INFLAMMATION ALTERS ENDOTHELIAL PROGENITOR CELL-DERIVED EXOSOME CONTENTS AND THERAPEUTIC EFFECT ON MYOCARDIAL REPAIR

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ABSTRACT

Cardiovascular disease remains the leading cause of morbidity and mortality worldwide and Myocardial Infarction (MI) and subsequent heart failure remains the leading cause for death. Despite the improvement in prognosis and treatment of acute MI patients, the underlying causes including loss of cardiomyocytes and microvasculature remain potential risk and lack proper and efficient solutions. Stem cell-based therapies for repair and regeneration have evolved and have been applied in clinical trials. Different types of stem cells, including Endothelial progenitor cell (EPC), Mesenchymal Stem Cell (MSC), induced Pluripotent Stem Cell (iPSC) and cardiac progenitor cells etc. have been used for potential long term recovery and cardiac regeneration. However, results from the clinical trials have been largely disappointing and improvement in cardiac functions have been modest likely due to the limitations of cell therapy including low integration in myocardium, poor survival, cellular dysfunction and limited differentiation ability. It is therefore necessary and urgent to develop cell free alternatives as next generation regenerative therapies. There is a consensus that the beneficial effect of stem cell therapy is largely due to paracrine effects. Exosomes have recently emerged as important functional units mediating stem cell paracrine effects. Exosomes are the family of extracellular vesicles (EV) which are 30-150nm in size, secreted by almost all types of cells and responsible for cell-cell communication via delivering their cargo including RNAs and proteins to host cells. Studies from our and other labs have shown that exosomes mimic parental stem cell in improving post-MI functions. The essential feature of exosome is decided by their cargo including RNA and protein, which are subject to dynamic changes depending on the environment of parental cells. Our studies

were focused on Endothelial Progenitor Cell (EPC)-derived exosomes. EPCs are generated in bone marrow, and home to the site of tissue injury and orchestrate neovascularization and tissue repair. Patients with ischemic heart disease, are usually accompanied with comorbidities such as systemic inflammation, aging, diabetes, etc. which are known to compromise EPC functions. We hypothesized that EPCs under inflammatory stress produce dysfunctional exosomes with altered RNA and protein content, leading to impaired cardiac reparative properties. We chose interleukin-10 knockout (IL-10KO) mice as a model of systemic inflammation. EPCs were isolated from IL-10KO and wild-type (WT) mice, and their exosomes (Exo) were compared for their reparative properties both *in vitro* and *in vivo*. Our in vitro studies showed WT-EPC-Exo treatment attenuated recipient cell apoptosis, enhanced cell mobilization and tube formation, whereas IL-10KO-EPC-Exo were functionally deficient or even had detrimental effects. We used MI mouse model to compare the *in vivo* function of two groups of exosomes and found WT-EPC-Exo treatment significantly improved left ventricular (LV) cardiac function, inhibited cell death, promoted angiogenesis and attenuated cardiac remodeling; while these cardioprotective effects were lost in IL-10KO-EPC-Exo treated group. Both *in vitro* and *in vivo* studies proved that even the same progenitor cell type (EPCs), under inflammatory stimulus (IL-10KO), secretes exosomes with different reparative properties. Next, we explored whether the observed difference in exosome function is caused by altered exosome content. Using Next Generation RNA Sequencing (NGS RNAseq) and mass spectrometry we found RNA and protein expression patterns were drastically different in wild type and IL-10 knockout EPC derived exosomes. This evidence leads to the conclusion that alteration in exosome

content is fundamental for exosome function. We picked two candidates that are highly enriched in IL-10KO-EPC-Exo for further study, miR-375 and Integrin-Linked Kinase (ILK). We treated IL-10KO-EPC with anti-miR against miR-375 and siRNA against ILK separately, and successfully decreased the expression of miR-375 and ILK in both EPCs and EPC derived exosomes. Then we explored the function of those miR and protein 'modified exosomes' with similar *in vitro* and *in vivo* experiments as previously described. Compared to IL-10KO-EPC-Exo, miR-375 knockdown exosomes showed enhanced angiogenesis and inhibited cell apoptosis, while ILK knockdown in exosomes rescued functions in both in vitro and in vivo experiments. These results suggested the possibility that exosome manipulation of identified factors may partially rescue their reparative functionality.

In summary, our studies revealed that stem cell derived exosomes are capable for independent cardiac repair in ischemic heart disease, however, parental stem cells under pathological stimulus secrete dysfunctional exosomes with altered RNA and protein content. Exosome function can be rescued or enhanced through RNA and protein content modification.

This work is dedicated to my parents,

for their love and support!

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TABLE OF CONTENTS

LIST OF ABBREVIATIONS

LIST OF FIGURES

CHAPTER 1

CARDIOVASCULAR DISEASE AND INFLAMMATION

Overview of Cardiovascular Disease

Cardiovascular disease (CVD) is the leading cause of death in the United States for both men and women. American Heart Association (AHA) updates statistics of heart disease and stroke every year; 2018 data suggests with the development of modern therapy and new drug discovery, CVD increasing rate is gradually under control but is still the top killer of humans. Death attributable to disease of the heart and CVD in the United States increased steadily during the 1900s to the 1980s and declined into the 2010s [3]. However, still about 630,000 Americans die from heart disease, that is 1 in every 4 deaths.(https://www.cdc.gov/dhdsp/data_statistics/fact_sheets/fs_heart_disease.htm) Coronary heart disease (43.8%) is the leading cause of deaths attributable to CVD in the United States, followed by stroke (16.8%), high blood pressure (9.4%), heart failure (9.0%), diseases of the arteries (3.1%), and other CVDs (17.9%). In United States, one person suffers heart attack every 40 seconds, and each minute more than one person dies from a heart disease related event. From the global data, CVD is also the leading cause of death worldwide, and is expected to account for >23.6 million deaths by 2030.

Not only does CVD lead to high mortality and morbidity, related healthcare cost is a huge burden. The estimated direct and indirect cost of CVD for 2013 to 2014 was $329.7 billion. AHA estimated that by 2035, 45.1% of the US population would have some form of CVD. Total costs of CVD are expected to reach $1.1 trillion in

2035, with direct medical cost projected to reach $748.7 billion and indirect costs estimated to reach $368 billion [3].

Coronary Heart Disease and Myocardial Infarction

Coronary Heart Disease (CHD) also known as Ischemic Heart Disease (IHD) is the most prevalent CVDs. It is caused by building up of plaques in coronary arteries, gradually narrowing arteries lead to limited blood flow supply to the heart and finally cause heart attack, also called Myocardial Infarction (MI). Every year, about 790,000 Americans have a heart attack and AHA estimated that every 40 seconds, there is one person who suffers heart attack. Risk factors of CHD include high blood pressure, smoking, diabetes, lack of exercise, obesity, high blood cholesterol, poor diet, depression, and excessive alcohol. CHD and MI both have very high mortality. Based on the data of 2015, CHD mortality was 366 801, and CHD any-cause mortality was 536 339. MI mortality was 114 023. MI any-cause mortality was 151 863. Approximately 35% of the people who experience a coronary event in a given year will die from it, and ≈14% who experience a heart attack (MI) will die of it. Globally, it is estimated that 110.6 million people live with IHD, and it is more prevalent in males than in females (64.4 and 46.1 million people, respectively). The number of people with IHD increased by 73.3% from 1990 to 2015. Additionally, CHD and MI are both very costly, the estimated direct costs of CHD in 2013 to 2014 (average annual) were $100.9 billion; the estimated direct and indirect cost of CHD in 2013 to 2014 (average annual) was $204.8 billion. MI ($12.1 billion) and CHD ($9.0 billion) were 2 of the 10 most expensive conditions treated in US hospitals in 2013. Between

2013 and 2030, medical costs of CHD are projected to increase by ≈100% [3], (https://www.cdc.gov/heartdisease/coronary_ad.htm).

Inflammation response post-MI

Myocardial Infarction is associated with inflammatory response both locally and systemically, which is prerequisite for healing and scar formation[4-6]. The immune response post MI can be divided into pro-inflammatory phase and inflammatory resolution/reparative phase, involving components of both the innate and adaptive immune systems [7, 8]. Inflammation after cardiac injury is mainly triggered by molecules released from necrotic cells and it is programmed to be resolved when the cell debris is cleared [9]. Of note, prolonged and unresolved inflammation seems to enhance the fibrotic response and worsen functional recovery during the recovery phase [10]. In fact, clinical trials revealed that almost one fifth patients after MI display severe systemic inflammation, some of the patients presented with culture-positive sepsis have twice the risk of death [11]. Appropriate and timely containment and resolution of inflammation are important determinants for preserving cardiac function. An inflammatory phase that is disproportionately prolonged, of excessive magnitude, or insufficiently suppressed can lead to sustained tissue damage and improper healing, defective scar formation, and heightened cell loss and contractile dysfunction, thereby promoting infarct expansion, adverse remodeling and chamber dilatation [12]. To date, there has been no large-scale immunomodulatory or anti-inflammatory therapeutic strategy post-MI that has been successfully translated into clinical practice, no doubt understanding the role of inflammation response post-MI is necessary and urgent.

The initiation of inflammation response during MI is triggered by endogenous molecule damage associated molecular patterns (DAMPs). Necrotic and stressed/injured cells, and the damaged extracellular matrix (ECM), release DAMPs. DAMPs bind to cognate pattern recognition receptors (PRRs) of the innate immune system on surveilling immune cells including resident macrophages and circulating monocytes as well as resident cells such as endothelial cells and further induce the expression and secretion of various inflammatory cytokines and chemokines, thereby propagating the inflammatory response [4]. In addition to being passively released upon cell necrosis or matrix damage, select DAMPs may also be upregulated and secreted by stressed cardiomyocytes, fibroblasts and activated leukocytes [13]. A number of discovered DAMPs can initiate inflammation response post-MI, including high mobility group box-1 (HMGB1), S100 proteins, fibronectin extra domain A, interleukin (IL)-1α, heat shock proteins (HSPs), low molecular weight hyaluronic acid, Adenosine Triphosphate (ATP), uric acid, mitochondrial DNA, dsRNA, ssRNA, and complement, among others. PRRs are primarily the membrane bound toll-like receptor/IL-1 receptor (TLR/IL-1Rs), as well as cytosolic nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and the cell-surface receptor for advanced glycation end-products (RAGE). After the binding of DAMPs and PRRs, downstream signaling converges on the activation of mitogen-activated protein kinases (MAPKs) and nuclear factor (NF)-κB [12]. These pathways (NF-κB in particular) drive the expression of a large panel of pro-inflammatory genes including inflammatory cytokines (e.g. tumor necrosis factor-α (TNFα), IL-1β, IL-6, IL-18); chemokines attract neutrophils, monocytes and T-lymphocytes. Subsequent leukocyte

recruitment further amplifies the inflammatory response, augments the production of DAMPs, and promotes digestion of dying cells and tissue via the release of proteases and oxidases [9].

Many cellular components are involved in inflammation response post-MI, including both resident cells (endothelial cells, fibroblasts, mast cells, tissue resident macrophages, cardiomyocytes and epicardial cells) as well as recruited cells (neutrophils, monocyte derived macrophages, lymphocytes and dendritic cells)[10]. Among cardiac resident cells, endothelial cells are the most abundant and constitute more than 60% of the non-myocytes in mouse heart. Endothelial activation is required for leukocyte extravasation during inflammation[14]. DAMPs released by dying cardiomyocytes induce rapid upregulation of endothelial adhesion molecules, triggering adhesive interactions with activated leukocytes. Preformed P-selectin is rapidly mobilized from Weibel-Palade bodies, and E-selectin is upregulated in the ischemic endothelium. Once expressed on the endothelial surface, selectins bind to their leukocyte ligands, capturing neutrophils and monocytes and mediating rolling along the venular endothelium[12]. Moreover, activated endothelial cells in the infarct zone serve as an important source of cytokines and chemokines[9]. Fibroblasts are the second most abundant cell population in mouse cardiac resident cells. DAMP-PRRs activation on fibroblasts leads to altered cellular function including changes in proliferation and migration, trans differentiation into myofibroblasts, ECM turnover, and production of fibrotic and inflammatory paracrine factors, play essential role in cardiac regeneration, remodeling and scar formation[4]. Recruited inflammatory cells are another component for cellular effects in inflammation response. Neutrophils are

the first immune cell type infiltrate myocardium in response to DAMPs, cytokines and chemokines, compliments and dominate infarcted myocardium in the first 2 days after MI[15]. Emigrated neutrophils release proteolytic enzymes and contribute to the clearance of the wound from dead cells and matrix debris. Due to their pro-inflammatory and cytotoxic properties, excessive neutrophil activity has been associated with poor prognosis, remodeling and mortality after MI[16]. Following the early appearance of neutrophils, monocytes and macrophages comprise the most abundant cells in the infarcted heart. A large compilation of studies suggest macrophages display different behaviors during different phases post-MI[9]. The initial phase (peak day ~3–4 post-MI) promotes tissue digestion and is characterized by $Ly6C^{hi}$ monocytes and M1 macrophages that are proteolytic, with augmented expression of proteinases (e.g., cathepsins and Matrix metalloproteinases(MMPs)), and pro-inflammatory, with augmented TNF expression. The second phase (peak day ~7 post-MI) promotes tissue repair, with a predominance of $Ly6C^{lo}$ monocytes and M2-like macrophages with augmented expression of anti-inflammatory, pro-fibrotic, and angiogenic factors (e.g., IL-10, Transforming growth factor beta (TGF-β), and Vascular endothelial growth factor (VEGF)). T-lymphocytes are demonstrated to be activated after MI and promote wound healing, resolution of inflammation and pro-inflammatory monocyte infiltration, and proper collagen matrix formation and scar formation, thereby limiting adverse remodeling. Invarient Natural Killer T-Lymphocytes (iNKT) cells enhance the expression of anti-inflammatory cytokine IL-10 and mediate immune suppression. CD4+Foxp3+ Tregs promote macrophage

polarization into M2-like phenotype. Hence, multiple T-lymphocyte subsets contribute to suppression of the inflammatory response[17-19].

Timely resolution and containment of inflammation are critical for cardiac healing. However, the precise spatial and temporal regulation of the inflammatory response toward scar-free regeneration after MI remains unclear. Therapeutic modulation of inflammation response may hold great promise for post-MI cardiac repair.

Interleukin-10

Interleukin-10 (IL-10) is a naturally occurring anti-inflammatory cytokine produced by a variety of immunologic cells, including Th2-type T cells, some T-regulatory cells, B cells, and macrophages[20]. It is released from these cells in response to both endogenous and exogenous mediators including LPS, catecholamines and c-AMP activating drugs. IL-10 exist as a homodimer and binds to IL-10 receptor complex, activate JAK/STAT and Akt cascade. IL-10 has been discovered involved in proliferation, anti-apoptotic and cell survival activities, dampens the inflammatory effects in overwhelming infections and attenuate tissue damage. There has been 37 molecules and 76 reactions annotated that are regulated or interact with IL-10.[1]

As for the role of IL-10 in heart, many studies suggest IL-10 is beneficial for cardiac repair and regeneration post-MI. After MI, IL-10-deficient mice have increased infarct size and heavier myocardial necrosis with more neutrophil infiltration. IL-10 treatment in MI mouse reduces inflammation, improves left ventricle cardiac physiology, stimulates M2 macrophage polarization and fibroblast activation and improves cardiac remodeling[21]. However, human studies are not very

conclusive. Some studies find that in patients with acute MI, higher serum IL-10 within 24 h after angioplasty is associated with reduced incidence of heart failure progression whereas others show that higher IL-10 predict recurrent cardiac events or heart failure[22, 23]. In particular to stem cell therapy in ischemic heart disease, many studies from our lab showed that IL-10 deficiency impairs bone marrow derived endothelial progenitor cell survival and function in ischemic myocardium and IL-10 treatment improves cardiac remodeling and function, inhibits fibroblast progenitor cell migration and trans differentiation, decreases cardiac fibrosis [24].

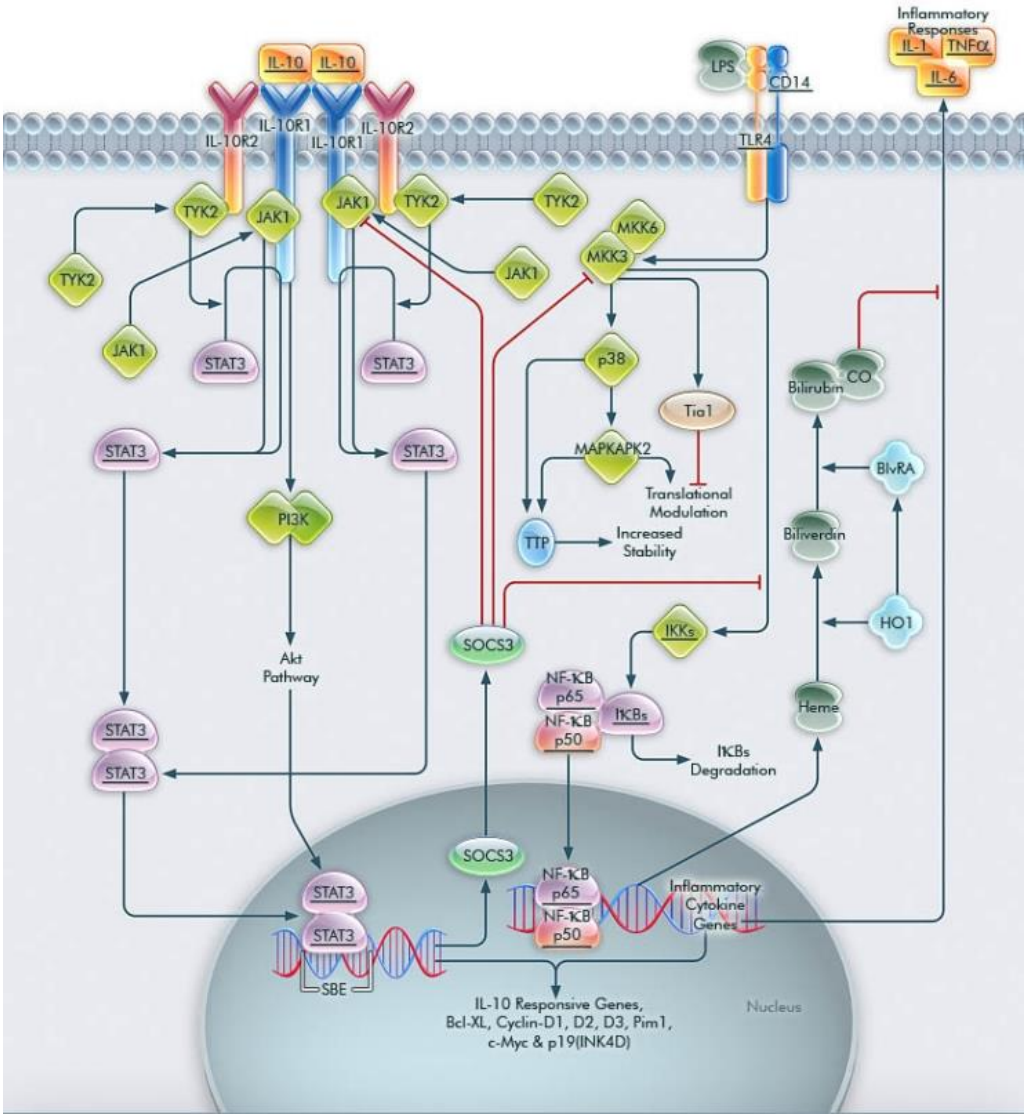


Figure1. A schematic representation of reactions induced by IL-10.
The signaling pathway map depicts molecules involved in protein-protein interactions, catalysis, activation/inhibition and translocation events induced upon treatment with IL-10 cytokine. Information regarding site and residue of post-translational modification is also shown in the pathway. Pathway legend is provided to identify the interactions, molecular associations and gene regulations on the map.[1] (Adapted from https://www.thermofisher.com/us/en/home/life-science/antibodies/antibodies-learning-center/antibodies-resource-library/cell-signaling-pathways/il-10-pathway.html)

NF-κB

Nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) is a protein complex found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, heavy metals, ultraviolet irradiation, oxidized LDL, and bacterial or viral antigens[25].

NF-κB plays a key role in regulating inflammation response. As transcription factor, after activation and nuclear translocation, it binds to DNA response element and upregulates expression of many genes including inflammatory cytokines, chemokines, adhesion molecules. The spatial and temporal expression of these mediators in resident and infiltrating cells choreographs events that further amplify the inflammatory response (cytokines) and attract and recruit specific leukocyte populations (chemokines and adhesion molecules) to injured myocardium[26]. NF-κB family contain five subunits, p65 (RelA), RelB, c-Rel, p50, and p52, these subunits

form homo- or heterodimers to modulate gene transcription. Activation of NF-κB family have been described as the canonical or classical pathway, and noncanonical or alternative pathway. In classical pathway, p65/p50 heterodimers are bound to inhibitor of κBα (IκBα) in the cytoplasm. Upon IκBα phosphorylation by IκB kinase (IKK), IκBα is rapidly ubiquitinated and degraded by the 26S proteasome, allowing for subunit nuclear translocation. In alternative pathway, activation of the NF-κB inducing kinase (NIK) upon receptor ligation led to the phosphorylation and subsequent proteasomal processing of the NF-κB precursor protein p100 into mature p52 subunit in an IKKα dependent manner. Then p52 dimerizes with RelB to appear as a nuclear RelB:p52 DNA binding activity and regulate a distinct class of genes[27].

The role of NF-κB in heart remains controversial. Regarding to its role in cardio protection, studies suggested that acute NF-κB activation is essential for late cardio protection induced by ischemic preconditioning[28]; B-cell lymphoma 2 (Bcl-2) suppresses cell death of ventricular myocytes through a mechanism involving IKKβ-mediated activation of NF-κB[29]; in cultured cardiac myocytes, forced expression of a nonphosphorylatable IκBα designed to inactivate NF-κB signaling resulted in more sensitive to TNFα induced cell apoptosis[30]; $p50^{-/-}$ mice display enhanced cardiac dysfunction following myocardial infarction[31]. All these evidences support the beneficial effect of NF-κB. However, there are also studies revealed conflicting results. Mice with cardiomyocyte-restricted overexpression of phosphorylation-resistant IκBα[32], cardiomyocyte-specific p65 deletion[33], IκBα overexpression via gene transfer[34], NF-κB double-stranded decoy DNA transfection[35], and pharmacological blockade of IκBα or IKKβ[36, 37] have demonstrated that NF-κB inhibition decreases

infarct size, reduces inflammatory responses including leukocyte infiltration, and improves cardiac function in ischemic heart disease. Perhaps neither overexpression nor eradicating NF-κB is good solution for cardiac disease treatment, maintaining the balance of NF-κB expression according to disease condition is more important for further study.

In our studies, we found inflamed exosomes, including both IL-10KO-EPC-Exo and exosomes derived from endothelial cells treated with TNFα induce inflammatory response of recipient cells via NF-κB signaling pathway. Exosomal Integrin Linked Kinase (ILK) appears to play an important role in this process by noncanonical activation of NFκB. We think the impaired function of inflamed exosomes can be partially explained by exaggerated inflammatory response contributed by inflamed exosomes. Through knocking down ILK in exosomes or recipient cells, we significantly blocked the inflammatory response of recipient cells and rescued the cardioprotective function of inflamed exosomes.

