyocardial implantation of EVs generated from BM-MSCs under hypoxia (1% O_2 for 72 h) significantly decreased scar formation and improved cardiac function 28 days post-MI, as compared to normoxia-derived EVs. Overall, the results from our findings highlight the therapeutic potential of physoxia generated EVs as compared to normoxia-derived EVs. Wound healing assays showed that EV treatment significantly increased migration as compared to No-EVs control. Similarly, 5% O_2 hCPC-derived exosomes showed significant increase in tube formation as assessed by the number of master junctions, segment length, and mesh area as compared to both 21 or 1% O_2 EVs. Ultimately, 5% O_2 CPC-derived EVs in this study displayed the greatest therapeutic effect *in vitro* as a result of enhanced angiogenic behavior.

CONCLUSION

Promising results have stemmed from our study by assessing the therapeutic potential of hCPC-derived EVs under physoxia. Culturing hCPCs under physoxia showed increased EVs secretion and minimal changes in cellular expression of hypoxia-related genes. Future studies will focus on functional outcome of hCPC-derived EVs for myocardial repair *in vivo* and to perform proteomics and miRNA profiling of EV cargo to identify novel proteins and miRNAs modulated under physoxia.

DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

AUTHOR CONTRIBUTIONS

MK, NP, and JD conceived and designed the experiments. MK, NP, JD, NK, SR, and HS performed the experiments and analyzed the data. MK, CC, HS, and MA contributed reagents, materials, and analysis tools. JD, NP, and MK wrote the first draft of the manuscript. All authors contributed to manuscript revision, read and approved the submitted version.

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SUPPLEMENTARY MATERIAL

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ORIGINAL RESEARCH
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The Small GTPases Rab27b Regulates Mitochondrial Fatty Acid Oxidative Metabolism of Cardiac Mesenchymal Stem Cells

*Yue Jin[1], Yan Shen[1], Xuan Su[1], Jingwen Cai[2], Yutao Liu[2], Neal L. Weintraub[1] and Yaoliang Tang[1]**

[1] Vascular Biology Center, Medical College of Georgia, Augusta University, Augusta, GA, United States, [2] Cellular Biology and Anatomy, Medical College of Georgia, Augusta University, Augusta, GA, United States

Cardiac mesenchymal stem cells (C-MSCs) are endogenous cardiac stromal cells that play a crucial role in maintaining normal cardiac function. Rab27b is a member of the small GTPase Rab family that controls membrane trafficking and the secretion of exosomes. However, its role in regulating energy metabolism in C-MSC is unclear. In this study, we analyzed mitochondrial oxidative phosphorylation by quantifying cellular oxygen consumption rate (OCR) and quantified the extracellular acidification rate (ECAR) in C-MSC with/without Rab27b knockdown. Knockdown of Rab27b increased glycolysis, but significantly reduced mitochondrial oxidative phosphorylation (OXPHOS) with loss of mitochondrial membrane potential in C-MSC. Furthermore, knockdown of Rab27b reduced H3k4me3 expression in C-MSC and selectively decreased the expression of the essential genes involved in β-oxidation, tricarboxylic acid cycle (TCA), and electron transport chain (ETC). Taken together, our findings highlight a novel role for Rab27b in maintaining fatty acid oxidation in C-MSCs.

Keywords: Rab27b, mitochondrial oxidative metabolism, cardiac mesenchymal stem cells, fatty acid oxidation, exosome

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****Correspondence:***
Yaoliang Tang
yaotang@augusta.edu

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INTRODUCTION

Cardiovascular disease (CVD) is the number one cause of mortality and morbidity worldwide, and its prevalence will increase with the progressive aging of the general population (Smith et al., 2012). CVD is characterized by a spectrum of alterations in cardiac energy and substrate metabolism (van Bilsen et al., 2004; Bertero and Maack, 2018). The heart consumes large amounts of energy in the generation of ATP, which is mainly supplied by oxidative phosphorylation (OXPHOS) in mitochondria (Bertero and Maack, 2018). Under normal conditions, about 60–90% of ATP required for the continuous contractile activity of the heart is primarily produced by oxidation of fatty acids (FA), while the remainder is derived from the oxidation of pyruvate (van der Vusse et al., 1992). Fatty acid oxidation (FAO) is a complex biological process involving mitochondrial β-oxidation, tricarboxylic acid (TCA) cycle activity, and the electron transport chain (ETC) (Lopaschuk et al., 2010). Changes in fatty acid metabolism can lead to a variety of CVDs. For example, excessive intake of fatty acids and beta-oxidation in obesity and diabetes can impair heart function. Additionally, changes in fatty acid β-oxidation during and after ischemia and in nutrient-depleted hearts may also contribute to cardiac pathology, affecting cardiac systolic function and cardiac efficiency (Liu et al., 2002; Lopaschuk et al., 2010).

Cardiac mesenchymal stem cells (C-MSC) that reside in adult hearts express the cardiac transcription factor GATA-4 and mesenchymal stem cell markers, including CD105, CD140, and Sca-1 (Ruan et al., 2018a,b). These C-MSC have cardiac reparative properties via paracrine mechanisms mediated by angiogenic factors (Tang et al., 2004, 2005) and/or exosomes (Chen et al., 2013; Wang et al., 2015; Campbell et al., 2016). Exosomes secreted by C-MSC are nano-size bilayer membrane vesicles (around 30∼150 nm in diameter) that can be absorbed by surrounding tissues (Vestad et al., 2017). Thus, proteins, RNA, and lipids from exosomes of C-MSC can enter adjacent cells (such as cardiomyocytes) and regulate their cellular signaling. We have reported that C-MSC-derived exosomes could protect cardiomyocytes from acute myocardial ischemia/reperfusion injury (Chen et al., 2013). Furthermore, recent studies have suggested that exosomes that are secreted from adipocytes and other cells in adipose tissue influence whole-body glucose and lipid metabolism (Ying et al., 2017; Flaherty et al., 2019). However, little is known about the metabolism of C-MSC contained in adult hearts.

The small GTPase family member Rab27b controls membrane trafficking and microvesicle transport, particularly the secretion of exosomes (Fukuda, 2013). While Rab27b can control cell–cell communications mediated by exosomes, including metabolism regulation, little is known about the role of Rab27b in regulating self-cell metabolism in C-MSC.

In this study, we first evaluated the role of Rab27b in the metabolism of adult C-MSCs and found that knockdown of Rab27b inhibits mitochondrial fatty acid β-oxidation, TCA, and ETC by decreasing the expression of related genes, resulting in mitochondrial respiratory depression in C-MSC.

MATERIALS AND METHODS

C-MSC Isolation and Culture

Cardiac mesenchymal stem cells were isolated from the hearts of male C57BL/6 mice (The Jackson Laboratory, Bar Harbor, ME, United States) of 2 to 3 months old via an established protocol with modification (Tang et al., 2007; Ju et al., 2018a,b; Ruan et al., 2018a,b). Briefly, in step 1, ventricular tissue was minced into approximately 1-mm^3-sized pieces and digested using 0.1% collagenase IV and 1 U/ml dispase in DMEM/F-12 for 1 h at 37°C. Then, cardiac explants were collected and incubated on fibronectin/gelatin-coated plates (0.5 mg fibronectin in 100 ml 0.1% gelatin) in DMEM containing 10% fetal bovine serum, 100 U/ml penicillin G, and 100 μg/ml streptomycin. Cultured cardiac explants were maintained until the small round phase-bright cells migrated from the adherent explants and proliferated over a fibroblast layer. In step 2, Sca-1 + cells were enriched from the phase-bright cells through a mouse hematopoietic lineage-depletion cocktail kit (STEMCELL Technologies, Vancouver, Canada), followed by enrichment for Sca-1 + cells via magnetic-activated cell sorting (MACS) with Sca-1 magnetic beads (Miltenyi Biotec Inc., Auburn, CA, United States) according to the manufacturers' protocols. The selected Sca-1 cells were cultured and maintained in complete DMEM medium containing 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin, 200 mmol/L L-glutamine, 55 nmol/L β-mercaptoethanol, and 1% MEM non-essential amino acids. Animal treatment protocols were approved by, and conducted in accordance with, animal welfare regulations of the Institutional Animal Care and Use Committee of the Medical College of Georgia.

Flow Cytometry

Cardiac mesenchymal stem cells were first blocked with 5% rat serum (Sigma) and stained, respectively, with conjugated antibodies, including anti-CD105-APC (BioLegend), anti-CD140b-PE (BioLegend), or isotype-matched control antibody (BD Biosciences). Flow cytometry analysis of cultured C-MSC was performed with a BD LSRII flow cytometer from Augusta University.

Exosome Purification

Exosomes released by the C-MSC were purified as previously described (Wang et al., 2015; Tang et al., 2017; Ju et al., 2018b; Ruan et al., 2018a,b; Su et al., 2018, 2019a). Briefly, after 48 h of cell culture in exosome-free medium, the supernatant was harvested and centrifuged at 150 × *g* for 10 min to eliminate cells, followed by filtration via 0.22-μm filter to remove cell debris. The filtered supernatant was ultracentrifuged by an SW-28 Ti rotor (Beckman Coulter Instruments, United States) at 100,000 × *g* for 120 min at 4°C to pellet the exosomes. The exosome pellets were resuspended in 1 ml PBS.

Zeta Analysis

We measured the exosome particle size and concentration with nanoparticle tracking analysis (NTA) using ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany) and the corresponding software ZetaView as previously described (Helwa et al., 2017; Shah et al., 2018; Rashid et al., 2019). Isolated exosome samples were appropriately diluted using 1 × PBS buffer (Life Technologies, Carlsbad, CA, United States) to measure the particle size distribution and concentration. NTA measurement was recorded and analyzed. The ZetaView system was calibrated using 100-nm polystyrene particles. The temperature was maintained at approximately 23°C.

Immunofluorescent Staining

For cell staining, C-MSCs plated on an 8-well chamber slide (Thermo Fisher Scientific, United States) were fixed with 4% paraformaldehyde, followed by the permeabilization with 1% Triton X-100™. After blocking with 5% goat serum, cells were incubated with rabbit anti-GATA4 (1:100; Aviva System Biology), rabbit anti-Rab27a (1:500; Cell Signaling), or rabbit anti-Rab27b (1:100; Millipore) at 4°C overnight. Secondary antibody incubation with goat anti-rabbit Alexa Fluor 555-conjugated (1:400, Invitrogen) was performed the following day, after which slides were mounted using VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, United States).

Lentiviral Vectors and Transfection

Lentiviral plasmids encoding shRNA targeting Rab27b mRNA (clone ID, MSH036525-31-LVRU6GH, MSH036525-32-LVRU6GH, MSH036525-33-LVRU6GH, and MSH036525-34-LVRU6GH) were purchased from Gene Copoeia. Lentiviral particles were produced in HEK293FT cells by cotransfecting the LVRU6GH shRNA plasmids, together with helper plasmids including pMD2.G and psPAX2 using lipofectamine 3000 reagents (Invitrogen). Viral supernatant was collected after 48 h. The lentiviral vectors were purified by adding PEG6000 (8.5% final concentration) and NaCl (0.4 M final concentration) to the 0.45 μM syringe filtered supernatant as previously reported (Su et al., 2019b). When C-MSCs reached 80% confluence, the purified lentivirus was added into medium containing 8 μg/ml of polypropylene for transduction. After 3 days of infection with lentiviral cells, hygromycin B (100 μg/ml) was added for cell selection.

Isolation and Quantification of Messenger RNA

Total RNA was extracted by RNAzol RT (Molecular Research Center) according to the manufacturer's instructions. cDNA was synthesized from total RNA using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific). Quantitative PCR was performed using a PowerUp SYBR Green Master Mix (Thermo Fisher) on a CFX96 Touch real-time PCR detection system (Bio-Rad Laboratories, United States). The amplification was performed at 50°C for 2 min, at 95°C for 2 min, followed by 50 cycles of 95°C for 15 s, and at 60°C for 1 min, using the primers listed in **Table 1**.

TABLE 1 | Primer sequences.

Gene	Sequence (5′–3′)
β-Actin FWD	AGAGCATAGCCCTCGTAGAT
β-Actin REV	GCTGTGCTGTCCCTGTATG
GAPDH FWD	TGACAAGCTTCCCATTCTCG
GAPDH REV	CCCTTCATTGACCTCAACTACAT
Rab27a FWD	CAGGAGAGGTTTCGTAGCTTAAC
Rab27a REV	GGCTTATCCAGTTTCGGACAT
Rab27b FWD	GTCCAGCAGTGTCCCAAAG
Rab27b REV	ATGACACACAAGGAGCAGATG
Sirt1 FWD	GTTGGTGGCAACTCTGATAAATG
Sirt1 REV	GTCATAGGCTAGGTGGTGAATATG
Ppargc1b FWD	AGGTGTGAGGGAAGCATAGA
Ppargc1b REV	CAAAGCCTTCTGGACTGAGTT
Acox1 FWD	CCTTGGCCAATGCTCTCATTA
Acox1 REV	CGCAGCAGTATAAACTCTTCCC
Acox3 FWD	CCCTAGAGAAGCTACGAGAACT
Acox3 REV	CAGGCAGTTAATCAGCACTAGAA
Hadha FWD	CCATGTCGGCCTTCTCAAA
Hadha REV	AGTGAAGAAGAAAGCTCTCACAT
Hadhb FWD	AGACCATGGGCCACTCT
Hadhb REV	CTTCTTGGCCAGACTATGAGAAC
Idh3a FWD	GGCCATCCATCTATGAATCTGT
Idh3a REV	GTATTCTCCTTCCGTGTTCTCTC
Ogdh FWD	CATGTATCACCGCAGGATCAA
Ogdh REV	GGTCTTTCCCATCACGACAG
Sdhd FWD	GATGCCGACATCGTGGTAAT
Sdhd REV	GTTACCGACTACGTTCATGGG
Uqcrq FWD	CTTTGCTGAAATAGCTTGGGAAG
Uqcrq REV	GAACCTGGCGCGGATAC

Mitochondrial Membrane Potential Assay

We use a mitochondrial membrane potential detection JC-1 kit™ (BD MitoScreen) to measure deltapsim according to instructions. The fluorescence intensity was measured at green fluorescence for JC-1 monomer and red fluorescence for JC-1 aggregate under a fluorescent microscope (Evos FL, Thermo). The deltapsim is represented by the ratio of JC-1 (red/green) on picture.

Western Blotting Assay

Western blotting was performed as described before (Ruan et al., 2018a). Briefly, proteins (normalized for concentration) were resolved on 10% SDS–polyacrylamide gels and transferred onto Odyssey® nitrocellulose membranes (LI-COR Biosciences). The membranes were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, United States) and probed with rabbit anti-Rab27a (1:1000; Cell Signaling), rabbit anti-Rab27b (1:1000; Millipore), rabbit anti-Tri-methyl-Histone H3 (Lys4) (1:1000; Cell Signaling), mouse anti-TBP (1:1000, Proteintech), and mouse anti-β-actin (1:5000, Novus Biologicals) at 4°C overnight. After washing with 1 × TBST, the membrane was incubated for 1 h at room temperature with IRDye 680 goat anti-rabbit IgG and IRDye 800 goat anti-mouse IgG (1:10,000, LI-COR Biosciences). The probed blot was scanned using an Odyssey infrared imager (LI-COR Biosciences).

Cell Metabolism Assays

The Mito Stress Test Kit (Agilent) was used to measure the oxygen consumption rate (OCR). The Glycolytic Rate Assay Kit (Agilent) was used for measuring the extracellular acidification rate (ECAR). On the day prior to experimentation, the sensor cartridge for XF analyzer was hydrated in a 37°C non-CO_2 incubator and cells were seeded at the density of 10,000 cells/well into XF96 cell culture microplates (for measurement of OCR) or 30,000 cells/well into XF24 cell culture microplates (Seahorse Bioscience) (for measurement of OCR and ECAR) and allowed to adhere to the plate overnight. On the day of the Seahorse assay, the cell culture medium was replaced with Seahorse XF DMEM base medium, without phenol red, supplemented with 10 mM glucose, 2 mM L-glutamine, and 1 mM pyruvate pH 7.4 and placed in a 37°C CO_2-free incubator for 1 h. For the measurement of OCR value, oligomycin, phenylhydrazone (FCCP), and rotenone/antimycin A (Rot/AA, inhibitors of mitochondrial ETC), respectively, were added according to the manufacturer's instructions and protocols. To determine effects of wild-type C-MSC-derived exosomes on OCR of C-MSC$^{sh-Rab27b}$, we seeded C-MSC^{sh-NC} and

C-MSC$^{sh-Rab27b}$ at 30,000 cells/well into XF24 cell culture microplates using culture medium with exosome-depleted FBS overnight, and prime cells with wild-type C-MSC-derived exosomes (25 μg/well) for 1 h before OCR assay. For Palmitate-BSA FAO experiments, etomoxir (40 μM/well, Millipore) was added at 15 min before OCR analysis. BSA or Palmitate-BSA (Agilent Technologies, United States) was added to the wells immediately prior to initiate XF assay.

For the detection of ECAR value, Rot/AA and 2-deoxy-D-glucose (2-DG, an inhibitor of glycolysis) were added according to the manufacturer's instructions and protocols. Finally, ECAR were determined and analyzed on the Agilent's Seahorse Bioscience XF24 Extracellular Flux Analyzer (Agilent Technologies, United States) according to the manufacturer's instructions and protocols (Agilent Technologies, United States). We first evaluated ECAR of C-MSC in the absence of glucose following by addition of glucose in 5 and 10 mM to compare the dosage of glucose on ECAR. Then, we compared the dosage of rotenone at a titration of 0.5 μM and 1 μM on ECAR in C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$.

Statistics

Results are presented as the mean ± standard deviation (SD). Differences between two groups were analyzed using unpaired Student's *t*-test, and the differences between three or more groups were analyzed using one-way analysis of variance (ANOVA). A value of $p < 0.05$ was considered statistically significant. Statistical analyses were conducted with GraphPad Prism 8.0 software.

RESULTS

Characterization of C-MSC

Cardiac mesenchymal stem cells were obtained using a two-step procedure: cardiac-derived cells were grown from enzymatically digested minced adult mouse hearts and expanded, and then the C-MSCs were isolated using a hematopoietic lineage-depletion cocktail followed by enrichment for Sca-1 + cells via MACS sorting (**Figure 1A**). GATA4, an early cardiac transcription factor (Takeuchi and Bruneau, 2009), was positive in C-MSCs by immunofluorescent staining (**Figure 1B**). Surface marker expression was profiled by flow cytometry. Over 93.8% cells were positive for CD105, and 93.6% cells were positive for CD140b (**Figure 1C**). These data indicate that C-MSC represents a subpopulation of cardiac-derived mesenchymal cells (Nery et al., 2013).

Lentiviral RNAi Vector-Mediated Knockdown of Rab27b in C-MSC

To knock down the expression of Rab27b in C-MSCs, four lentiviral vectors with Rab27b small hairpin RNA (sh-Rab27b) were transfected into C-MSC, and a non-targeting shRNA (NC) was employed as control. The gene silencing efficiency of these shRNAs was evaluated by RT-PCR. As shown in **Figure 2A**,

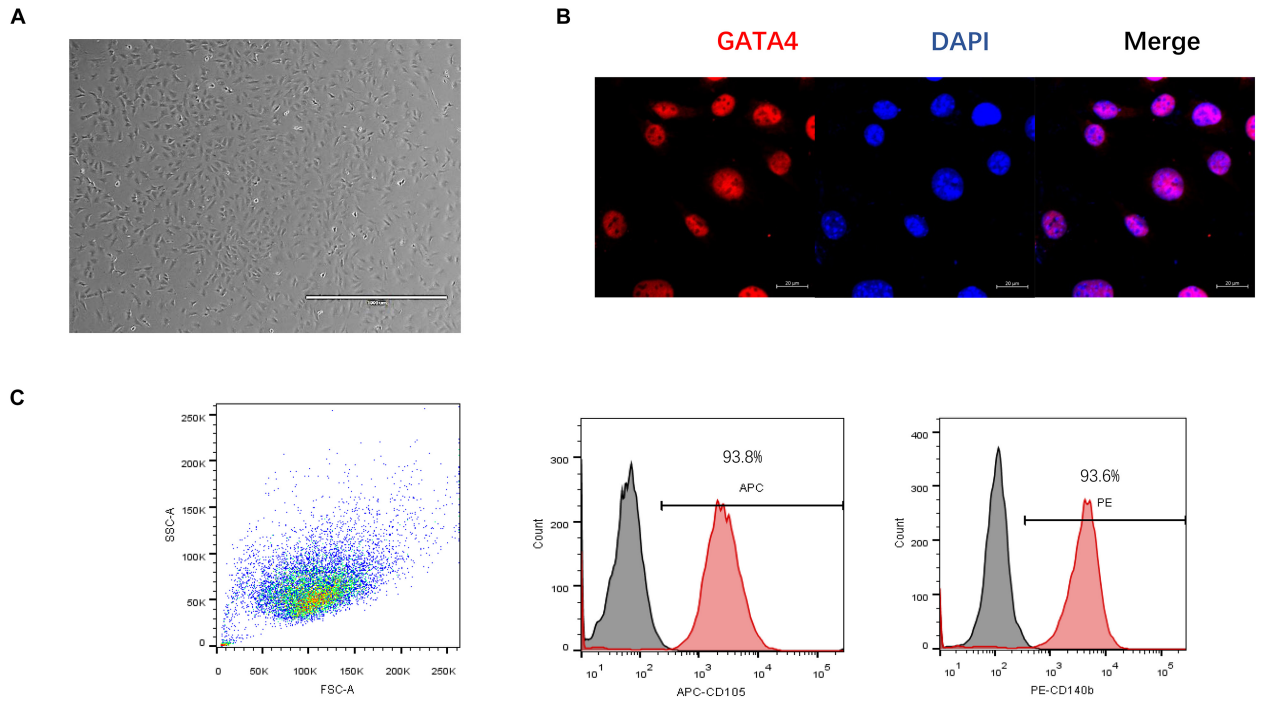

FIGURE 1 | Phenotypic characterization of C-MSCs. **(A)** Cultured C-MSCs at passage 10, scale bar = 1000 μm. **(B)** Immunofluorescent staining of GATA4, a marker for early cardiac transcription factor (red); cell nuclei were counterstained with DAPI (blue) (scale bar = 20 μm). **(C)** Flow cytometric analyses of C-MSCs for the profile of the cell surface markers CD105 and CD140b.

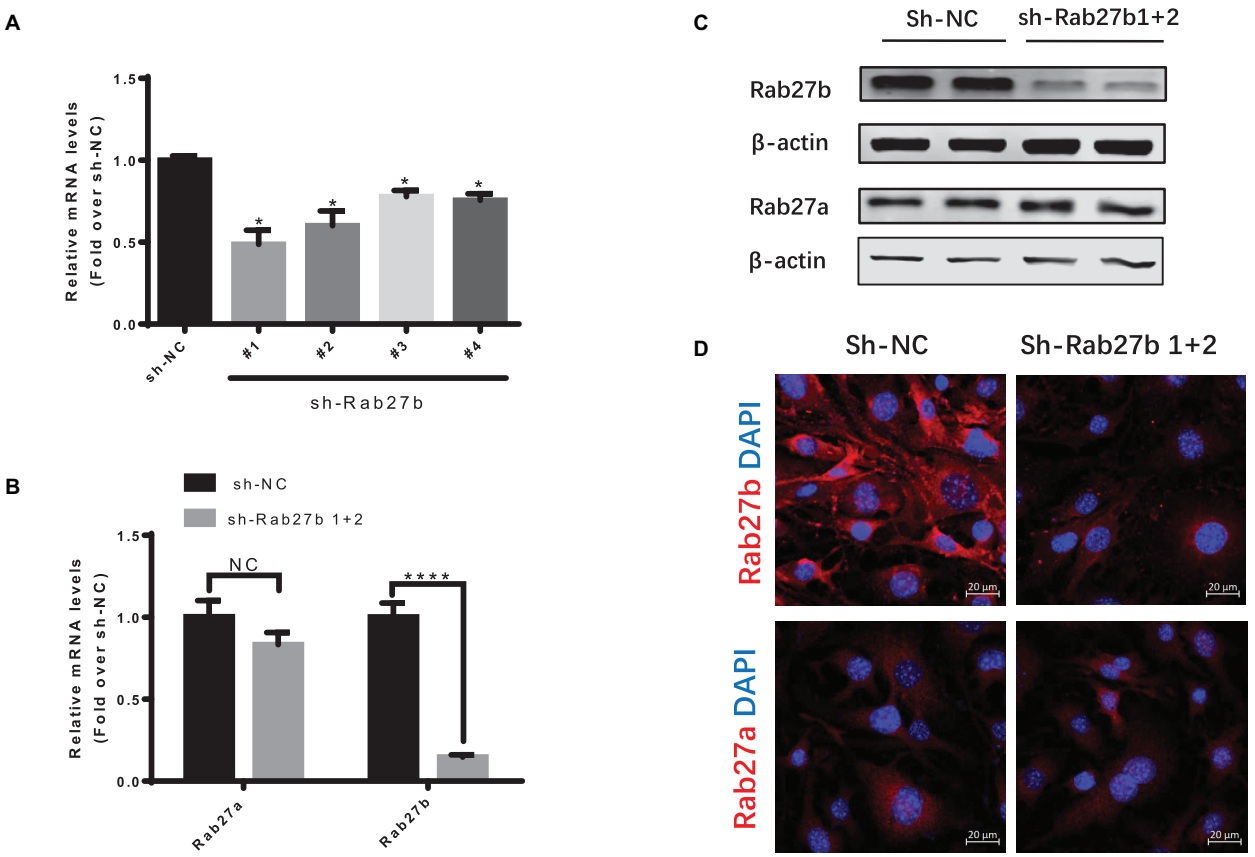

FIGURE 2 | Knockdown of Rab27b in C-MSCs via lentiviral shRNAs vector transduction. **(A)** qRT-PCR analysis of Rab27b in C-MSCs cells infected with lentiviral vectors encoding shRNA targeting Rab27b mRNA (sh-Rab27b-1, sh-Rab27b-2, sh-Rab27b-3 and sh-Rab27b-4) or negative control (sh-NC). β-Actin was used as endogenous control. Results are shown as mean ± SD (*$p < 0.05$ vs. sh-NC, $n = 3$). **(B)** qRT-PCR analysis of Rab27a and Rab27b in C-MSCs infected with sh-Rab27b1 + 2 (lentiviral vectors encoding sh-Rab27b1 and lentiviral vectors encoding sh-Rab27b2) or negative control (sh-NC). GAPDH was used as endogenous control. Results are shown as mean ± SD (****$p < 0.0001$ vs. sh-NC, $n = 3$). **(C)** Western blotting of Rab27b and Rab27a protein using β-actin as a loading control. **(D)** Immunofluorescent staining of Rab27b and Rab27a (red) in C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b1+2}$; cell nuclei were counterstained with DAPI (blue) (scale bar = 20 μm).

the lentiviral shRNA#1 and #2 efficiently downregulated the expression of Rab27b mRNA in C-MSCs. Thus, we analyzed C-MSCs that were stably double-transduced with lentiviral vectors encoding both sh-Rab27b#1 and sh-Rab27b#2 (sh-Rab27b1 + 2). Infecting with both lentiviral vectors resulted in a significant decrease of Rab27b at the mRNA and protein level, with no influence on Rab27a at the mRNA and protein level (**Figures 2B–D**). Cells transfected with sh-NC or sh-Rab27b1 + 2 were subsequently passaged for use in experiments.

The Effect of Rab27b Depletion on Exosome Secretion

To determine the effect of Rab27b on exosome release, we analyzed the exosomes released from C-MSCs infected with sh-NC or sh-Rab27b1 + 2. The size and concentration of pelleted structures were determined with nanoparticle tracking analysis using a ZetaView® nanoparticle tracking analyzer for hydrodynamic particle size. The pellets consisted of particles with an average size of approximately 140–150 nm in diameter, consistent with the characteristic size range of exosomes. Furthermore, knockdown of Rab27b in C-MSC cells significantly reduced the concentration of exosomes released by C-MSC in culture medium (**Figure 3**), demonstrating that Rab27b plays a vital role in exosome secretion.

Knockdown of Rab27b Impairs Mitochondrial Oxidative Phosphorylation in C-MSC

To determine the impact of Rab27b downregulation on cell metabolism, we quantified cellular OCR. **Figure 4A** shows the time course of the protocol with the injection of each compound and the impact on OCR; the results demonstrate that Rab27b deletion reduced mitochondrial oxidative phosphorylation, as evidenced by decreased basal respiration, maximal respiration, and ATP production in comparison with C-MSC^{sh-NC}.

To determine the effect of wild-type C-MSC derived Exosomes on both C-MSC with/without Rab27b knockdown, we cultured

	Exo from C-MSC sh-NC	Exo from C-MSC sh-Rab27b
Size (nm)	140.5	152.6
Concentration (particles/ml)	3.3E+8	1.9E+8

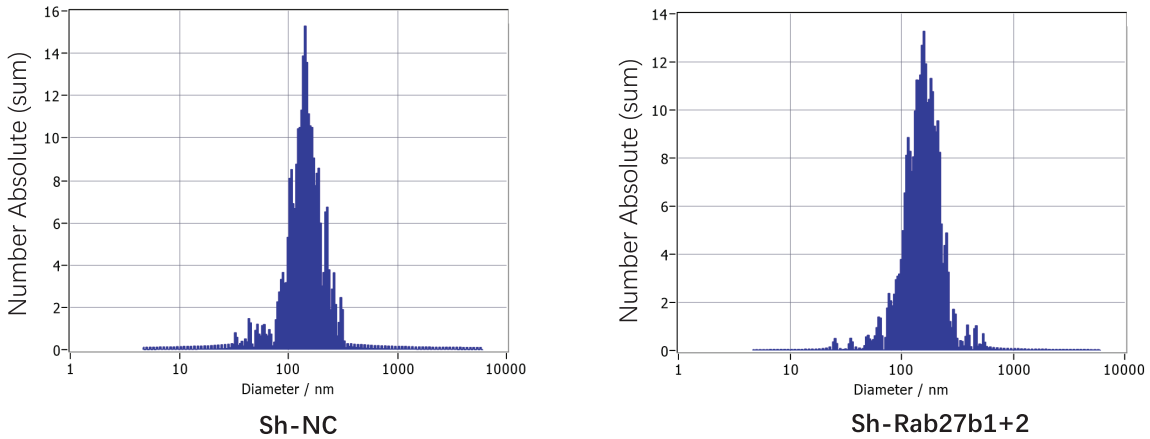

FIGURE 3 | Characterization of exosomes derived from C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b1+2}$. Particle concentration and size distribution in purified particles are consistent with the size range of exosomes (average size, 140–150 nm), as measured by ZetaView® Particle Tracking Analyzer.

both C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$ in media with Exo-depleted FBS and primed cells with 25 μg exosomes per well from wild-type C-MSC for 1 h before OCR assay. As shown in **Figures 4B,C**, exosome treatment reduced maximal respiration in both C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$, suggesting that external stem cell-derived exosome treatment impacts the mitochondrial respiration of C-MSC. To evaluate the FAO capacity of C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$, we measured OCR for beta-oxidation in C-MSC treated with BSA, FAO-BSA, FAO-BSA plus ETO. As shown in **Supplementary Figure S1**, both C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$ do not respond well to exogenous substrate (BSA-conjugated FAO); however, knockdown of Rab27b shows increased FAO-associated OCR.

Next, we evaluated the ECAR in the absence of any substrate with further addition of glucose with the dosage of 5 and 10 mM, respectively. As shown in **Supplementary Figure S2**, addition of glucose in low and high dosage does not impact glycolysis and glycolytic capacity. Finally, we measured ECAR at 0.5 and 1 μM rotenone. As shown in **Figure 5**, knockdown of Rab27b increased basal and compensatory glycolysis at 1 μM rotenone compared to control C-MSC, suggesting higher glycolytic activity of C-MSC$^{sh-Rab27b}$.

Taken together, these findings suggest that knockdown of Rab27b results in selectively decreased mitochondrial oxidative phosphorylation and increased glycolytic rate.

Knockdown of Rab27b Inhibits Mitochondrial Fatty Acid β-oxidation and ETC in C-MSC

To further investigate the inhibitory effect of Rab27b knockdown on mitochondrial oxidative phosphorylation, we examined the expression of essential genes related to FAO, TCA cycle, and ETC. As shown in **Figure 6**, the expression of SIRT1, HADHA and HADHB (related to fatty acid β-oxidation), IDH3A (involved in TCA), UQCRQ, and SDHD (the components of the mitochondrial ETC), was decreased in C-MSC$^{sh-Rab27b}$ in comparison to C-MSC^{sh-NC}. No difference in the expression of other genes related to fatty acid β-oxidation (PPARGC1B, ACOX1, ACOX3) and TCA (OGDH) was detected between C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$. These data indicate that Rab27b might play a role in mitochondrial oxidative phosphorylation primarily by regulating genes that promote fatty acid β-oxidation, TCA, and ETC in C-MSCs.

To identify the mechanism of reduced expression of several genes involved in FAO, TCA, and ETC in Rab27b knockdown C-MSC, we measured the protein level of H3K4me3, a gene activation mark, in CMSC^{sh-NC} and CMSC$^{sh-Rab27b}$. As shown in **Figure 6B**, knockdown of Rab27b significantly reduces the H3K4me3 level in C-MSC, suggesting that the knockdown of Rab27b can change gene expression via epigenetic regulation.

Finally, we measured the mitochondrial membrane potential between C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$. The mitochondrial membrane potential (deltapsim) was calculated by red/green fluorescence ratio. As shown in **Figure 6C**, the Rab27b knockdown decreased mitochondrial membrane potential in C-MSC.

DISCUSSION

This is the first observation that the small GTPase Rab27b, a regulator of exosome secretion, can affect metabolism in C-MSC. In this study, we compared cellular metabolism

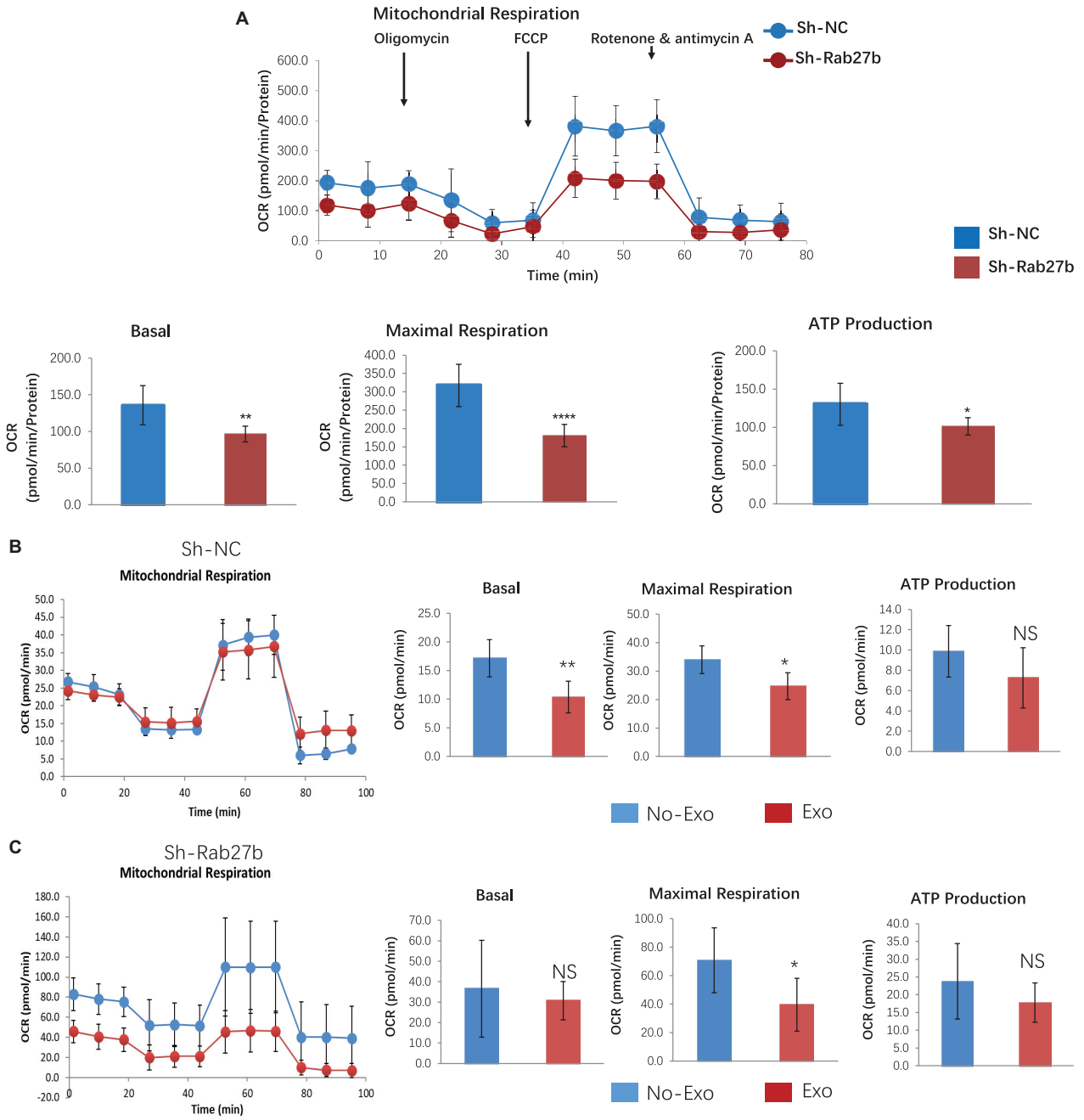

FIGURE 4 | Assessment of oxygen consumption rate (OCR) in C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$. **(A)** Cells were exposed sequentially to oligomycin, FCCP, and rotenone/antimycin A. Vertical lines indicate time of addition of mitochondrial inhibitors. Oxygen consumption rate was measured over time using a Seahorse XFe96 Analyzer. Cell mito stress test was performed according to manufacturer's protocol, results are normalized to total cellular protein (n = 7–8). **(B)** C-MSC^{sh-NC} were seeded in media with Exo-depleted FBS overnight, and primed cells with 25-μg exosomes from wild-type C-MSC for 1 h before OCR assay using a Seahorse XF24 analyzer (n = 5).**(C)** C-MSC$^{sh-Rab27b}$ were seeded in media with Exo-depleted FBS overnight, and primed cells with 25-μg exosomes from wild-type C-MSC for 1 h before OCR assay using a Seahorse XF24 analyzer (n = 5). Results are presented as mean ± SD (*p < 0.05; **p < 0.01; ****p < 0.0001).

and energetics in adult C-MSC following knockdown of Rab27b. Knockdown of the Rab27b gene increased cell glycolysis, but significantly inhibited oxidative phosphorylation in C-MSCs and selectively down-regulated the expression of the key genes involved in fatty acid β-oxidation, TCA and ETC, indicating that Rab27b plays a regulatory role in C-MSCs metabolism.

Under normal conditions, about 60–90% of ATP required for the continuous contractile activity of the heart is primarily produced by oxidation of fatty acids (FA), while the rest is derived

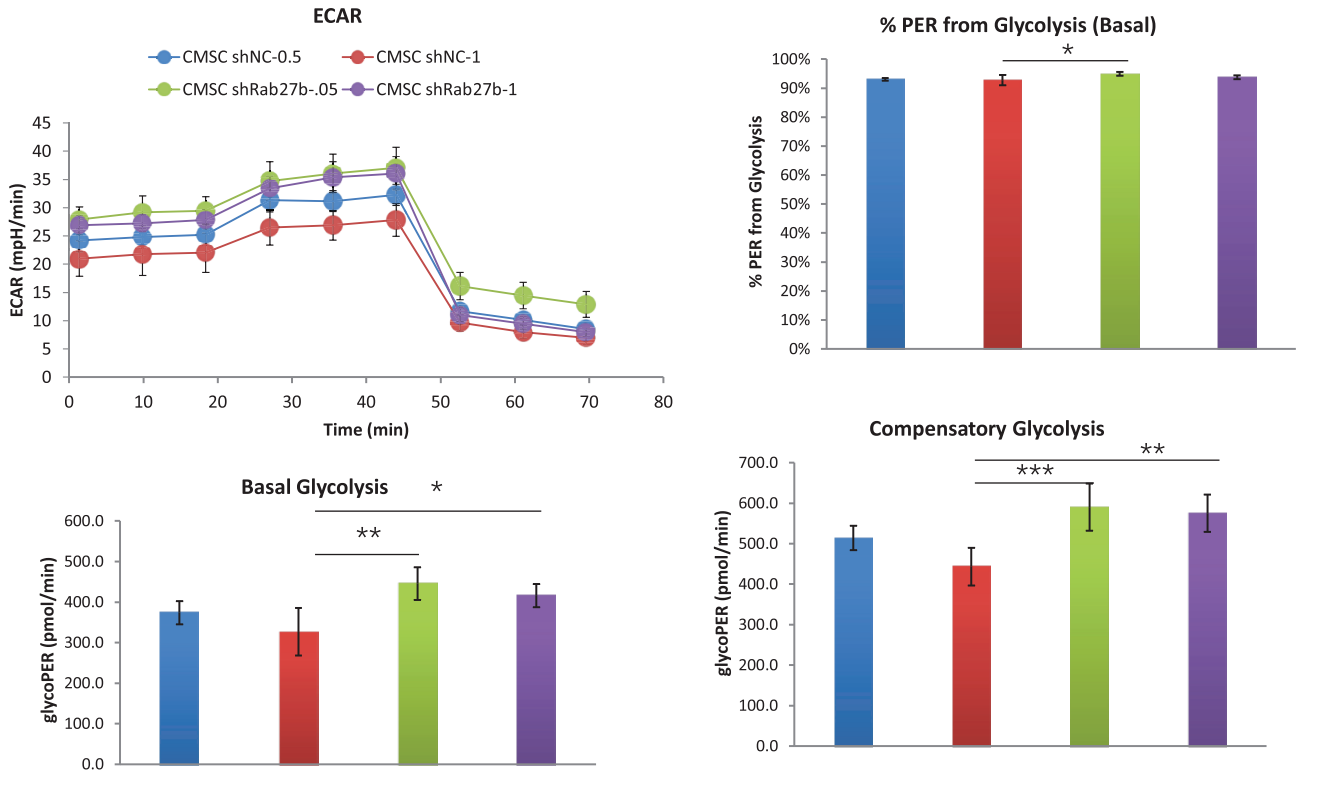

FIGURE 5 | Assessment of extracellular acidification rate (ECAR) in C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$. Cells were exposed sequentially to 0.5 or 1 μM rotenone/antimycin A and 2-DG. Extracellular acidification rate (ECAR) was measured over time using a Seahorse XFe24 Analyzer. Measurements of basal glycolysis,% PER from glycolysis and compensatory glycolysis were calculated from the ECAR data. Results are presented as mean ± SD (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, $n = 5$).

from the oxidation of pyruvate (van der Vusse et al., 1992). Fatty acids are completely oxidized to water and carbon dioxide through β-oxidation, the TCA cycle, and the ETC, producing ATP (Wanders et al., 2010). At the same time, TCA cycle and ETC are also involved in the oxidative metabolism of carbohydrates and proteins (Akram, 2014). Flaherty et al. (2019) reported that fat cells can regulate fat metabolism by direct release of neutral lipids from exosomes. Ying et al. (2017) demonstrated that exosome microRNAs from adipose tissue macrophages were shown to regulate systemic glucose metabolism by regulating fat cell function.