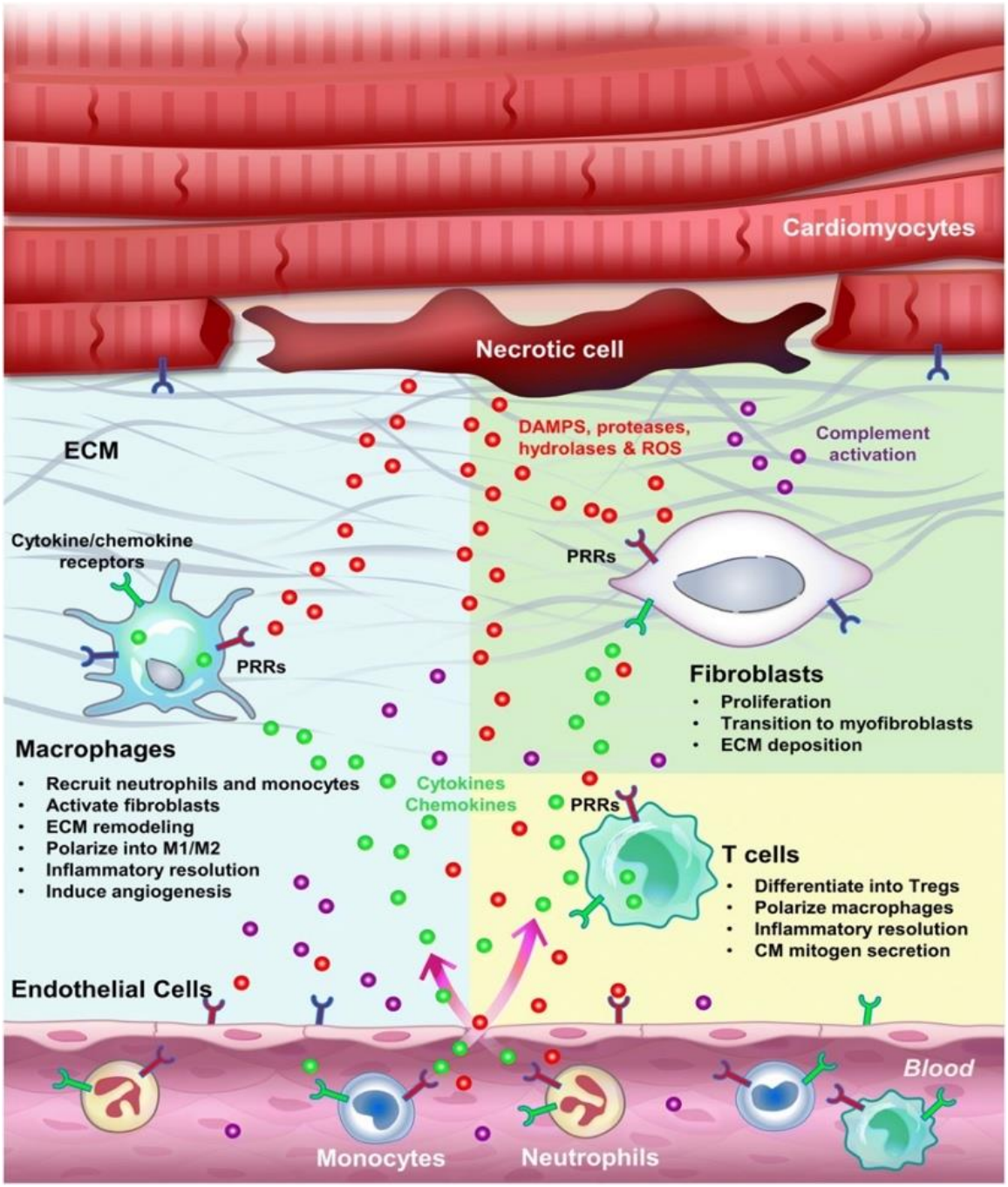

Cardiomyocytes
Necrotic cell
ECM
DAMPS, proteases, hydrolases & ROS
Complement activation
Cytokine/chemokine receptors
PRRs
PRRs
Fibroblasts
• Proliferation
• Transition to myofibroblasts
• ECM deposition
Macrophages
• Recruit neutrophils and monocytes
• Activate fibroblasts
• ECM remodeling
• Polarize into M1/M2
• Inflammatory resolution
• Induce angiogenesis
Cytokines
Chemokines
PRRs
T cells
• Differentiate into Tregs
• Polarize macrophages
• Inflammatory resolution
• CM mitogen secretion
Endothelial Cells
Blood
Monocytes
Neutrophils

Figure 2. Inflammation induced by cardiac injury.

Sterile inflammation can be triggered by various components released by necrotic cells, including DAMPs, proteases, hydrolases and mitochondrial ROS. DAMPs directly activate PRRs on surveillant cells, including tissue macrophages, circulating monocytes and neutrophils, as well as on resident cells, including endothelial cells, fibroblasts and CMs. Proteases, hydrolases and ROS activate the complement system as well as inflammasomes, and degrade the ECM, altogether further propagating the inflammatory response. Activated tissue resident macrophages secrete cytokines to attract monocytes and neutrophils, activate endothelial cells to promote cell adhesion and permeability, and remodel the ECM. Infiltrating monocytes and neutrophils clear cell debris by phagocytosis and help terminate the initial insult. After wound clearance, myofibroblasts secrete ECM to help prevent the injured heart from rupturing. Differentiated Tregs tune down the inflammation by secreting anti-inflammatory cytokines, in parallel with M1–M2 macrophage polarization and programmed neutrophil apoptosis. Inflammation initiation, propagation and resolution can occur in both regenerative and non-regenerative models. However, in the regenerative models, these processes seem to facilitate CM dedifferentiation and proliferation and scar resolution by mechanisms yet to be determined. (Adapted from https://link.springer.com/article/10.1007/s00018-018-2995-5)

CHAPTER 2

STEM CELL THERAPY AND EXOSOME

Introduction of Stem Cell Therapy

Stem cells are defined as undifferentiated cells with characteristics of self-renewing, clonogenic (form identical clones), and multipotent (able to differentiate into a wide array of specialized cell types). There is no standard categorization of stem cells, most labs use anatomical sites, functionality, cell surface markers, transcription factors for protein expression to describe a population of stem cells. It is quite common in research field that even under the same name (such as endothelial progenitor cells (EPCs)), different labs have different definition and using different cell population for experiment and might lead to inconsistent result between labs.

Cardiovascular disease is the leading cause of death worldwide, despite the improvement of prognosis due to advanced diagnosis and new drug discovery, the underlying cause, loss of cardiomyocytes and microvasculature remain unsolved. In recent decades, stem cell therapy is gradually getting popular in clinical trials aiming at preserving or regenerating myocardial tissue[38]. The first goal of cardiac stem cell research appears to identify a population of stem cells that can become cardiomyocyte which when transplanted to the ischemic myocardium may replenish the damaged cardiomyocytes. That goal also includes identification of a specific cell type that is derived from autologous source, which can override immunological rejection on one hand and is plastic enough to differentiate desired cell type on the other. However, clinical trials using various autologous stem/progenitor cells in patients with heart disease were largely disappointing with transient or inconsistent improvement in left

ventricle symptoms and functions. Recently, the ability of stem/progenitor cells derived from adult autologous sources, to transdifferentiate into cardiomyocytes has been vigorously contested with the consensus emerging that adult stem/progenitor cells do not form cardiomyocytes in any meaningful way [39]. Moreover, numerous studies including findings from our lab, have demonstrated that the beneficial effect of adult stem cells in cardiovascular disease is largely due to paracrine effects[40]. More and more studies are now focusing on stem cell paracrine derivatives such as cytokines, chemokines, growth factors, cell derived spheres, secretomes, exosomes and so on.

In general, autologous adult stem cell therapy is a promising strategy, feasible and safe. Although no firm evidence that transplanted cells replace dead myocytes has emerged, beneficial effect is supported by modest gain in function in clinical trials and in preclinical studies and deserve more exploration and newer strategies to improve functional outcome of stem cell-based therapies. Our studies focused on paracrine effect, especially on stem cell derived exosomes, are therefore of potential clinical significance.

Types of Stem Cells and Limitations

Bone Marrow Derived Stem Cells

BM represents a highly heterogeneous tissue, harboring numerous cell populations, including differentiated cells, such as monocytes, lymphocytes, fibroblasts, adipocytes, chondroblasts, osteoblasts, and osteoclasts, as well as undifferentiated cells composed of hematopoietic stem cells (HSCs), which include hemangioblasts and endothelial progenitor cells (EPCs), and nonhematopoietic

mesenchymal precursor cells that give rise to stromal cells referred to as mesenchymal stem cells (MSCs)[39]. The subpopulation of bone marrow stem cells is very complex and varies according to labs. In general, through density gradient centrifugation, a subgroup of undifferentiated cells can be isolated and referred as bone marrow mononuclear cells (BMMNCs), with only 2-4% comprising HSC/EPC and approximately 0.01% of MSC. Then HSC, EPC and MSC can be differed by their surface markers[41].

Bone marrow stem cells can be recruited to damage area and contribute to tissue regeneration which leads to the basic idea of bone marrow stem cell therapy. As early as 2001, there has been study demonstrated that intramyocardially injected murine BM-derived cells improved cardiac function in a murine model of MI[42]. Likewise, many early clinical trials reported beneficial effects of BM-derived cells for the treatment of heart disease[43-45]. With low ethical concern, relatively large number of autologous cells, bone marrow stem cells therapy rapidly developed and broadly used in clinical experiments.

Bone marrow derived Endothelial Progenitor Cells (BM-EPCs)

In 1997, Asahara et al discovered BM-EPCs as a population of cells in human peripheral blood are capable of differentiation in vitro into endothelial cells[46]. These cells arise from the bone marrow and circulate in blood; injury can trigger the recruitment of these cells to damaged tissue. EPCs retain the characteristics of self-renewing, clonogenic, and multipotent. Proper and unambiguous molecular characterization of these cells is still lacking, generally, EPCs express common HSC markers CD34 and CD133 as well as vascular endothelial growth factor receptor 2

(VEGF2 or KDR)[47]. Some studies propose EPCs in particular "early" and "late" EPCs, based on the appearance in culture of these circulating mononuclear cells[48]. Early EPCs are believed to promote endothelial cell repair and angiogenesis through paracrine effects, whereas late EPCs are believed to transdifferentiate into endothelial cells. In our study, we define EPCs as CD31 and CD34 positive and lineage negative cells isolated from bone marrow with density gradient centrifugation, and we culture them for less than 10 days[49].

Over the past decades, many animal studies and clinical trials indicate the protective role of EPCs in cardiovascular homeostasis. In 2001, a German group initiated a phase I clinical trial injecting autologous bone marrow CD31 positive cells into the infarcted border zone during coronary artery bypass grafting in 6 patients and observed enhanced left ventricle ejection fraction (LVEF) in 4 patients and improved infarcted tissue perfusion in 5 patients after following for 3 months[43]. Since then, several phase I and II clinical trials were conducted and reported the safety, feasibility and beneficial effects of intramyocardially and intracoronary injected BM-derived CD133+ cells for the treatment of cardiac diseases[50-52]. However, long term follow-up studies for several years failed to show an improved cardiac function. There are several other noteworthy clinal trials with EPCs and receive inconsistent effects on heart diseases. TOPCARE-AMI was one of the earliest clinical trials of stem cell therapy[53]. Assmus et al randomized 20 patients in an open-label clinical trial to assess the safety, feasibility, and efficacy of unfractionated bone marrow-derived stem cells (n = 9) or circulating blood-derived EPCs (n = 11) injected into the infarct-related artery a mean 4.3 ± 1.5 days after an acute MI. The bone marrow-derived cells were

harvested on the morning of infusion whereas the blood-derived EPCs were harvested approximately 3-4 days before planned infusion. Importantly, neither population of cells was a pure progenitor cell population. There was no difference in efficacy between the patients receiving bone marrow derived stem cells or blood-derived EPCs. At 4-month follow-up, these cells were associated with a modest increase in global left ventricular ejection fraction (LVEF), minimally improved regional wall motion, and mildly reduced end-systolic LV volume. After 1 year of follow-up, MRI studies showed improved LVEF, smaller infarct size, and improved LV hypertrophy. Wollert et al conducted a randomized control clinical trial on a similar but larger population of patients than in the TOPCARE-AMI trial[54]. The Bone marrow transfer to enhance ST-elevation infarct regeneration trial (BOOST) enlisted 60 patients, randomly assigned to receive either unfractionated BMMNCs (n = 30) or standard therapy with percutaneous coronary intervention (PCI; n = 30). BMMNCs were infused 4-8 days following PCI reperfusion for an acute ST-elevation MI. As in TOPCARE-AMI, a heterogeneous population of BMMNCs were injected into the infarct-related artery on the same day of harvest, but 4-8 days after PCI reperfusion for an acute ST-elevation MI. After 6 months, mean global LVEF had increased by 7% in the BMMNC group and by 0.7% in the control group (P = 0.0026). By 18months, the increase in LVEF was lost.

Overall, the BM-EPCs transplantation therapy benefits patients with MI in short time treatment but does not show advantages compare to other therapy in long term follow up. Several other potential limitations of using BM-EPCs have been identified and therefore must be acknowledged. Risk factors for coronary artery

disease are reported to be associated with a reduced number and functional activity of EPCs in the peripheral blood of patients[55]. Likewise, patients with diabetes showed lower EPC numbers. Additionally, patients with systemic inflammation, diabetes, aging or smoking habits showed not only a smaller number of EPCs but also functional defect EPCs[56].

In our lab, we showed EPCs functional defects isolated from IL-10 knockout mice and diabetes mice, and these defects are reflected in their secreted exosomes[49]. Those evidence composed partially of our study rationale.

Mesenchymal Stem Cells (MSCs)

MSCs represent a very small fraction of BMMNCs, ranging from 0.001% to 0.01%. These cells are characterized by their ability to differentiate into osteoblasts, adipocytes and chondrocytes under defined in vitro conditions[38]. They uniquely adhere to plastic in cell culture and are characterized primarily by the cell surface antigens CD73, CD90 and CD105, but also will express CD 17, CD29, CD44, CD106, CD120a, and CD124. MSCs are negative for CD31, CD34, CD45, CD133, CD14 (CD11b), CD19, and kinase insert domain receptor (KDR) [57]. Although MSCs are primarily thought to arise from the bone marrow, other tissues and organs may also produce cells of the MSC phenotype. These organs include the heart, blood, brain, liver, testis, prostate, skeletal muscle, and fatty tissue. MSCs are also human leukocyte antigen (HLA) DR negative and, therefore, are believed to evade immune recognition[58]. Several reports suggest that MSCs also differentiate into cardiomyocytes under specific culture conditions in vitro and following myocardial transplantation in vivo[59]. From a feasibility standpoint, MSCs can be potentially

harvested in advance, expanded in culture, and stored for use after an acute MI in the future.

Many animal studies and clinical trials revealed the beneficial effect of MSC transplantation therapy for heart disease. Typically, in animal experiments, researchers found MSCs as cardiomyogenic. Toma et al demonstrated the ability of human MSC to differentiate into cardiomyocytes when injected into the murine myocardium[60]. Mangi et al injected genetically modified autologous MSC into the ischemic rat myocardium[61]. Transplanted cells inhibited the process of cardiac remodeling by reducing inflammation, collagen deposition, and cardiomyocyte hypertrophy. Moreover, these investigators demonstrated over 80% regeneration of lost rat myocardial volume and the normalization of systolic and diastolic cardiac functions. Tang et al demonstrated improved LV function in a rodent infarct model and increased neovasculature alongside newly formed myocardial cells[62]. In clinical trials, intracoronary injection of bone marrow- derived MSCs resulted in improvement in global LVEF and regional wall motion, and reduction in infarct size, LVESV, as well as LVEDV in patients with acute MI[63]. A recent meta-analysis, including 58 pre-clinical studies, indicated an overall reduced infarct size of ~7% as well as an improved heart function of ~11% after MSC transplantation in animal models of acute myocardial infarction (AMI) and chronic ischemic cardiomyopathy (ICM)[64]. In consistence with this, clinical study suggested that intramyocardial injection of MSCs in patients undergoing Coronary Artery Bypass Grafting (CABG) reduced scar size and improved tissue perfusion as well as regional function predominantly at the site of injection[45]. In patients with ischemic cardiomyopathy, intracoronary injection of

MSCs resulted in improved perfusion and improved exercise tolerance and New York Heart Association (NYHA) class[65]. However, the beneficial effects of MSCs on global cardiac function in a clinical setting remain uncertain. In the study by Katritsis et al., intracoronary injection of two to four million MSCs enhanced myocardial perfusion and viability, but failed to improve global Left Ventricular Ejection Fraction (LVEF), Left Ventricular End-Systolic Volume (LVESV), or Left Ventricular End-Diastolic Volume (LVEDV) in patients with acute or old MI[66].

In general, MSC is a very good source of stem cell and presented cardioprotective effect in specific heart disease. There is still controversy whether MSCs become cardiomyocytes in human study and whether MSCs therapy improve global cardiac function.

Embryonic Stem Cell (ESCs)

ESCs are totipotent cells that possess the ability to differentiate into cells derived from the three germ layers: ectoderm, endoderm, and mesoderm. ESCs are derived from the inner cell mass of the blastocyst of a 3- to 5-day-old embryo. ESCs were first isolated from murine blastocysts by Evans, Kaufman, and Martin in 1981[67], these cells have been shown to grow for an indefinite period in vitro while maintaining the potential to differentiate into derivatives of all three embryonic germ layers. Thompson et al developed the first successful ESC line, opening the field of embryonic stem cell research[68]. In particular to cardiac field, Boheler et al proved that ESCs are able to differentiate into cardiomyocytes represented by all specialized cell types of the heart, such as atrial-, ventricular-, sinus nodal-, and Purkinje-like cells[69]. These cardiomyocytes exhibit not only cell morphology similar to that of adult cardiac

cells, but also have similar physiology; cultured ESCs beat spontaneously and synchronously, almost fully mimicking the behavior of mature cardiomyocytes. As a good source for cell transplantation therapy, many animal studies have been applied. Min et al injected ESCs into severely damaged ischemic rat hearts in an experimental model of MI[70]. Cardiac function in the ESC-treated animals improved significantly 6 weeks after cell transplantation. The area of infarcted myocardium was diminished after treatment, as was the severity of LV hypertrophy, resulting in improved hemodynamics. Nelson et al used ESC to evaluate cardiac regeneration in a mouse model of MI. MI was followed 1 h later by injection of a relatively low number (5 × 105) of ESCs[71]. Although geometry and hemodynamics of the LV improved, over 20% of the animals developed teratomas in the pericardial space. Similar findings published by Riess et al and others raise serious safety concerns with the use of ESC in human patients[72]. Additionally, the ethical concerns, the risk of immune rejection and genetic instability all limited the broad application of ESCs in human experiments. Recently, many studies try to use ESC differentiated cardiomyocytes as alternative strategy but also generated our concerns including arrhythmia complications in large animal experiment[73].

Induced Pluripotent Stem Cells (iPSCs)

In 2006, Takahashi and Yamanaka were the first reporting the generation of pluripotent stem cells from mouse fibroblasts by retroviral introduction of 4 defined transcription factors (c-Myc, octamer-binding transcription factor (Oct)3/4, sex determining region Y box 2 (Sox2), Kruppel-like factor (Klf4))[74].1 year later, human iPSCs were established by applying the same technology to human fibroblasts[75].

Nowadays, the field of iPSCs has moved forward with unprecedented speed, and we have come a long way in both the methods available to generate iPSCs and our understanding of their molecular biology. It is now well established that iPSCs can differentiate into all three germ-layer-derived cells. The seminal achievement of induced pluripotency holds great promise for regenerative medicine.

Many studies have successfully generated cardiovascular lineage cells from iPSCs and applied to various heart diseases. It has been shown that cardiomyocytes and endothelial cells (ECs) can be successfully differentiated from murine iPSCs in vitro as well as in vivo when these iPSCs are transplanted in a murine MI model[76]. Zhang et al. successfully characterized the cardiac differentiation potential of human iPSCs compared to human embryonic stem cells (hES) cells[77]. Electrophysiology studies indicated that iPSCs have a capacity similar to ES cells for differentiation into nodal-, atrial-, and ventricular-like phenotypes based on their action potential characteristics[78]. Another study showed that iPSCs-derived cardiomyocytes display spontaneous rhythmic intracellular Ca^{2+}fluctuations with amplitudes of Ca^{2+} transients comparable to those of ES-cell-derived cardiomyocytes, indicating functional coupling of the newly generated cardiomyocytes[79]. Electrophysiological studies with multielectrode arrays demonstrated the functionality and presence of the beta-adrenergic and muscarinic signaling cascade in these cells.

With all these amazing features of iPSCs and iPSCs derived cardiac lineage cells, many studies have been conducted to check their cardioprotective roles. In animal MI models, iPSCs derived cardiomyocyte like cells are able to integrate into myocardium as well as to improve cardiac function and alleviate adverse remodeling

processes[80]. Although limited human studies of iPSCs, iPSCs provide significant advantages such as no ethical concern and less immune response compared to human ES[81]. However, limitations exist in iPSCs applications, various studies described genomic instabilities in iPSC lines resulting either from pre-existing variations in parental adult cells or from mutations occurring during the reprogramming process and culturing time[82]. Moreover, transplanted iPSC-derived cells might possess immunogenic capabilities raised additional major concerns about the safety of iPSCs[83]. Many new protocols were established to overcome potential issues, in order to minimizing the potential for mutagenesis during the reprogramming process, non-integrating gene delivery approaches[84], modified messenger ribonucleic acids (mRNAs) and microRNAs (miRs) have been used[85, 86]. Recently, chemically induced iPSCs have been generated by applying a cocktail of small molecules[87].

There has been significant improvement in iPSCs discovery and development, but there is still long way to go before it can be used in clinical cases.

Exosome

Introduction of Exosome

Most of the extracellular and body fluids, such as blood, urine, saliva, and breast milk, are known to contain secreted vesicles, called extracellular vesicles (EVs). Although known for several decades, extracellular membrane vesicles have long been thought of as mere cell debris, signs of cell death, or structures specific to a unique organ. The importance of EVs and the recognition of its wider involvement in intercellular signaling began to emerge in the last decade. EVs with size of 30-150nm bearing CD63 and CD9/CD81 were defined as exosomes and were shown crucial for

preserving vascular integrity and in the development of cardiovascular and other diseases. Exosomes are generated from almost all types of cells such as blood cells, endothelial cells, immune cells, smooth muscle cells, etc., work as natural carrier for small molecules and modify a multitude of cellular bioactivities. They circulate in body fluids and extracellular space and shuttle lipids, proteins and nucleic acids between donor and recipient cells. A recent surge of interest in the field of exosome biology is in part due to the recognition that the molecules they carry can act as facilitators of both physiological and pathological signaling. Exosomes have been described to modulate immune-regulatory processes, tumor escape mechanisms and to mediate both regenerative and degenerative processes. To date, a large number of reports dealing with circulating exosomes and their cargos defines them to be a potential source of valuable diagnostic and prognostic biomarkers and holds a great therapeutic potential to serve as vehicles for targeted therapy for cardiovascular and other diseases.[2, 88-91]

Biogenesis and Secretion of Exosome

The biogenesis and secretion of exosomes involve several crucial steps: formation of intraluminal vesicles (ILVs) in multi vesicular bodies (MVBs), transport of MVBs to the plasma membrane and fusion of MVBs with the plasma membrane. Formation and secretion of exosomes require enzymes and ATP and can be both endosomal sorting complexes required for transport (ESCRT) dependent and independent.

Exosome biogenesis starts within the endosomal system. The endocytic pathway consists of highly dynamic membrane compartments involved in the

internalization of extracellular ligands or cellular components. Early endosomes mature into late endosomes, and during this process, they accumulate ILVs in their lumen. Because of their morphological features, they are generally referred to as multivesicular endosomes or MVBs. The ILVs that are formed by inward budding of the early endosomal membrane sequester proteins, lipids, and cytosol that are specifically sorted. In most cells, the main fate of MVBs is to fuse with lysosomes, acidic compartments that contain lysosomal hydrolases, ensuring the degradation of their content. However, organelles with hallmarks of MVBs, bearing the tetraspanin CD63, lysosomal-associated membrane proteins (LAMP) LAMP1 and LAMP2, and other molecules that are generally present in late endosomes, can also fuse with the plasma membrane, releasing their content into the extracellular milieu and fated for exocytosis.

The formation of MVBs and ILVs is driven by the endosomal sorting complex required for transport (ESCRT), which is composed of approximately thirty proteins that assemble into four complexes (ESCRT-0, -I, -II and -III) with associated proteins (Vacuolar protein sorting-associated protein 4A (VPS4), Vesicle Trafficking 1 (VTA1), ALIX also called Programmed cell death 6-interacting protein (PDCD6IP)). The ESCRT-0 complex recognizes and sequesters ubiquitinated transmembrane proteins in the endosomal membrane, whereas the ESCRT-I and -II complexes appear to be responsible for membrane deformation into buds with sorted cargo, and ESCRT-III components subsequently drive vesicle scission. ESCRT-0 consists of HRS (hepatocyte growth factor–regulated tyrosine kinase substrate) that recognizes the monoubiquitinated cargo proteins and associates with signal transducing adaptor

molecule(STAM) (the other ESCRT-0 component) and Epidermal growth factor receptor substrate 15 (Eps15) and clathrin (two non-ESCRT proteins). HRS recruits tumor susceptibility 101 (TSG101) of the ESCRT-I complex, and ESCRT-I is then involved in the recruitment of ESCRT-III via ESCRT-II or ALIX. Finally, the dissociation and recycling of the ESCRT machinery require interaction with the AAA-ATPase VPS4.

MVBs and ILVs can also form in absence of ESCRT function. For example, it has been shown that despite simultaneously silencing of key subunits of all four ESCRT-complexes, ILVs are still formed in MVBs, thus indicating the presence of ESCRT-independent mechanisms. Tetraspanins, transmembrane proteins enriched in exosomes, are also involved in ESCRT-independent exosome release. Expression of the tetraspanins CD9 and CD82 has been shown to enhance the exosomal release of β-catenin from HEK293 cells. Recently, the tetraspanin CD63 was shown to play a role in exosome biogenesis as well. CRISPR/Cas9 knockout of CD63 resulted in reduced secretion of EVs, measured by NTA. Besides proteins, two lipid metabolism enzymes have been shown to generate lipids in the limiting membrane of MVBs, which induce inward budding and thus formation of ILVs in an ESCRT-independent manner: neutral sphingomyelinase (nSMase) allowing hydrolysis of sphingomyelin into ceramide, and phospholipase D2 allowing hydrolysis of phosphatidylcholine into phosphatidic acid. Therefore, MVBs and their ILVs can be formed by both ESCRT-dependent and -independent mechanisms certainly related to the cargo that is sorted within a given cell.

Exosomes are secreted by multiple cell types and cell lines spontaneously, some exosome-stimulating conditions have also been described, although the intracellular signals involved are not known. For instance, the secretion of exosomes by rat cortical neurons can be stimulated by depolarization of the cells or stimulation by neurotransmitters, a signal that also promotes exosome secretion by oligodendrocytes. γ-Irradiation-induced DNA damage can also promote EV secretion by tumor cells or fibroblasts through the activation of the p53-regulated protein Metalloreductase STEAP3 (TSAP6). In these studies, however, whether the vesicles recovered were MVB-derived exosomes or other EVs was not clearly determined. More recently, silencing of papilloma virus E6/E7 oncogenes in HeLa cells was clearly shown to induce senescence and a concomitant increase of p53 and TSAP6 expression, as well as a large increase in secretion of EVs, including those bearing endosomal markers (CD63, TSG101). Finally, some cells, such as B or T lymphocytes, secrete very little EVs at the steady state, but MVB-derived exosome secretion is strongly enhanced by activation through interactions with T cells or the B cell receptor.[92, 93]

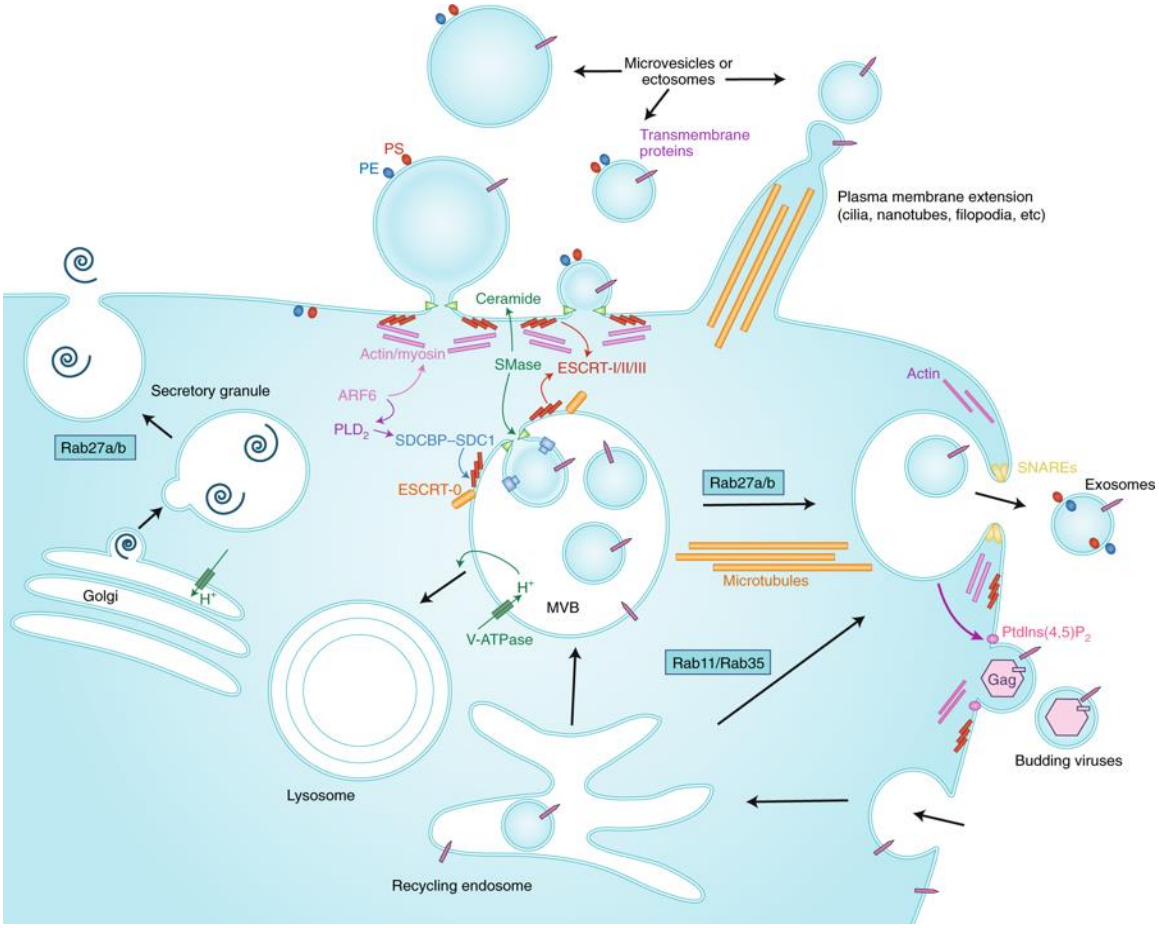


Figure 3. Biogenesis and Secretion of Exosome.

Selected examples of the crosstalk between the intracellular molecular machineries involved in the biogenesis and secretion of EVs forming at the plasma membrane (microvesicles, microparticles or ectosomes) or in late endosomal MVBs (exosomes).[2]

Exosome Transport and Targeting

Detailed mechanisms of exosome trafficking, targeting as well as the modes of internalization are still not well understood. It is known that secreted exosomes can

either be rapidly internalized by neighboring cells or travel via body fluids to deliver their informational cargo to distant cells or possibly remove it from the organism. Despite the lack knowledge of detailed mechanisms concerning exosomal interaction with target cells, some reports suggest that lipid rafts and exosomal membrane proteins are directly responsible for their trafficking, mode of interaction with cells and dictate their uptake specificity. In several studies, dendritic cells and macrophages were designed as the major recipients of EVs, for example, activated T cells bind DC-derived EVs through Lymphocyte function-associated antigen 1 (LFA-1). α4 Integrin expression in Tspan8-expressing tumor cells redirects their secreted EVs to endothelial and lymph node stromal cell lines, whereas fibroblasts are the major target of non-α4-bearing EVs. A cardiomyocytic cell line captures mesenchymal-derived EVs. DC-derived EVs engineered to present a surface receptor containing an amino acid sequence recognized by receptors specifically expressed on either neuronal or muscle cells were readily bound to a neuron or muscle cell line in vitro.[89, 93]

Isolation and Characterization of Exosome

Exosomes can be isolated from any biological fluid or from the supernatant of cells grown in a media free of exosomes. Experimental procedures used to purify exosomes are mostly based on its unique size and density. The most rigorous and accepted protocols involve clearing the sample fluid by sequential centrifugations and pelleting the exosomes at 100 000 to 110 000g, followed by resuspensions and repelleting. For purer preparations, a sucrose gradient is used, because exosomes float at a density that ranges from 1.13 to 1.19 g/mL. Recently, several alternate methods,

such as microfluidic devices, antibody-coated magnetic beads, and precipitation-based, filtration-based isolation, have been developed[94].

Initial characterization of exosomes is typically based on electron microscopy analysis because their size, <100 nm, is below the resolution of a light microscope. Beside electron microscopy, both dynamic light scattering analysis and nanoparticle tracking analysis (NTA) allow the determination of the size of exosomes; nanoparticle tracking analysis also allows the determination of concentration. Further characterization of isolated exosomes requires complementary biochemical (immunoblotting and flowcytometry), mass spectrometry, and imaging techniques[40, 95].

Exosome Composition and Cargo

Exosomes have been shown to carry a unique cargo of lipids, proteins, and RNAs, which are often distinct from the cell of its origin. Because of its endosomal origin, all exosomes contain membrane transport and fusion proteins (GTPases, annexins, flotillin), tetraspannins (CD9, CD63, CD81, CD82), heat shock proteins (HSC70, HSP90), proteins involved in multivesicular body biogenesis (Alix, TSG101), as well as lipid-related proteins and phospholipases[89]. Additionally, exosomes also contain unique molecules specific to their cells of origin. It is this type, variety and abundance of donor cell specific cargo that is a topic of extensive studies, in hopes to shed light on their potential application for development of diagnostic and therapeutic tools. The exact pathways which regulate the packing of exosomal content do not appear to be a random collection of cytoplasmic proteins and nucleic acids from donor cells. For example, certain miRNAs are enriched in exosomes relative to

cells, indicating that miRNAs can be sorted into exosomes. It has been shown that KRAS may play a role in miRNA sorting into exosomes[96]. Exosomes from mutant KRAS colorectal cancer cells show a distinct miRNA profile compared to wild type cells. Furthermore, inhibition of nSMase caused cellular accumulation of certain miRNAs in KRAS mutant, but not wild type cells. Another study has shown that overactive mutated KRAS inhibits localization of the RISC component Argonaute 2 (Ago2) to MVBs and decreases Ago2 secretion in exosomes[97]. Valadi et al identified many exosomal components not expressed in the cytoplasm of donor cells, indicating a highly regulated and selective cargo sorting mechanism that delivers and guides specific intracellular miRNAs into exosomes[98]. Similar conclusions were made based on the observation that miRNA profiles of exosomes and their parent cells can significantly differ from one another[96]. Lipids may also be important for sorting of specific proteins into exosomes, for example sphingosine 1-phosphate (SP1), a lipid formed by the phosphorylation of sphingosine by sphingosine kinase 1 (Sphk1) and 2 (Sphk2), has been shown to regulate cargo (such as CD63, CD81 and flotillin) sorting into exosomes via inhibitory G protein (Gi)-coupled S1P receptors located on MVB membranes[99].

Interestingly, the profiles of exosomes from same cell types can be significantly altered based on physiological state of cells during synthesis. Quantity and quality of constitutively secreted exosomes is regulated by the physiology of the donor cells as well as by stimuli such as calcium, mitogens, cytokines and stress. Our lab compared the exosome content of EPCs exosomes generated from wild type, IL-10 knockout and diabetic mice, and found the drastically different expression pattern of

RNAs and proteins[49]. And this alteration in exosomal content partially explains the dysfunction of exosomes under high glucose and inflammatory stimulus.

As for my project, there are two molecules enriched in IL-10 knockout EPC exosomes chosen for further study, miR-375 and integrin-linked kinase (ILK). Their brief introduction is as follows:

miR-375

The discovery of microRNAs (miRs) has opened a new approach regarding regulation of cellular processes such as proliferation, differentiation, cell metabolism, apoptosis and angiogenesis. Initially, miR-375 was considered to be a pancreatic islet-specific miRNA and its role in regulation of glucose homeostasis was demonstrated[100]; further expression profiling studies revealed that miR-375 is widely expressed in other normal tissues[101]. By today, there is an increasing evidence that miR-375 is important cancer-related miRNA; miR-375 was shown to be downregulated in tumor tissues and served as tumor suppressor miRNA[102, 103].

As for stem cell functions, miR-375 generally favors the differentiation of stem cells and enhance stem cell therapeutic efficiency in different disease. In diabetic studies, overexpression of miR-375 promotes the differentiation of insulin generation cells from both MSCs and iPSCs, greatly improve therapeutic outcome[104]. In scar formation, transplantation of miR-375-expressing MSCs significantly reduced the fibrosis in the scar region of the mice, possibly through reduction of reactive oxygen species (ROS), suppression of transition of myofibroblasts from fibroblasts, and increases in hepatic growth factor (HGF)[105]. miR-375 also promote osteogenic differentiation of MSCs and improve bone regeneration[106]. However, He L et.al found

miR-375 inhibits HGF-elicited migration of MSCs through downregulating the expression of Pyruvate Dehydrogenase Kinase 1(PDK1) and suppressing the activation of Akt, as well as influencing the tyrosine phosphorylation of focal adhesion kinase (FAK) and paxillin and FA periphery distribution, which further affect MSC based therapy[102]. Our previous study found miR-375 expression is significantly up regulated in EPCs upon exposure to inflammatory/hypoxic stimulus. IL-10 KO mice display significantly elevated miR-375 levels. miR-375 knock down in EPCs significantly improved the survival and retention of transplanted EPCs in MI mouse model, anti-miR-375 therapy in MI mouse study reduced inflammatory response, decreased cardiomyocyte death, improved LV function, and enhanced angiogenesis through PDK-1/AKT signaling[107].

Moreover, many studies revealed the unfavorable role of miR-375 in heart disease. Increased miR-375 expression was identified in mice hearts after acute MI and in myocardium of patients with heart failure[107]. Inhibition of miR-375 was shown to enhance angiogenesis and reduce inflammatory response after MI that reflected in limiting infarct size and preserving post-MI cardiac function and integrity in mice. In murine macrophage cell line and in murine MI model miR-375 knockdown promoted M2 polarization and enhanced cardiac recovery post MI[108]. However, there is also study could not repeat the previous study and found miR-375 presented low expression in MI patients[101].

Despite the inconsistent role of miR-375 in both stem cells and heart disease, our NGS analysis of IL-10KO EPC exosomes found high level of miR-375 expression which is consistent with previous studies. And using antimir-375 therapy we

successfully inhibited miR-375 expression in exosomes and enhanced exosome mediated angiogenesis[49].

Integrin-Linked-Kinase (ILK)

Integrin-linked kinase (ILK) is a widely expressed serine/threonine protein kinase that binds to the cytoplasmic tail of β integrins, linking cell–matrix interactions to signals regulating cytoskeletal remodeling and cellular processes such as growth, proliferation, survival, and differentiation. ILK functions as a molecular scaffold, assembling multiprotein complexes that maintain the integrity of integrin adhesion points. ILK also plays a central role in transducing biochemical signals initiated by cell–matrix interaction. Aside from cell matrix interaction with integrin, ILK can be activated by inflammation stimulus (LPS, TNFα, etc.) through PI3K pathway, then activated ILK triggers NFκB signaling and promotes pro-inflammatory gene expression as well as cardiac deleterious miRs such as miR-375[109].

ILK is widely distributed in mammalian tissues, but highest in the heart suggesting its essential role in cardiac function regulation[110]. Cardiac-specific ablation of ILK causes cardiomyopathy and sudden death in mice. ILK protein complexes are found in the sarcomere, which is the basic contractile unit of myocytes. A natural inactivating mutation in the kinase domain of ILK disrupts ILK protein interactions in the sarcomere, causing a contractile defect in the zebrafish heart. Cardiac-specific expression of ILK in transgenic mice induces a hypertrophic program, pointing to ILK as a proximal regulator of multiple hypertrophic signal transduction pathways. Mayuko Nishimura et. al found relationship between cardiac aging with ILK in studies with Drosophila[111]. Decreasing ILK expression prevent typical dysfunction changes

resulted from cardiac aging and flies liver longer. ILK overexpression cause aging like cardiac dysfunction. Another study demonstrated that ILK is involved in fibrosis formation and inhibition of ILK attenuates interstitial fibrosis[112].

ILK plays important role regulating stem cell function in heart disease. ILK overexpressed MSC transplantation improves ventricular remodeling and cardiac function in pigs after MI[113]. And associated with increased angiogenesis, reduced apoptosis, and increased cardiomyocyte proliferation. Mao Q et.al found MSCs overexpressing ILK attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions[114]. Seung-Pyo Lee et.al found ILK is upregulated in endothelial cells under hypoxia condition and further control EPC homing to hypoxia and ischemic tissue. Overexpressing ILK in EPCs significantly improved blood flow recovery and neovascularization in high limb ischemia model[115, 116].

In our project, we found ILK is highly enriched in IL-10KO EPC exosomes and is responsible for stimulating inflammation response in recipient cells through NF-κB activation. Blocking ILK expression in exosomes successfully attenuated inflammation response. ILK knockdown exosome therapy in MI mouse study improved cardiac function.

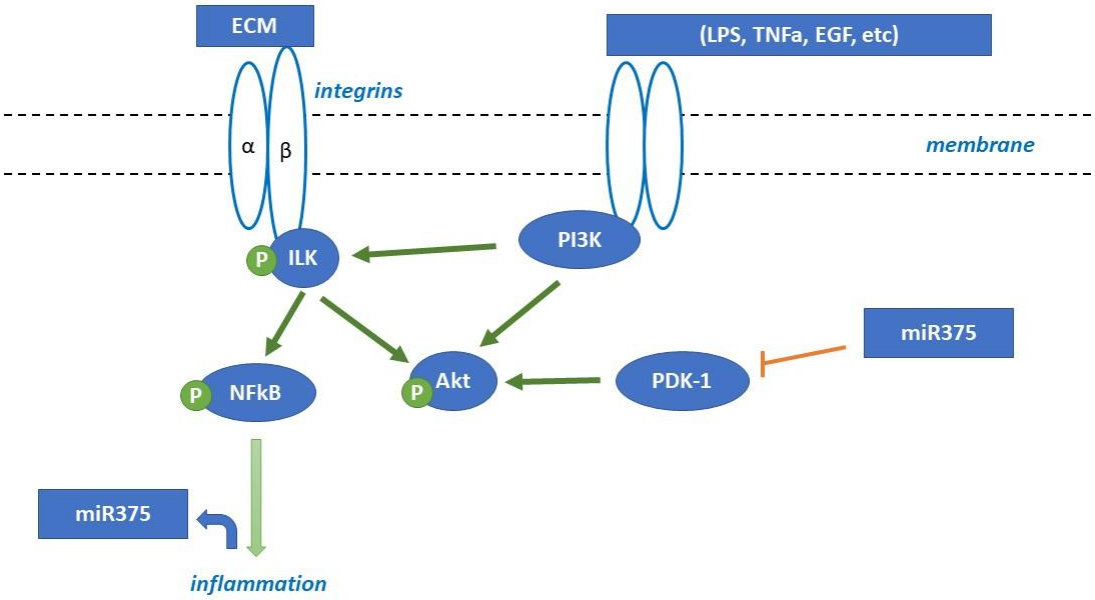


Figure 4. ILK activation pathway and interaction with miR-375.

ILK can be activated through interaction between extracellular matrix with integrin or inflammatory stimulus through PI3K. Activated ILK phosphorylate p65 subunit of NFκB, induce NFκB nuclear translocation and increase downstream inflammation response related gene expression as well as miR-375 expression. miR-375 has been revealed inhibiting cell survival Akt pathway through directly targeting PDK-1.

Exosome in Cardiovascular Disease

Intercellular communication mediated by extracellular vesicles is crucial for preserving vascular integrity and in the development of cardiovascular and other diseases. Many published reports suggest that major cardiovascular and metabolic pathologies like coronary artery disease, myocardial infarction, heart failure, and diabetes are highly influenced by the exosome directed transfer of molecules. There

are two major ways exosomes are involved in cardiovascular disease. First is the exosome secreted by injured cells. Exosomes act as vectors for cell communication and deliver out the information of cell injury or disease and recruit stem cells for repair and regeneration. Second is the exosome secreted from stem cell, they pass RNAs and proteins to recipient cells and modulate relative biological process and rescue or enhance recipient cell function.

Evidence from multiple studies suggest that cardiomyocytes secrete exosomes that shuttle proteins and genetic information to other cells. In one of the earliest studies, Gupta and Knowlton demonstrated that highly differentiated adult cardiomyocytes, generally not considered a secretory cell type, release a cytosolic protein, HSP60, within exosomes and cause damage to surrounding cardiomyocytes via activation of apoptosis signaling[117]. A recent work from the same group demonstrated that pathological changes in the environment, including fever, changes in pH, hypoxia, and ethanol treatment, alter the rate of exosomes secretion and the protein content of exosomes from cardiomyocytes[118]. Waldenström et al detected nucleic acid–containing exosomes in the media of cultured cardiomyocyte cell line HL-1, which could reprogram the fibroblast gene expression[119].

Exosome content alteration indicate potential disease and be used as biomarkers. Recently, it was reported that the levels of muscle-specific miRs increased in the plasma or serum of patients with MI. Kuwabara et al demonstrated that muscle-specific miR-1 and miR-133a are increased in the serum of patients with acute coronary syndrome, and that these miRNA levels correlate with serum cardiac troponin T levels[120]. Cheng et al detected significantly higher levels of miR-1 and

miR-208 in exosomes from the urine of acute MI patients and in the circulating blood of rats after acute MI[121]. Furthermore, a cardioprotective miR-214 has been shown to be upregulated in the heart after ischemia and altered in the plasma from coronary artery disease patients, indicating the severity of the disease. Zampetaki et al detected miR-126 to be part of a miRNA signature associated with MI in general patient population[122].

Besides the role in information delivery, stem cell exosomes have independent repair and regeneration ability in cardiovascular disease. It has been widely accepted that beneficial effects of stem cell therapy in cardiovascular disease is largely due to paracrine effects. Many animal studies including the findings from our lab supported the idea. In a very first demonstration that stem/progenitor cell derived exosomes can mimic angiogenesis responses just like their parent cell of origin, we reported that exosomes from human CD34+ hematopoietic cells augment angiogenesis both in vitro and in vivo[123]. Furthermore, we demonstrated ex vivo modification of human CD34+ cells with sonic hedgehog can preserve cardiac function in face of ischemic injury. Importantly, the cardio-protective effect of CD34+ cells is mediated by Shh packaging in exosomes produced by the CD34+ cells that not only protected existing cardiomyocytes but enhanced angiogenic response of the heart. Moreover, exosomes derived from MSCs were demonstrated to enhance myocardial viability and adverse remodeling by increasing ATP levels, reducing oxidative stress and promoting AKT activation in a myocardial infarction model[124]. Recently, we have shown that mouse embryonic stem cell exosomes augment myocardial repair by activating cardiomyocyte cell cycle re-entry[125].

In general, mounting evidence suggests that exosomes released from a damaged or diseased heart could be a potential intercellular communicator and carry signaling molecules to activate distant organs such as bone marrow. RNAs and proteins packed in those exosomes could be potential biomarkers indicating specific disease. Stem cell derived exosomes preserve their repair and regeneration ability and will be novel cell free therapeutic strategy for cardiovascular disease.

Rationale and Objective for this study

Stem cells hold great promise for potential clinical therapeutic strategies. The ability of injured myocardium to recruit extra-cardiac stem cells like bone marrow-derived stem/progenitor cells (e.g.EPC) is critical to aid in myocardial repair and regeneration. Increasing evidence from animal models of experimental ischemic injuries[126], including those from our labs[127, 128], suggest that EPC participate in the process of neo-vascularization and tissue repair leading to enhanced recovery of ischemic myocardium[129]. Furthermore, clinical trials involving bone marrow derived progenitor cell transplantation for ischemic myocardium has confirmed this possibility[129]. Despite these encouraging data, however, a great deal remains unknown regarding the adverse effects of ischemic tissue (hostile microenvironment) like inflammation and hypoxia on the survival of the mobilized/transplanted stem cells and the functional properties of EPCs during angio/vasculogenesis and tissue repair. Rheumatoid arthritis patients with high serum levels of TNFα, a potent pro-inflammatory cytokine, was associated with significantly lower EPC counts as compared to normal counts in patients treated with TNFα blocker[130]. Our preliminary studies established the cardio-protective role of anti-inflammatory cytokine, interleukin-10 (IL-10) in mouse models of acute myocardial infarction (AMI) and pressure-overload[131, 132]. We found recombinant IL-10 treatment enhances the benefits of EPC-mediated myocardial repair[24, 107]. However, EPCs derived from IL-10 knockout (IL-10 KO) mice are functionally impaired in mobilization, angiogenic capacity and post-MI repair ability. Apoptosis and poor retention of

transplanted EPCs leads to failed neovascularization, which itself is strongly associated with poor cardiovascular prognosis in stem cell-based therapy[24]. These studies provide solid evidence for the deleterious effects of inflammation on EPC survival and function, therefore, there is a critical need for the development of alternate strategies to enhance efficiency of cell-based therapies. Stem cell/EPC derived exosomes provide one such alternate cell-free therapeutic modality**.** Novel, non-traditional use of cell-free components of stem cells such as exosomes, loaded with stem cell-specific miRs and proteins may allow for harnessing the regenerative power of these cells to augment and modulate endogenous protection repair processes in the ischemic myocardium. Our proposed studies therefore put-forth a novel concept and significant step-forward.