Furthermore, a recent study suggested that exosome secretion can attenuate cellular stress and maintain cellular homeostasis by exporting various unnecessary or harmful materials (Takahashi et al., 2017). Thus, in addition to participating in paracrine signaling, exosome formation and secretion may play a fundamental role in regulating intracellular functions. However, Rab27b, which regulates exosome secretion (Ostrowski et al., 2010), has not been demonstrated to modulate cellular metabolism. In this study, we first confirmed that knockdown of Rab27b decreased exosome secretion in C-MSCs. Next, we found that knockdown of Rab27b in C-MSC results in a significant reduction in mitochondrial oxidative metabolism, suggesting that Rab27b might be important to maintain fatty acid oxidative metabolism in C-MSC.

To investigate the mechanism by which knockdown of Rab27b inhibits mitochondrial oxidative metabolism in C-MSCs, we analyzed the expression of β-oxidation, TCA, and ETC-related genes in C-MSC^{sh-NC} and C-MSC$^{sh-Rab27b}$. We observed that knockdown of Rab27b not only reduced the expression of SIRT1, HADHA, and HADHB related to β-oxidation, IDH3A related to TCA, but also decreased the expression of UQCRQ and SDHD involved in ETC. SIRT1 is a highly conserved member of the family of NAD + -dependent Sir2 histone deacetylases (Rodgers et al., 2005) and has also been reported to sense the redox shifts and integrate mitochondrial metabolism through post-transcriptional regulation of the transcription factors and histones (Vachharajani et al., 2016). HADHA and HADHB, respectively, encode the α and β subunits of the mitochondrial trifunctional protein, catalyzing the last three steps of mitochondrial beta-oxidation of long-chain fatty acids (Diebold et al., 2019; Ljubkovic et al., 2019) also observed that in diabetic myocardium, a decrease in HADHA reduces the mitochondrial β-oxidation capacity and increases intracellular lipid accumulation, resulting in a negative impact on the heart. Moreover, Vasilescu et al. (2018) demonstrated that HADHB is directly associated with severe childhood-onset cardiomyopathies.

IDH3A encodes the alpha subunit of IDH3, which is one of three isozymes of NAD (+)-dependent isocitrate dehydrogenase

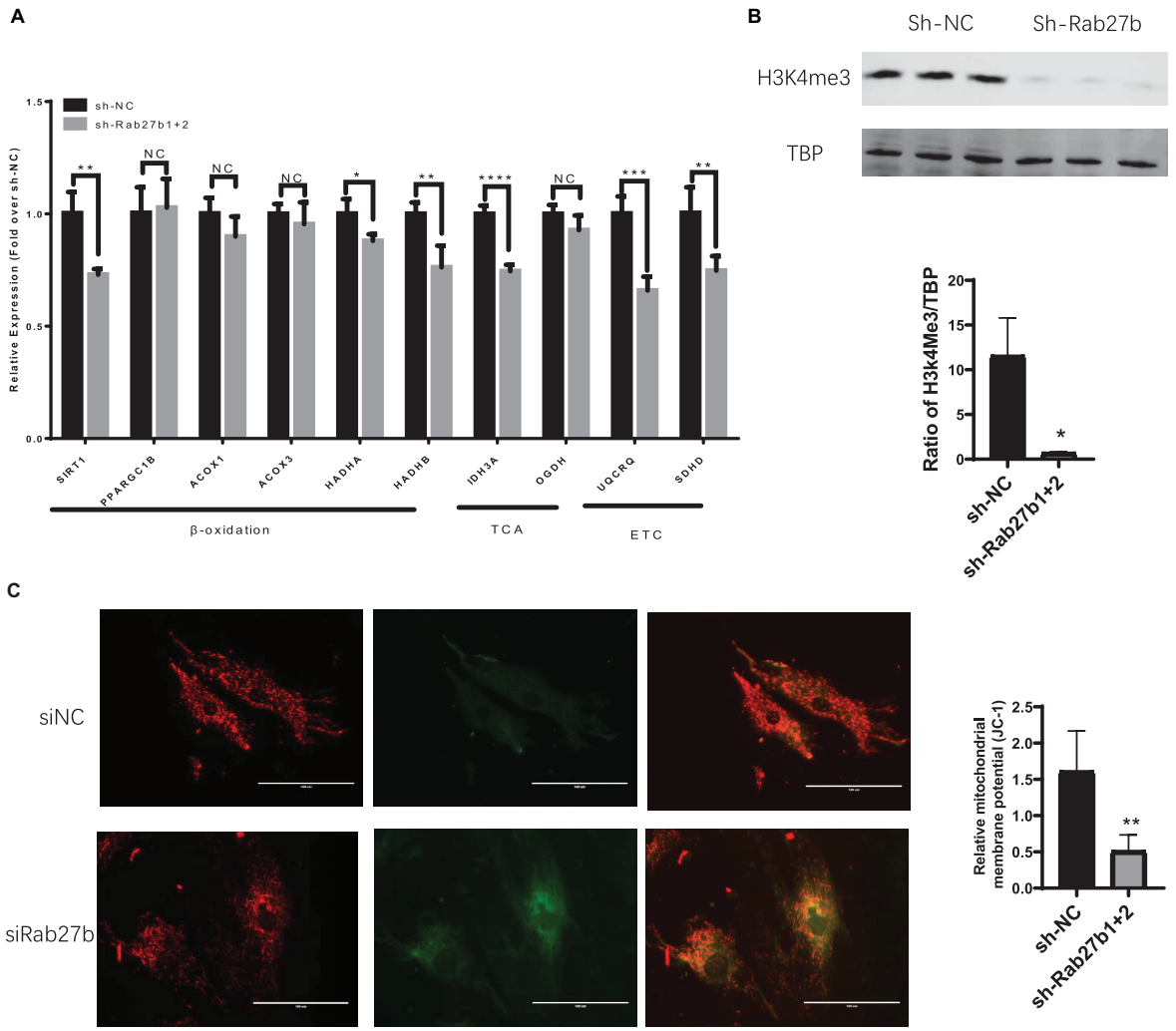

FIGURE 6 | (A) qRT-PCR analysis of genes related to mitochondrial fatty acid oxidation; TCA, tricarboxylic acid cycle; ETC, electron transport chain. The amount of mRNA was normalized using GAPDH. (n = 3–4). **(B)** Western blotting of H3k4me3 protein using TBP as a loading control (n = 3). **(C)** Mitochondrial membrane potential was measured by JC-1 staining and the images were obtained by fluorescent microscopy. Scale bars = 100 μm. The deltapsim is expressed as the ratio of red fluorescence to green fluorescence (n = 5–6). Results are shown as mean ± SD (NS, $p > 0.05$, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

that localize in the mitochondrial matrix and is an allosteric regulatory rate-limiting step that catalyzes the TCA cycle. It is necessary for the TCA cycle to progress and generate NADH, which feeds into oxidative phosphorylation to generate ATP (Findlay et al., 2018). SDHD encodes a member of complex II of the respiratory chain, which is responsible for the oxidation of succinate and represents an intersection between the mitochondrial respiratory chain for which an important function in cardiopulmonary oxygen sensing has been demonstrated, and the Krebs cycle, a central element of α-KA metabolism (Muhling et al., 2010). UQCRQ encodes a subunit of ubiquinol-cytochrome c reductase complex III, which is a part of the mitochondrial respiratory chain (Wen and Garg, 2004). Furthermore, cytochrome C reductase complex III has been identified as a major producer of superoxide and derived reactive oxygen species (ROS) in the mitochondrial respiratory chain (Drose and Brandt, 2012; Bleier and Drose, 2013). Mitochondrial ROS can lead to oxidative damage, cell death and abnormal immune responses in the heart after ischemia–reperfusion injury (Chouchani et al., 2014). Therefore, UQCRQ might also be involved in mitochondrial ROS production and myocardial dysfunction (Lu et al., 2019).

Our study shows that knockdown of Rab27b significantly reduces the level of H3K4me3, a gene activation marker, in C-MSC$^{sh-Rab27b}$ compared to C-MSC^{sh-NC}, suggesting that the knockdown of Rab27b can change gene expression via epigenetic regulation. Vizoso et al. (2015) recently reported that the epigenetically reactivated TBC1D16-47KD transcript is able to reduce EGFR activity in metastatic cells through members of Rab family. We will identify the mechanism how Rab27b modifies histone H3 methylation in our future study.

CONCLUSION

Our findings highlight, for the first time, a novel role for Rab27b in maintaining oxidation of fatty acids in C-MSCs, possibly by regulating the expression of key genes involved in fatty acid β-oxidation, TCA, and ETC. Future studies will be needed to determine the underlying epigenetic and gene specific mechanism whereby Rab27b controls metabolism and regulates oxidation of fatty acids in C-MSCs.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

Animal treatment protocols were approved by, and conducted in accordance with, animal welfare regulations of the Institutional Animal Care and Use Committee of the Medical College of Georgia.

AUTHOR CONTRIBUTIONS

YT: conceptualization. YJ: writing. YJ, XS, YS, JC, and YL: experiments. NW and YT: writing-review and editing.

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The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00209/full#supplementary-material

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The Immunomodulatory Signature of Extracellular Vesicles From Cardiosphere-Derived Cells: A Proteomic and miRNA Profiling

Esther López[1†], Federica Marinaro[1†], María de los Ángeles de Pedro[1], Francisco Miguel Sánchez-Margallo[1,2], María Gómez-Serrano[2,3,4*], Viviane Ponath[5,6], Elke Pogge von Strandmann[5,6], Inmaculada Jorge[2,3], Jesús Vázquez[2,3], Luis Miguel Fernández-Pereira[7], Verónica Crisóstomo[1,2], Verónica Álvarez[1] and Javier G. Casado[1,2]*

[1] Stem Cell Therapy Unit, Jesús Usón Minimally Invasive Surgery Centre, Cáceres, Spain, [2] CIBER de Enfermedades Cardiovasculares (CIBERCV), Madrid, Spain, [3] Laboratory of Cardiovascular Proteomics, Centro Nacional de Investigaciones Cardiovasculares (CNIC), Madrid, Spain, [4] Institute of Molecular Biology and Tumor Research (IMT), Center for Tumor Biology and Immunology (ZTI), Philipps University, Marburg, Germany, [5] Institute for Tumor Immunology, Center for Tumor Biology and Immunology (ZTI), Philipps University, Marburg, Germany, [6] Clinic for Hematology, Oncology, and Immunology, Philipps University, Marburg, Germany, [7] Immunology Department, Hospital San Pedro de Alcántara, Cáceres, Spain

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Mahmood Khan, The Ohio State University, United States

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Lucio Barile, University of Zurich, Switzerland
Venkata Naga Srikanth Garikipati, Temple University, United States

***Correspondence:**
Francisco Miguel Sánchez-Margallo msanchez@ccmijesususon.com
María Gómez-Serrano maria.gomezserrano@imt.uni-marburg.de

[†]These authors have contributed equally to this work and share first authorship

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Experimental data demonstrated that the regenerative potential and immunomodulatory capacity of cardiosphere-derived cells (CDCs) is mediated by paracrine mechanisms. In this process, extracellular vesicles derived from CDCs (EV-CDCs) are key mediators of their therapeutic effect. Considering the future applicability of these vesicles in human diseases, an accurate preclinical-to-clinical translation is needed, as well as an exhaustive molecular characterization of animal-derived therapeutic products. Based on that, the main goal of this study was to perform a comprehensive characterization of proteins and miRNAs in extracellular vesicles from porcine CDCs as a clinically relevant animal model. The analysis was performed by identification and quantification of proteins and miRNA expression profiles. Our results revealed the presence of clusters of immune-related and cardiac-related molecular biomarkers in EV-CDCs. Additionally, considering that priming stem cells with inflammatory stimuli may increase the therapeutic potential of released vesicles, here we studied the dynamic changes that occur in the extracellular vesicles from IFNγ-primed CDCs. These analyses detected statistically significant changes in several miRNAs and proteins. Notably, the increase in interleukin 6 (IL6) protein, as well as the increase in mir-125b (that targets IL6 receptor) was especially relevant. These results suggest a potential involvement of EV-CDCs in the regulation of the IL6/IL6R axis, with implications in inflammatory-mediated diseases.

Keywords: cardiosphere-derived cells, cardiac stem cells, proteomic analyses, quantitative polymerase chain reaction, interferon-γ, extracellular vesicles, miRNA–microRNA, priming

Abbreviations: CDCs, cardiosphere-derived cells; DMEM, Dulbecco's modified Eagle's medium; EV-CDCs, extracellular vesicles from cardiosphere-derived cells; EVs, extracellular vesicles; FDR, false discovery rate; IFNγ, interferon gamma; IFNγ/EV-end MSCs, extracellular vesicles from IFNγ-primed cardiosphere-derived cells; IPA, ingenuity pathway analysis; iTRAQ, isobaric Tags for Relative and Absolute Quantitation; LC-MS/MS, liquid chromatography-tandem mass spectrometry; MSCs, mesenchymal stem cells; Np, number of peptides; PBS, phosphate-buffered saline; PCA, principal component analysis; SBT, Systems Biology Triangle; WSPP, weighted spectrum, peptide, protein.

INTRODUCTION

Cardiac-derived stem cells have been considered as one of the most promising therapeutic options for myocardial regeneration (Zhang et al., 2015; Lader et al., 2017). However, more than 30 top-cited articles have been retracted in the last year (Chien et al., 2019). In the early years of stem cell-based therapies, several disappointing results were reported after the administration of MSCs in myocardial infarction (Miao et al., 2017). Some years later, clinical trials were focused on the administration of cardiac stem cells, and 5 years ago, the clinical trial CADUCEUS (ClinicalTrials.gov Identifier: NCT00893360) opened an optimistic scenario in cardiology, demonstrating the regenerative potential of autologous CDCs (Malliaras et al., 2014).

Nowadays, accumulating pieces of evidence have demonstrated that paracrine mechanisms have a major impact on immunomodulation and tissue regeneration capacity of stem cells (Epstein, 2018). In this sense, exosomes derived from CDCs have demonstrated a therapeutic effect (Ibrahim et al., 2014). This was further confirmed in a clinically relevant animal model of acute and chronic myocardial infarction, where exosomes also demonstrated a relevant clinical outcome (Gallet et al., 2016).

Considering these results, different groups have tried to unravel the molecular mechanisms underlying the therapeutic effects of CDCs and their EVs. In this sense, *in vitro* and *in vivo* studies in murine models using EV-CDCs and their most abundant small RNA constituent, the Y RNA fragment YF1, produced an increase in the anti-inflammatory cytokine IL10 levels, inducing cardioprotection and attenuating hypertension-associated damage (Cambier et al., 2017, 2018).

In vivo studies in rats and pigs have also demonstrated that exosomes from CDCs reduce the presence of infiltrating macrophages in the infarcted tissue and mediate macrophage polarization through miRNAs, such as mir-181b (de Couto et al., 2017). Furthermore, the analysis of miRNAs in exosomes from CDCs cultured under hypoxic conditions increased pro-angiogenic miRNAs (mir-126, mir-130a, and mir-210) (Namazi et al., 2018a) as well as helped in the release of exosomes with anti-apoptotic properties (Namazi et al., 2018b).

Taking together the therapeutic effect of CDC-derived EVs and their promising application in different diseases, such as Duchenne muscular dystrophy (Aminzadeh et al., 2018), the first goal of this study was to identify biomarkers, or clusters of biomarkers, that might be associated with the therapeutic efficacy of EV-CDCs. A detailed characterization and classification of the proteome was performed by high-throughput proteomic screening, followed by bioinformatic analyses.

Furthermore, an innovative aspect of our study lies in the characterization of EVs isolated from IFNγ-primed CDCs (IFNγ/EV-CDCs). The idea of priming adult stem cells with IFNγ to increase their immunomodulatory or pro-regenerative effect is not new, and this effect has been experimentally demonstrated in MSCs from umbilical cord blood (Oh et al., 2008) and human adipose tissue (DelaRosa et al., 2009). More recently, several studies have been focused on *in vitro* stimulation protocols to trigger the release of vesicles loaded with therapeutic agents. In this regard, primed MSCs (exposed to hypoxia and serum deprivation) released exosomes with increase in the immunomodulatory potential (Showalter et al., 2019), inflammation-primed MSCs amplified EVs' immunosuppression against T-cell proliferation (Di Trapani et al., 2016), and interleukin-1β-primed MSCs produced exosomes with an increased expression of mir-146a with immunomodulatory properties (Song et al., 2017). It is important to note that the inflammatory priming of MSCs has been recently used for donor selection using miRNAs as biomarkers (Ragni et al., 2019).

Apart from protein characterization and considering that miRNA cargo has a key role in the effector function of EVs (Qiu et al., 2018), this study has been also focused on the characterization of a large panel of miRNAs. These miRNAs were selected for their involvement in cardiac regeneration, immune response, and expression in EVs derived from adult stem cells.

To our knowledge, this is the first study describing the proteomic and miRNA profiling of IFNγ/EV-CDCs from a clinically relevant animal model. Here, we show the identification, quantification, and classification of proteins according to immune-related and cardiac-related categories. The presence of interleukin 6 (IL6) in the proteomic analysis is especially relevant, as well as the expression of different miRNAs targeting interleukin 6 receptor (IL6R). Altogether, these results highlight a critical role for IL6/IL6R axis in the therapeutic effect of EV-CDCs.

MATERIALS AND METHODS

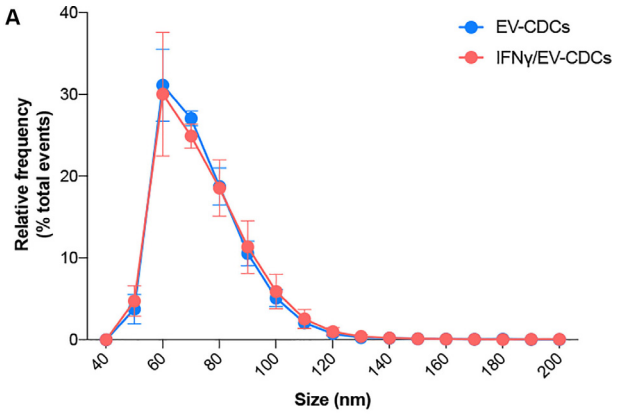
Isolation and Characterization of CDCs

CDCs were isolated from cardiac explants of four euthanized healthy large white pigs. This procedure was authorized by the Animal Welfare and Ethics Committee of the Jesús Usón Minimally Invasive Surgery Centre, in accordance with the recommendations outlined by the local government (Junta de Extremadura), and the EU Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

Briefly, explants were mechanically disaggregated and subjected to three successive enzymatic digestions with a solution of 0.2% trypsin (Lonza, Basel, Switzerland) and 0.2% collagenase IV (Sigma, St. Louis, MO, United States). Cell culture, isolation, and *in vitro* expansion were performed as previously described by our group (Blázquez et al., 2016).