Exosomes as functional paracrine units of stem cells: Exosomes are small micro vesicles secreted by different cells and have been the subject of intense research in recent times. Exosomes were first described in sheep reticulocytes 50 years back[133, 134] have been since then described in a number of cells types such as T-cells[135], dendritic cells[136], mast cells[137], tumor cells[138], cardiovascular lineage cells[123], sperm[139] and various stem cells type[140, 141]. The function of exosomes is not fully known but they are believed to be important for inter-cellular communication[142], immune-modulation[143] and as biomarkers for disease progression[144]. Recent reports, including those from our laboratories, have suggested that exosomes secreted by different stem cells enhance post-injury cardiac repair[125]. In a very first demonstration that stem/progenitor cell derived exosomes can mimic angiogenesis responses just like their parent cell of origin, we reported that exosomes from human CD34+ hematopoietic cells augment angiogenesis both in vitro and in vivo[123]. Furthermore, we demonstrated ex vivo modification of human CD34+ cells with sonic hedgehog can preserve cardiac function in face of ischemic injury[145]. Importantly, the cardio-protective effect of CD34+ cells is mediated by Shh packaging in exosomes produced by the CD34+ cells that not only protected existing cardiomyocytes but enhanced angiogenic

response of the heart. Since our early reports, a number of studies, including additional studies from our laboratories have established that exosomes derived from a wide variety of stem cells including mesenchymal stem cells, resident cardiac progenitor cells as well as pluripotent stem cells possess ischemic tissue reparative properties and augment ischemic myocardium/other tissue repair and regeneration following ischemic insult[146, 147]. The mechanism of action of exosome mediated repair processes largely include transfer of exosomal cargo including parent cell specific microRNAs (miRs), mRNA and proteins to the target cell/tissue resulting in several protective processes[148]. These processes include cell survival, cell protection and proliferation, augmentation of protective signaling pathways, cell cycle activation, epigenetic modulation, immune regulation and activation of resident stem cell populations[149]. Exosomes derived from MSCs were demonstrated to enhance myocardial viability and adverse remodeling by increasing ATP levels, reducing oxidative stress and promoting AKT activation in a myocardial infarction model[150]. Similarly, exosomes secreted by cardiomyocyte progenitor cells were reported to stimulate migration of endothelial cells and protect ischemic myocardium from ischemia/reperfusion injury validating the cardio protective effect of stem cell derived exosomes[140]. Recently, we have shown that mouse embryonic stem cell exosomes augment myocardial repair by augmenting resident cardiac progenitor cells (CPC) proliferation and differentiation as well by activating cardiomyocyte cell cycle re-entry[125]. Very interestingly, a recent study has demonstrated that inhibition of exosome secretion in cardiosphere derived stem cells (CDC) renders them ineffective for myocardial repair process[151]. Since these same cells have been repeatedly shown to augment post-MI myocardial repair in multiple pre-clinical and phase I clinical trial, this new data suggests that exosomes are the functional component of these stem cells. When combined with available stem cell-exosome studies for myocardial repair in literature, two things become amply evident:1) stem cell exosomes mimic reparative properties of their parental stem cells and 2) inhibition of exosome secretion renders stem cell ineffective suggesting exosomes as major paracrine mediators of stem cell function. However, the exact

molecular mechanisms of exosome action are poorly understood and may differ depending upon of parental cell population, exosomal content and modulation of those contents by various stimuli. Our studies are uniquely placed to further the understanding of mechanisms that mediate stem cell exosome function and dysfunction are therefore a significant step forward.

Exosomes are not created equal: Exosome therapy may represent a multi-faceted strategy for promoting regeneration and repair in the heart after pathological damage. However, recent excitement over these powerful cell-free components of stem cells must be channeled into meaningful research efforts towards fully understanding the role of these micro-vesicles in cardiac repair. The recent evidence on stem cell exosomes as potential therapeutic modality is exciting since exosome could possibly be used as a surrogate for the stem cell-based therapy and this regimen may overcome the roadblocks identified with the use of cell themselves. However, several important questions need to be answered before stem cell exosomes could potentially be employed in clinic. The overarching hypothesis driving our studies is that exosomes, even when derived from same defined stem cell population (EPC as model stem cells in this project), are not created equal- and the constitution of their contents as well as their biological activity is largely dependent upon physiological state of the parental cell of origin[152]. Systemic inflammation, one of the major symptoms presented in patients with cardiac disease, is an important stimulus causing EPCs functional deficiencies[153-155]. It is well established that stem cells obtained from patients with systemic inflammation have vastly reduced reparative activity[154]. Our preliminary studies also revealed the same results, by using IL-10 KO mice as a model of systemic inflammation, we reported that EPCs from IL-10KO mice have diminished angiogenic activity and depressed post-MI repair ability[24]. Since the therapeutic efficiency of EPCs is largely due to paracrine effects, especially exosomes, it is reasonable to predict that EPCs under inflammatory stimuli secrete dysfunctional exosomes by altering miR/protein exosomal contents, and finally compromise exosomal repair ability in ischemic cardiac tissue. However, it remains unknown

how systemic inflammation alter both the contents and the function of stem cell-derived exosome and whether exosomes could be engineered to deliver proteins/miR etc. that are present in healthy stem cells but are dysregulated in diseased stem cells. Our studies tested the mechanisms of EPC-derived exosomal dysfunction under inflammatory stimuli, and we provide novel information for possible rescue and/or alteration of these exosomes and hopefully towards designing "super-exosomes" for possible clinical applications.

Overall significance of our studies: Cell based therapy for myocardial repair is limited by low retention, low viability after transplantation, and adverse effects of hostile microenvironment of ischemic myocardium. Data from our lab have raised exciting possibility that the benefits of cell therapy can be replicated in an exosome based novel cell-free system. However, a few questions remain to be answered before potential translational application of exosomes in the clinic. These questions include: 1) whether exosomes obtained from BM-EPCs under inflammatory stress are as functionally competent as those from WT mice? Whether exosomal function/dysfunction changes the miR/ protein contents of the exosomes? Whether loss and gain in function of identified miR/proteins/signaling molecules in the deficient cells can rescue exosomal function? Thus, our studies are of great significance since we partially answered many of these questions in detail and we believe our studies not only significantly enhanced the fundamental biological insights to the mechanisms of exosome reparative function but also paved way for clinical applications of stem cell exosomes.

CHAPTER 3

INTERLEUKIN-10 DEFICIENCY IMPAIRS REPARATIVE PROPERTIES OF BONE MARROW-DERIVED ENDOTHELIAL PROGENITOR CELL EXOSOMES

Study aims

Aim1: IL-10 deficiency alters EPC derived exosome function.

Our previous studies revealed that IL-10 is essential for EPC cardioprotective functions. As exosomes reflect the characteristics of parental cells, we hypothesized IL-10 deficiency impairs EPC derived exosome function. We designed a series of *in vitro* (tube formation, cell migration, TUNEL assays) and *in vivo* (MI mouse model with exosome treatment) studies to compare the functions of wild type and IL-10 knockout EPC derived exosomes.

Aim2: IL-10 deficiency alters EPC derived exosome content.

Exosome functions are dependent on exosome content. We especially explored the RNA content with next generation sequencing and protein content with mass spectrometry. We further explored miRs and kinases manipulation in exosomes in aim3.

Aim3: exosome function can be enhanced or rescued through exosome content modulation.

We chose two candidates from the results in aim2, miR-375 and integrin-linked kinase for further study, both are highly enriched in IL-10KO-EPC exosomes. We used anti-miR and siRNA targeting ILK as strategies for miR-375 and ILK knockdown in IL-10KO-EPCs and derived exosomes. We compared modulated exosome function through a series of *in vitro* and *in vivo* experiments.

Aim4: ILK mediates exosome induced inflammation response.

ILK is the upstream of NFκB-pathway and can be activated through either direct cell matrix interaction with integrin or inflammation stimulus through PI3K pathway. NFκB activation triggers pro-inflammatory gene expression as well as miR-375 upregulation. We hypothesized overwhelmed inflammation response is mediated by ILK enrichment in exosomes and can be attenuated through ILK knockdown. ILK knockdown not only decreased inflammation response in recipient cells but also reduced deleterious miRs (such as miR-375) delivered by exosomes.

Materials and methods

Vertebrate Animals and reagents

All experiments conform to the protocols approved by the Institutional Animal Care and Use Committee at Temple University. Eight-week-old wild-type (WT) and IL-10 knockout (KO; $IL10^{tm1Cgn}$) male mice of C57BL/6J background were procured from Jackson Research Laboratory (Bar Harbor, ME) for EPC isolation. Ten-to-twelve-week-old Wild-type (WT) male mice of C57BL/6J background were procured from Jackson Research Laboratory (Bar Harbor, ME) for myocardial infarction surgery. Lipopolysaccharide (LPS) was obtained from Sigma Aldrich Inc (St. Louis, MO). Recombinant Mouse TNF-alpha (aa 80-235) Protein was obtained from R&D Systems Inc.

Cell isolation, culture, supernatant collection and treatment.

EPC isolation, ex vivo expansion and culture were performed as previously described[41]. Bone marrow extracted from the femurs, tibiae and hip-bones of 8-10-week-old C57BL/6J or IL-10 knockout mice were stained with labeled antibodies against lineage markers (CD3e(145-2C11), CD11b(M1/70), B220(RA3-6B2), Ter119(Ly76),

Ly6G/C(RB6-8C5)) Sca-1 (D7) and CD31 (MEC13.3), then sorted to retain lineage-negative cells on a FACS Aria-II (BD Biosciences). Sorted cells were cultured in phenol red–free endothelial cell basal medium-2 (EBM-2, Clonetics) supplemented with exosome-free 10% FBS, vascular endothelial growth factor (VEGF)-A, fibroblast growth factor-2, epidermal growth factor, insulin-like growth factor-1, ascorbic acid, and antibiotics. Cells were cultured at 37 °C with 5% CO2 in a humidified atmosphere. After 4 days in culture, non-adherent cells were removed by washing with PBS, new medium was applied, and the culture was maintained through day 7. Notice that for exosome study, all Fetal Bovine Serum (FBS) used in cell culture are Exosome depleted FBS (ThermoFisher Scientific A2720801). For WT-EPC and IL-10KO-EPC, supernatant was collected daily from day 7 to day 10 for exosome isolation. For ILK-KD-EPC-Exo group, siRNA transduction was administrated on day 7 IL-10KO-EPCs and supernatant was collected on day 8, day 9 and day 10 for exosome isolation (Notice that for western blotting confirmation, we used 24h, 48h, 72h exosomes, however, there was observable cell apoptosis of EPCs after 48h of siRNA induction, for angiogenesis study and in vivo experiment, only 24h exosomes were used to minimize deleterious effect from EPC apoptosis). SVEC4-10 (ATCC® CRL-2181™) and Homo sapiens, umbilical vein/vascular endothelial cells (HUVECs, ATCC® CRL-1730™) were obtained from ATCC and cultured following manufacturer's instructions. Immortalized mouse cardiac endothelial cell (MCEC) line was purchased from CEDARLANE (CLU510) and managed according to the manufacture instructions. Supernatant was collected daily for MCEC-Exo group exosome isolation. For MCEC+ TNFα-Exo group, MCECs were treated with recombinant mouse tumor necrosis factor-α (TNFα) 100ng/ml for 24 hours, then washed with PBS and replenished with fresh media,

incubated for another 24 hours, then collected supernatant for exosome isolation. For ILKKD-Exo group, MCECs were first treated with 100ng/ml TNFα for 24hours, then washed with PBS, replenished with fresh medium, and conducted the transduction of siRNA, incubated for another 24hours, collected supernatant for exosome isolation. For experiments involving MCECs treated with exosomes, exosome number was 100-fold of cell number.

Exosome isolation and characterization

The exosome isolation, purification and storage was performed as previously described[156]. Collected exosomes were suspended in Phosphate-Buffered Saline (PBS), aliquoted and stored at -80 ℃or used immediately for experiments. The size and shape of exosomes were observed by electron microscopy supported by Electron Microscopy Resource Laboratory (EMRL) at University of Pennsylvania. Exosomes docking on Dynabeads (Sigma) coated with anti-mouse CD63 and CD81 was used to check exosome surface markers by flow cytometry. Three exosome markers, including HSP70(HSP 70/HSC 70 Antibody (W27): sc-24), CD63(CD63 Antibody (H-193): sc-15363) and Flotillin-1(Flotillin-1 Antibody (K-19): sc-16640) were verified by western blotting with antibodies from Santa Cruz. Exosome protein level was quantified with Pierce BCA Protein Assay Kit (Thermo Fisher) as manufacturer's instructions. Exosome particle number was quantified by NanoSight running Nanoparticle Tracking Analysis (NTA) software. The RNAs and proteins in exosomes were labeled and tracked by Exo-Glow (SBI) as manufacturer's instructions to check the delivery function of exosomes.

Migration assay

The migration of SVECs toward a gradient of growth factors was performed in a 24-well Transwell Chamber (8.0μm pore size, polycarbonate membrane, Corning Costar, Corning Incorporated Life Sciences, Acton, MA) as described earlier[131]. Briefly, SVEC culture media (DMEM with 10% exosome-depleted FBS, Life Technologies) was added to the lower compartment. SVECs (1×10^5 cells) in 200μL of OPTIMEM (Thermo Fisher Scientific) were added to the upper compartment and simultaneously treated with 10ug WT-EPC-Exo, IL-10KO-EPC-Exo or 20ul of PBS. After incubation at 37 °C for 18 h, the filters were removed and the cells that migrated through the pores of the membrane to the bottom chamber were stained with Giemsa stain (Sigma) and counted manually under phase contrast microscopy (Microscope model and manufacturer; ×10 magnification) in each well. Data are expressed as average number of cells per well that migrated through or invaded pores. All groups were studied at least in duplicate.

Tube formation assay

4×10^4 human umbilical vein endothelial cells (HUVECs) were plated on 130ul Matrigel (BD Falcon) per well in a 48-well plate and simultaneously treated with $4x10^6$ particle number of WT-EPC-Exo, IL-10KO-EPC-Exo, MCEC-Exo, MCEC+TNFα-Exo, ILKKD-Exo or 10ul of PBS. After incubation at 37°C in an atmosphere of 5% CO_2 for 16h, gels were observed by using a phase contrast microscope (×10; model & manufacturer). The total branch points were counted in each well and normalized against the PBS control group. Results are represented as mean +/- s.e.m. for three independent experiments.

Terminal deoxynucleotidyl transferase–mediated dUTP nick end-labeling (TUNEL) staining assay

200,000/chamber SVEC4-10 endothelial cells (SVECs) were seeded in chamber slides (Thermo Fisher Scientific) and simultaneously treated with 10ug of WT-EPC-Exo, IL-10KO-EPC-Exo, miR-375 modified EPC-Exo or 20ul of PBS. After 16h of culture, cells were stimulated with 100ng/ml LPS or 100nM H2O2 for 24h. Cells then were washed with PBS and fixed with 4% paraformaldehyde fixative (PFA). Survival of SVECs was determined by TUNEL staining as per manufacturer's instructions (Cell death detection assay, Roche, Indianapolis, IN). 4′,6-diamidino-2-phenylindole (DAPI) staining was used to count the total number of nuclei. Apoptosis of SVECs was assessed by counting the total number of TUNEL+ cells and DAPI+ cells in each slide and calculated as percentage of TUNEL+/DAPI+ per slide.

Exosome RNA and protein content analysis

100ug each of WT and IL-10KO-EPC derived exosomes solved in PBS (duplicates in each group) were sent to SBI for Exo-RNA Next-Generation Sequencing according to manufacturer's instructions. Deep sequencing data were analyzed with Maverix software provided by SBI. 50ug of WT and IL-10KO-EPC derived exosomes solved in PBS were sent to Quantitative Proteomics Resource Core (QPRC) in U Penn for mass-spectrometry to characterize and quantify proteins from exosomes.

Real time Polymerase chain reaction (PCR)

Expression levels of miR-375 were measured using quantitative miRNA stem loop RT-PCR technology (TaqMan miRNA assays; Applied Biosystems). This assay uses gene-specific stem loop RT primers and Taq-Man probes to detect mRNA or mature miRNA

transcripts. Transcription was performed using 2ug or 10 ng total RNA and the TaqMan miRNA RT kit (Applied Biosystems). Real-Time PCR was performed on an Applied Biosystems 770 Step One Plus using the TaqMan Universal PCR Master Mix, No AmpErase UNG (Applied Biosystems). The amplification steps consisted of initial denaturation at 95°C, followed by 40 cycles of denaturation at 95°C for 15 s and annealing at 60°C for 1 min. The TaqMan specific primer 18S or U6 small nucleolar RNA was used for normalization with the threshold delta-delta cycle method [Gene Expression Macro; Bio-Rad, Hercules, CA, USA].

miR-375 modulation in exosomes

30nM anti-miR-375 (Applied Biosystems) or a negative control (siRNA-NC, Applied Biosystems) was introduced using Lipofectamine™ RNAiMAX (Invitrogen) in IL-10KO-EPCs following the manufacturer's instruction, and the cells were transfected for 24h. Subsequently, the knockdown efficiency in EPCs was determined by RT-PCR assays.

siRNA knockdown experiments

Small interfering RNA (siRNA) sequences targeting mouse ILK was synthesized by Santa Cruz (sc-35667) or a negative control (siRNA-NC) was used at a final concentration of 100nmmol/L according to the manufacturer's instructions, and the cells were transfected for 24h, 48h or 72h. Subsequently, the knockdown efficiency in MCECs and IL-10KO-EPCs was determined by Western blot assays.

Preparation of cell lysates, exosome lysates and western blotting

EPCs, MCECs and exosomes lysates preparation and western blotting were conducted as previously described[49, 125]. As for antibody usage, beta-actin (sc-47778) was

used for cell loading control while flotilin-1 (sc-74566) and CD63 (sc-15363) were used for exosome loading control. ILK antibody was purchased from abcam (ab76468).

Induction of acute myocardial infarction (AMI)

Mice underwent surgery to ligate the left anterior descending coronary artery as reported previously[24] followed by administration of exosomes (WT-EPC-Exo, IL-10KO-EPC-Exo, ILK-KD-EPC-Exo, all 10^9 particles per group) or PBS intramyocardially into the left ventricular wall (border zone) at three different locations immediately after left anterior descending ligation. The sham group underwent the same open chest procedure without ligation. Each group has 10-15 animals for measurement. Heart tissue was harvested on day5 or day 30 after AMI for histological analysis.

Echocardiography (Echo)

Transthoracic two-dimensional M-mode echocardiography was performed using the Vevo770 (VisualSonics, Toronto, ON, Canada) equipped with a 30-MHz transducer as described previously[24].

Tissue preparation, TUNEL assay, immunohistochemistry and Masson's trichrome staining

Heart tissue preparation, TUNEL assay, immunohistochemistry and Masson's trichrome staining were performed as previously described[24, 157]. CD31 staining on week 4 heart samples were used for neovascularization detection.

Statistics

Statistical analysis is performed using Student t test. Comparison of 2 or more groups is performed by 1-way ANOVA or 2-way ANOVA with Bonferroni post hoc test.

$P < 0.05$ is considered statistically significant. Error bars represent ±SEM. Statistical analysis is performed using Graph Pad prism v 7.0 software.

Results

Characterization of EPCs from WT and IL-10KO C57BL/6J mice.

Our published studies described bone marrow derived EPCs as Lineage negative, Sca1+ and CD31+ [41] In our experiments, WT and IL-10KO mouse bone marrow cells was FACS sorted for the population of cells characterized as lineage Lin (CD11b, Ly6G/C, B220, CD3e, Ter119) negative, Sca-1+ CD31+, approximately 4% of total mononuclear cells in mice of both strains (Figure 5A). These cells were referred to as EPCs henceforth, and they showed spindle-like morphology (Figure 5B).

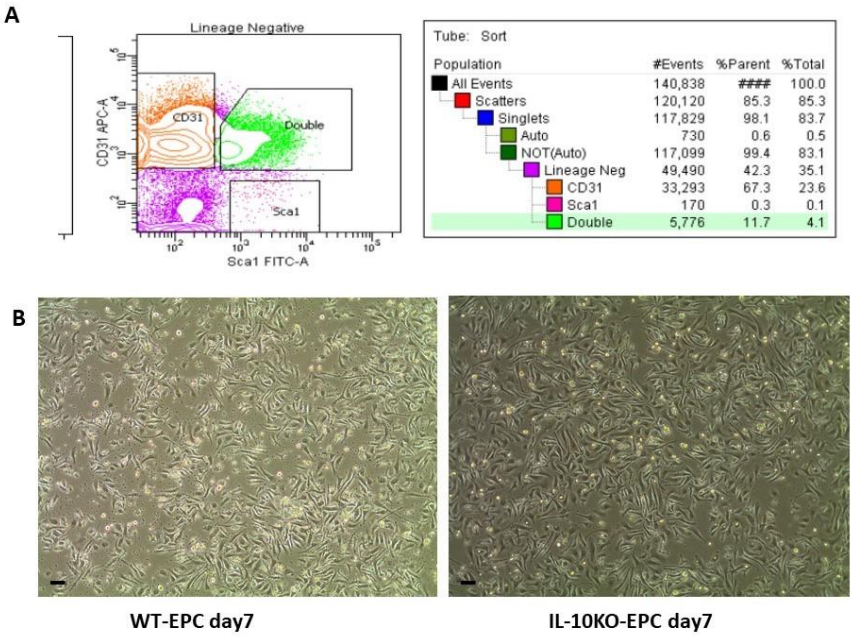


Figure 5. Characterization of EPCs from C57BL/6J mouse bone marrow.

(A) FACS sorting showed about 4% of bone marrow mononuclear cells are lineage negative, CD31 and Scar1 double positive. (B) The morphology of isolated EPCs showed a typical spindle-like shape. (scale bar: 100 μm)

Characterization of EPC-derived exosomes.

We harvested exosomes from the supernatant collected from cultured WT and IL-10KO-EPCs by differential centrifugation with sucrose cushion[156]. Electron microscopy and NanoSight analysis of WT and IL-10KO-EPCs exosomes exhibited a range of 30-100 nm particles (Figures 6A and 6B). Western blotting and flow cytometry results confirmed expression of exosome surface marker proteins including HSP70, flotillin-1, CD63 and CD81 (Figures 6C, D and E). Cellular uptake of both exosome RNA and exosome protein was confirmed using Exo-Glow kits (EXOC300A-1, SBI) that exclusively label exosome RNA with RFP and proteins with GFP. Exosomes were quickly internalized by cells (as early as 2-30 min after addition to the culture) and delivered both RNA and protein to the target SVECs (Figure 6F and G).

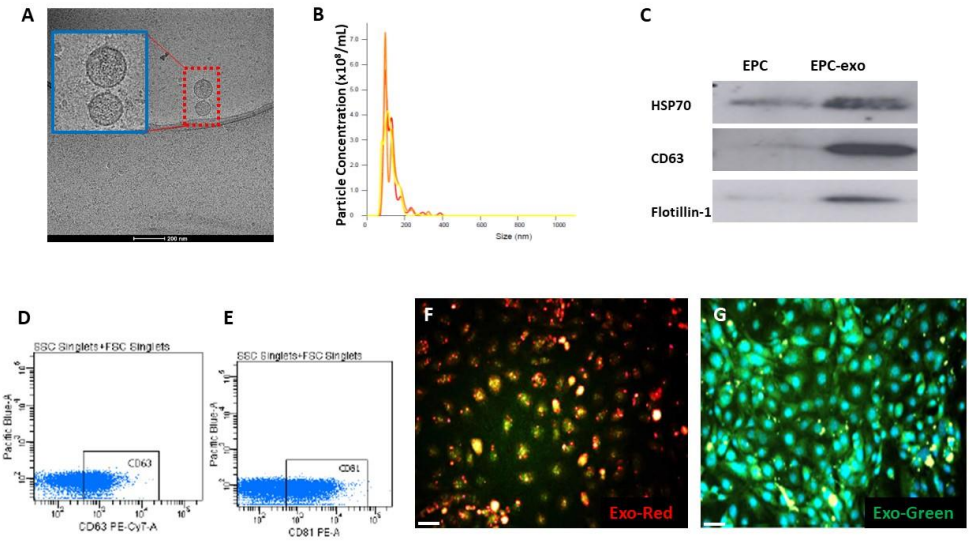


Figure 6. Characterization of EPC derived exosomes.