IFNγ Treatment, Isolation, and Characterization of EV-CDCs

EV-CDCs were isolated from expanded CDCs at passages 12–15 and 80% confluence. For preconditioning, cells were treated with 3 ng/ml swine IFN gamma Recombinant Protein (IFNγ, catalog number RP0126S-025; Kingfisher Biotech, Saint Paul, MN, United States) for 3 days in standard culture medium. Controls and preconditioned cells were washed with PBS and incubated with DMEM containing 1% insulin–transferrin–selenium (product code: 41400045; Thermo Fisher Scientific, Waltham, MA, United States). This conditioned



Final concentration (No. Particles E+08/mL)	Median size (nm)	
4.69 ± 1.20	71.00 ± 2.19	EV-CDCs
3.53 ± 1.80	72.50 ± 5.45	IFNγ/EV-CDCs

FIGURE 1 | High-sensitivity flow cytometry analysis of extracellular vesicles derived from CDCs (EV-CDCs) at the single-particle level. **(A)** Histogram of particle size with a bin width of 10 nm for control EV-CDCs (blue, $n = 4$) and EVs isolated from IFNγ-primed CDCs (IFNγ/EV-CDCs) (red, $n = 4$) analyzed by NanoFCM. Data show average (±SD) of the relative frequency of total events from representative technical replicates. **(B)** Concentration and median size values of EV-CDC ($n = 4$) and IFNγ/EV-CDC ($n = 4$) samples characterized in this study. Dilution range was tested for every sample, and two technical replicates were recorded for at least 3,000 and up to 12,000 events. Data show the average (±SD) of group samples.

medium was collected at day 4 and centrifuged first at 1,000 × *g* for 10 min at 4°C, and then 5,000 × *g* for 20 min at 4°C. Supernatants were filtered through a 0.22-μm mesh to eliminate dead cells and debris. The filtrate was used to concentrate the EV-CDCs through a 3-kDa MWCO Amicon® Ultra device (Merck-Millipore, MA, United States) by centrifugation at 4,000 × *g* for 1 h at 4°C. Concentrate samples were recovered from the device and stored at −20°C until further analyses.

The characterization of EV-CDCs was performed by high-throughput proteomic analysis, and proteins were classified following the MISEV2018 guidelines of the International Society for Extracellular Vesicles (ISEV) (Théry et al., 2018). In compliance with ISEV recommendations, EV preparations should be characterized by at least three positive protein markers. Accordingly, a total of 88 proteins from our EV-CDCs were grouped in the following categories: "Transmembrane or GPI-anchored proteins associated to plasma membrane and/or endosomes", "Cytosolic proteins recovered in EVs", "Major components of non-EV co-isolated structures", "Transmembrane, lipid-bound, and soluble proteins associated to other intracellular compartments than PM/endosomes", and "Secreted proteins recovered with EVs". **Supplementary Table S1** shows the classification of proteins according to these categories.

In addition to proteomics, a Flow NanoAnalyzer (NanoFCM INC., United Kingdom) was used for the multiparameter analysis of EV-CDCs samples. The Flow NanoAnalyzer platform enables quantitative and multiparameter analysis of single EVs down to 40 nm, which is distinctively sensitive and high throughput. All experiments were performed in compliance with the NanoFCM system's recommendations (more information on http://www.nanofcm.com/). Briefly, dilution of all samples was individually tested in order to record a total number of events in between 3,000 and 12,000. Concentrated DMEM 1% insulin–transferrin–selenium medium was used for threshold setting and as a blank. Monodisperse silica nanoparticles cocktail (68–155 nm. Cat. No. S16M-Exo; NanoFCM INC.) were employed as the reference to calibrate the size of EVs and polystyrene 210 nm beads (QC Beads; Cat. No. S08210; NanoFCM INC.) at 1:100 dilution for particle concentration estimation. Light scattering was used for the measurement of nanoparticle size and size distributions. The EV size range was set at 40-200 nm. All samples were measured with at least two technical replicates.

Protein Identification by High-Resolution Liquid Chromatography Coupled to Mass Spectrometry

Protein characterization of EV-CDCs and their comparison with IFNγ/EV-CDCs was performed by a high-throughput multiplexed quantitative proteomic approach according to previously described protocols (Jorge et al., 2009; Navarro and Vázquez, 2009; Bonzon-Kulichenko et al., 2011; Navarro et al., 2014; García-Marqués et al., 2016). Protein extracts were incubated with trypsin using the Filter Aided Sample Preparation (FASP) digestion kit (Expedeon, San Diego, CA, United States), as previously described (Wiśniewski et al., 2011). The resulting peptides were labeled using 8plex-iTRAQ reagents, according to the manufacturer's instructions, and desalted on OASIS HLB extraction cartridges (Waters Corporation, Milford, MA, United States). Half of the tagged peptides were directly analyzed by liquid chromatography tandem mass spectrometry (LC-MS/MS) in different acquisition runs, and the remaining peptides were separated into three fractions using the high pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific). Samples were analyzed using an Easy nLC 1000 nano-HPLC coupled to a Q Exactive mass spectrometer (Thermo Fisher Scientific). Peptides were injected onto a C18 reversed-phase nano-column (75 μm I.D. and 50 cm; Acclaim PepMap100 from Thermo Fisher Scientific) in buffer A [0.1% formic acid (v/v)] and eluted with a 300-min lineal gradient of buffer B [90% acetonitrile, 0.1% formic acid (v/v)], at 200 nl/min. Mass spectrometry (MS) runs consisted of 140,000 enhanced FT-resolution spectra in the 390 to 1,500-m/z wide range and separated 390–700 m/z (range 1), 650–900 m/z (range 2), and 850–1500 m/z (range 3) followed by data-dependent MS/MS spectra of the 15 most intense parent ions acquired along the chromatographic run. HCD fragmentation was performed at 30% of normalized collision energy. A total of 14 MS

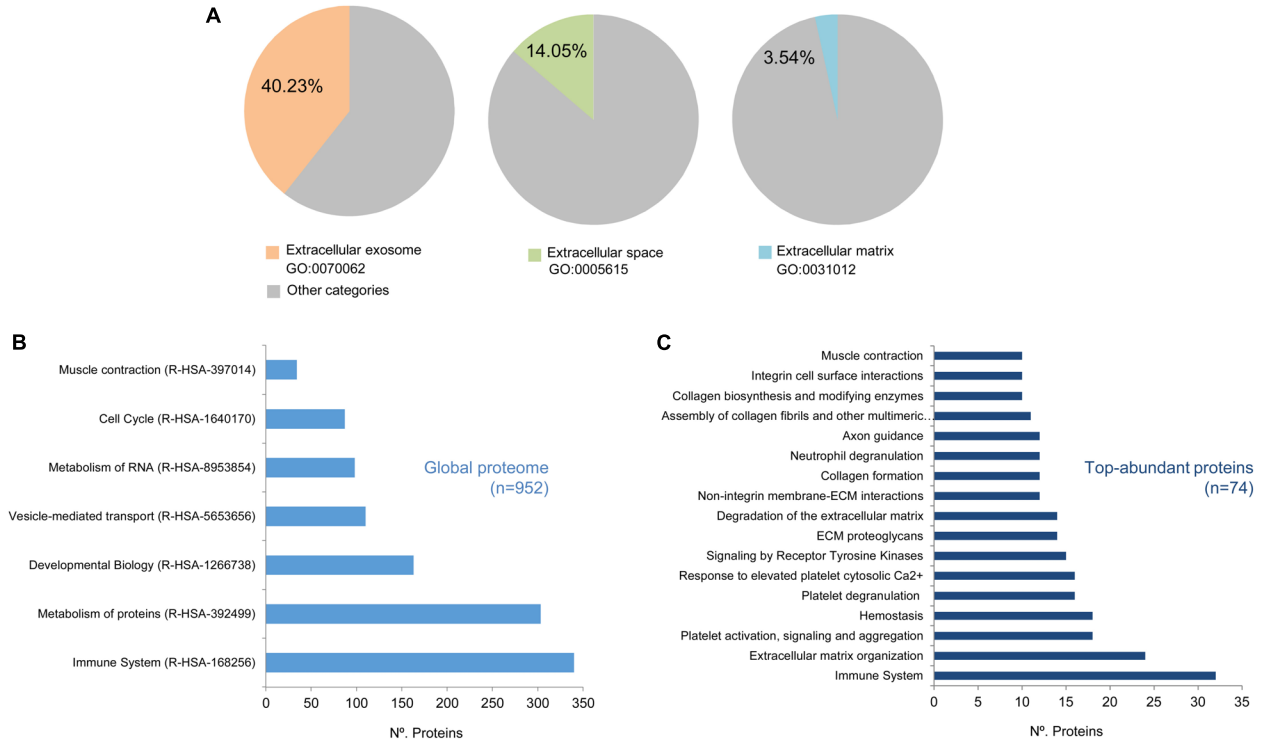


FIGURE 2 | High-throughput proteomic analysis on EV-CDCs. Protein extracts from EV-CDCs (n = 4) and IFNγ/EV-CDCs (n = 4) were processed by high-resolution liquid chromatography coupled to tandem mass spectrometry analyses. Those proteins were identified by at least two peptides (n = 952) and were classified according to their biological function using annotations from the Gene Ontology **(A)** or Reactome **(B,C)** databases. **(A)** Pie charts representing the percentage of proteins (% of total pig annotated proteins) classified as *Extracellular exosome* (GO:0070062, orange), *Extracellular space* (GO:0005615, green), and *Extracellular matrix* (GO:0031012, blue) proteins. Significantly enriched Reactome top-level pathways [enrichment value of $p < 0.01$, 5% false discovery rate (FDR)] including **(B)** the global EV-CDC proteome (n = 952, Np ≥ 2) and **(C)** particular most abundant proteins (n = 74, Np ≥ 20).

data sets, eight from unfractionated material and six from the corresponding fractions, were registered with 80 h total acquisition time.

Peptide Identification, Protein Quantification, and Statistical Analysis

For peptide identification, MS/MS scans were searched as previously described by Binek et al. (2017) using a combined pig and human database (UniProtKB/Swiss-ProtUniProtKB/Swiss-Prot 20147_02 07 Release). *Sus scrofa* gene and protein annotation is not complete; hence, pig proteins were given priority when they shared peptides with human proteins. The Proteome Discoverer 2.1 software (Thermo Fisher) was used for database searching with the following parameters: trypsin digestion with two maximum missed cleavage sites, precursor mass tolerance of 800 ppm, fragment mass tolerance of 0.02 Da. Variable methionine oxidation (+15.994915 Da) and fixed cysteine carbamidomethylation (+57.021 Da), and 8plex-iTRAQ labeling at lysine and N-terminal modification (+304.2054) were chosen.

For peptide identification, the MS/MS spectra were searched using the probability ratio method (Martínez-Bartolomé et al., 2008), and the FDR of peptide identification was calculated based on the search results against a decoy database using the refined method (Navarro and Vázquez, 2009). Peptide and scan counting were performed assuming as positive events those with an FDR equal or lower than 1%.

Quantitative information of 8plex-iTRAQ reporter ions was extracted from MS/MS spectra using an in-house developed program (SanXoT) as already described (Trevisan-Herraz et al., 2019), and protein abundance changes were analyzed using the WSPP statistical model (Navarro et al., 2014).

Briefly, the $\log_2$-ratio of concentration in the two samples being compared, A and B, determined by spectrum s of peptide p derived from protein q in experiment e is expressed as $X_{eqps} = \log_2(A/B)$. The $\log_2$-ratio value associated with each peptide, X_{eqp}, is then calculated as a weighted average of the spectra used to quantify the peptide, and the value associated with each protein, X_{eq}, is similarly the weighted average of its peptides. In addition, a grand mean, X_e, is calculated in each experiment as a weighted average of the protein values. In this study, we calculated X_e by the integration of the four biological replicates, both from control and IFNγ samples, and determined $\log_2$ - (X_eIFNγ/X_e control). WSSP was applied in the SBT workflow that detects significant protein abundance by performing the protein to category integration and taking into account the protein outliers within each category (García-Marqués et al.,

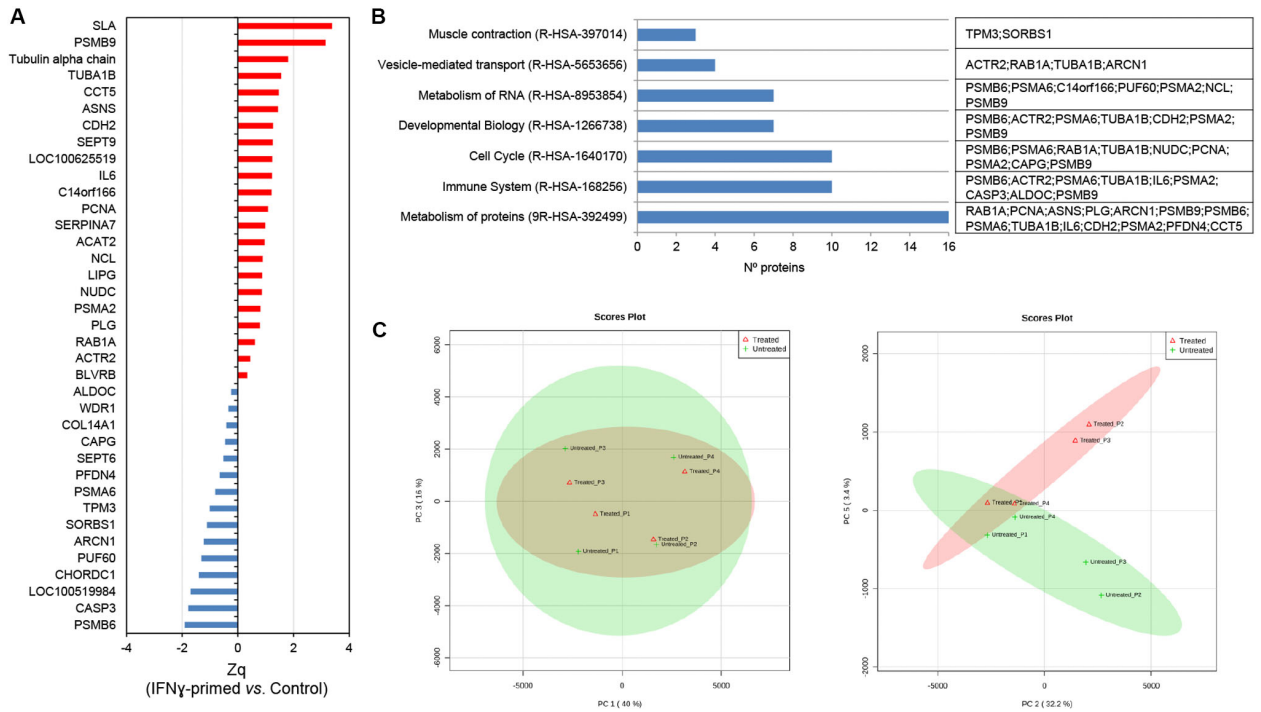


FIGURE 3 | Quantitative proteomic analysis of IFNγ/EV-CDCs. **(A)** Quantitative proteomic results. Protein profile changes were analyzed by the WSPP model (Navarro et al., 2014) to identify significantly altered proteins comparing IFNγ/EV-CDCs and EV-CDCs samples. Since a combined database (human and pig) for peptide searching was used, data were integrated at the gene coding level. Protein (gene) values (Zq) are reported as the standardized variable, which is defined as the mean corrected $\log_2$-ratio expressed in units of standard deviation. The protein ratio of each sample was calculated against an internal standard (IS) based on the average of iTRAQ reporters from EV-CDCs control samples. Statistical differences between Zq values of sample groups were evaluated by paired *t*-test. Significant protein abundance change was set at a value of $p < 0.05$. Plots show the integrated Zq values from the sample groups for those proteins significantly up- (red) or down-regulated (blue) in IFNγ/EV-CDCs vs. EV-CDCs. **(B)** Significantly changed proteins were classified and annotated by Reactome in the top level pathways. **(C)** A total of 952 proteins (number of peptides, Np ≥ 2 at 1% FDR) quantified by iTRAQ-based proteomics were subjected to PCA. Left: Score plot for PC1 (40% variance explained) vs. PC3 (16% variance explained). Right: Score plot for PC2 (32.2% variance explained) vs. PC5 (3.4% variance explained). Data display 95% confidence regions. Animal origin ($n = 4$) is indicated on the plots as p1–p4. IFNγ/EV-CDCs (treated) and EV-CDCs (untreated, control) samples are indicated in red and green, respectively.

2016). For that, proteins were previously annotated based on Gene Ontology database (The Gene Ontology Consortium, 2017). The algorithm provides a standardized variable, Z_q, defined as the mean-corrected $\log_2$(A/B) expressed in units of standard deviation at the protein level. Student *t*-test was used to compare Z_q values from EV-CDCs and IFNγ/EV-CDCs, and the statistical significance was set at a value of $p < 0.05$. Enrichment analysis of proteins was performed by DAVID functional annotation database[1] (Huang et al., 2009a,b) and Benjamini–Hochberg FDR was used for multiple test correction (FDR < 0.05). For biological data interpretation, proteins were classified using the Reactome pathway database[2] (Fabregat et al., 2018). The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium via the PRIDE (Perez-Riverol et al., 2019) partner repository with the dataset identifier PXD016434.

Additionally, PCA was performed on proteins with two or more peptides (number of peptides or Np ≥ 2) quantified after iTRAQ proteomic analysis and at 1% FDR. For PCA, Metaboanalyst software version 4.0[3] (Chong et al., 2018) was used.

To validate the differential expression patterns shown by proteomic analysis, the expression of IL6 in EV-CDCs and IFNγ/EV-CDCs from three pigs was determined using Porcine IL6 DuoSet ELISA kit (R&D SYSTEMS, Minneapolis, MN, United States). EV samples were normalized by total particle concentration measured by NanoFCM system. ELISA protocol was performed following the manufacturer's instructions. IL6 concentrations between EV-CDCs and IFNγ/EV-CDCs were compared through a paired *t*-test.

miRNAs Expression in EV-CDCs and Target Interactions

Expression of the selected miRNAs in EV-CDCs was evaluated by real-time quantitative PCR (qPCR). Total RNAs from EV-CDCs were isolated using mirVANA miRNA isolation

[1]https://david.ncifcrf.gov/home.jsp
[2]https://reactome.org/
[3]https://www.metaboanalyst.ca/

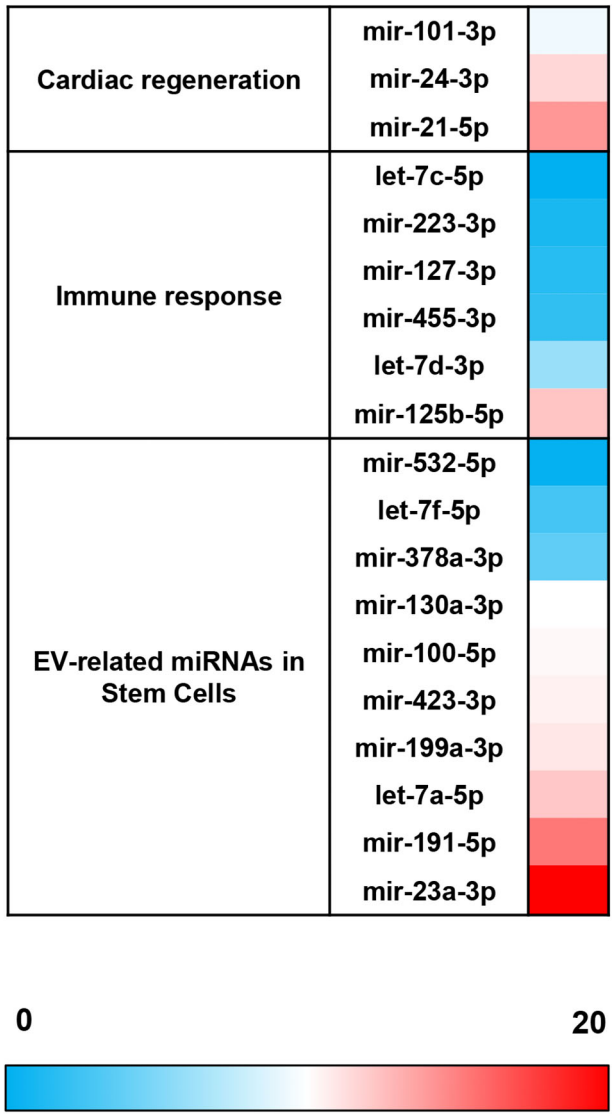


FIGURE 4 | miRNA expression abundance in EV-CDCs. miRNA expression was analyzed by qPCR. Total RNA was isolated from EV-CDCs, and the amplification products were analyzed by the $2^{-\Delta Ct}$ method using the most stable miRNAs as endogenous controls. The statistical analysis was performed using a Thermo Fisher Cloud Analysis version 1.1. The figure shows the abundance of miRNAs in terms of $2^{-\Delta Ct}$, from less (blue) to more abundant (red), grouped by our selected miRNA panel.

kit (Applied Biosystems, Foster City, CA, United States), following the manufacturer's protocol for total RNA extraction. Quality and concentration of total RNAs were evaluated by spectrophotometry. For reverse transcription, 10 ng of total RNA was used to synthesize miRNAs' cDNA using TaqMan® Advanced miRNA cDNA Synthesis kit (Cat. No. A28007; Thermo-Fisher Scientific Inc., Waltham, MA, United States), according to the manufacturer's instructions. Five microliters of diluted cDNA (1:100) was then employed as template for qPCR amplification with the TaqMan™ Fast Advanced Master Mix (Cat. No. 4444964; Thermo-Fisher Scientific Inc., Waltham, MA, United States). Commercial TaqMan® Gene Expression Assays probes (Thermo-Fisher Scientific Inc., Waltham, MA, United States) were used, according to the manufacturer's recommendations, to evaluate the relative expression of 44 miRNAs (**Supplementary Table S2**). qPCR reactions were performed in duplicate, and molecular biology-grade water replaced cDNA in no template control reactions. Data from individual TaqMan Assays were acquired by QuantStudio 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc.) and quantified with the Relative Quantification Application (Thermo Fisher) tool in the Thermo Fisher Cloud software. Levels of each miRNA were normalized to three endogenous controls selected by their score variation. The quantification of miRNAs was performed by $2^{-\Delta Ct}$ calculation. Moreover, EV-CDC and IFNγ/EV-CDC differences were compared through paired *t*-test and $2^{-\Delta\Delta Ct}$ calculation (Livak and Schmittgen, 2001).