(A) EPCs secrete exosomes as evident from electron micrograph (inset magnified) and by expression of exosomal proteins by western blot (C) and FACS (D and E). (B) Size range of EPC derived exosomes measured with Nanosight. (F and G) EPC derived exosomes delivered RNA (tagged with RFP) and protein (tagged with GFP) to SVECs. (scale bar: 100 μm)

IL-10 deficiency impaired EPC-derived exosome function in vitro.

Our published study demonstrated functional impairment in EPCs obtained from IL-10KO mice[131, 132], we hypothesized IL-10KO EPCs secrete dysfunctional exosomes. We performed TUNEL, tube formation and cell migration assays with endothelial cells (HUVECs or SVECs) treated with exosomes collected from WT or IL-10KO EPCs. Our results showed that WT-EPC-Exo treatment significantly decreased LPS-induced endothelial cell apoptosis whereas IL-10KO-EPC derived exosomes showed decreased anti-apoptotic effects (Figures 7A and 7B). Further, in tube formation assay, IL10-KO-EPC-Exo treatment on human umbilical vein endothelial cell (HUVECs) inhibited tubular structure formation (Figures 7C and 7D). Both WT and IL-10KO-EPC-Exo treatment promoted cell mobilization, but less migrated cells observed in the IL-10KO-EPC-Exo treated group (Figures 7E and 7F). Our data revealed that under IL-10 deficiency, EPC-derived exosomes are dysfunctional in terms of preserving cell survival, promoting angiogenesis and cell mobilization.

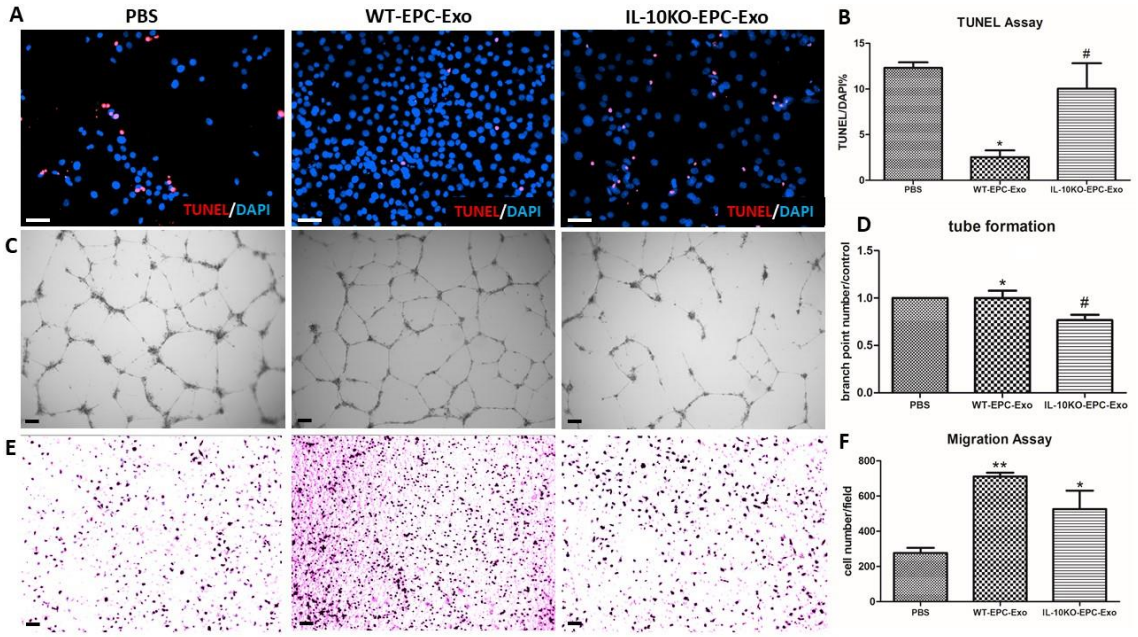


Figure 7. IL-10 deficiency impaired EPC-Exo *in vitro*.

(A) Compared to WT-EPC-Exo treated group, IL-10KO-EPC-Exo treated group showed more apoptotic cells (scale bar: 100 μm) (B) Quantification of TUNEL assay. (C)All three groups showed tube like structures. (scale bar: 100 μm) (D) IL-10KO-EPC-Exo treatment significantly inhibited tube formation. (E) Both WT and IL-10KO-EPC-Exo treatment promoted cell mobilization. (scale bar: 100 μm) (F) IL-10KO-EPC-Exo significantly decreased cell migration. *vs PBS group (*$p<0.05$, **$p<0.01$), #vs WT-EPC-Exo group (#$p<0.05$)

IL-10 deficiency drastically altered EPC exosome contents.

Several studies implicated that exosome contents (RNAs and proteins) are essential for exosome functions[152]. Therefore, we hypothesized that the functional differences between WT and IL-10KO-EPC derived exosomes are caused by content alteration. Exosomes isolated from WT and IL-10KO-EPCs were subjected to Next Generation deep sequencing (NGS) as well as mass spectrometric analysis. Our deep sequencing data identified various types of RNAs packaged in exosomes suggesting linkage to specific functions (Figure 8A). We further selected miRs for analysis and found miR-375 highly enriched in IL-10KO-EPC-Exo (Figure 8B). Proteomic analysis showed different expression pattern of proteins in WT and IL-10KO-EPC exosomes (Figure 9A). IL-10KO-EPC-Exo were enriched with proteins involving inflammation, apoptosis and angiogenesis (Figure 9B). These data provided evidence that IL-10 deficiency altered EPC derived exosome contents (RNAs and proteins).

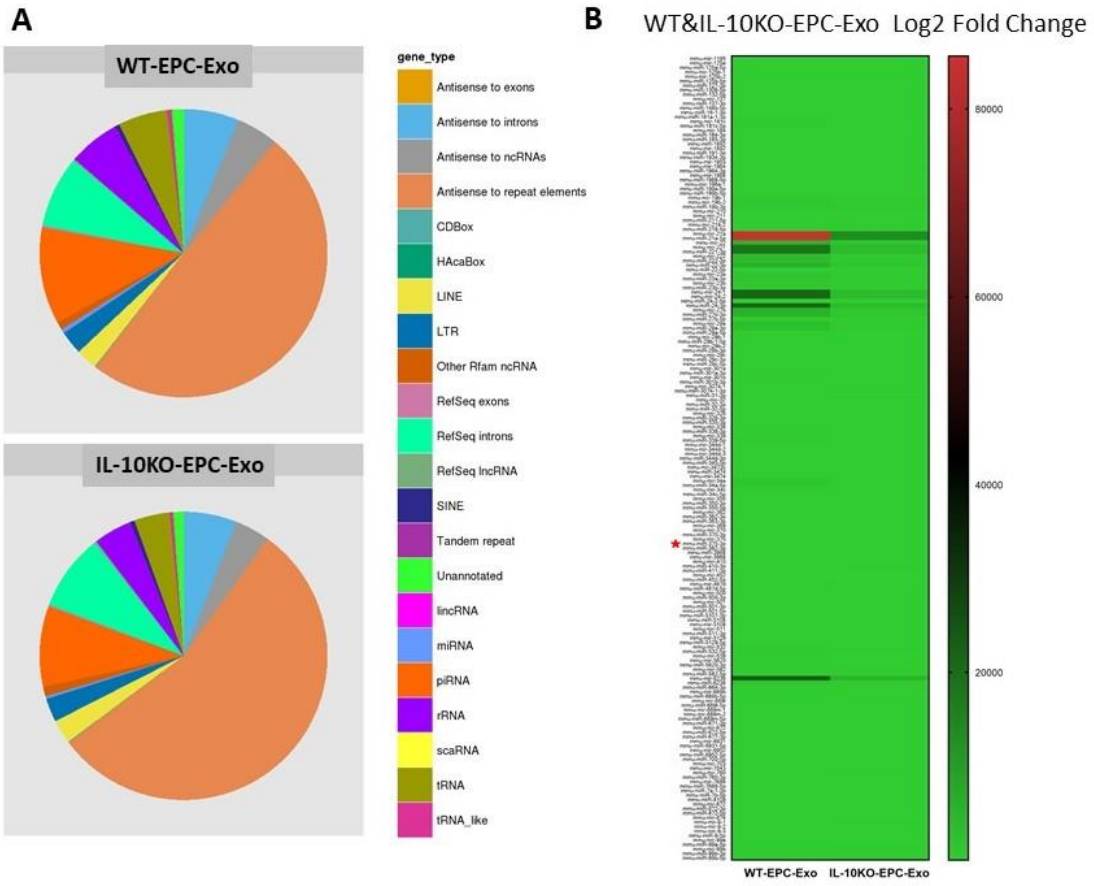
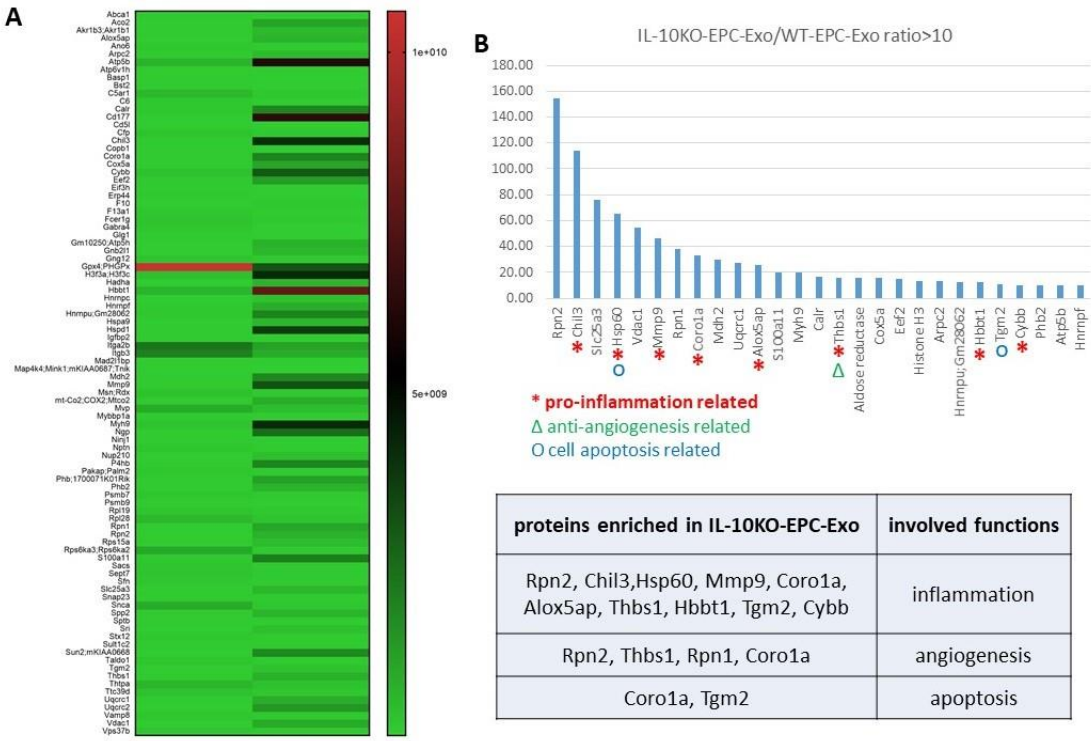


Figure 8. NGS analysis of RNAs in EPC derived exosomes.

(A) Pie graph showed EPC derived exosomes contain many types of RNAs and the ratio of different types of RNAs were different in WT and IL-10KO EPC derived exosomes. (B) Heat map of deep sequencing reveals WT-EPC-Exo and IL-10KO-EPC-Exo exhibit different miR expression pattern and miR-375 is enriched in IL-10KO-EPC-Exo.



proteins enriched in IL-10KO-EPC-Exo	involved functions
Rpn2, Chil3,Hsp60, Mmp9, Coro1a, Alox5ap, Thbs1, Hbbt1, Tgm2, Cybb	inflammation
Rpn2, Thbs1, Rpn1, Coro1a	angiogenesis
Coro1a, Tgm2	apoptosis

Figure 9. Proteomic analysis of proteins in EPC derived exosomes.

(A) Heatmap of proteomic analysis showed different expression pattern of protein content in WT-EPC-Exo and IL-10KO-EPC-Exo. (B) IL-10KO-EPC exosomes are highly enriched with proteins involving inflammation, angiogenesis and apoptosis.

IL-10KO-EPC-Exo dysfunction was partially mediated by miR-375

We have published that IL-10KO-EPCs and LPS-treated WT-EPCs expressed high levels of miR-375 [107]. Inhibition of miR-375 in IL-10KO-EPCs rescued their defective phenotype by enhancing the expression of PDK1 (a direct target of miR-375) and PDK1-mediated activation of PI3K/Akt pathway[107]. From NGS analysis of EPC exosomes, we observed enrichment of miR-375 in IL-10KO-EPC-Exo, which might be responsible for IL-10KO-EPC-Exo dysfunction. To modulate miR-375 levels in IL-10KO-EPC-Exo, IL-10KO-EPCs were transfected with miR-375 antagomir before exosomes were isolated. Quantitative RT-PCR analysis of exosomes confirmed that inhibition of miR-375 in IL-10 KO-EPCs significantly diminished miR-375 expression in IL10KO-EPC-Exo. (Figures 10A and 10B). TUNEL assay further showed that miR-375 knockdown rescued IL-10KO-EPC-Exo dysfunction in preserving cell survival (Figure10C to 10F). These experiments revealed the promising strategy of molecule manipulation in modulating exosome functions.

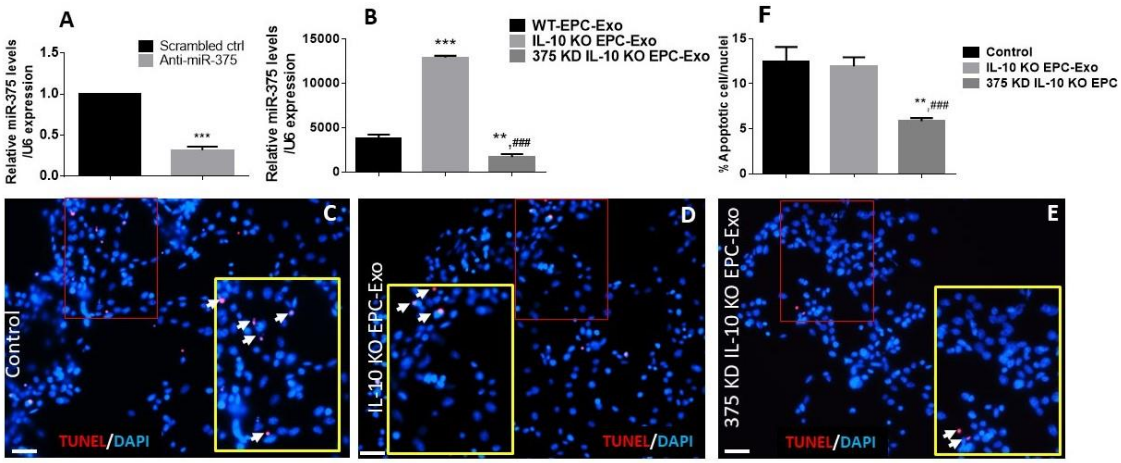


Figure 10. IL-10KO-EPC-Exo dysfunction was partially mediated by miR-375

(A) Knockdown efficiency of anti-miR-375. (B)IL-10KO-EPC-Exo expressed high levels of miR-375 and antimir strategy greatly decreased miR-375 expression. (C, D, E) miR-375 knockdown in IL-10KO-EPC-Exo rescued exosome function in preventing cell apoptosis. (scale bar: 100 μm) (F) Quantification of TUNEL assay showed significantly decreased apoptotic cells in miR-375 knockdown exosome treated group. *vs PBS group (**$p<0.01$, ***$p<0.005$); #vs WT-EPC-Exo group (###$p<0.005$).

IL-10KO-EPC-Exo showed compromised cardiac repair and regeneration ability in MI.

To assess therapeutic effect of EPC derived exosomes in myocardial infarction, WT-EPC-Exo and IL-10KO-EPC-Exo were intramyocardially administered in mouse MI model, whereas PBS was served as control. Left ventricular function was increased with WT-EPC-Exo treatment as evidenced by significantly improved ejection fraction and fractional shortening measurements 4 weeks after infarction (Figure 11A and 11B). TUNEL analysis of day5 heart showed decreased cell apoptosis in WT-EPC-Exo treated, but diminished effect observed in IL-10KO-EPC-Exo group (Figure 11C and 11D). Histological analysis of the heart 4 weeks post infarction indicated decreased scar size and enhanced capillary density in WT-EPC-Exo treated group. However, no left ventricular function improvement is observed in IL-10KO-EPC-Exo group and IL-10KO-EPC-Exo treatment even inhibited neovascularization (Figure 11E to 11H). Together these results provided evidence for compromised cardiac repair and regeneration ability of IL-10KO-EPC-Exo in MI model.

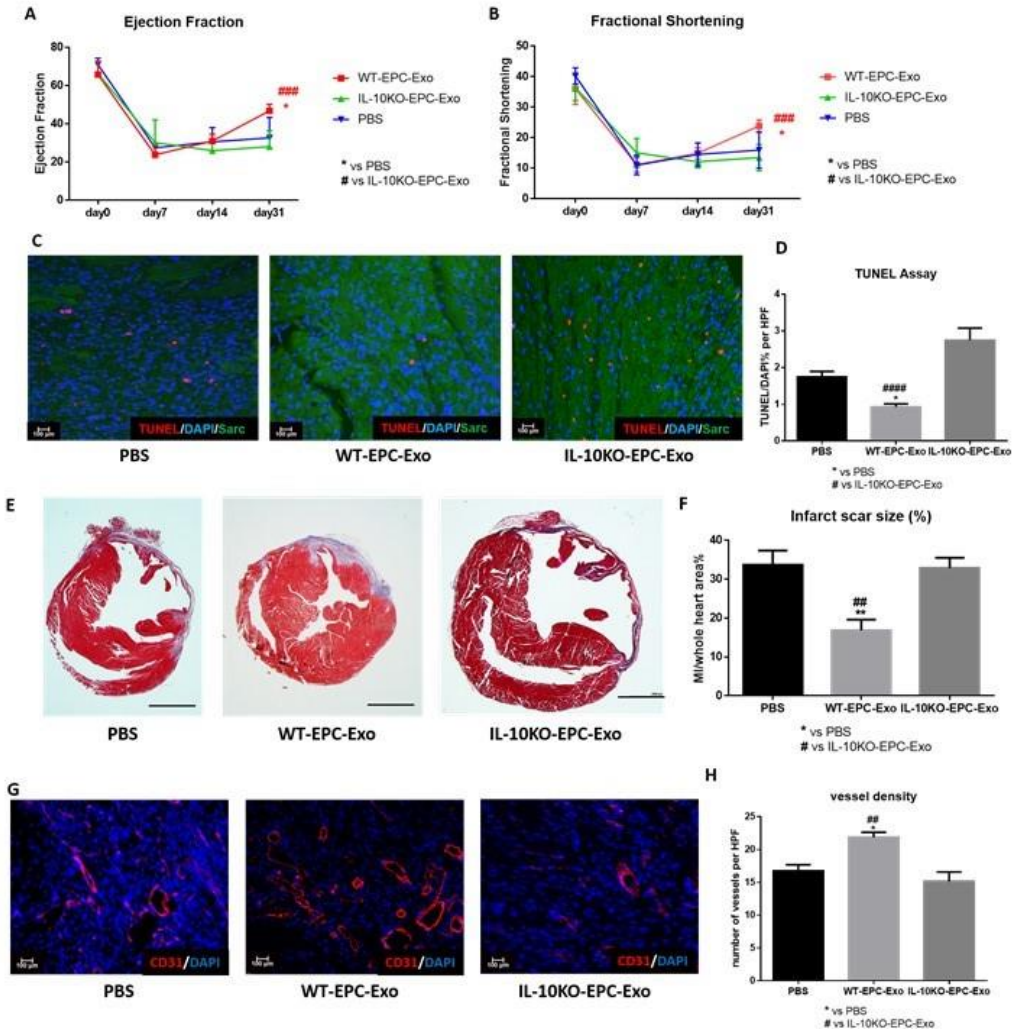


Figure 11. IL-10KO-EPC-Exo showed compromised cardiac repair and regeneration ability post-MI.

A and B, echo analysis of left ventricle cardiac function. **C and D,** TUNEL analysis on day 5 heart samples (scale bar: 10μm). **E and F,** Trichrome Staining on day 31 heart samples (scale bar: 2000 μm). **G and H,** CD31 staining for neovascularization detection (scale bar:100 μm). *vs PBS group (*p<0.05, **p<0.01); #vs IL-10KO-EPC-Exo group (##p<0.01, ####p<0.001).

IL-10 deficiency altered exosome content, ILK was highly expressed in both IL-10KO-EPC-Exo and MCEC+TNFα-Exo.

Proteomic analysis suggested WT-EPC-Exo and IL-10KO-EPC-Exo have different protein expression pattern (Figure12A). We particularly selected protein kinase candidates involved in inflammation pathway and chose ILK for further study (Figure 12B). Western blotting confirmed there was about 2 to 4-fold increase in ILK expression in both IL-10KO-EPC-Exo and MCEC+TNFα-Exo (Figure 12C and 12D). These data suggested that ILK was generally enriched in inflamed cell derived exosomes.

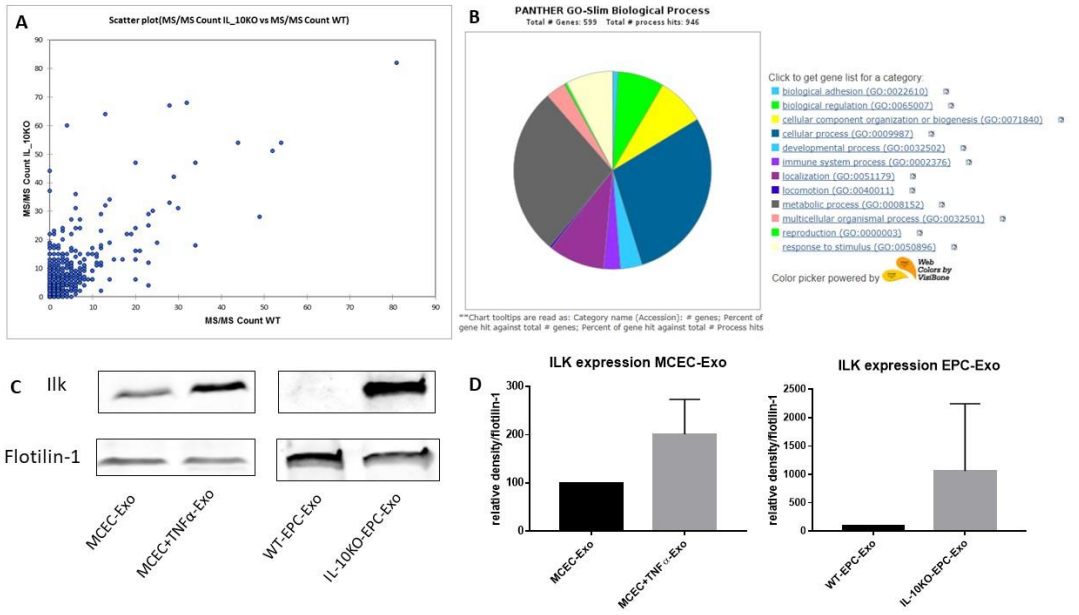


Figure 12. ILK enrichment in both IL-10KO-EPC-Exo and MCEC+TNFα-Exo

A, scatter plot showed different protein expression pattern in WT-EPC-Exo and IL-10KO-EPC-Exo. **B**, Gene Ontology analysis for EPC exosome proteins. **C and D**, western blotting analysis verified ILK enrichment in both IL-10KO-EPC-Exo and MCEC+TNFα-Exo.