The miRNet web tool[4] (Fan and Xia, 2018), that integrates *Sus scrofa* database, was used for miRNA target interaction analysis. Subsequently, Reactome was used to classify the targeted genes according to their biological function.

In vitro Differentiation and Activation of Peripheral Blood Lymphocytes (PBLs), Co-culture With EV-CDCs and IFNγ/EV-CDCs, and Flow Cytometry Analysis

Extracellular vesicles from cardiosphere-derived cells and IFNγ/EV-CDCs were co-cultured *in vitro* with peripheral blood lymphocytes (PBLs) in order to evaluate their immunomodulatory effect. Peripheral blood from one large white pig was collected in EDTA-containing tubes. The blood was diluted in PBS, layered over Histopaque-1077 (Sigma, St. Louis, MO, United States), centrifuged, washed twice with PBS, and seeded in V-bottom 96 well plates at a total density of 200,000 cells per well in RPMI medium. EV-CDCs (n = 4) and IFNγ/EV-CDCs (n = 4) from four different animals were added to different wells at different concentrations (50, 100, and 200 μg/ml), and analyzed at day 3 by flow cytometry. PBLs without EVs were used as negative control.

For flow cytometry analyses, cells were incubated for 30 min at 4°C with fluorescence-labeled porcine monoclonal antibodies against porcine CD4, CD8α, CD14, CD16, CD27, CD45RA, and Swine Leukocyte Antigen class II (SLAII; AbD Serotec, Kidlington, United Kingdom). Cells were then washed and re-suspended in PBS. Analyses were performed in a FACScalibur cytometer (BD Biosciences, San Jose, CA, United States) after acquisition of 10,000 events. Cells were primarily selected using forward and side scatter characteristics, and fluorescence was analyzed using CellQuest software (BD Biosciences). Appropriate isotype-matched negative control antibodies were used in all the experiments. Paired *t*-test

[4]https://www.mirnet.ca/miRNet/home.xhtml

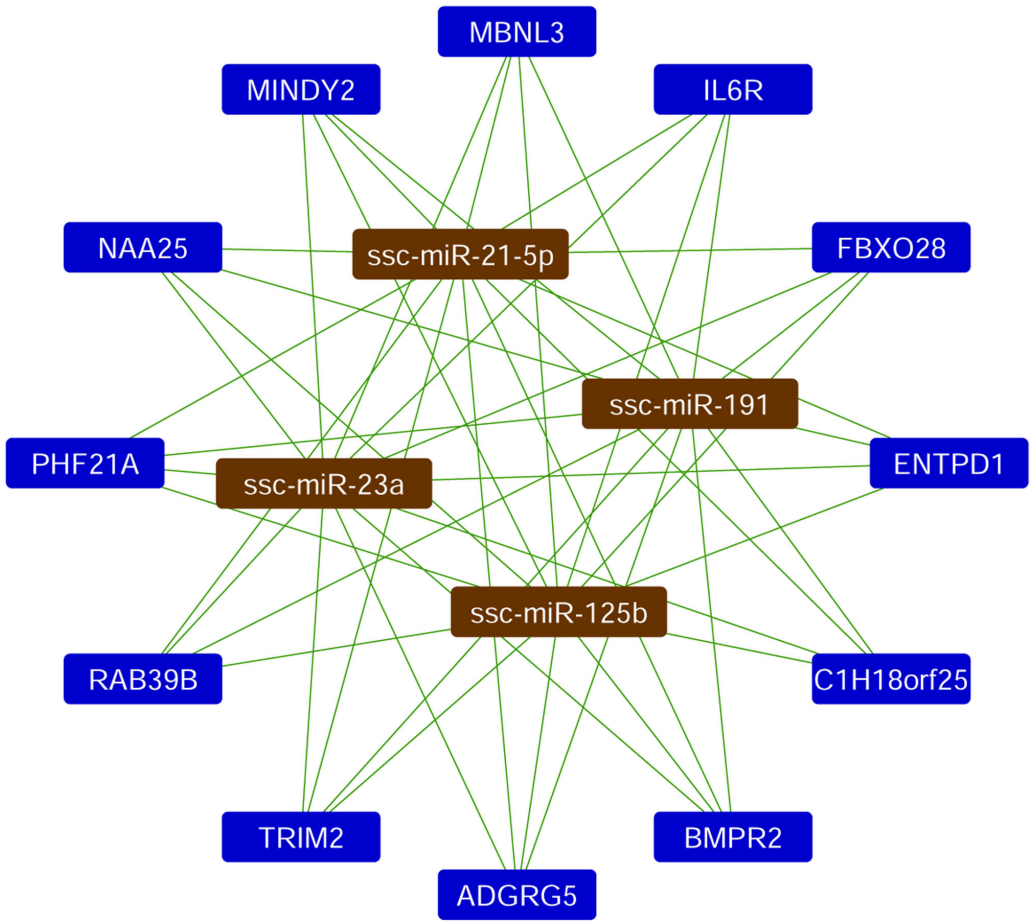


FIGURE 5 | Analysis of miRNA target network. The miRNet analysis was performed in the four top-abundant expression analyzed miRNAs. The network shows the interactions with the *Sus scrofa* target genes.

was used to compare each EV dose to the corresponding negative control.

RESULTS

High-Throughput Analysis of EV-CDCs and IFNγ/EV-CDCs

Proteomic profiling provides a global view of subcellular fractions, offering a better understanding of protein abundance. Moreover, quantitative proteomics is a valuable technique for a better characterization of biological products, such as stem cells or stem cell-derived vesicles. In this work, the identification and quantification of EV-CDCs proteins were performed by high-throughput quantitative proteomics using multiplex peptide stable isotope labeling, a useful technique for the characterization of these vesicles (Cypryk et al., 2014). Additionally, high-sensitivity flow cytometry analyses on EV-CDCs samples were performed. Sizing profile of the samples demonstrated that preparations were enriched in small EVs (ranging from 40 to 200 nm) (**Figure 1**). Median size and concentration of the released EVs showed no significant differences between EV-CDCs and IFNγ-primed CDCs. Besides, nano-FCM analyses performed on non-concentrated conditioned media did not show significant differences in particle releasing between CDCs and IFNγ/CDCs (*data not shown*).

Unfortunately, protein and gene annotation databases for *Sus scrofa* are not as complete as databases for *Homo sapiens*. Thus, MS/MS scans were searched against a combined pig and human database, giving priority to pig identifications when peptide sequences were identified in both (Binek et al., 2017). Quantification of each protein was calculated at the gene-coding level. Of note, 1,205 protein identifications were retrieved only from the pig database. This combined strategy allowed us to increase the depth of the study, depicting around 30% of the identifications (remarkably, 369 identifications were retrieved exclusively from the human database).

Our study was limited to those proteins represented by at least two peptides (Np $\geq$ 2). Using this cut-off value, a total of n = 952 proteins were analyzed and classified by the DAVID

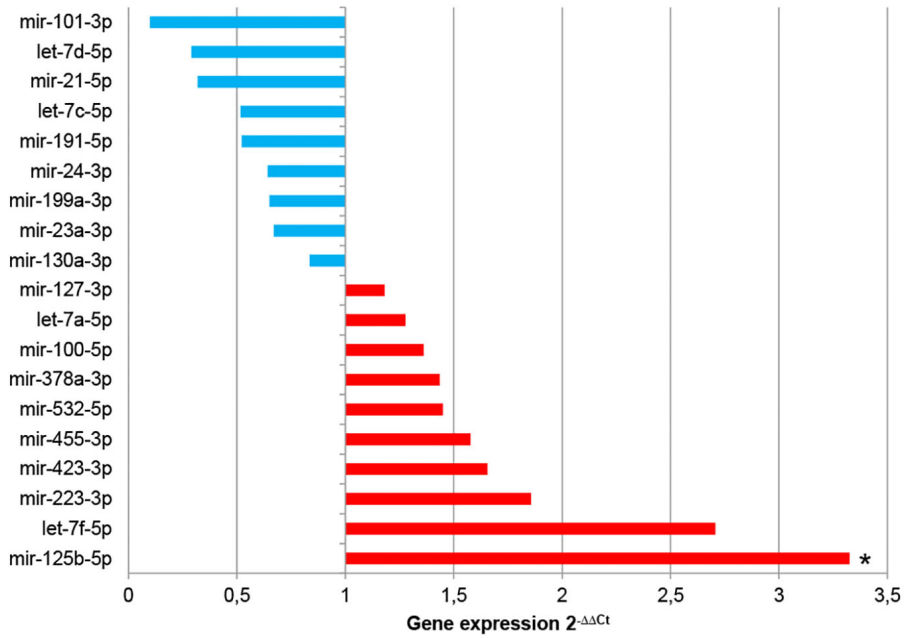


FIGURE 6 | Comparative analysis of miRNA expression between EV-CDCs and IFNγ/EV-CDCs. The comparative analysis of miRNA expression was performed by qPCR. Total RNA was isolated from EV-CDCs and IFNγ/EV-CDCs, and amplification products were compared by the $2^{-\Delta\Delta Ct}$ method using the most stable miRNAs as endogenous controls. The statistical analysis was performed using a Thermo Fisher Cloud Analysis version 1.1. Graphs show $2^{-\Delta\Delta Ct}$. *$p \leq 0.05$.

software[5] (**Supplementary Table S3**) (Huang et al., 2009a,b). As shown in **Figure 2A**, this classification revealed that $n = 375$ annotations (40.23% from total annotations) were comprised in the *extracellular exosome* category (GO:0070062), $n = 131$ (14.05%) were comprised in the *extracellular space* category (GO:0005615), and $n = 33$ (3.54%) in the *extracellular matrix* category (GO:0031012). Additionally, the EV-CDCs proteome included 75 proteins from the 100 top-identified proteins in ExoCarta database[6] (Keerthikumar et al., 2016).

The 952 proteins were then classified according to the Reactome database (Fabregat et al., 2018) to elucidate the functional pathways. Reactome is a hierarchically classified database divided in 24 top-level pathways (such as *Metabolism of Proteins*, *Signal Transduction*, *Immune System*) that serve as "roots" for thousands of more specific pathways. **Figure 2B** represents the number of proteins classified in the following top-level pathways: *Metabolism of Proteins* (R-HSA-392499), *Signal Transduction* (R-HSA-162582), *Immune System* (R-HSA-168256), *Cell Cycle* (R-HSA-1640170), *Metabolism* (R-HSA-1430728), *Developmental Biology* (R-HSA-1266738), *Metabolism of RNA* (R-HSA-8953854), *Transport of Small Molecules* (R-HSA-382551), *Vesicle-Mediated Transport* (R-HSA-5653656), and *Muscle Contraction* (R-HSA-397014). It is important to note that the number of proteins included in these top-level pathways is directly correlated with the total amount of proteins pre-classified in the Reactome database. Taking into account this observation, an enrichment analysis of the top-abundant proteins ($n = 74$, Np ≥ 20) was performed to identify over-represented pathways (enrichment analyses were calculated using a value (−Log) adjusted by Benjamini–Hochberg FDR correction of $p \leq 0.05$). Our analysis for the global EV-CDC proteome ($n = 952$, Np ≥ 2) demonstrated an enrichment of several top-level pathways: *Immune System*, *Vesicle Transport*, and *Muscle Contraction* (**Figure 2B**). The identification of more than 300 proteins in the *immune system* pathway was especially relevant. Additionally, the enrichment analysis of top-abundant proteins highlighted several subcategories, such as *Neutrophil Degranulation*, *Platelet Activation*, *Signaling and Aggregation*, and *Degradation of the Extracellular Matrix*, among others (**Figure 2C**).

Once the EV-CDC protein cargo was classified, we resorted to a multiplexed quantitative proteomic approach, which offers an extensive dynamic range and great proteome coverage, allowing the simultaneous identification and quantification of hundreds of proteins in the same experiment. This methodology offers an important advantage for the analysis of limited sample amounts (Edwards and Haas, 2016; Jylhä et al., 2018), as in EV-CDC case. In this analysis, protein abundance changes in the IFNγ/EV-CDCs were calculated in relation to the average values of each protein quantified in EV-CDCs (log2-ratio) and expressed in units of standard deviation (Zq). Our results showed that a total of 37 proteins were differentially expressed when EV-CDCs and IFNγ/EV-CDCs were compared ($p \leq 0.05$). Among the significantly increased proteins in IFNγ/EV-CDCs, we identified SLA, PSMB9, Tubulin alpha chain, TUBA1B, CCT5, ASNS, CDH2, SEPT9, COC100625519, IL6, C14orf166, PCNA, SERPINA7, ACAT2, NCL, LIPG, NUDC, PSMA2, PLG, RAB1A,

[5]http://david.abcc.ncifcrf.gov

[6]http://www.exocarta.org/

ACTR2, and BLVRB ($n = 22$). Conversely, among the significantly decreased proteins in IFNγ/EV-CDCs, we identified ALDOC, WDR1, COL14A1, CAPG, SEPT6, PFDN4, PSMA6, TPM3, SORBS1, ARCN1, PUF60, CHORDC1, LOC100519984, CASP3, and PSMB6 ($n = 15$) (**Figure 3A**).

In order to validate these proteomic results, ELISA tests were performed in EV-CDCs and IFNγ/EV-CDCs. Unfortunately, there are few available reagents for swine protein detection, and this study was limited to the validation of IL6 expression in EVs from three pigs by ELISA. According to proteomic analysis, ELISA tests showed an increase in IL6 in IFNγ/EV-CDCs samples: $9.52 \times 10^{-8} \pm 4.68 \times 10^{-8}$ ng/particle in EV-CDCs ($n = 3$) and $1.37 \times 10^{-7} \pm 8.43 \times 10^{-8}$ in IFNγ/EV-CDCs ($n = 3$). It is important to note that the number of samples did not allow a proper statistical analysis, and further validations are required.

The differentially expressed proteins were then classified according to top-level Reactome pathways (**Figure 3B**). Of note, proteins such as PSMB6, ACTR2, PSMA6, TUBA1B, IL6, PSMA2, CASP3, ALDOC, and PSMB9 were classified in the pathway *Immune System* where six of them were found to be increased (PSMB6, ACTR2, TUBA1B, IL6, PSMA2, and PSMB9) and three decreased (PSMA6, CASP3, and ALDOC) in IFNγ/EV-CDCs vs. control EV-CDCs.

Finally, the unsupervised evaluation of proteomic results through principal component analyses (PCA) showed considerable differences between EV-CDCs and IFNγ/EV-CDCs (**Figure 3C**). Additionally, PCA analyses revealed that the distribution of main protein components (PC1 vs. PC2) from the same animal under both treatments behaved similarly, highlighting a distinctive individual EV-CDC proteome background regardless of IFNγ priming (**Figure 3C**, left). Despite individual differences among animals, IFNγ-priming of CDCs caused an important effect on the EV proteome (**Figure 3C**, right).

These proteomic results prompted us to complete the characterization of EV-CDCs and IFNγ/EV-CDCs based on miRNA analysis.

Real-Time Quantification of miRNAs on EV-CDCs and IFNγ/EV-CDCs

The comparative analysis of miRNAs was performed in a selected panel of cardiac-related miRNAs, immune-related miRNAs, and miRNAs associated to EVs from adult/mesenchymal and stem/stromal cells. The evaluation of miRNAs by qPCR revealed that 25 of 44 total evaluated miRNAs were not expressed (or expressed below the detection limit) in EV-CDCs (**Table 1**). Interestingly, mir-23a-3p, mir-191-5p, mir-21-5p, mir-125b-5p, and let-7a-5p were abundantly expressed ($2^{-\Delta Ct} > 4$) in EV-CDCs (**Figure 4**).

Based on the quantification of miRNAs, the top four most abundant miRNAs in EV-CDCs (mir-23a-3p, mir-191-5p, mir-21-5p, and mir-125b-5p) were further analyzed using a *Sus scrofa* database with the miRNet tool. As shown in **Figure 5**, this analysis revealed that the following genes could be targeted by each of these top-abundant miRNAs: *IL6R*, *ADGRG5*, *C1H18orf25*, *NAA25*, *PHF21A*, *MINDY2*, *RAB39B*, *TRIM2*, *MBNL3*, *ENTPD1*, *FBXO28*, and *BMPR2*.

TABLE 1 | Panel of miRNA transcriptomic analysis of extracellular vesicles derived from CDCs (EV-CDCs).

	miRNAs		References
	Expressed in EV-CDCs	**Not expressed in EV-CDCs**	
Cardiac-related miRNAs	mir-101-3p mir-21-5p mir-24-3p	mir-133a-5p mir-15b-5p mir-208b-3p mir-29b-3p mir-29c-3p mir-34a-5p mir-34c-5p mir-92a-3p	Bernardo et al., 2015; Chistiakov et al., 2016; Zhu et al., 2016
Immune-related miRNAs	let-7c-5p let-7d-3p mir-125b-5p mir-127-3p mir-223-3p mir-455-3p	let-7d-3p mir-126-3p mir-126-5p mir-132-3p mir-137-3p mir-139-3p mir-142-5p mir-145-3p mir-150-5p mir-487b-5p	O'Neill et al., 2011; Marques-Rocha et al., 2015
EV-MSCs-related miRNAs	let-7a-5p let-7f-5p mir-100-5p mir-130a-3p mir-191-5p mir-199a-3p mir-23a-3p mir-378a-3p mir-423-3p mir-532-5p	let-7i-3p mir-146a-5p mir-148a-3p mir-148a-5p mir-29a-5p mir-424-5p mir-451a	Eirin et al., 2014; Fernández-Messina et al., 2015; Fafián-Labora et al., 2017; Zhao et al., 2017; Ferguson et al., 2018; Namazi et al., 2018a

A deep bibliographic research was performed to select a list of miRNAs for transcriptomic analysis of EV-CDCs. These miRNAs were classified into different groups: cardiac-related miRNAs, immune-related miRNAs, and miRNAs associated with extracellular vesicles from adult/mesenchymal and stem/stromal cells. Real-time quantitative PCR (qPCR) was carried out to analyze miRNA expression. The table shows miRNA classification according to their expression in EV-CDCs.

The analysis of miRNA expression levels was also used to compare EV-CDCs and IFNγ/EV-CDCs. With this aim, $2^{-\Delta\Delta Ct}$ calculation was performed using EV-CDCs as the "control group." Paired *t*-test analysis demonstrated a significant increase in mir-125b-5p in IFNγ/EV-CDCs. Additionally, this analysis showed an increase (although non-significant) in IFNγ/EV-CDCs of let-7f-5p, mir-223-3p, mir-423-3p, mir-455-3p, mir-532-5p, mir-378a-3p, mir-100-5p, let-7a-5p, and mir-127-3p, together with a non-significant decrease in mir-130a-3p, mir-23a-3p, mir-199a-3p, mir-24-3p, mir-191-5p, let-7c-5p, mir-21-5p, and let-7d-5p, and mir-101-3p (**Figure 6**).

Finally, miRNet analysis was carried out for the differentially expressed mir-125b. This analysis revealed a total of 1,367 interactions with *Sus scrofa* target genes. The 510 genes with more than 150 *experiment scores* were further classified by the webserver g:Profiler in the Reactome pathways (**Figure 7**). This classification showed that 59 targeted genes were categorized in *Metabolism*; 51 targeted genes were categorized in *Immune*

System; 22 targeted genes in *Transport of Small Molecules*; 20 targeted genes in *Cytokine Signaling in Immune System*; 17 targeted genes in *Signaling by Interleukins*, 12 targeted genes in *Extracellular Matrix Organization*; and 8 targeted genes in *Toll-Like Receptor 4 (TLR4) Cascade.*

In vitro Effect of EV-CDCs and IFNγ/EV-CDCs in Lymphocyte Differentiation and Activation

The flow cytometry analysis of PBLs co-cultured with EV-CDCs and IFNγ/EV-CDCs was performed on day 3. Activation/differentiation markers were analyzed on CD4+ and CD8+ T-cell subsets. The first analysis was focused on CD45RA and CD27 expression. The percentages of naïve CD8+ T cells (CD45RA+/CD27+) and naïve CD4+ T cells (CD45RA+) were compared in a paired *t*-test using PBLs without EVs as negative controls. Our results demonstrated that EV-CDCs at 200 μg/ml counteracted the *in vitro* differentiation of CD4+ T cells and CD8+ T cells toward effector/memory cells. The percentages of naïve CD4+ T cells and CD8+ T cells were significantly higher than the negative controls (PBLs without EVs) (**Figures 8A,B**, respectively). Similarly, IFNγ/EV-CDCs partially counteracted the *in vitro* differentiation of CD4+ T cells and CD8+ T cells. However, the differences of percentage of naïve T cells between EV-CDCs and IFNγ/EV-CDCs were not statistically significant (**Figures 8A,B**).

The second analysis was focused on Swine Leukocyte Antigen class II (SLAII) expressed in CD4+ and CD8+ T-cell subsets. The surface expression of MHC class II can be defined as an activation marker on T-cell subsets (Revenfeld et al., 2017). In this study, we quantified the percentages of CD4+ SLAII+ T cells and CD8+ SLAII+ T cells on PBLs co-cultured with EVs. Our results showed a significant decrease in activated CD4+ T cells in PBLs co-cultured with EV-CDCs and IFNγ/EV-CDCs at 200 μg/ml (**Figure 8C**). Additionally, PBLs co-cultured with different EVs showed a decrease in CD8+ SLAII+ T cells (**Figure 8D**), although no significant difference was found when EV-CDCs and IFNγ/EV-CDCs were compared.

DISCUSSION

Large animal models in preclinical research are essential for a successful clinical translation of advanced therapies. Porcine models have been widely used in cardiovascular research to evaluate different administration routes (McCall et al., 2012), stem cell-based therapies (Bolli et al., 2013; Ye et al., 2014; Crisostomo et al., 2015), and to identify biomarkers under controlled experimental conditions (Koudstaal et al., 2014; Blázquez et al., 2018; López et al., 2019). Obviously, the translation of preclinical results to clinical trials is an arduous and challenging process. First, animal models cannot fully represent human disease, where risk factors and comorbidities play important roles. Second, therapeutic products (such as stem cells) are usually different in animal studies and clinical trials. For this reason, an exhaustive analysis of animal-derived therapeutic products is necessary prior to be used in preclinical models.

Nowadays, human-derived EVs from adult stem cells are very well studied; however, animal-derived EVs are poorly characterized, and this characterization is mandatory for a successful translation from animal models to humans. Based on that, the first goal of this study was to perform a deep proteomic and genomic analysis of porcine-derived EV-CDCs. Additionally, we hypothesized that priming *in vitro* cultured CDCs with inflammatory stimuli (such as IFNγ) may increase the therapeutic potential (immunomodulatory and/or pro-regenerative) of released vesicles. This hypothesis is based on previous studies in which primed MSCs with hypoxia, serum deprivation, or inflammatory cytokines produced soluble factors with immunomodulatory and pro-angiogenic properties (Oh et al., 2008; DelaRosa et al., 2009; Di Trapani et al., 2016; Song et al., 2017; Ragni et al., 2019; Showalter et al., 2019).

Our first set of results has demonstrated that *in vitro* culture conditions for CDCs and vesicle isolations were optimal and provided a satisfactory enrichment of EV-CDCs. The nano flow-cytometry analyses led us to demonstrate that our EV-CDC preparations were enriched in small EVs (ranging from 40 to 200 nm). Of note, high-sensitivity nano-flow cytometry has recently demonstrated to be comparable to electron microscopy, notably reducing costs, sample preparation time, and increasing statistical power of analysis (Tian et al., 2018). In our case, although the isolation protocols demonstrated significant enrichment of *extracellular exosome* proteins, this methodology did not exclude the co-purification of extracellular matrix proteins, such as collagens, and other proteins, such as Vinculin, Filamin A, or Fibronectin 1.

The enrichment analysis of top-abundant protein ($Np \geq 20$) in EV-CDCs by Reactome revealed an over-representation in three top-level categories: *Immune System*, *Homeostasis*, and *Muscle Contraction.* This enrichment analysis highlights the hypothetical involvement of proteins and clusters of proteins in the therapeutic effect of these vesicles. For example, HSP90 (classified in *Immune System*) has been found to be involved in the modulation of cardiac ventricular hypertrophy (Tamura et al., 2019), and SPARC (classified in *Hemostasis*) has been found to be involved in the improvement of cardiac function after myocardial infarction (Deckx et al., 2019).

This study was also focused on the comparative analysis between EVs released from *in vitro* cultured CDCs and EVs from IFNγ-primed CDCs. NanoFCM results did not show significant differences between IFNγ-primed and control EV-CDCs in terms of size profile, nor particle concentration (**Figure 1**). The idea of inflammatory priming to increase the immunomodulatory effect of cells has been first described to generate anti-inflammatory cells and, more recently, to generate anti-inflammatory vesicles (Di Trapani et al., 2016; Song et al., 2017; Showalter et al., 2019). Our matched-paired comparative analysis revealed significant differences in 37 proteins and, although many of these proteins may deserve a proper discussion, we focused our interest in some of the proteins categorized in the *Immune System* pathway by Reactome.

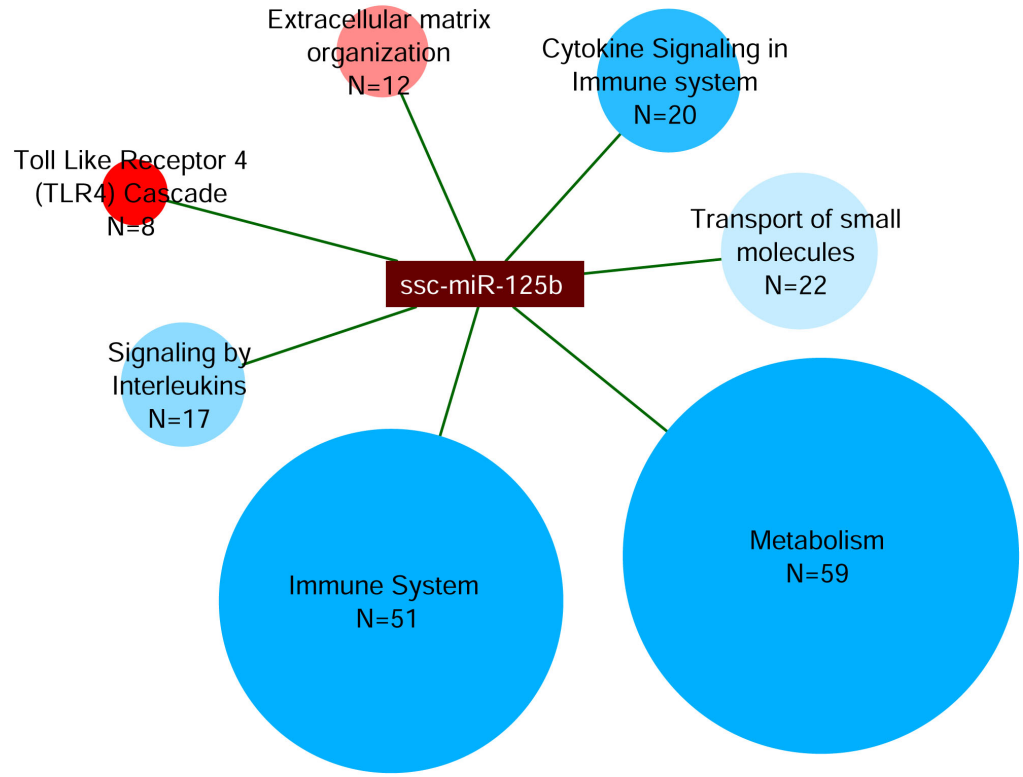


FIGURE 7 | Analysis of miRNA target network of mir-125b. The miRNet analysis was performed on the up-regulated mir-125b in IFNγ/EV-CDCs vs. EV-CDCs. Targeted genes were classified by g:Profiler in Reactome pathways. The network shows the interactions between mir-125b and the Reactome terms (ellipses). Ellipsis size represents the number of proteins in each category (N), while their color represents the significance level of each category, from less (blue) to more statistically significant (red).

Our results showed differential expressions in four different proteasome subunits: PSMA2, PSMB9 (increased), PSMB6, and PSMA6 (decreased). It is well known that IFNγ and other pro-inflammatory signals are involved in the formation of immunoproteasome, which is derived from the constitutive proteasome (Strehl et al., 2005). Moreover, the presence of co-purified proteasome subunits has been described in exosomes derived from MSCs, and the authors suggested that these proteasome subunits "*could synergize with other constituents to ameliorate tissue damage*" (Lai et al., 2012).

Apart from proteasome subunits, the comparative analysis showed a significant increase in ACTR2 (actin-related protein 2) in IFNγ/EV-CDCs. This protein is also classified in the *Immune System* pathway (*R-SSC-168256*) and is a key component of the Arp2/3 complex, which is involved in actin polymerization (Suetsugu and Takenawa, 2003). According to Exocarta (Mathivanan and Simpson, 2009), this protein was previously described in very different tissues and cell types. Moreover, Reactome pathway analysis demonstrated that this protein is functionally classified in very different pathways, such as *EPHB-Mediated Forward Signaling*, *Regulation of Actin Dynamics for Phagocytic Cup Formation*, and *RHO GTPases Activate WASPs and WAVEs* (Mathivanan and Simpson, 2009). Unfortunately, it is difficult to elucidate the consequences of this change, and the functional relevance of this protein requires further investigations.

Among the significantly increased proteins in IFNγ/EV-CDCs classified in the *Immune System* pathway, the increase in IL6 is especially relevant. The biological role of this cytokine has always been contradictory. On the one hand, IL6 has been considered as a pro-inflammatory cytokine, participating in the development of coronary heart disease, obesity or diabetes (Fuster and Walsh, 2014). In contrast, it has been associated with the alternative activation of macrophages (Mauer et al., 2014) and with an atheroprotective effect against inflammatory vascular disorders (Elhage et al., 2001; Schieffer et al., 2004). Additionally, it was defined as a "myokine" with anti-inflammatory effects during exercise (Pedersen, 2006). In the context of inflammatory-primed MSCs, IL6 secretion has been considered as an anti-inflammatory molecule (Xing et al., 1998). So, here, we hypothesize that the presence of IL6 in EV-CDCs, as well as the increase in IFNγ/EV-CDCs, may have a therapeutic effect in the control of acute inflammatory responses in myocardial infarction. However, we should also keep in mind that many different studies have experimentally demonstrated that IL6R signaling has an adverse effect and a key role in the development of stroke (IL6R Genetics Consortium Emerging Risk Factors Collaboration et al., 2012; Schuett et al., 2012).

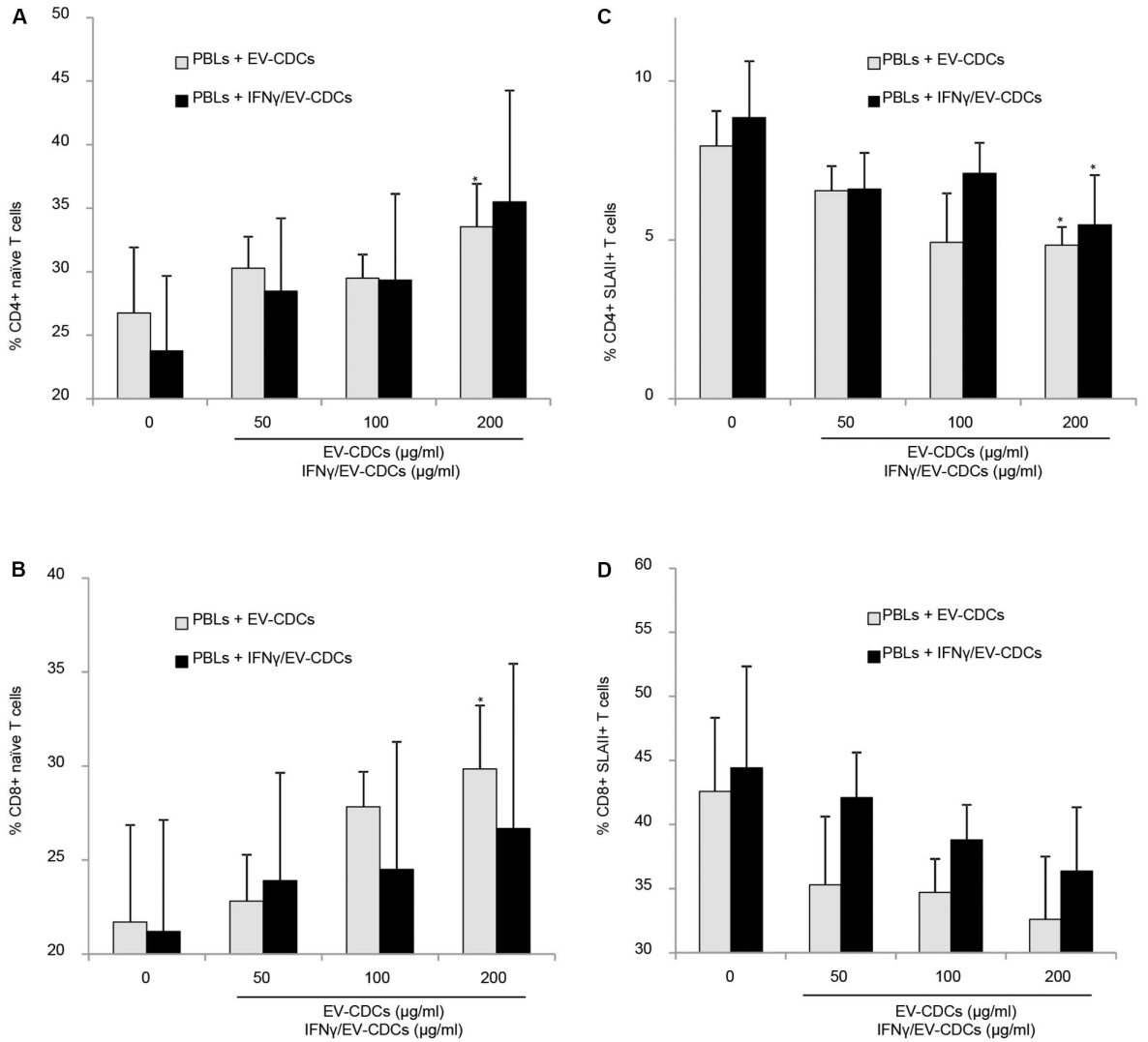


FIGURE 8 | *In vitro* peripheral blood lymphocyte (PBL) activation and differentiation assays in co-culture with EV-CDCs and IFNγ/EV-CDCs. Peripheral blood lymphocytes (PBLs) were isolated from blood samples by density gradient and co-cultured with 0 (control), 50, 100, and 200 µg/ml of EV-CDC (gray bars) and IFNγ/EV-CDC (black bars) protein for 3 days. Lymphocyte activation/differentiation was analyzed by flow cytometry on CD4+ **(A,C)** and CD8+ T-cell subpopulations **(B,D)**, using naïve T-cell markers (CD45RA+, CD27+) and activation marker (SLAII). Paired *t*-test was used to compare doses of EVs to negative controls (*$p < 0.05$).

In short, the proteomic analysis of EV-CDCs and IFNγ/EV-CDCs showed a myriad of different proteins with different functions. The bioinformatic and biostatistical analyses in top-abundant proteins (Np $\geq$ 20) suggest the contribution of clusters of proteins in immune-related and cardiac-related process. Once the proteomic profile of vesicles was identified, and considering that the therapeutic effect of exosomes from CDCs is also mediated by miRNAs (Namazi et al., 2018b), here, we performed a quantitative and comparative analysis in a panel of miRNAs. This panel was selected for their association with cardiac regeneration, immune response, and expression in EVs.

In this analysis, mir-23a-3p, mir-191-5p, mir-21-5p, mir-125b-5p, and let-7a-5p were identified as the top-abundant miRNAs in EV-CDCs. Although further experimental validations are needed to assess the impact of these miRNAs, the *in silico* analysis by miRNet revealed that *IL6R* is a target gene of the four top-abundant miRNAs.

The comparative analysis of miRNAs in EV-CDCs and IFNγ/EV-CDCs showed a statistically significant difference in the expression of mir-125b-5p. Previous studies demonstrated that the presence of this miRNA is positively correlated with circulating inflammatory cytokines in patients with chronic obstructive pulmonary disease (Hu et al., 2017). This may explain, at least in part, the increased release of mir-125b-5p under IFNγ stimuli. Additionally, mir-125b has been described as a cardioprotective miRNA that participates in cardiac regeneration after myocardial infarction (Wang et al., 2014), having a key role in the regulation of cardiomyocyte survival during acute myocardial

infarction (Bayoumi et al., 2018). So, the local administration of IFNγ-primed vesicles may have increased therapeutic potential in infarcted patients.

The target network for mir-125b-5p was finally analyzed by miRNet. This analysis identified 1,367 miRNA–gene target interactions. Although this *in silico* study needs to be corroborated by functional studies, the 510 genes with the highest *experiment score* (>150) were categorized by Reactome. Most of the targeted genes were classified in the terms of *Metabolism* and *Immune System*.

Our *in silico* analysis also suggested the hypothetical involvement of the four top-abundant miRNAs in EV-CDCs, including mir-125b-5p (differentially expressed between EV-CDCs and IFNγ/EV-CDCs), in the regulation of the *IL6R* gene. According to the Interleukin-6 Receptor Mendelian Randomisation Consortium, *IL6R* has been considered a target for coronary heart disease: "*IL6R blockade could provide a novel therapeutic approach to prevention of coronary heart disease.*" Nowadays, monoclonal antibodies against IL6R, such as tocilizumab, have been considered as a therapeutic strategy for prevention of coronary heart disease (Carroll, 2018). Based on that, the optimization of *in vitro* culture conditions for EV-CDC isolation may have a therapeutic relevance for targeting IL6R and subsequently in inflammatory-mediated diseases. Moreover, it would be interesting to analyze additional miRNA targeting *IL6R* in EV-CDCs (i.e., mir-34b-3p, mir-124-3p).

Our proteomic and genomic analysis was finally completed with an *in vitro* study to determine the immunomodulatory effect of EV-CDCs and IFNγ/EV-CDCs on lymphocyte subsets. Owing to the limited availability of reagents for this animal model, this study was focused on CD4+ and CD8+ T-cell subsets. The *in vitro* differentiation and activation markers were analyzed in PBLs co-cultured with EV-CDCs and IFNγ/EV-CDCs. Our results demonstrated that EV-CDCs counteracted the *in vitro* differentiation of CD4+ and CD8+ T-cells toward an effector memory phenotype and reduced the expression of activation markers. This result agrees with our previous studies using EVs derived from endometrial stem cells (Álvarez et al., 2018; Marinaro et al., 2019). In fact, similar to Álvarez et al. (2018) here, we could also assert that EV-CDCs have an "*inhibitory effect against CD4+ T cell activation.*" It is important to note that, under these experimental conditions, paired *t*-test did not reveal significant differences between control EV-CDCs and IFNγ/EV-CDCs.

In summary, here, we demonstrate that *in vitro* cultured CDCs release vesicles that are enriched in immune-related proteins. On the one hand, the content of these EV-CDCs can modify *in vitro* the immunomodulatory status of PBLs. Within the protein content, the abundance and the increased expression of IL6 in IFNγ/EV-CDCs are especially relevant. According to preclinical models, which demonstrated an IL6-dependent M2b polarization using IFNγ pre-conditioned MSCs (Philipp et al., 2018), here, we hypothesize that IL6 expression in EV-CDCs may have a key role in the regulation of macrophage and/or neutrophil polarization. On the other hand, miRNA analyses pinpoint the abundance and the differential expression of mir-125b-5p, which target genes involved in the *Immune System* process, including *IL6R*. Altogether, the proteomic and genomic results point out the hypothetical involvement of these vesicles in the regulation of IL6/IL6R axis and, subsequently, in inflammatory-mediated diseases.