ILK enriched exosome mediated NFκB activation in recipient cells.

To elucidate the role of ILK enriched exosome on inflammation response, MCECs were treated with MCEC-Exo, MCEC+TNFα-Exo, PBS or TNFα. Within short time treatment, MCEC+TNFα-Exo activated the nuclear translocation of NFκB (Figure 13A and 13B), phosphorylated NFκB p65 subunit (Supplemental Figure1), decreased iκBα expression (Figure 14A and 14B). Long time treatment increased the NFκB downstream gene expression (Figure 14C). Similar results observed in TNFα treated group which was designed as positive control. No significant differences observed between MCEC-Exo and PBS group. Collectively, these results indicated that inflamed exosomes deliver larger amount of ILK and activate NFκB pathway in recipient cells.

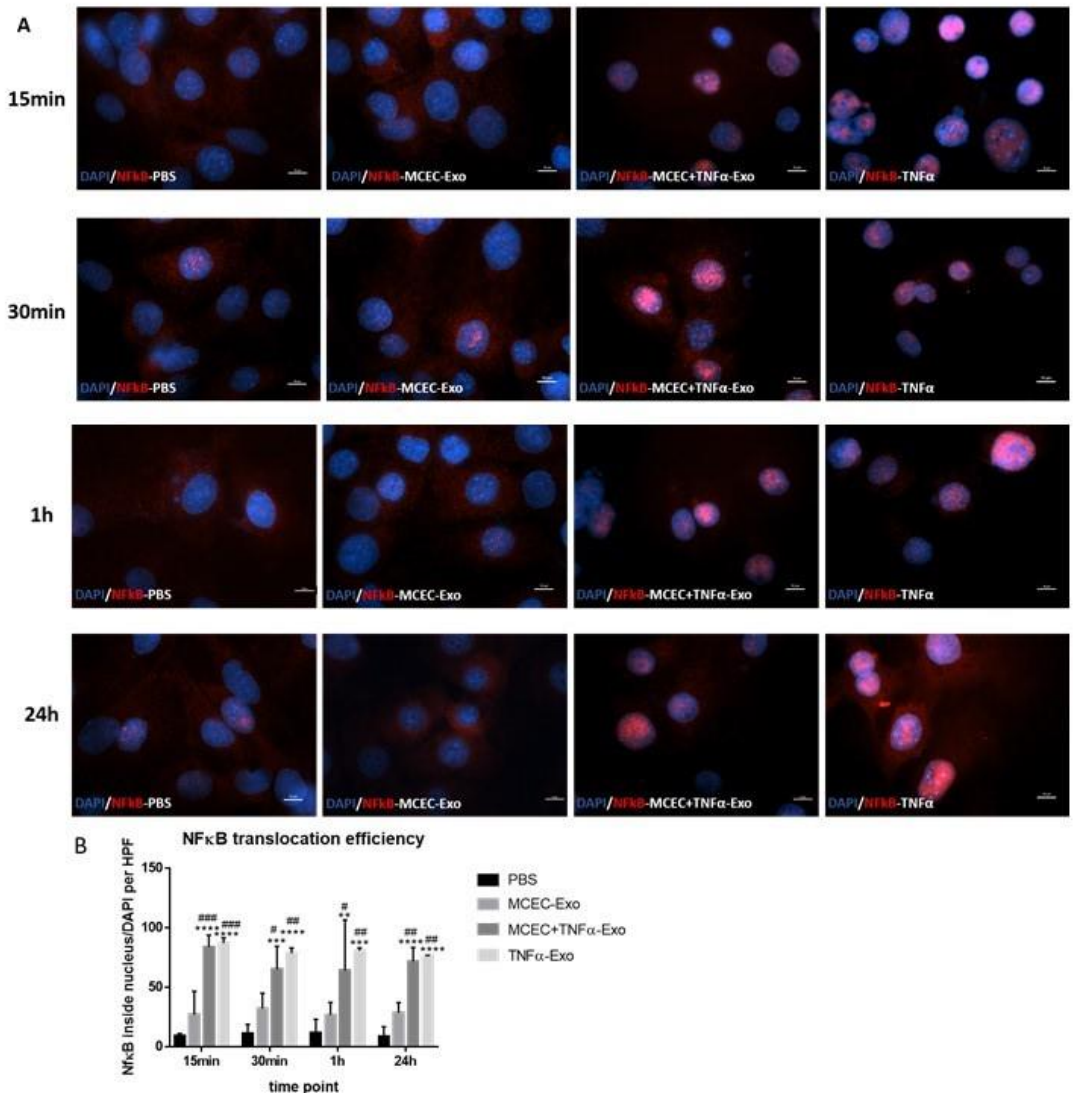


Figure 13. MCEC+TNFα-Exo activated nuclear NFκB translocation in recipient cells. **A and B**, NFκB staining analysis showed both MCEC+TNFα-Exo and TNFα treatment triggered NFκB nuclear translocation in recipient cells. *vs PBS group (**p<0.01, ***p<0.005, ****p<0.001); #vs MCEC-Exo group (#p<0.05, ##p<0.01, ###p<0.005) (scale bar:10 μm)

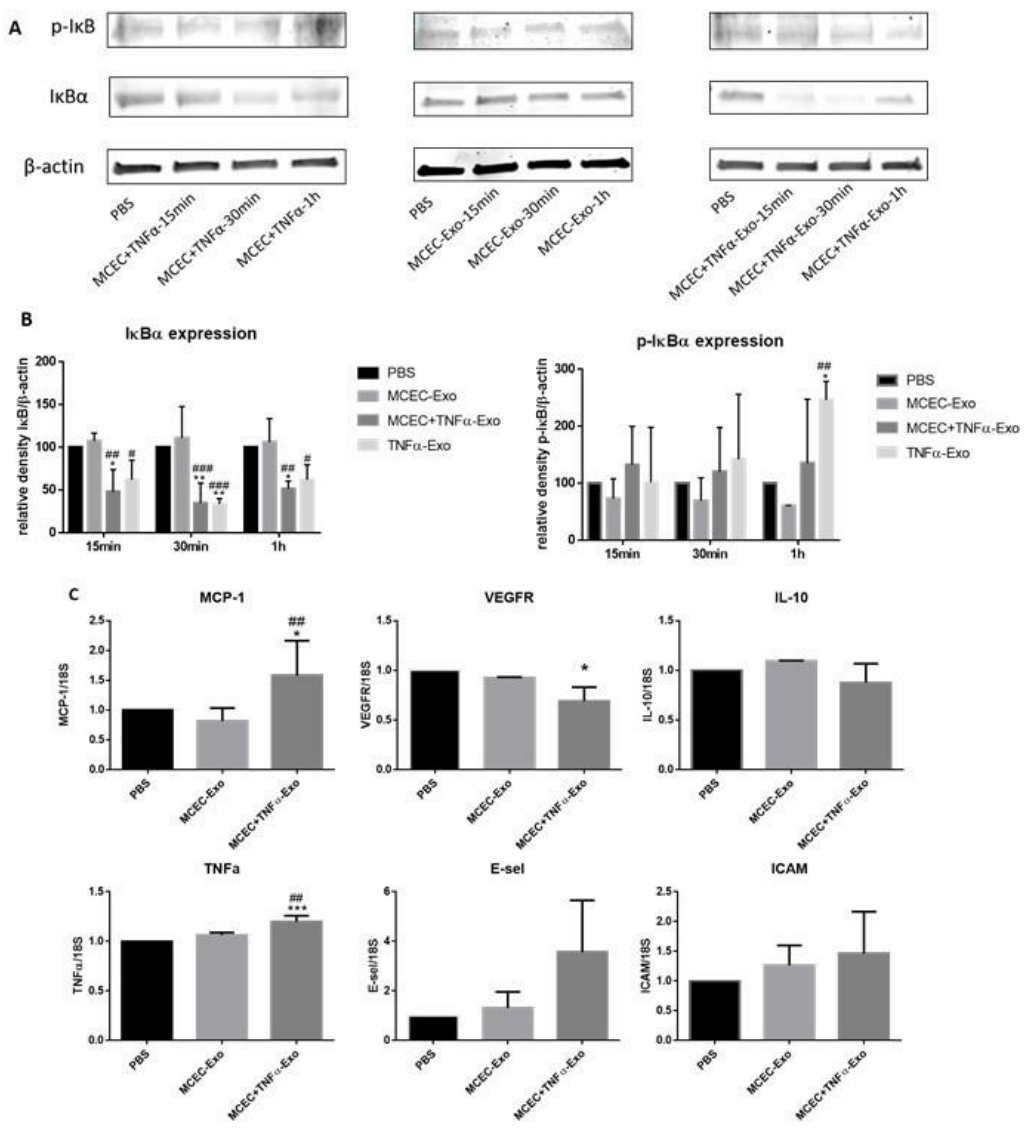


Figure 14. MCEC+TNFα-Exo activated NFκB signaling pathway.

A and B, western blotting showed decreased expression of iκBα in both MCEC+TNFα-Exo and TNFα treated groups, suggested NFκB signaling pathway activation. **C and D** qRT-PCR analysis showed increased expression of NFκB downstream genes with MCEC+TNFα-Exo treatment. *vs PBS group (*p<0.05, **p<0.01, ***p<0.005); #vs MCEC-Exo group (#p<0.05, ##p<0.01, ###p<0.005)

ILK knockdown exosome showed attenuated NFκB activation effect.

siRNA strategy was used for ILK knockdown with 70% to 90% knockdown efficiency (Supplemental Figure2). Interestingly, ILK knockdown in exosomes also decreased the expression of miR-375 in exosomes, suggesting ILK is involved in miR-375 generation (shown in supplemental figure 4). To investigate functional differences in exosomes with or without ILK knockdown, MCEC were treated MCEC-Exo, MCEC+TNFα-Exo, or ILK knockdown exosome (ILKKD-Exo). PBS and TNFα (not shown) are designed as control. The nuclear NFκB translocation was not activated with ILKKD-Exo shot time treatment but activated with long time treatment (Figure 15A and 15B). iκBα expression was not decreased with short time ILKKD-Exo treatment (Figure 16A and 16B). ILKKD-Exo also decreased the expression of NFκB downstream genes (Figure 16C). All these results suggested ILK enriched exosomes mediate inflammation response through NFκB activation in recipient cells. ILK knockdown in either exosomes or recipient cells (here as MCECs) greatly inhibited NFκB activation and downstream gene expression (shown in supplemental data).

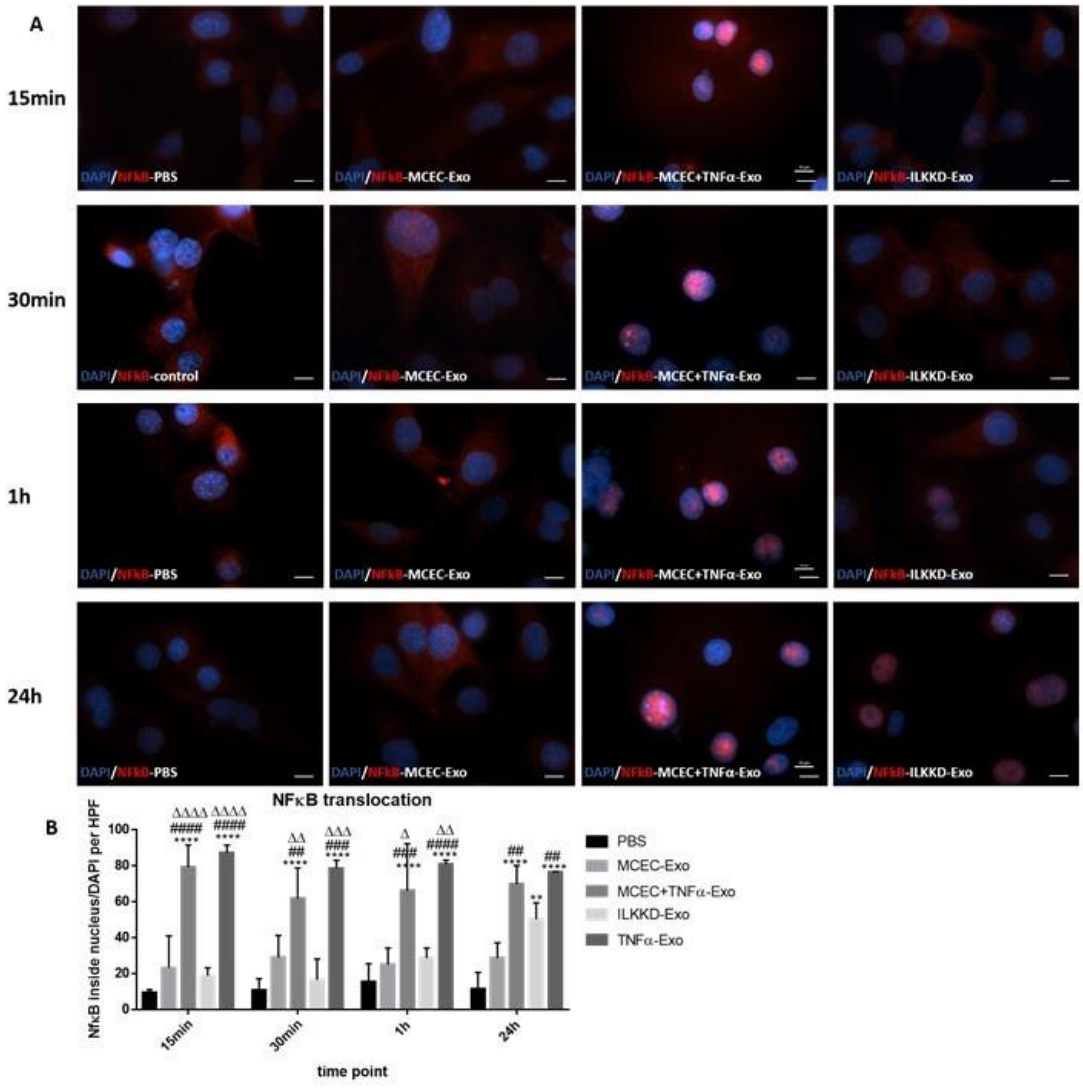


Figure 15. ILK knockdown exosomes activated less NFκB nuclear translocation in recipient cells.

A and B, NFκB staining analysis indicated nuclear NFκB translocation is not activated with ILKKD-Exo short time treatment but activated with long time treatment. *vs PBS group (**p<0.01, ****p<0.001); #vs MCEC-Exo group (##p<0.01, ###p<0.005, ####p<0.001); Δvs ILKKD-Exo group (Δp<0.05, ΔΔp<0.01, ΔΔΔp<0.005, ΔΔΔΔp<0.001) (scale bar:10 μm).

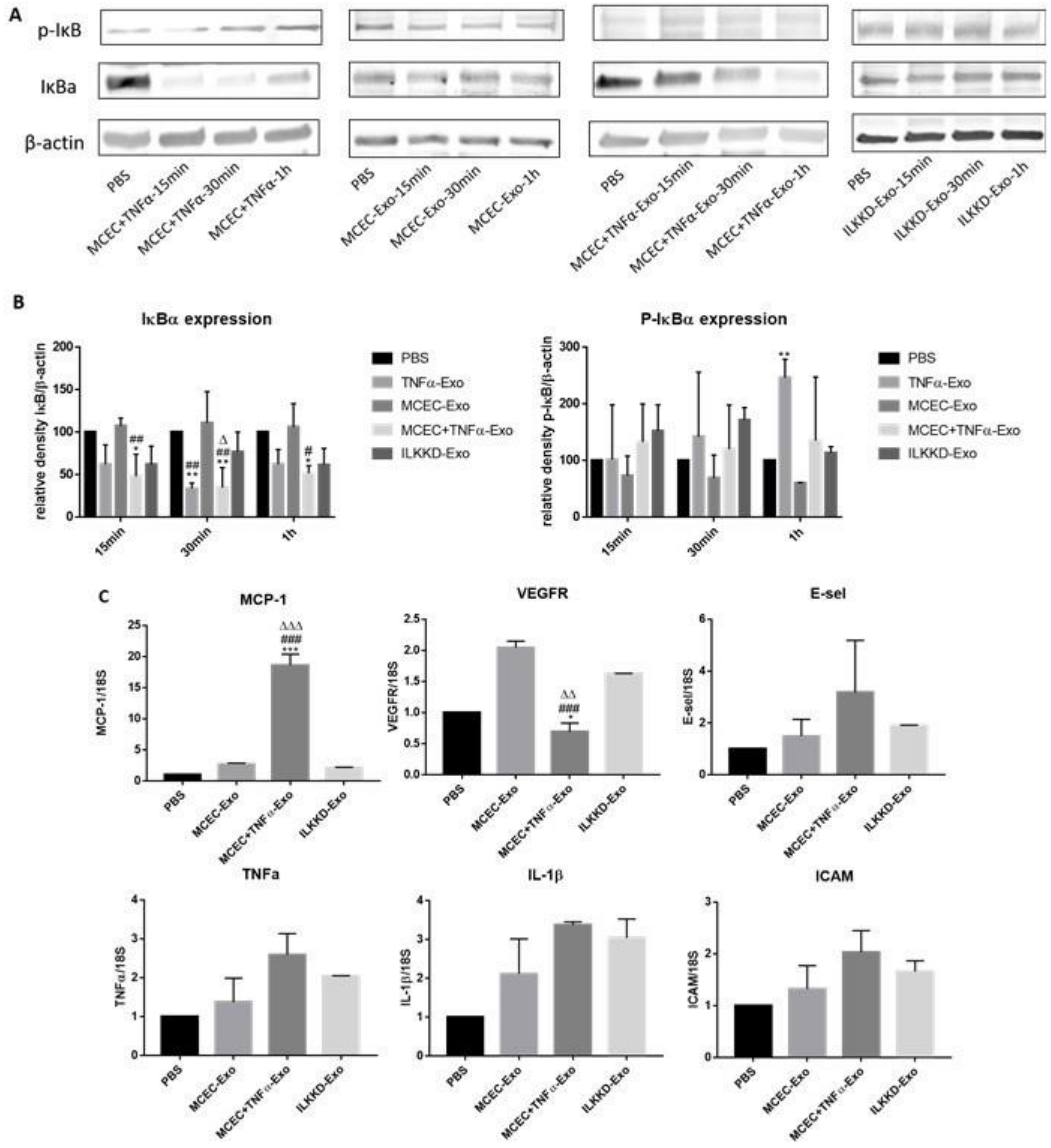


Figure 16. ILK knockdown exosomes attenuated NFκB signaling pathway activation in recipient cells.

A and B, western blotting showed iκBα expression was not greatly decreased with short time ILKKD-Exo treatment. **C and D** qRT-PCR analysis showed ILKKD-Exo group had relatively decreased expression level of NFκB downstream genes. *vs PBS group (*p<0.05, **p<0.01, ***p<0.005); #vs MCEC-Exo group (#p<0.05, ##p<0.01, ###p<0.005), Δvs ILKKD-Exo group (Δp<0.05, ΔΔp<0.01, ΔΔΔp<0.005).

ILK knockdown in MCEC+TNFα-Exo rescued dysfunction in promoting angiogenesis.

Previous study indicated IL-10KO-EPC-Exo inhibited tube formation *in vitro*. Similar effect is observed in MCEC+TNFα-Exo (Figure 7A and 7B). However, MCEC+TNFα-Exo with ILK knockdown significantly increased branch point number in tube formation assay suggesting rescued angiogenic capacity (Figure 7C and 7D). Therefore, intense inflammatory stress inhibits angiogenesis via ILK delivery in exosome which can be rescued through ILK knockdown in exosome.

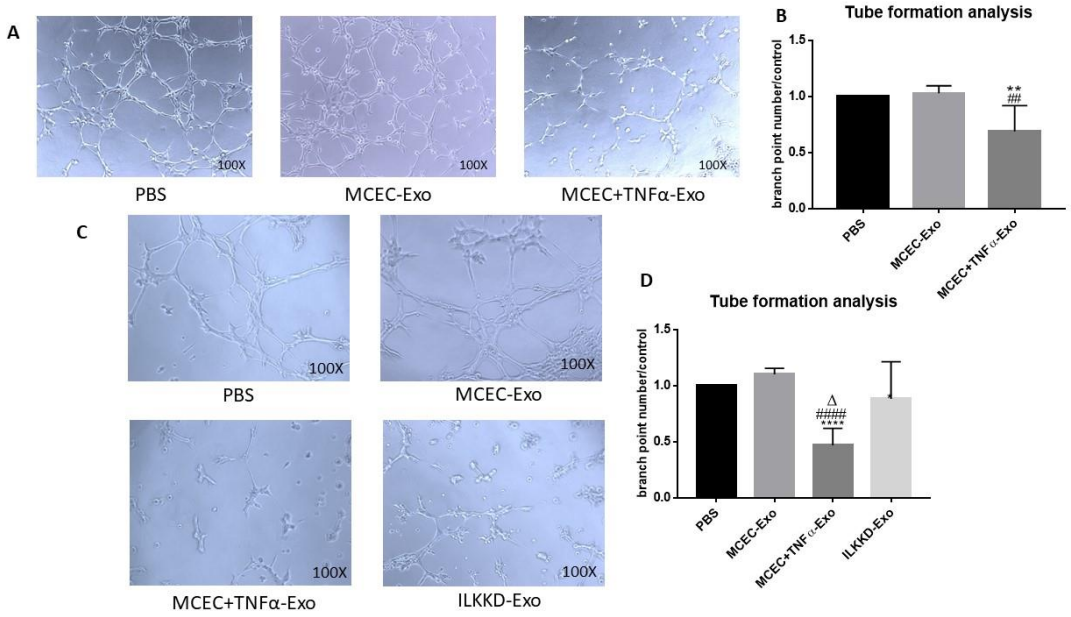


Figure 17. Knocking down ILK in MCEC+TNFα-Exo rescued defect in promoting angiogenesis.

MCEC+TNFα-Exo inhibited tube formation while knocking down ILK attenuated the inhibition effect. *vs PBS group (*p<0.05, **p<0.01, ****p<0.001); #vs MCEC-Exo group (##p<0.01, ####p<0.001), Δvs ILKKD-Exo group (Δp<0.05).

ILK knockdown in IL-10KO-EPC-Exo rescued compromised cardiac repair and regeneration benefit post-MI.

To further explore whether post-MI cardiac repair and regeneration effect of IL-10KO-EPC-Exo can be rescued with ILK knockdown, we applicated same siRNA strategy on EPCs. MI mice were treated with 3 groups of exosomes (WT-EPC-Exo, IL-10KO-EPC-Exo, ILK-KD-EPC-Exo) and PBS as control. After 4 weeks of following, we found increased LVEF and LVFS in ILK-KD-EPC-Exo group, suggesting improved left ventricle function (Figure 8A and 8B). Histological analysis indicated enhanced neovascularization and decreased scar size with ILK-KD-EPC-Exo treatment (Figure 8D to 8F). Notice that during cell culture, there was observable cell apoptosis in IL-10KO-EPCs after siRNA administration, but the *in vivo* results did not show deleterious effect in cardiac repair since exosomes were collected in very short time window.