DATA AVAILABILITY STATEMENT

The mass spectrometry proteomics data can be found in ProteomeXchange (http://www.proteomexchange.org/) with identifier PXD016434.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Welfare and Ethics Committee of the Jesús Usón Minimally Invasive Surgery Centre, in accordance with the recommendations outlined by the local government (Junta de Extremadura), and the EU Directive 2010/63/EU of the European Parliament on the protection of animals used for scientific purposes.

AUTHOR CONTRIBUTIONS

EL, FM, MP, MG-S, FS-M, IJ, and JC conceived and designed the experiments. EL, FM, MP, MG-S, IJ, JV, VÁ, VC, LF-P, VP, EP, and JC performed the experiments and analyzed the data. EL, FM, MG-S, IJ, and JC wrote the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcell.2020.00321/full#supplementary-material

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Cortical Bone Derived Stem Cells Modulate Cardiac Fibroblast Response via miR-18a in the Heart After Injury

Lindsay Kraus[1†], Lena Ma[1†], Yijun Yang[1], Faustina Nguyen[1], Robert C. Hoy[2], Tomoko Okuno[1], Mohsin Khan[2] and Sadia Mohsin[1]*

[1] Independence Blue Cross Cardiovascular Research Center, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, United States, [2] Center for Metabolic Disease, Lewis Katz School of Medicine, Temple University, Philadelphia, PA, United States

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Charles K. Thodeti,
Northeast Ohio Medical University, United States

***Correspondence:**
Sadia Mohsin
sadia.mohsin@temple.edu

[†] These authors have contributed equally to this work

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The adult heart following injury such as a myocardial infarction forms a fibrotic scar associated with transformation of resident cardiac fibroblasts into myofibroblast, accelerating cardiac remodeling and dysfunction. Cell therapies provide a novel direction for the enhancement of cardiac structure and function but remain poorly described in terms of the effect on resident cardiac fibroblasts. We have shown cortical bone derived stem cells (CBSCs) exhibit an ability to repair the heart after myocardial injury together with reduced scar formation. Nevertheless, whether CBSCs possess ability to modulate resident fibroblast response after myocardial injury remains untested.

Objective: To determine the effect of secreted factors from CSBCs to attenuate myofibroblast formation in the heart after injury.

Methods and Results: CBSCs were injected in mice after myocardial infarction which demonstrated reduced fibrosis as determined by Masson's trichrome and Picro-Sirius red staining. In parallel, decreased expression of myofibroblast markers such as Acta2 was observed compared to PBS injected mice. To determine the effect of CBSCs on cardiac fibrosis, adult mouse cardiac fibroblasts were isolated from C57BL/6 mice, primed with CBSC pre-conditioned media for 12 h, and treated with 10ng TGF-β for 48 h to mimic cardiac injury. Decreased expression of Acta2, periostin and CTGF was observed in adult cardiac fibroblasts cultured in CBSC medium compared to control cells. Additionally, analysis of myofibroblast markers such as vimentin and pSMAD/SMAD was also decreased compared to control cells. To determine the mechanism, we looked for enriched miRNA in CBSCs that can mediate anti fibrotic response after injury. Results showed significantly increased expression of miR-18a in CBSCs. The upregulation of miR-18a was also validated in adult fibroblasts treated with CBSCs compared to control cells. Adult fibroblasts treated with mimic for miR-18a followed by TGF-β showed significant decrease in myofibroblast formation while miR-18a inhibitor completely inhibited the effect of CBSC medium.

Conclusion: CBSCs reduce fibroblast to myofibroblast transition and differentiation in adult cardiac fibroblasts via miR-18a-5p. This finding reveals a new avenue for cell therapies to target myocardial scar modulation and provides a resolution for the cardiac repair response after injury in the adult myocardium.

Keywords: stem cells, cardiac fibrosis, myocardial infarction, fibroblasts, miR-18a

Nomenclature: ACF, adult cardiac fibroblasts; Acta2, alpha smooth muscle actin; CBSC, cortical bone stem cells; CF, cardiac fibroblast; Col1a1, collagen type 1 alpha 1; CTGF, connective tissue growth factor; ECM, extracellular matrix; FMT, fibroblast-myofibroblast transition; HBSS, Hank's balanced salt solution; LAD, left anterior descending artery; LV, left ventricular; MF, myofibroblast; MI, myocardial infarction; PBS, phosphate buffered saline; TGF, transforming growth factor beta.

INTRODUCTION

Cardiac fibrosis is an outcome of most cardiac injuries promoting stiffness in the heart by excessive accumulation of the extracellular matrix (ECM). Deposition of increased ECM results in cardiac remodeling and scar formation leading to compromised cardiac contractility after injury (Berk et al., 2007; Kong et al., 2014). Since the myocardium is comprised of about 70% nonmyocytes with the majority including fibroblasts (Banerjee et al., 2007) it is therefore crucial to study pathways that can promote pro-reparative responses in fibroblasts to enhance repair after cardiac ischemic event. Cell-based therapies have recently provided a new direction for enhancement of cardiac structure and function after myocardial injury (Laflamme et al., 2005; Schachinger et al., 2006; Tang et al., 2010; Makkar et al., 2012; Hong S. J. et al., 2014; Karantalis et al., 2014). Transplanted stem cells can promote cardiac repair in the heart after injury by release of paracrine factors at the site of injury (Gnecchi et al., 2006, 2008; Duran et al., 2013; Hodgkinson et al., 2016). Studies have shown that stem cell derived paracrine factors promote cardioprotection (Sharp et al., 2017), myocyte cell cycle (Khan et al., 2015), and angiogenesis (Hatzistergos et al., 2010; Khan et al., 2015). Nevertheless, the effect of paracrine factors on the ability to modulate cardiac fibrosis and myofibroblast formation in the heart after injury remains poorly characterized.

Several studies recently have shown the salutary effects of multiple stem cell secretome on cardiac structure and function (Ibrahim et al., 2014; Gallina et al., 2015). Nevertheless, the main question is the selection of the optimal stem cell type for cardiac wound healing. We have recently shown that Cortical Bone-Derived Stem Cells (CBSCs) isolated and cultured from the cortical bone, preserved left ventricular (LV) volumes and ejection fraction in the hearts, as shown by echocardiogram in small (Duran et al., 2013) and large animal (Sharp et al., 2017) heart failure models. While these studies showed cardioprotective effects of CBSCs in vivo, the underlying mechanism of this effect is not yet known. One of the most significant observations during the animal studies with CBSCs were reduced fibrotic scar size after treatment with CBSCs. We hypothesized that CBSCs secretome possesses the ability to augment pro-reparative changes by suppressing the fibrotic response after injury which would explain the beneficial effects seen in the transplanted animals in previous studies. Since miRNAs represents one of the major components in the secretome that can ultimately affect cellular processes (Chiang et al., 2019), such as protection, survival and proliferation of cardiac cells (Borden et al., 2019), we looked for enriched miRNAs in CBSC the secretome with potential cardioprotective and anti-fibrotic effects. We observed expression of miR-18a-5p upregulated in animals after CBSC treatment.

In the article, we identify a novel role for CBSC secreted factors in modulating cardiac scar and fibrosis. Moreover, CBSCs attenuate myofibroblast formation via secretion of miR-18a-5p that represses CTGF mediated myofibroblast formation in the heart after injury. Our data suggests, CBSC secretome to possess potent scar remodeling and wound healing abilities providing a possible mechanism for the salutary effects of CBSC therapy for myocardial injury.

MATERIALS AND METHODS

Primary Adult Cardiac Fibroblast Isolation and Treatment

Adult cardiac fibroblasts (ACFs) were isolated from the ventricular myocardium of 8–12-week-old adult C57/B6 mice. Briefly, ventricles were cut and washed with sterile 1X HBSS, and cut into fine pieces in digestion buffer (HBSS buffer, 100 U/mL of collagenase II, 2.5% trypsin) and transferred to a 50 mL sterile Falcon tube for digestion. The supernatant from digested tissue was then centrifuged, and pellets were combined and cultured in adult cardiac fibroblast media consisting of filtered DMEM/F12, 10%FBS, 100 U/mL pen/strep, 20 mM L-glutamine, and 0.1 mM 2-mercaptoethnanol, on collaged coated plates for 3 days until confluency. Cells were plated at 50,000 cells at passage 1 for 24 h in DMEM supplemented with 20% FBS on collagen coated plates and primed with CBSC pre-conditioned media (500,000 cells plated for 48 h) for 12 h and stimulated with 10 ng TGF-β for 48 h before harvesting.

Cortical Bone Derived Stem Cell Culture and Characterization

Cortical bone derived stem cells are isolated from tibias and femurs of C57BL/6 mice and characterized as described previously (Duran et al., 2013; Mohsin et al., 2015). Briefly, tibias and femurs are flushed to get rid of all the bone marrow and then

digested in collagenase at 37°C. The digested cells are washed and plated in CBSC media till colonies of CBSCs appear. CBSC media consists of DMEM-F12 mix supplemented with; 10% Embryonic Stem Cell Qualified FBS, 10 ml/L Pen/Strep/Glytamine (100× stock), 10 μL/L Leukemia Inhibitory Factor (1 mL/107 units), 10 mL/L ITS (100×), 40 ng/mL EGF, 20 ng/mL bFGF. The CBSCs are used from passage 12–16. The cells characterized for CBSC markers including CD44, CD105, CD106, Sca-1 and negative for CD45and then expanded for experiments.

Quantitative Real-Time PCR and RT2 Profiler PCR Arrays

Fibroblasts and transplanted hearts were tested for expression of fibrotic genes by using RT2 profiler PCR arrays (Qiagen). Briefly, RNA was isolated from cells using the miRNeasy Kit (Qiagen) according to manufacturer's protocol. Single-stranded cDNA was synthesized from all samples using the RT2 First Strand Kit (Qiagen) as described in the Qiagen protocol for RT2 profiler array sample preparation on an ABI stepOneplus system (Applied Biosystems). The primer sets used during the study is listed is in **Table 1**.

Western Blot

Western Blot analysis was performed as described previously (Borden et al., 2019). Briefly, sample concentrations were determined using the Bicinchoninic assay (BCA) according to manufacturer's protocol and ran on a Mini-PROTEAN TGX Gels (Bio-rad). Primary antibodies against Acta2 (1:1000, rabbit polyclonal, Abcam, catalog ab5694), GAPDH (1:1000, mouse monoclonal, Millipore Sigma, catalog MAB374), CTGF (1:000, rabbit polyclonal, Abcam, catalog ab6992), P-SMAD2 (1:1000, rabbit polyclonal, Millipore Sigma, catalog ZRB04953), SMAD2 (1:1000, rabbit polyclonal, Abcam, catalog EP784Y), and Vimentin (1:1000, mouse monoclonal, Abcam, catalog ab8978) overnight at 4°C, and incubated with the appropriately conjugated light-sensitive IRDye secondary antibodies (1:5000, LiCOR) for 1 h at room temperature, and visualized.

Immunocytochemistry

Cells were prepared for immunocytochemistry as described earlier (Mohsin et al., 2015). Briefly, cells were fixed on tissue-chamber slides with 4% paraformaldehyde and washed with PBS and 0.1% Triton X-100 and blocked for 30 min with 0.5% Horse serum. Cells were then incubated with rabbit anti-Acta2 (1:100, rabbit polyclonal, Abcam, catalog ab5694) and DAPI (0.1 μg/mL in PBS) (EMD Millipore, catalog 268298-10MG) (light-sensitive) for 10 min at room temperature and mounted on slides for imaging.

TABLE 1 | List of primers.

Primer set	Forward	Reverse
Acta2	ACTCTCTTCCAGCCATCTTTC	GCTGTTATAGGTGGTTTCGTGG
Vimentin	TTCAAGAACACCCGCAGGAA	TTGGCAAAGCGGTCATTCAG
Collagen 1	AAGGAGACACTGGTGCCAAA	GGACCTTGAACTCCAGTAGC
Periostin	TGGAAGGGATGAAAGGCTGC	CCCAGCGTGCCATAAACATG
CTFG	CCCTCGCGGCTTACCGACTGG	CACAGG TCTTGGA ACAGGCGC
SMAD2	ACTAACTTCCCAGCAGGAAT	GTTGGTCACTTGTTTCTCC
18s	GGTCTTCGTCGGTAGGCATC	ACACCGACACGAGAGAGAGA
GAPDH	CATGGCCTTCCGTGTTCCTA	TACTTGGCAGGTTTCTCCAGG

Induction of Acute MI and Fibrosis Detection

Animals (C57BL/6 8–12 weeks old male mice) n = 30 were divided into two groups PBS and CBSC treated). All surgical procedures and animal care protocols were approved by the Temple University Animal Care and Use Committee. Animals underwent myocardial infarction procedure by permanent ligation of the left anterior descending artery (LAD) as described previously (Duran et al., 2013). 100,000 CBSCs were injected in the border zone area at the time of infarction. The animals were sacrificed 2 weeks after MI for Masson Trichome and Picro-Sirius red staining following manufacturer's protocol. Briefly, formalin-fixed heart tissues were routinely processed, embedded in paraffin, and sectioned for histochemistry. Masson's Trichrome staining was performed with Trichrome stain kit (HT 15-1 KT, Sigma-Aldrich) and Weigert's Iron Hematoxylin (HT1079-1SET Sigma Aldrich). Picro-Sirius red staining was performed with the kit (ab150681, Abcam) briefly following the steps of section deparaffinization, incubation with staining solution and dehydration. Pictures were taken with light microscopy. Fibrosis and non-fibrosis areas were calculated with 'color threshold' tool from ImageJ software (version 1.49v; National Institutes of Health).

microRNA Treatments

Adult cardiac fibroblasts were transiently transfected with 50 nM miR-18a-5p mimic (Thermo Fisher Scientific, catalog 4464066), miR-18a-5p inhibitor (Thermo Fisher Scientific, catalog MH12973), or negative control (Thermo Scientific, catalog 4464058) using Invitrogen Lipofectamine 3000 (Thermo Fisher Scientific, catalog L3000015) in serum free Gibco Opti-MEM media (Thermo Fisher Scientific, catalog 31985062) according to the manufacturer's recommendations. After 24 h post transfections, cells were used for protein and RNA analysis or stimulated with 10ng TGF-β for 48 h before harvesting.

Luciferase Assay

Adult cardiac fibroblast (30,000 cells on 12 well plate) were plated and the following day transiently transfected with Lipofectamine 3000 (Thermo Fisher Scientific, catalog L3000015) and CTGF miRNA (GeneCopoeia) in Gibco Opti-MEM media (Thermo Fisher Scientific). Cells were treated with 50 nM miR-18a-5p mimic or inhibitor as described previously. The luciferase assay was performed using the GeneCopoeia Luc-Pair Duo-Luciferase Assay Kit 2.0 (GeneCopoeia). Cells were prepared and washed according to the manufacturer's recommendations. The ratio of luminescence was measured on luminometer.

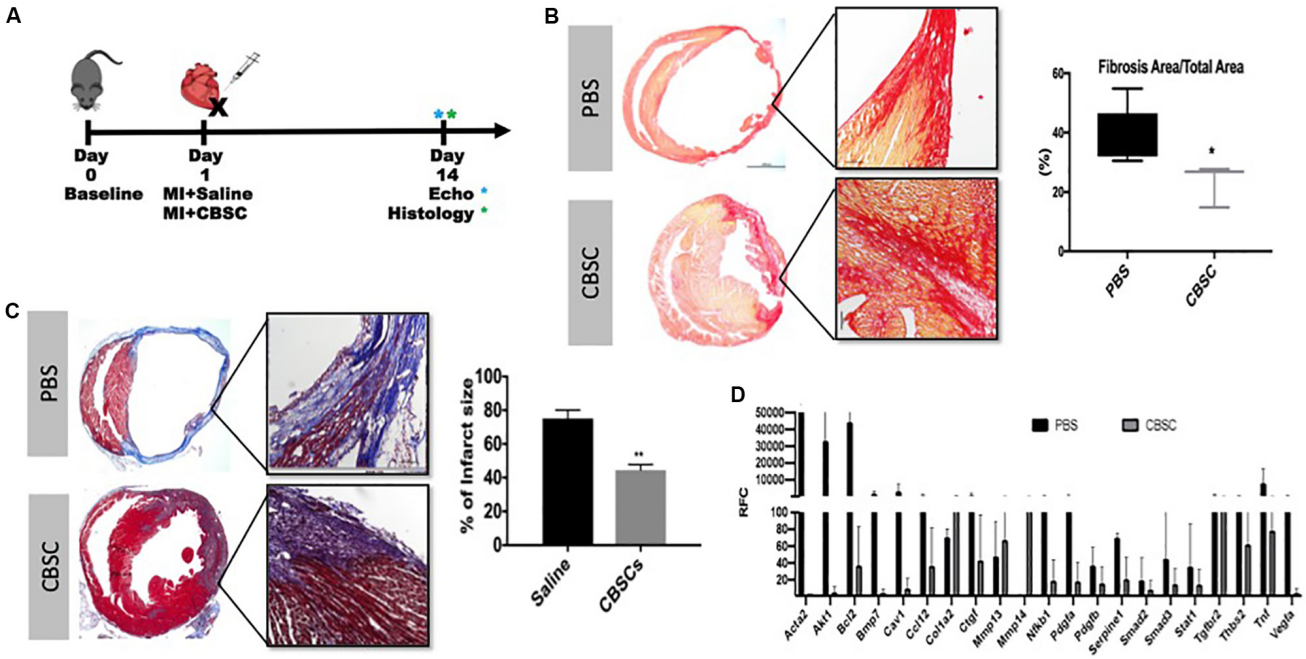

FIGURE 1 | CBSCs treatment restrict fibrotic scar after MI. **(A)** Outline of experimental design using an adult mouse heart given a myocardial infarction (MI) and treated with CBSC or Saline injection with measurements taken 3, 7, and 14 days after injection. **(B)** Fibrosis was decreased in hearts injected with CBSCs compared to PBS using Picrosirius Red stain for fibrosis and quantified based on the amount of fibrosis relative to the total area 14 days after injection, $n = 5$, $^{*}P < 0.05$. **(C)** A decrease in percent of infarct size in heart injected with CBSCs compared to PBS hearts using Masson's trichrome staining and quantified based on the percentage of infarct size 14 days after injection, $n = 5$, $^{**}P < 0.01$. **(D)** A fibrotic screen of a MI heart treated with PBS or CBSCs showing lower myofibroblast marker expression in CBSC treated hearts compared to PBS treatment. Black bars indicating the control PBS or Saline treatment with adult cardiac fibroblasts, gray bars indicating CBSC treatment with adult cardiac fibroblasts.

Animal Housing and Husbandry Care

All animals are housed within our AAALAC accredited animal facility. The facility is staffed with veterinary technicians and husbandry staff. The mice are housed 4–5 mice/cage. All animals are purchased through Jackson Laboratories. Each suite contains housing rooms and small animal surgical wards to perform necessary testing in a centralized, controlled environment to minimize animal stress. The animals are provided with comprehensive preventative medicine and veterinary care programs including daily observation of animals (weekends and holidays) by husbandry staffing, veterinary technicians, and on-site veterinarians. The primary method of euthanasia that will be used is CO_2 inhalation. Euthanasia procedures will be conducted in accordance to the recommendations published by the Panel of Euthanasia of the American Veterinary Medical Association.

Statistical Analysis

Data are represented as mean ± SD. Two-sided testing was used for all statistical tests. *P* values of ≤0.05 was used to determine significance for all statistical tests. Comparisons for data with a single measurement were performed using the unpaired *t*-test. Analysis was performed by One-or two way ANOVA (analysis of variance), followed by Tukey's multiple comparison test using the GraphPad Prism software (GraphPad Inc., La Jolla, CA, United States).

RESULTS

CBSCs Treatment Restrict Fibrotic Scar After MI

Animals treated with CBSCs after myocardial infarction (experimental design illustrated in **Figure 1A**) showed reduction in scar size measured by Masson's Trichrome staining and Picro-Sirius red staining after treatment (**Figures 1B,C**). The quantitative analysis showed 1.6-fold decrease in fibrotic scar versus saline treated animals ($p < 0.05$) by the histological analysis. Concurrent with reduced scar size, we showed expression of fibroblast and myofibroblast markers including Acta2, CTGF, SMAD2 and SMAD 3 are down regulated compared to Saline treated group (**Figure 1D** and **Supplementary Figure 1**). These findings conclude that CBSCs treatment helps with restriction of fibrotic scars explaining the functional benefits observed in the earlier studies.

CBSC Secretome Reduces Myofibroblast Markers in Adult Cardiac Fibroblasts

Adult cardiac fibroblasts were cultured as demonstrated in **Supplementary Figure 1**. The purity of our adult cardiac fibroblast cultures and their transition to myofibroblasts was confirmed by staining with Acta2 after TGF-β stimulation. As anticipated stress fibers were formed and stained with Acta2

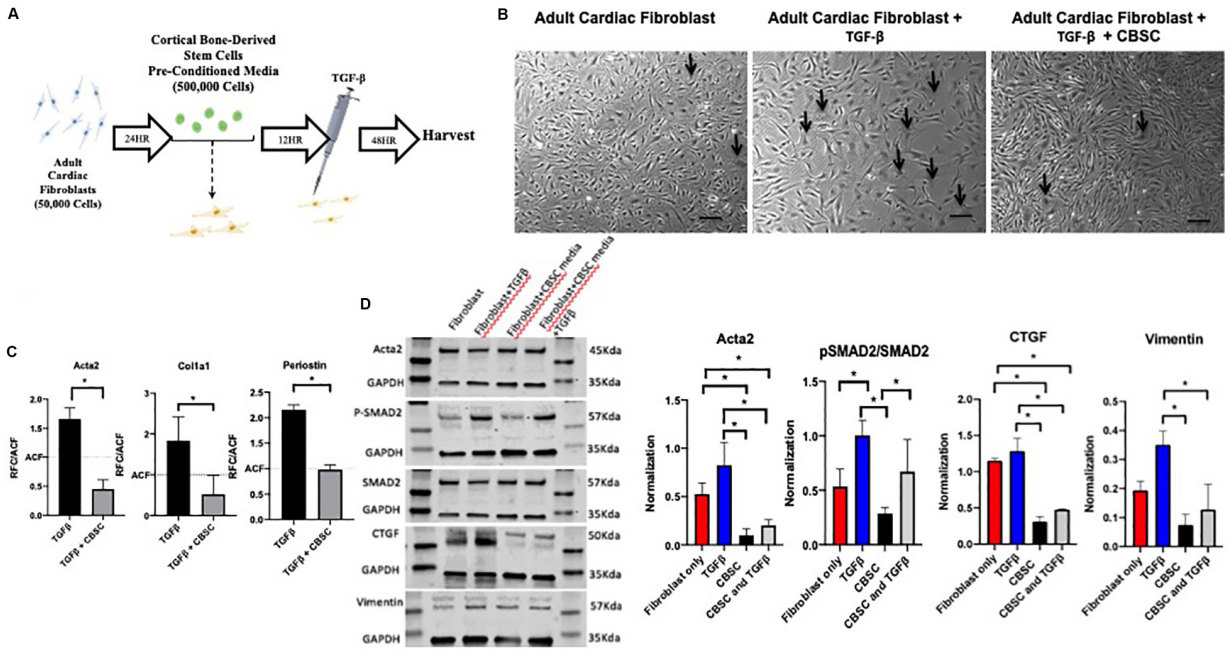

FIGURE 2 | CBSC secretome reduces myofibroblast markers in adult cardiac fibroblasts. **(A)** Outline of experimental design showing adult cardiac fibroblasts isolation **(B)** Representative image of untreated fibroblasts that have a (spindle-like shaped), fibroblast treated with TGFβ (flattened and broad) indicating myofibroblast transition. The fibroblast reverts their morphology back to untreated fibroblasts after CBSC treatment scale bar represents 20 μM. **(C)** Decreased myofibroblast marker expression is observed in fibroblast treated with CBSCs and TGFβ measured by quantitative reverse transcription polymerase chain reaction analysis, $n = 3$, $^*P < 0.05$. **(D)** Decreased myofibroblast and TGFβ pathway maker expression are observed in fibroblasts treated with CBSCs and CBSCs and TGFβ using Western blot analysis, normalized to GAPDH, $^*P < 0.05$.

indicating myofibroblast transition (**Supplementary Figure 3**). Concurrently, markers including Acta2, Periostin and Col1a was significantly increased after TGF-β treatment as measured by RT-PCR analysis (**Supplementary Figure 3**). We wanted to confirm whether CBSCs secretome is inhibiting fibroblasts to myofibroblasts transition, therefore, we treated adult cardiac fibroblasts with CBSC pre-conditioned media for 12 h, followed by stimulation with 10 ng TGF-β for 48 h (**Figure 2A**). Untreated fibroblasts have a spindle-like shape cells as confirmed by a light microscopy which is significantly changed to flattened broad cells after introduction of TGF-β stimulation. However, we see a reversal of morphology of stimulated fibroblasts after pretreatment with CBSCs (**Figure 2B**). These findings were further confirmed by analyzing the stimulated and CBSC treated adult cardiac fibroblast for the myofibroblast markers using RT PCR analysis. There was a significant reduction in the gene expression of myofibroblast markers including Acta2, Cola1a1 and Periostin ($p < 0.05$) (**Figure 2C**). Simultaneously, there was a decrease protein expression of Acta2 (3-fold with $p < 0.05$) and CTGF (3.6-fold with $p < 0.05$) in adult cardiac fibroblast versus CBSCs treated fibroblasts after TGF-β stimulation. Additionally, expression of the ratio between PSMAD2 to SMAD2 (1.66-fold) and Vimentin (3-fold) also showed significant decrease after CBSC treatment alone and after TGF-β stimulation in adult cardiac fibroblasts (**Figure 2D**), indicating a decrease in TGF-β signaling pathway with CBSC treatment. These findings clearly demonstrate the role of CBSC secretome in reducing fibrotic genes/proteins, suggesting the CBSC secretome's role in suppressing fibrogenic response after injury.

miR-18a-5p Prevents Adult Cardiac Fibroblast Differentiation

After confirming that CBSC secretome significantly reduce the fibrotic response after injury in vivo and in vitro using adult cardiac fibroblast, we wanted to look for the potential mechanism. Since stem cell secretome represents a large fraction of miR's we screened the hearts treated with CBSCs for miRNAs with roles in the fibrotic response. Expression of mir-18a-5p was 3.25-fold higher in hearts treated CBSC versus saline treated hearts after MI (**Figure 3A**). We additionally confirmed the increased expression of miR-18a-5p in fibroblasts treated with CBSC pre-conditioned media after TGF-β stimulation (**Figure 3B**). When we screened for highly expressed miRNA's in cultured CBSCs, that can mediate an anti-fibrotic response we identified miR-18a-5p (**Supplementary Figure 4A**). Additionally, we also confirmed expression of miR-18a-5p physiological levels in mouse hearts, the expression goes down 6-fold from 2 days versus 2 weeks after birth (**Supplementary Figure 4B**). Similarly, in an isolated cardiac fibroblast the expression of miR18a-6p was significantly reduced (2-fold) in fibroblasts isolated from neonatal versus adult heart (**Supplementary Figure 4C**). To further validate

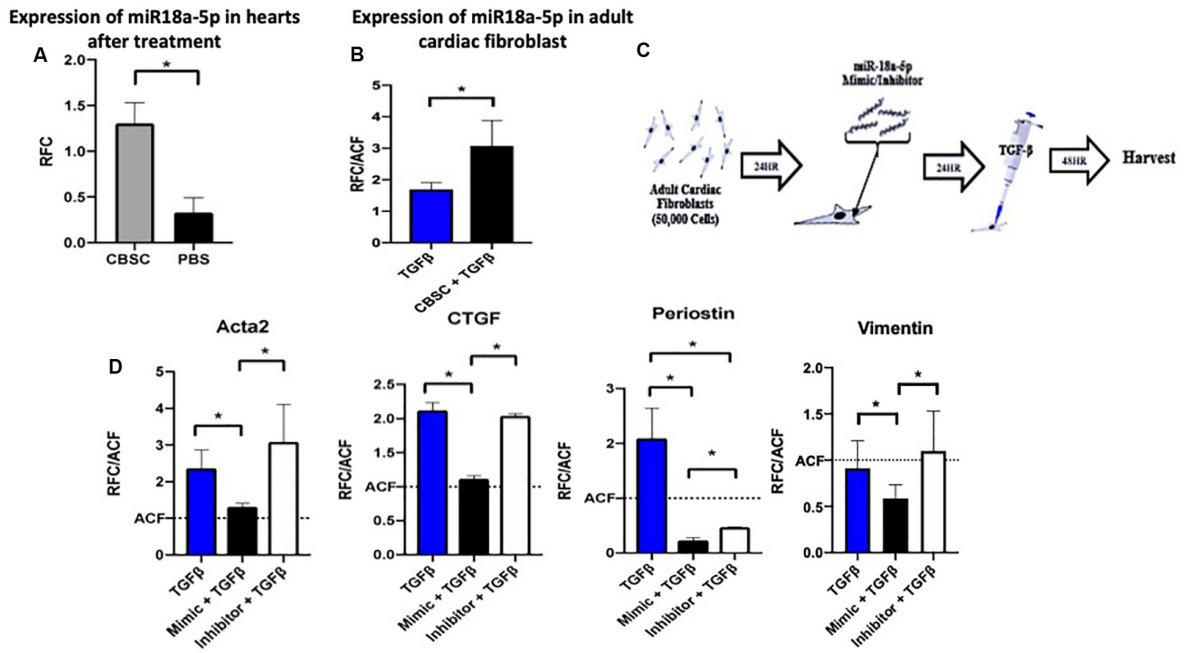

FIGURE 3 | The miR-18a-5p prevents adult cardiac fibroblast differentiation and TGF-β stimulation. **(A)** The increased expression of miR-18-5p in adult hearts after treatment with CBSCs compared to PBS treatment using quantitative reverse transcription polymerase chain reaction analysis, $n = 4$, $^*P < 0.05$. **(B)** The increased expression of miR-18-5p in adult cardiac fibroblast treated with CBSC and TGFβ compared to TGFβ alone using quantitative reverse transcription polymerase chain reaction analysis, $n = 3$, $^*P < 0.05$. **(C)** Outline of experimental design using adult cardiac fibroblasts treated with the miR-18-5p mimic or inhibitor for 24 h. **(D)** Decrease in myofibroblast marker expression in fibroblast treated with the mimic of miR-18-5p with TGFβ compared to TGFβ alone and/or the inhibitor of miR-18-5p and TGFβ using quantitative reverse transcription polymerase chain reaction analysis, $n = 3$, $^*P < 0.05$ All qRT-PCR data is represented as RFC (relative fold change).

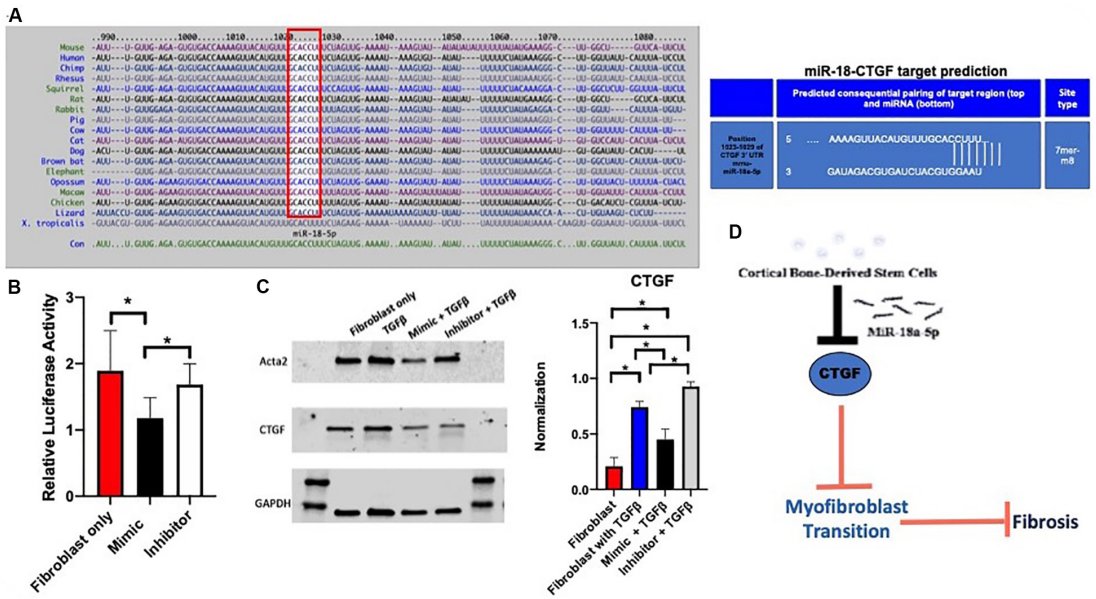

FIGURE 4 | The miR-18a-5p prevents fibrosis in the adult heart. **(A)** A target scan search of the miR-18-5p sequence indicating conserved sequence across species and a miR-18-5p target predication position for pairing of the target region of the myofibroblast marker, CTGF. **(B)** Decrease in relative luciferase activity of CTGF with the treatment of the mimic of miR-18-5p using a Luc-Pair Duo-Luciferase assay, $n = 6$. **(C)** A decrease in myofibroblast marker expression of Acta2 and CTGF respectively with the treatment of the mimic of miR-18-5p with TGFβ using western blot analysis, normalized to GAPDH, $n = 6$, $^*P < 0.05$. **(D)** The mechanism of action of CBSCs and the miR-18-5p inhibiting CTGF, preventing the myofibroblast transition and fibrosis.

the finding, the adult cardiac fibroblasts were dosed with miR-18a-5p mimic or inhibitor overnight before treated the cells with TGF-β for 48 h (**Figure 3C**). The effect of the mimic and inhibitor with TGF-β was measured using RT-PCR (**Figure 3D**). TGF-β stimulated fibroblast with the miR-18a-5p mimic had decreased expression levels of myofibroblast markers including Acta 2, CTGF, Periostin and Vimentin (**Figure 3D**) versus miR-18a-5p inhibitor treatment indicating a more differentiated state. This helped elucidate the importance of miR-18a-5p in preventing adult cardiac fibroblast differentiation into myofibroblasts.

miR-18a-5p Prevents Fibrosis by Targeting CTGF After Injury

miRNAs act through transcriptional repression and targeting of mRNA 3′-UTR, so a miRNA target prediction search was conducted using TargetScan 7.2 that identified CTGF as a potential target with only 1 putative 7mer site within CTGF-3′-UTR (**Figure 4A**) among the fibrotic genes altered. To confirm whether miR-18a targets CTGF-3′-UTR, a reporter assay was performed using 3′-UTR of CTGF that drives luciferase expression. Adult cardiac fibroblasts were transfected with mouse CTGF 3′-UTR luciferase reporter plasmid together with control plasmid and treated with miR-18a mimic or inhibitor. Treatment with miR-18a mimic reduced luciferase activity validating miR-18 targeting of CTGF ($p < 0.05$) (**Figure 4B**). After confirming the CTGF as a target for miR-18a, western blot analysis was performed which showed a significant decrease in myofibroblast markers including Acta2 and CTGF ($p < 0.05$) after treatment of TGF-β and miR-18a-5p mimic, while there was an increase with the miR18a inhibitor treatment (**Figure 4C**). Therefore, we conclude that CBSC secretome release miR18a-5p which blocks CTGF and inhibits the fibrogenic response (**Figure 4D**).

DISCUSSION

Our findings here identify a novel role for cortical bone derived stem cells (CBSCs) secreted factors in modulating cardiac fibrosis in the heart after myocardial injury. In particular, CBSCs secreted factors attenuate myofibroblast formation via release of miR-18-5p which represses connective tissue growth factor (CTGF) leading to concurrent reduction in signaling pathways associated with myofibroblast formation.

Cardiac fibrosis is one of the most predominant outcomes of heart failure during ischemia (Sutton and Sharpe, 2000). Cardiomyocyte death triggers a massive inflammatory and fibrogenic response that initially is largely an adaptive response leading to scar formation designed to prevent cardiac rupture (van den Borne et al., 2010; Shinde and Frangogiannis, 2014). However, cardiomyocyte death leads to adverse cardiac remodeling and fibrosis continues to progress and transition into replacement fibrosis, which is associated with further cardiomyocyte loss leading to ventricular dilation and heart failure (Janicki and Brower, 2002; Berk et al., 2007; Kong et al., 2014). During this later phase, tissue-resident cardiac fibroblasts (CFs) become activated into stellate-shaped cells called myofibroblasts (MFs), characterized by increased expression of α-smooth muscle actin (Acta2), Collagen I, and Periostin (van den Borne et al., 2010). Current available therapies and treatments improve cardiac function and slow down the progression of the disease but are unable to reverse cardiac scar as a consequence of cardiomyocyte death. In the past decade, cell-based therapies have offered a new way to address cardiac repair and regeneration after injury (Bolli et al., 2011; Kanazawa et al., 2015). Several adult stem cells have demonstrated that donated cells in the heart improve cardiac structure and function after myocardial injury (Karantalis et al., 2014; Kanazawa et al., 2015). Nevertheless, adoptively transferred stem cells are lost in the heart early yet the effects persist for up to a year indicating additional mechanisms at play (Hong K. U. et al., 2014). Moreover, salutary effects of cell therapy are linked to the secretion of paracrine factors at the site of injury that mimic similar responses as the parent cell in enhancing cardiac function (Mayourian et al., 2018). Stem cell derived paracrine factors have been shown to exert cardioprotective effects together with promoting cardiomyocyte proliferation and angiogenesis. Interestingly, the effect of cell-based therapies to modulate cardiac wound healing and cardiac fibroblast response have been largely overlooked. We have previously employed CBSCs isolated from the bone cortex for myocardial repair and the data shows CBSC therapy enhanced cardiac structure and function (Duran et al., 2013; Sharp et al., 2017). Importantly, CBSCs transplanted animals showed significantly decreased infarct size and scar suggesting a possible role for CBSCs in modulation of cardiac wound healing. In this context, we show here that CBSC secretome releases factors that target adult cardiac fibroblasts preventing transformation into myofibroblasts and thereby promoting cardiac function.

Our results show that CBSC secrete a number of cardioprotective factors including miR-18a-5p. miR-18a5p belongs to the miR-17-92 cluster with seed sequence AAGGUG and participates in a number of physiological processes regulating proliferation, migration and differentiation of various cell types (Hayashita et al., 2005; Mendell, 2008; Gu et al., 2017). In cardiac biology, miR-18a-5p expression declines in patients with heart failure and is considered to be a biomarker for cardiovascular diseases (Ovchinnikova et al., 2016). Nevertheless, there is not much evidence of miR-18a-5p in cardiac fibroblasts or cardiac fibrosis after myocardial injury. Our results show that adult cardiac fibroblasts treated with CBSCs medium increase miR-18a-5p expression suggesting the ability of CBSCs to transfer miR-18a-5p to adult fibroblasts. Moreover, miR-18a-5p expression declines in the heart after myocardial infarction together with reduction of cardiac fibrosis and decreased fibroblast-myofibroblast transition in response to TGF-β treatment. During cardiac injury myofibroblast transformations rely heavily on the transforming growth factor (TGF-β) signaling pathway (Khalil et al., 2017). Fibroblast-myofibroblast transition (FMT) pathways have been shown to be controlled by TGF-β signaling, that upregulates mesenchymal markers such as Acta2 and fibronectin, markers associated with the increase

in differentiation expression (Kim, 2018). We observe that CBSCs reduce the fibroblast-myofibroblast transition in adult cardiac fibroblasts by overexpressing miR-18a-5p. We found that CBSCs counteract increased differentiation created by TGF-β stimulation, reducing genes overexpressed by the extracellular matrix and promoting wound healing processes. Mechanistically, our results showed that miR-18a-5p targets connective tissue growth factor (CTGF) in the adult cardiac fibroblasts. Several studies recently have implicated a central role of CTGF in regulating tissue remodeling and fibrosis (Sakai et al., 2017; Dorn et al., 2018; Ramazani et al., 2018). CTGF expression is induced by many cytokines and conditions associated with pathophysiology of different organs (Holbourn et al., 2008; Jaffa et al., 2008; Chen and Lau, 2009). Its presence induces formation of myofibroblasts by differentiation of other cell types, activates myofibroblasts and stimulates their deposition and remodeling of ECM protein. In the heart, CTGF expression is induced after myocardial injury and its blockade is associated with reduced cardiac fibrosis (Chatzifrangkeskou et al., 2016; Tan et al., 2019). In accordance, our results suggest that CBSC mediated enhancement of the cardiac structure and attenuation of cardiac fibrosis is mediated via secretion of miR-18a-5p that in turn blocks CTGF expression in adult cardiac fibroblasts leading to reduced myofibroblast formation and ECM remodeling.

In conclusion, we report here a novel role of CBSC secretome in modulating cardiac fibrosis via release of miR-18a-5p that targets adult cardiac fibroblasts attenuating their ability to form myofibroblasts, thereby enhancing cardiac structure and function.

DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

ETHICS STATEMENT

The animal study was reviewed and approved by Temple University Animal Care and Use Committee.

AUTHOR CONTRIBUTIONS

LK and LM contributed equally to the design, data, and writing of the study. YY helped with data in a section. FN helped with formatting and data. RH and TO helped with data analysis. MK and SM helped with design and sections of the manuscript. All authors contributed to the manuscript revision, read 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/fcell.2020.00494/full#supplementary-material

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Conflict of Interest: SM is a named inventor on intellectual property fillings that are related to cells used in the manuscript.

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