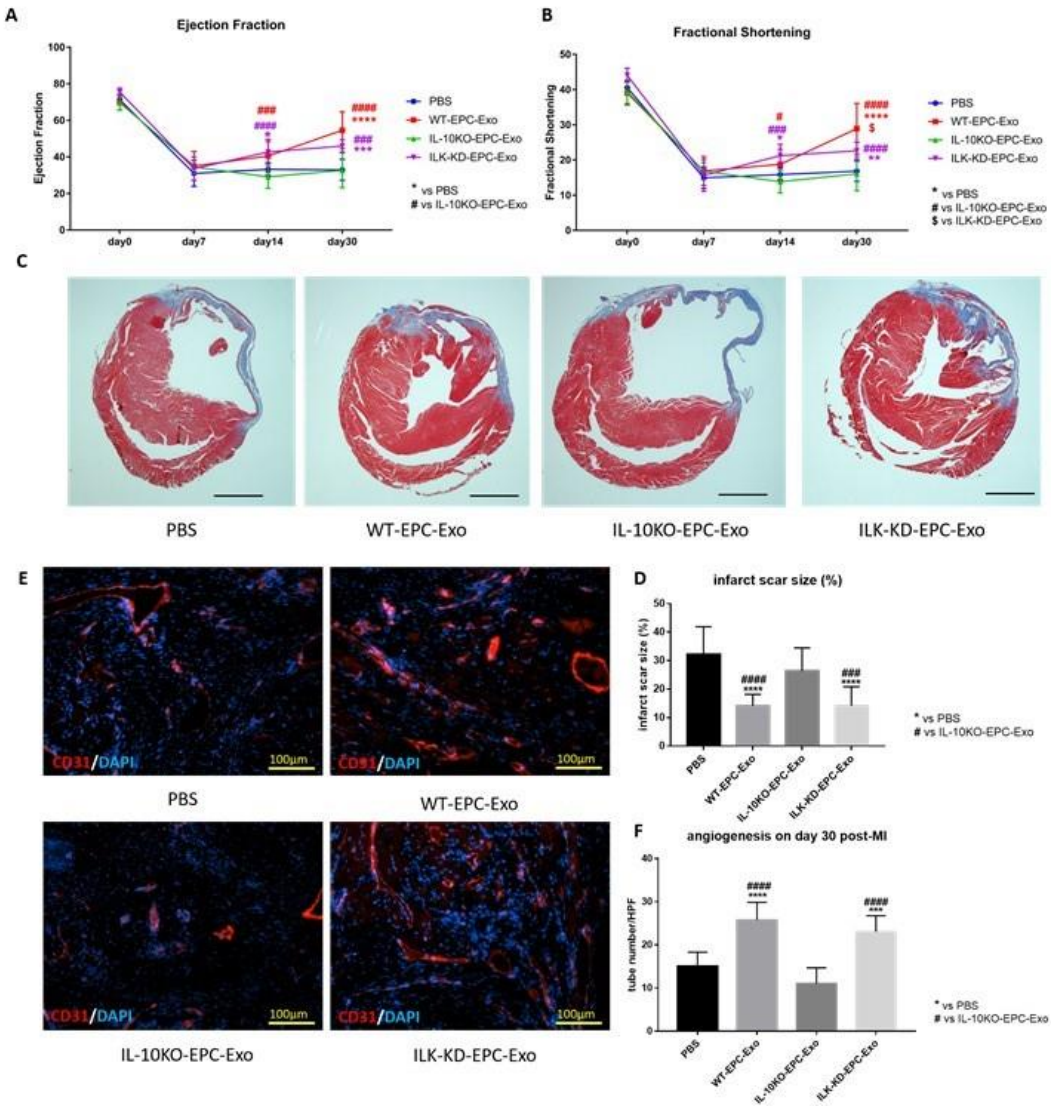


Figure 18. ILK knockdown in IL-10KO-EPC-Exo rescued the compromised cardiac repair and regeneration ability in MI model.

A and B, Echo analysis of left ventricle cardiac function. **C and D**, Trichrome staining analysis on day 30 post-MI heart (scale bar: 2000 μm). **E and F**, CD31 staining on day 30 post-MI heart (scale bar: 100 μm). *vs PBS group (**p<0.01, ****p<0.001); #vs IL-10KO-EPC-Exo group (##p<0.01, ###p<0.005, ####p<0.001), $vs ILK-KD-EPC-Exo group ($p<0.05).

Discussion

Over the past few decades, stem cell-based therapy has emerged as a promising therapeutic approach for ischemic cardiac repair and regeneration[158]. Endothelial progenitor cells derived from bone marrow or peripheral blood have been tested in clinical studies. However, myocardial infarction, the leading cause of death in US patients, triggers an intense immune response and greatly compromises the therapeutic effects of EPC transplantation[159]. Our published studies have established that EPCs are functionally impaired under inflammatory stimulus. Using IL-10KO mice as a model of systemic inflammation, we reported that EPCs from IL-10KO mice have diminished *in vitro* angiogenic activity; when transplanted *in vivo* in MI mouse model, IL10KO-EPCs did not activate angiogenic or protective responses and did not elevate post-injury LV function[24]. Recent studies revealed that functional benefits of stem cell-based therapy are largely mediated via paracrine effects[123, 160, 161] while exosome is major paracrine functional unit.

Exosomes are secreted from most cells and characterized with cell-specific cargo of proteins and RNAs, mediate intracellular communication in physiological and pathological conditions. Profound studies have been done exploring cardiac repair and regeneration benefits of exosomes from induced pluripotent stem cell(iPSCs)[162], mesenchymal stem cell(MSCs)[163] and bone marrow cells[49] in myocardial infarction[164], heart failure[165], ischemia/reperfusion injury[166] and many disease types. However, exosomes are not created equal and their content is actively under alteration reflecting the physiological or pathological state of parental cells[40]. Patients with cardiovascular disease have dysfunctional stem cells as well exosomes[49, 167]. In our study, EPCs under hostile

environment such as systemic inflammation produces defective exosomes with compromised ability preserving cell survival, promoting cell mobilization and angiogenesis[49].

Exosome function is mainly dependent on exosome content including RNAs and proteins. Several microRNAs (miRs) in stem cell derived exosomes have been identified playing important roles in cell proliferation, differentiation, angiogenesis, anti-apoptosis, adaptation to hypoxic stress, regulation of cell cycle, self-renewal, and pluripotency and their modification in exosome significantly changed performance of exosomes in cardiac repair and regeneration[162, 164, 168]. Our lab also found knocking down miR-375 in IL-10KO-EPC-Exo enhanced endothelial cells ability against hypoxia stress[49, 107]. Despite excellent progress made in miRs, not many studies explored exosome proteins. The difficulty in extracting enough amount of protein from stem cell exosome, huge cost and great variation of protein expression from different batches of exosomes all lead to limitations in exosome protein study. In our experiment, BM-EPC-Exo and MCEC-Exo were both used for mechanism study due to mentioned limitations. We consider using MCEC-Exo is a reasonable alternative strategy for several reasons, in context to protein content, both IL-10KO-EPC-Exo and MCEC+TNFα-Exo were enriched with ILK; in terms of functional study, both IL-10KO-EPC-Exo and MCEC+TNFα-Exo showed same inhibiting effect on angiogenesis. We think experiments conducted with MCEC-Exo should partially reflect EPC-Exo behaviors. Interestingly, we observed cell apoptosis in EPCs after siRNA administration but not in MCECs, suggesting exosome therapy may not be restricted to stem cell origin only, mature cell exosomes might have even superior therapeutic potential.

The role of ILK in heart and stem cells has been investigated for years but not fully addressed. In physiological state, overexpressing ILK upregulate reparative capacity of stem cells in cardiac disease. There have been studies showing ILK engineered MSCs significantly improved regional LV contractile function, reduced scar size, fibrosis, cell apoptosis, and increased regional myocardial perfusion and cell proliferation post-MI[113]. On the contrary, ILK plays opposing role under pathological state especially in heart. ILK is highly expressed in human failing heart and cardiomyopathy[169]. In myocardial hypertrophy, ILK is central mediator of oxidative stress, inflammatory cell recruitment and cardiac remodeling[170]; It promotes pro-fibrotic process and play critical role in cardiac fibrosis under angiotensin II stimulation[171]; moderate down regulation of ILK in heart prevented the decline in cardiac performance with aging[111, 171]. It is not clear what role ILK might play in stem cell derived exosomes under inflammation stimulus. In our study, we found ILK was highly enriched in both IL-10KO-EPC-Exo and MCEC+TNFα-Exo. Under inflammation stimulus, ILK activates NFκB pathway and affects post-MI cardiac repair process. NFκB super family of transcription factors has been implicated in the regulation of immune cell maturation, cell survival, and inflammation in many cell types, including cardiac myocytes[26, 172]. Recent studies have shown that NFκB is cardioprotective during acute hypoxia and reperfusion injury[173, 174]. However, prolonged activation of NFκB appears to be detrimental and promotes heart failure by eliciting signals that trigger chronic inflammation through enhanced elaboration of cytokines including TNFα, interleukin-1, and interleukin-6, leading to endoplasmic reticulum stress responses and cell death[173, 175]. In our study, ILK knockdown in exosomes decreased the NFκB activation in recipient endothelial cells and reduced the downstream gene

expression suggesting ILK blocking attenuated the overwhelming inflammation response after myocardial infarction and improve cardiac function. Additionally, we also found ILK knockdown decreased miR-375 expression in exosomes, indicating ILK knockdown protected heart via down-regulating other detrimental miRs delivered through exosomes.

In sum, our study demonstrated that IL-10 deficiency altered EPC derived exosome content and function. miR-375 and ILK are both enriched in inflamed exosomes and partially mediate overwhelming inflammation response post MI. Molecule manipulation in exosomes can enhance or rescue exosome function.

CHAPTER 4 SUMMARY

Cell therapy in cardiovascular disease showed promising but modest results. Low survival, poor integration and limited resources harnessed cell therapy application. Exosome is a cell free component that inherited fully benefit of stem cell and emerging as promising tool for disease treatment. Exosomes generated from stem cells have independent therapeutic effect and exosomal content is essential for exosome function. However, both the content and function of the exosomes, even from the same cell source, may differ in context of cellular stresses such as systemic inflammation, known co-morbid factor in heart disease patients. Our central hypothesis is that inflammation stimulus alters EPC derived exosome content and function in myocardial infarction treatment. We tested our central hypothesis by conducting experiments organized under following aims: 1) inflammatory stimulus alters EPC derived exosome function; 2) inflammatory stimulus alters EPC derived exosome content; 3) EPC derived exosome function can be enhanced or rescued through exosome molecule manipulation; 4) ILK plays essential role in exosome mediated inflammatory response.

Our study showed that under IL-10 deficiency, EPC derived exosomes have compromised ability preserving cell survival, promoting angiogenesis and cell mobilization *in vitro*, decreased cardiac repair and regeneration capacity *in vivo*. We also found IL-10 deficiency greatly changed RNA and protein expression patterns in EPC derived exosomes. Through manipulation of exosome molecules (miR-375 and ILK), we enhanced and rescued IL-10KO-EPC functions.

The significance of this study is to develop a novel understanding of the role played by EPC exosomes in supporting endogenous reparative processes in the heart.

Specific molecules are delivered through exosomes and mediate inflammation and reparative process in myocardial infarction. Exosomes are promising therapeutic tool and identified molecules in exosomes are potential therapeutic targets in cardiovascular diseases.

Conclusion

IL-10 deficiency alters EPC derived exosome content and functions in cardiac repair, therapeutic effect of exosomes can be rescued or enhanced through exosome content manipulation.

REFERENCE

1. Verma R, Balakrishnan L, Sharma K, Khan AA, Advani J, Gowda H, Tripathy SP, Suar M, Pandey A, Gandotra S, Prasad TS and Shankar S. A network map of Interleukin-10 signaling pathway. *J Cell Commun Signal*. 2016;10:61-7.

2. Mathieu M, Martin-Jaular L, Lavieu G and Thery C. Specificities of secretion and uptake of exosomes and other extracellular vesicles for cell-to-cell communication. *Nat Cell Biol*. 2019;21:9-17.

3. Benjamin EJ, Virani SS, Callaway CW, Chamberlain AM, Chang AR, Cheng S, Chiuve SE, Cushman M, Delling FN, Deo R, de Ferranti SD, Ferguson JF, Fornage M, Gillespie C, Isasi CR, Jimenez MC, Jordan LC, Judd SE, Lackland D, Lichtman JH, Lisabeth L, Liu S, Longenecker CT, Lutsey PL, Mackey JS, Matchar DB, Matsushita K, Mussolino ME, Nasir K, O'Flaherty M, Palaniappan LP, Pandey A, Pandey DK, Reeves MJ, Ritchey MD, Rodriguez CJ, Roth GA, Rosamond WD, Sampson UKA, Satou GM, Shah SH, Spartano NL, Tirschwell DL, Tsao CW, Voeks JH, Willey JZ, Wilkins JT, Wu JH, Alger HM, Wong SS, Muntner P, American Heart Association Council on E, Prevention Statistics C and Stroke Statistics S. Heart Disease and Stroke Statistics-2018 Update: A Report From the American Heart Association. *Circulation*. 2018;137:e67-e492.

4. Frangogiannis NG, Smith CW and Entman ML. The inflammatory response in myocardial infarction. *Cardiovasc Res*. 2002;53:31-47.

5. Mehta JL and Li DY. Inflammation in ischemic heart disease: response to tissue injury or a pathogenetic villain? *Cardiovasc Res*. 1999;43:291-9.

6. Entman ML and Smith CW. Postreperfusion inflammation: a model for reaction to injury in cardiovascular disease. *Cardiovasc Res*. 1994;28:1301-11.

7. Sansbury BE and Spite M. Resolution of Acute Inflammation and the Role of Resolvins in Immunity, Thrombosis, and Vascular Biology. *Circ Res*. 2016;119:113-30.

8. Epelman S, Liu PP and Mann DL. Role of innate and adaptive immune mechanisms in cardiac injury and repair. *Nat Rev Immunol*. 2015;15:117-29.

9. Lai SL, Marin-Juez R and Stainier DYR. Immune responses in cardiac repair and regeneration: a comparative point of view. *Cell Mol Life Sci*. 2018.

10. Eming SA, Wynn TA and Martin P. Inflammation and metabolism in tissue repair and regeneration. *Science*. 2017;356:1026-1030.

11. Kohsaka S, Menon V, Lowe AM, Lange M, Dzavik V, Sleeper LA, Hochman JS and Investigators S. Systemic inflammatory response syndrome after acute myocardial infarction complicated by cardiogenic shock. *Arch Intern Med*. 2005;165:1643-50.

12. Prabhu SD and Frangogiannis NG. The Biological Basis for Cardiac Repair After Myocardial Infarction: From Inflammation to Fibrosis. *Circ Res*. 2016;119:91-112.

13. Frangogiannis NG. The Functional Pluralism of Fibroblasts in the Infarcted Myocardium. *Circ Res*. 2016;119:1049-1051.

14. Pinto AR, Chandran A, Rosenthal NA and Godwin JW. Isolation and analysis of single cells from the mouse heart. *J Immunol Methods*. 2013;393:74-80.

15. Soehnlein O and Lindbom L. Phagocyte partnership during the onset and resolution of inflammation. *Nat Rev Immunol*. 2010;10:427-39.

16. Yan X, Anzai A, Katsumata Y, Matsuhashi T, Ito K, Endo J, Yamamoto T, Takeshima A, Shinmura K, Shen W, Fukuda K and Sano M. Temporal dynamics of cardiac immune cell accumulation following acute myocardial infarction. *J Mol Cell Cardiol*. 2013;62:24-35.

17. Hilgendorf I, Gerhardt LM, Tan TC, Winter C, Holderried TA, Chousterman BG, Iwamoto Y, Liao R, Zirlik A, Scherer-Crosbie M, Hedrick CC, Libby P, Nahrendorf M, Weissleder R and Swirski FK. Ly-6Chigh monocytes depend on Nr4a1 to balance both inflammatory and reparative phases in the infarcted myocardium. *Circ Res*. 2014;114:1611-22.

18. Courties G, Heidt T, Sebas M, Iwamoto Y, Jeon D, Truelove J, Tricot B, Wojtkiewicz G, Dutta P, Sager HB, Borodovsky A, Novobrantseva T, Klebanov B, Fitzgerald K, Anderson DG, Libby P, Swirski FK, Weissleder R and Nahrendorf M. In vivo silencing of the transcription factor IRF5 reprograms the macrophage phenotype and improves infarct healing. *J Am Coll Cardiol*. 2014;63:1556-66.

19. Leuschner F, Rauch PJ, Ueno T, Gorbatov R, Marinelli B, Lee WW, Dutta P, Wei Y, Robbins C, Iwamoto Y, Sena B, Chudnovskiy A, Panizzi P, Keliher E, Higgins JM, Libby P, Moskowitz MA, Pittet MJ, Swirski FK, Weissleder R and Nahrendorf M. Rapid monocyte kinetics in acute myocardial infarction are sustained by extramedullary monocytopoiesis. *J Exp Med*. 2012;209:123-37.

20. Iyer SS and Cheng G. Role of interleukin 10 transcriptional regulation in inflammation and autoimmune disease. *Crit Rev Immunol*. 2012;32:23-63.

21. Jung M, Ma Y, Iyer RP, DeLeon-Pennell KY, Yabluchanskiy A, Garrett MR and Lindsey ML. IL-10 improves cardiac remodeling after myocardial infarction by stimulating M2 macrophage polarization and fibroblast activation. *Basic Res Cardiol*. 2017;112:33.

22. Falcao RA, Christopher S, Oddi C, Reznikov L, Grizzard JD, Abouzaki NA, Varma A, Van Tassell BW, Dinarello CA and Abbate A. Interleukin-10 in patients with ST-segment elevation myocardial infarction. *Int J Cardiol*. 2014;172:e6-8.

23. Welsh P, Murray HM, Ford I, Trompet S, de Craen AJ, Jukema JW, Stott DJ, McInnes IB, Packard CJ, Westendorp RG, Sattar N and Group PS. Circulating interleukin-10 and risk of cardiovascular events: a prospective study in the elderly at risk. *Arterioscler Thromb Vasc Biol*. 2011;31:2338-44.

24. Krishnamurthy P, Thal M, Verma S, Hoxha E, Lambers E, Ramirez V, Qin G, Losordo D and Kishore R. Interleukin-10 deficiency impairs bone marrow-derived endothelial progenitor cell survival and function in ischemic myocardium. *Circ Res*. 2011;109:1280-9.

25. Gilmore TD. Introduction to NF-kappaB: players, pathways, perspectives. *Oncogene*. 2006;25:6680-4.

26. Gordon JW, Shaw JA and Kirshenbaum LA. Multiple facets of NF-kappaB in the heart: to be or not to NF-kappaB. *Circ Res*. 2011;108:1122-32.

27. Oeckinghaus A and Ghosh S. The NF-kappaB family of transcription factors and its regulation. *Cold Spring Harb Perspect Biol*. 2009;1:a000034.

28. Li G, Labruto F, Sirsjo A, Chen F, Vaage J and Valen G. Myocardial protection by remote preconditioning: the role of nuclear factor kappa-B p105 and inducible nitric oxide synthase. *Eur J Cardiothorac Surg*. 2004;26:968-73.

29. Regula KM, Ens K and Kirshenbaum LA. IKK beta is required for Bcl-2-mediated NF-kappa B activation in ventricular myocytes. *J Biol Chem*. 2002;277:38676-82.

30. Yan J, Xiang J, Lin Y, Ma J, Zhang J, Zhang H, Sun J, Danial NN, Liu J and Lin A. Inactivation of BAD by IKK inhibits TNFalpha-induced apoptosis independently of NF-kappaB activation. *Cell*. 2013;152:304-15.

31. Hamid T, Guo SZ, Kingery JR, Xiang X, Dawn B and Prabhu SD. Cardiomyocyte NF-kappaB p65 promotes adverse remodelling, apoptosis, and endoplasmic reticulum stress in heart failure. *Cardiovasc Res*. 2011;89:129-38.

32. Onai Y, Suzuki J, Kakuta T, Maejima Y, Haraguchi G, Fukasawa H, Muto S, Itai A and Isobe M. Inhibition of IkappaB phosphorylation in cardiomyocytes attenuates myocardial ischemia/reperfusion injury. *Cardiovasc Res*. 2004;63:51-9.

33. Zhang XQ, Tang R, Li L, Szucsik A, Javan H, Saegusa N, Spitzer KW and Selzman CH. Cardiomyocyte-specific p65 NF-kappaB deletion protects the injured heart by preservation of calcium handling. *Am J Physiol Heart Circ Physiol*. 2013;305:H1089-97.

34. Squadrito F, Deodato B, Squadrito G, Seminara P, Passaniti M, Venuti FS, Giacca M, Minutoli L, Adamo EB, Bellomo M, Marini R, Galeano M, Marini H and Altavilla D. Gene transfer of IkappaBalpha limits infarct size in a mouse model of myocardial ischemia-reperfusion injury. *Lab Invest*. 2003;83:1097-104.

35. Morishita R, Sugimoto T, Aoki M, Kida I, Tomita N, Moriguchi A, Maeda K, Sawa Y, Kaneda Y, Higaki J and Ogihara T. In vivo transfection of cis element "decoy" against nuclear factor-kappaB binding site prevents myocardial infarction. *Nat Med*. 1997;3:894-9.

36. Frantz S, Hu K, Bayer B, Gerondakis S, Strotmann J, Adamek A, Ertl G and Bauersachs J. Absence of NF-kappaB subunit p50 improves heart failure after myocardial infarction. *FASEB J*. 2006;20:1918-20.

37. Kawano S, Kubota T, Monden Y, Tsutsumi T, Inoue T, Kawamura N, Tsutsui H and Sunagawa K. Blockade of NF-kappaB improves cardiac function and survival after myocardial infarction. *Am J Physiol Heart Circ Physiol*. 2006;291:H1337-44.

38. Muller P, Lemcke H and David R. Stem Cell Therapy in Heart Diseases - Cell Types, Mechanisms and Improvement Strategies. *Cell Physiol Biochem*. 2018;48:2607-2655.

39. Michler RE. The current status of stem cell therapy in ischemic heart disease. *J Card Surg*. 2018;33:520-531.

40. Khan M and Kishore R. Stem Cell Exosomes: Cell-FreeTherapy for Organ Repair. *Methods Mol Biol*. 2017;1553:315-321.

41. Garikipati VNS and Kishore R. Endothelial Progenitor Cells: Procedure for Cell Isolation and Applications. *Methods Mol Biol*. 2017;1553:85-89.

42. Orlic D, Kajstura J, Chimenti S, Jakoniuk I, Anderson SM, Li B, Pickel J, McKay R, Nadal-Ginard B, Bodine DM, Leri A and Anversa P. Bone marrow cells regenerate infarcted myocardium. *Nature*. 2001;410:701-5.

43. Stamm C, Westphal B, Kleine HD, Petzsch M, Kittner C, Klinge H, Schumichen C, Nienaber CA, Freund M and Steinhoff G. Autologous bone-marrow stem-cell transplantation for myocardial regeneration. *Lancet*. 2003;361:45-6.

44. Stamm C, Kleine HD, Westphal B, Petzsch M, Kittner C, Nienaber CA, Freund M and Steinhoff G. CABG and bone marrow stem cell transplantation after myocardial infarction. *Thorac Cardiovasc Surg*. 2004;52:152-8.

45. Mocini D, Staibano M, Mele L, Giannantoni P, Menichella G, Colivicchi F, Sordini P, Salera P, Tubaro M and Santini M. Autologous bone marrow mononuclear cell transplantation in patients undergoing coronary artery bypass grafting. *Am Heart J*. 2006;151:192-7.

46. Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G and Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science*. 1997;275:964-7.

47. Urbich C and Dimmeler S. Endothelial progenitor cells: characterization and role in vascular biology. *Circ Res*. 2004;95:343-53.

48. Hur J, Yoon CH, Kim HS, Choi JH, Kang HJ, Hwang KK, Oh BH, Lee MM and Park YB. Characterization of two types of endothelial progenitor cells and their different contributions to neovasculogenesis. *Arterioscler Thromb Vasc Biol*. 2004;24:288-93.

49. Yue Y, Garikipati VNS, Verma SK, Goukassian DA and Kishore R. Interleukin-10 Deficiency Impairs Reparative Properties of Bone Marrow-Derived Endothelial Progenitor Cell Exosomes. *Tissue Eng Part A*. 2017;23:1241-1250.

50. Ahmadi H, Baharvand H, Ashtiani SK, Soleimani M, Sadeghian H, Ardekani JM, Mehrjerdi NZ, Kouhkan A, Namiri M, Madani-Civi M, Fattahi F, Shahverdi A and Dizaji AV. Safety analysis and improved cardiac function following local autologous transplantation of CD133(+) enriched bone marrow cells after myocardial infarction. *Curr Neurovasc Res*. 2007;4:153-60.

51. Stamm C, Kleine HD, Choi YH, Dunkelmann S, Lauffs JA, Lorenzen B, David A, Liebold A, Nienaber C, Zurakowski D, Freund M and Steinhoff G. Intramyocardial delivery of CD133+ bone marrow cells and coronary artery bypass grafting for chronic ischemic heart disease: safety and efficacy studies. *J Thorac Cardiovasc Surg*. 2007;133:717-25.

52. Forcillo J, Stevens LM, Mansour S, Prieto I, Salem R, Baron C, Roy DC, Larose E, Masckauchan D and Noiseux N. Implantation of CD133+ stem cells in patients undergoing coronary bypass surgery: IMPACT-CABG pilot trial. *Can J Cardiol*. 2013;29:441-7.

53. Assmus B, Schachinger V, Teupe C, Britten M, Lehmann R, Dobert N, Grunwald F, Aicher A, Urbich C, Martin H, Hoelzer D, Dimmeler S and Zeiher AM. Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI). *Circulation*. 2002;106:3009-17.

54. Wollert KC, Meyer GP, Lotz J, Ringes-Lichtenberg S, Lippolt P, Breidenbach C, Fichtner S, Korte T, Hornig B, Messinger D, Arseniev L, Hertenstein B, Ganser A and Drexler H. Intracoronary autologous bone-marrow cell transfer after myocardial infarction: the BOOST randomised controlled clinical trial. *Lancet*. 2004;364:141-8.

55. Eizawa T, Ikeda U, Murakami Y, Matsui K, Yoshioka T, Takahashi M, Muroi K and Shimada K. Decrease in circulating endothelial progenitor cells in patients with stable coronary artery disease. *Heart*. 2004;90:685-6.

56. Huang PH, Chen JW and Lin SJ. Effects of Cardiovascular Risk Factors on Endothelial Progenitor Cell. *Acta Cardiol Sin*. 2014;30:375-81.

57. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D and Horwitz E. Minimal criteria for defining multipotent

mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8:315-7.

58. Gao F, Chiu SM, Motan DA, Zhang Z, Chen L, Ji HL, Tse HF, Fu QL and Lian Q. Mesenchymal stem cells and immunomodulation: current status and future prospects. *Cell Death Dis*. 2016;7:e2062.

59. Guo X, Bai Y, Zhang L, Zhang B, Zagidullin N, Carvalho K, Du Z and Cai B. Cardiomyocyte differentiation of mesenchymal stem cells from bone marrow: new regulators and its implications. *Stem Cell Res Ther*. 2018;9:44.

60. Toma C, Pittenger MF, Cahill KS, Byrne BJ and Kessler PD. Human mesenchymal stem cells differentiate to a cardiomyocyte phenotype in the adult murine heart. *Circulation*. 2002;105:93-8.

61. Mangi AA, Noiseux N, Kong D, He H, Rezvani M, Ingwall JS and Dzau VJ. Mesenchymal stem cells modified with Akt prevent remodeling and restore performance of infarcted hearts. *Nat Med*. 2003;9:1195-201.

62. Tang J, Xie Q, Pan G, Wang J and Wang M. Mesenchymal stem cells participate in angiogenesis and improve heart function in rat model of myocardial ischemia with reperfusion. *Eur J Cardiothorac Surg*. 2006;30:353-61.

63. Jeevanantham V, Afzal MR, Zuba-Surma EK and Dawn B. Clinical trials of cardiac repair with adult bone marrow- derived cells. *Methods Mol Biol*. 2013;1036:179-205.

64. Kanelidis AJ, Premer C, Lopez J, Balkan W and Hare JM. Route of Delivery Modulates the Efficacy of Mesenchymal Stem Cell Therapy for Myocardial Infarction: A Meta-Analysis of Preclinical Studies and Clinical Trials. *Circ Res*. 2017;120:1139-1150.

65. Hare JM, Fishman JE, Gerstenblith G, DiFede Velazquez DL, Zambrano JP, Suncion VY, Tracy M, Ghersin E, Johnston PV, Brinker JA, Breton E, Davis-Sproul J, Schulman IH, Byrnes J, Mendizabal AM, Lowery MH, Rouy D, Altman P, Wong Po Foo C, Ruiz P, Amador A, Da Silva J, McNiece IK, Heldman AW, George R and Lardo A. Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: the POSEIDON randomized trial. *JAMA*. 2012;308:2369-79.

66. Katritsis DG, Sotiropoulou PA, Karvouni E, Karabinos I, Korovesis S, Perez SA, Voridis EM and Papamichail M. Transcoronary transplantation of autologous mesenchymal stem cells and endothelial progenitors into infarcted human myocardium. *Catheter Cardiovasc Interv*. 2005;65:321-9.

67. Martin GR. Isolation of a pluripotent cell line from early mouse embryos cultured in medium conditioned by teratocarcinoma stem cells. *Proc Natl Acad Sci U S A*. 1981;78:7634-8.

68. Thompson S, Clarke AR, Pow AM, Hooper ML and Melton DW. Germ line transmission and expression of a corrected HPRT gene produced by gene targeting in embryonic stem cells. *Cell*. 1989;56:313-21.

69. Boheler KR, Czyz J, Tweedie D, Yang HT, Anisimov SV and Wobus AM. Differentiation of pluripotent embryonic stem cells into cardiomyocytes. *Circ Res*. 2002;91:189-201.

70. Min JY, Yang Y, Converso KL, Liu L, Huang Q, Morgan JP and Xiao YF. Transplantation of embryonic stem cells improves cardiac function in postinfarcted rats. *J Appl Physiol (1985)*. 2002;92:288-96.

71. Nelson TJ, Ge ZD, Van Orman J, Barron M, Rudy-Reil D, Hacker TA, Misra R, Duncan SA, Auchampach JA and Lough JW. Improved cardiac function in infarcted mice after treatment with pluripotent embryonic stem cells. *Anat Rec A Discov Mol Cell Evol Biol*. 2006;288:1216-24.

72. Riess P, Molcanyi M, Bentz K, Maegele M, Simanski C, Carlitscheck C, Schneider A, Hescheler J, Bouillon B, Schafer U and Neugebauer E. Embryonic stem cell transplantation after experimental traumatic brain injury dramatically improves neurological outcome, but may cause tumors. *J Neurotrauma*. 2007;24:216-25.

73. Chong JJ, Yang X, Don CW, Minami E, Liu YW, Weyers JJ, Mahoney WM, Van Biber B, Cook SM, Palpant NJ, Gantz JA, Fugate JA, Muskheli V, Gough GM, Vogel KW, Astley CA, Hotchkiss CE, Baldessari A, Pabon L, Reinecke H, Gill EA, Nelson V, Kiem HP, Laflamme MA and Murry CE. Human embryonic-stem-cell-derived cardiomyocytes regenerate non-human primate hearts. *Nature*. 2014;510:273-7.

74. Takahashi K and Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell*. 2006;126:663-76.

75. Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K and Yamanaka S. Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell*. 2007;131:861-72.

76. Rajasingh J, Lambers E, Hamada H, Bord E, Thorne T, Goukassian I, Krishnamurthy P, Rosen KM, Ahluwalia D, Zhu Y, Qin G, Losordo DW and Kishore R. Cell-free embryonic stem cell extract-mediated derivation of multipotent stem cells from NIH3T3 fibroblasts for functional and anatomical ischemic tissue repair. *Circ Res*. 2008;102:e107-17.

77. Zhang J, Wilson GF, Soerens AG, Koonce CH, Yu J, Palecek SP, Thomson JA and Kamp TJ. Functional cardiomyocytes derived from human induced pluripotent stem cells. *Circ Res*. 2009;104:e30-41.

78. Ma J, Guo L, Fiene SJ, Anson BD, Thomson JA, Kamp TJ, Kolaja KL, Swanson BJ and January CT. High purity human-induced pluripotent stem cell-derived

cardiomyocytes: electrophysiological properties of action potentials and ionic currents. *Am J Physiol Heart Circ Physiol*. 2011;301:H2006-17.

79. Hsieh CL. Stability of patch methylation and its impact in regions of transcriptional initiation and elongation. *Mol Cell Biol*. 1997;17:5897-904.

80. Kawamura M, Miyagawa S, Miki K, Saito A, Fukushima S, Higuchi T, Kawamura T, Kuratani T, Daimon T, Shimizu T, Okano T and Sawa Y. Feasibility, safety, and therapeutic efficacy of human induced pluripotent stem cell-derived cardiomyocyte sheets in a porcine ischemic cardiomyopathy model. *Circulation*. 2012;126:S29-37.

81. Citro L, Naidu S, Hassan F, Kuppusamy ML, Kuppusamy P, Angelos MG and Khan M. Comparison of human induced pluripotent stem-cell derived cardiomyocytes with human mesenchymal stem cells following acute myocardial infarction. *PLoS One*. 2014;9:e116281.

82. Yoshihara M, Hayashizaki Y and Murakawa Y. Genomic Instability of iPSCs: Challenges Towards Their Clinical Applications. *Stem Cell Rev*. 2017;13:7-16.

83. Liu X, Li W, Fu X and Xu Y. The Immunogenicity and Immune Tolerance of Pluripotent Stem Cell Derivatives. *Front Immunol*. 2017;8:645.

84. Stadtfeld M, Nagaya M, Utikal J, Weir G and Hochedlinger K. Induced pluripotent stem cells generated without viral integration. *Science*. 2008;322:945-9.

85. Zhou W and Freed CR. Adenoviral gene delivery can reprogram human fibroblasts to induced pluripotent stem cells. *Stem Cells*. 2009;27:2667-74.

86. Kaji K, Norrby K, Paca A, Mileikovsky M, Mohseni P and Woltjen K. Virus-free induction of pluripotency and subsequent excision of reprogramming factors. *Nature*. 2009;458:771-5.

87. Kang PJ, Moon JH, Yoon BS, Hyeon S, Jun EK, Park G, Yun W, Park J, Park M, Kim A, Whang KY, Koh GY, Oh S and You S. Reprogramming of mouse somatic cells into pluripotent stem-like cells using a combination of small molecules. *Biomaterials*. 2014;35:7336-45.

88. Yellon DM and Davidson SM. Exosomes: nanoparticles involved in cardioprotection? *Circ Res*. 2014;114:325-32.

89. Sahoo S and Losordo DW. Exosomes and cardiac repair after myocardial infarction. *Circ Res*. 2014;114:333-44.

90. Abdelwahid E, Kalvelyte A, Stulpinas A, de Carvalho KA, Guarita-Souza LC and Foldes G. Stem cell death and survival in heart regeneration and repair. *Apoptosis*. 2016;21:252-68.

91. Kowal J, Arras G, Colombo M, Jouve M, Morath JP, Primdal-Bengtson B, Dingli F, Loew D, Tkach M and Thery C. Proteomic comparison defines novel markers to characterize heterogeneous populations of extracellular vesicle subtypes. *Proc Natl Acad Sci U S A*. 2016;113:E968-77.

92. Hessvik NP and Llorente A. Current knowledge on exosome biogenesis and release. *Cell Mol Life Sci*. 2018;75:193-208.

93. Colombo M, Raposo G and Thery C. Biogenesis, secretion, and intercellular interactions of exosomes and other extracellular vesicles. *Annu Rev Cell Dev Biol*. 2014;30:255-89.

94. Greening DW, Xu R, Ji H, Tauro BJ and Simpson RJ. A protocol for exosome isolation and characterization: evaluation of ultracentrifugation, density-gradient separation, and immunoaffinity capture methods. *Methods Mol Biol*. 2015;1295:179-209.

95. Kishore R and Khan M. Cardiac cell-derived exosomes: changing face of regenerative biology. *Eur Heart J*. 2017;38:212-215.

96. Cha DJ, Franklin JL, Dou Y, Liu Q, Higginbotham JN, Demory Beckler M, Weaver AM, Vickers K, Prasad N, Levy S, Zhang B, Coffey RJ and Patton JG. KRAS-dependent sorting of miRNA to exosomes. *Elife*. 2015;4:e07197.

97. McKenzie AJ, Hoshino D, Hong NH, Cha DJ, Franklin JL, Coffey RJ, Patton JG and Weaver AM. KRAS-MEK Signaling Controls Ago2 Sorting into Exosomes. *Cell Rep*. 2016;15:978-987.

98. Valadi H, Ekstrom K, Bossios A, Sjostrand M, Lee JJ and Lotvall JO. Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat Cell Biol*. 2007;9:654-9.

99. Kajimoto T, Okada T, Miya S, Zhang L and Nakamura S. Ongoing activation of sphingosine 1-phosphate receptors mediates maturation of exosomal multivesicular endosomes. *Nat Commun*. 2013;4:2712.

100. Poy MN, Hausser J, Trajkovski M, Braun M, Collins S, Rorsman P, Zavolan M and Stoffel M. miR-375 maintains normal pancreatic alpha- and beta-cell mass. *Proc Natl Acad Sci U S A*. 2009;106:5813-8.

101. Baulina N, Osmak G, Kiselev I, Matveeva N, Kukava N, Shakhnovich R, Kulakova O and Favorova O. NGS-identified circulating miR-375 as a potential regulating component of myocardial infarction associated network. *J Mol Cell Cardiol*. 2018;121:173-179.

102. He L, Wang X, Kang N, Xu J, Dai N, Xu X and Zhang H. MiR-375 inhibits the hepatocyte growth factor-elicited migration of mesenchymal stem cells by downregulating Akt signaling. *Cell Tissue Res*. 2018;372:99-114.

103. Wu Y, Sun X, Song B, Qiu X and Zhao J. MiR-375/SLC7A11 axis regulates oral squamous cell carcinoma proliferation and invasion. *Cancer Med*. 2017;6:1686-1697.

104. Jafarian A, Taghikani M, Abroun S, Allahverdi A, Lamei M, Lakpour N and Soleimani M. The Generation of Insulin Producing Cells from Human Mesenchymal Stem Cells by MiR-375 and Anti-MiR-9. *PLoS One*. 2015;10:e0128650.

105. Sheng W, Feng Z, Song Q, Niu H and Miao G. Modulation of mesenchymal stem cells with miR-375 to improve their therapeutic outcome during scar formation. *Am J Transl Res*. 2016;8:2079-87.

106. Chen S, Zheng Y, Zhang S, Jia L and Zhou Y. Promotion Effects of miR-375 on the Osteogenic Differentiation of Human Adipose-Derived Mesenchymal Stem Cells. *Stem Cell Reports*. 2017;8:773-786.

107. Garikipati VN, Krishnamurthy P, Verma SK, Khan M, Abramova T, Mackie AR, Qin G, Benedict C, Nickoloff E, Johnson J, Gao E, Losordo DW, Houser SR, Koch WJ and Kishore R. Negative Regulation of miR-375 by Interleukin-10 Enhances Bone Marrow-Derived Progenitor Cell-Mediated Myocardial Repair and Function After Myocardial Infarction. *Stem Cells*. 2015;33:3519-29.

108. Wang L, Song G, Liu M, Chen B, Chen Y, Shen Y, Zhu J and Zhou X. MicroRNA-375 overexpression influences P19 cell proliferation, apoptosis and differentiation through the Notch signaling pathway. *Int J Mol Med*. 2016;37:47-55.

109. Hao J, Lou Q, Wei Q, Mei S, Li L, Wu G, Mi QS, Mei C and Dong Z. MicroRNA-375 Is Induced in Cisplatin Nephrotoxicity to Repress Hepatocyte Nuclear Factor 1-beta. *J Biol Chem*. 2017;292:4571-4582.

110. Hannigan GE, Coles JG and Dedhar S. Integrin-linked kinase at the heart of cardiac contractility, repair, and disease. *Circ Res*. 2007;100:1408-14.

111. Nishimura M, Kumsta C, Kaushik G, Diop SB, Ding Y, Bisharat-Kernizan J, Catan H, Cammarato A, Ross RS, Engler AJ, Bodmer R, Hansen M and Ocorr K. A dual role for integrin-linked kinase and beta1-integrin in modulating cardiac aging. *Aging Cell*. 2014;13:431-40.

112. Thakur S, Li L and Gupta S. NF-kappaB-mediated integrin-linked kinase regulation in angiotensin II-induced pro-fibrotic process in cardiac fibroblasts. *Life Sci*. 2014;107:68-75.

113. Song SW, Chang W, Song BW, Song H, Lim S, Kim HJ, Cha MJ, Choi E, Im SH, Chang BC, Chung N, Jang Y and Hwang KC. Integrin-linked kinase is required in hypoxic mesenchymal stem cells for strengthening cell adhesion to ischemic myocardium. *Stem Cells*. 2009;27:1358-65.

114. Mao Q, Lin CX, Liang XL, Gao JS and Xu B. Mesenchymal stem cells overexpressing integrin-linked kinase attenuate cardiac fibroblast proliferation and collagen synthesis through paracrine actions. *Mol Med Rep*. 2013;7:1617-23.

115. Lee SP, Youn SW, Cho HJ, Li L, Kim TY, Yook HS, Chung JW, Hur J, Yoon CH, Park KW, Oh BH, Park YB and Kim HS. Integrin-linked kinase, a hypoxia-responsive molecule, controls postnatal vasculogenesis by recruitment of endothelial progenitor cells to ischemic tissue. *Circulation*. 2006;114:150-9.

116. Cho HJ, Youn SW, Cheon SI, Kim TY, Hur J, Zhang SY, Lee SP, Park KW, Lee MM, Choi YS, Park YB and Kim HS. Regulation of endothelial cell and endothelial progenitor cell survival and vasculogenesis by integrin-linked kinase. *Arterioscler Thromb Vasc Biol*. 2005;25:1154-60.

117. Gupta S and Knowlton AA. HSP60 trafficking in adult cardiac myocytes: role of the exosomal pathway. *Am J Physiol Heart Circ Physiol*. 2007;292:H3052-6.

118. Malik ZA, Kott KS, Poe AJ, Kuo T, Chen L, Ferrara KW and Knowlton AA. Cardiac myocyte exosomes: stability, HSP60, and proteomics. *Am J Physiol Heart Circ Physiol*. 2013;304:H954-65.

119. Waldenstrom A, Genneback N, Hellman U and Ronquist G. Cardiomyocyte microvesicles contain DNA/RNA and convey biological messages to target cells. *PLoS One*. 2012;7:e34653.

120. Kuwabara Y, Ono K, Horie T, Nishi H, Nagao K, Kinoshita M, Watanabe S, Baba O, Kojima Y, Shizuta S, Imai M, Tamura T, Kita T and Kimura T. Increased microRNA-1 and microRNA-133a levels in serum of patients with cardiovascular disease indicate myocardial damage. *Circ Cardiovasc Genet*. 2011;4:446-54.

121. Cheng Y, Wang X, Yang J, Duan X, Yao Y, Shi X, Chen Z, Fan Z, Liu X, Qin S, Tang X and Zhang C. A translational study of urine miRNAs in acute myocardial infarction. *J Mol Cell Cardiol*. 2012;53:668-76.

122. Zampetaki A, Willeit P, Tilling L, Drozdov I, Prokopi M, Renard JM, Mayr A, Weger S, Schett G, Shah A, Boulanger CM, Willeit J, Chowienczyk PJ, Kiechl S and Mayr M. Prospective study on circulating MicroRNAs and risk of myocardial infarction. *J Am Coll Cardiol*. 2012;60:290-9.

123. Sahoo S, Klychko E, Thorne T, Misener S, Schultz KM, Millay M, Ito A, Liu T, Kamide C, Agrawal H, Perlman H, Qin G, Kishore R and Losordo DW. Exosomes from human CD34(+) stem cells mediate their proangiogenic paracrine activity. *Circ Res*. 2011;109:724-8.

124. Liang X, Ding Y, Zhang Y, Tse HF and Lian Q. Paracrine mechanisms of mesenchymal stem cell-based therapy: current status and perspectives. *Cell Transplant*. 2014;23:1045-59.

125. Khan M, Nickoloff E, Abramova T, Johnson J, Verma SK, Krishnamurthy P, Mackie AR, Vaughan E, Garikipati VN, Benedict C, Ramirez V, Lambers E, Ito A, Gao E, Misener S, Luongo T, Elrod J, Qin G, Houser SR, Koch WJ and Kishore R. Embryonic stem cell-derived exosomes promote endogenous repair mechanisms and enhance cardiac function following myocardial infarction. *Circ Res*. 2015;117:52-64.

126. Kishore R and Verma SK. Roles of STATs signaling in cardiovascular diseases. *Jak-Stat*. 2012;1:118-24.

127. Albrecht-Schgoer K, Schgoer W, Holfeld J, Theurl M, Wiedemann D, Steger C, Gupta R, Semsroth S, Fischer-Colbrie R, Beer AG, Stanzl U, Huber E, Misener S, Dejaco D, Kishore R, Pachinger O, Grimm M, Bonaros N and Kirchmair R. The angiogenic factor secretoneurin induces coronary angiogenesis in a model of myocardial infarction by stimulation of vascular endothelial growth factor signaling in endothelial cells. *Circulation*. 2012;126:2491-501.

128. Kishore R, Verma SK, Mackie AR, Vaughan EE, Abramova TV, Aiko I and Krishnamurthy P. Bone marrow progenitor cell therapy-mediated paracrine regulation of cardiac miRNA-155 modulates fibrotic response in diabetic hearts. *PloS one*. 2013;8:e60161.

129. Losordo DW and Kishore R. A big promise from the very small identification of circulating embryonic stem-like pluripotent cells in patients with acute myocardial infarction. *Journal of the American College of Cardiology*. 2009;53:10-2.

130. Sasi SP, Yan X, Enderling H, Park D, Gilbert HY, Curry C, Coleman C, Hlatky L, Qin G, Kishore R and Goukassian DA. Breaking the 'harmony' of TNF-alpha signaling for cancer treatment. *Oncogene*. 2012;31:4117-27.

131. Krishnamurthy P, Rajasingh J, Lambers E, Qin G, Losordo DW and Kishore R. IL-10 inhibits inflammation and attenuates left ventricular remodeling after myocardial infarction via activation of STAT3 and suppression of HuR. *Circulation research*. 2009;104:e9-18.

132. Verma SK, Krishnamurthy P, Barefield D, Singh N, Gupta R, Lambers E, Thal M, Mackie A, Hoxha E, Ramirez V, Qin G, Sadayappan S, Ghosh AK and Kishore R. Interleukin-10 treatment attenuates pressure overload-induced hypertrophic remodeling and improves heart function via signal transducers and activators of transcription 3-dependent inhibition of nuclear factor-kappaB. *Circulation*. 2012;126:418-29.

133. Pan BT and Johnstone RM. Fate of the transferrin receptor during maturation of sheep reticulocytes in vitro: selective externalization of the receptor. *Cell*. 1983;33:967-78.

134. Johnstone RM. Exosomes biological significance: A concise review. *Blood cells, molecules & diseases*. 2006;36:315-21.

135. Peters PJ, Geuze HJ, Van der Donk HA, Slot JW, Griffith JM, Stam NJ, Clevers HC and Borst J. Molecules relevant for T cell-target cell interaction are present in cytolytic granules of human T lymphocytes. *European journal of immunology*. 1989;19:1469-75.

136. Zitvogel L, Regnault A, Lozier A, Wolfers J, Flament C, Tenza D, Ricciardi-Castagnoli P, Raposo G and Amigorena S. Eradication of established murine tumors using a novel cell-free vaccine: dendritic cell-derived exosomes. *Nature medicine*. 1998;4:594-600.

137. Raposo G, Tenza D, Mecheri S, Peronet R, Bonnerot C and Desaymard C. Accumulation of major histocompatibility complex class II molecules in mast cell secretory granules and their release upon degranulation. *Molecular biology of the cell*. 1997;8:2631-45.

138. Wolfers J, Lozier A, Raposo G, Regnault A, Thery C, Masurier C, Flament C, Pouzieux S, Faure F, Tursz T, Angevin E, Amigorena S and Zitvogel L. Tumor-derived exosomes are a source of shared tumor rejection antigens for CTL cross-priming. *Nature medicine*. 2001;7:297-303.

139. Sullivan R, Saez F, Girouard J and Frenette G. Role of exosomes in sperm maturation during the transit along the male reproductive tract. *Blood cells, molecules & diseases*. 2005;35:1-10.

140. Vrijsen KR, Sluijter JP, Schuchardt MW, van Balkom BW, Noort WA, Chamuleau SA and Doevendans PA. Cardiomyocyte progenitor cell-derived exosomes stimulate migration of endothelial cells. *Journal of cellular and molecular medicine*. 2010;14:1064-70.

141. Xin H, Li Y, Liu Z, Wang X, Shang X, Cui Y, Zhang ZG and Chopp M. MiR-133b promotes neural plasticity and functional recovery after treatment of stroke with multipotent mesenchymal stromal cells in rats via transfer of exosome-enriched extracellular particles. *Stem cells*. 2013;31:2737-46.

142. Greco V, Hannus M and Eaton S. Argosomes: a potential vehicle for the spread of morphogens through epithelia. *Cell*. 2001;106:633-45.

143. Raposo G, Nijman HW, Stoorvogel W, Liejendekker R, Harding CV, Melief CJ and Geuze HJ. B lymphocytes secrete antigen-presenting vesicles. *The Journal of experimental medicine*. 1996;183:1161-72.

144. Taylor DD and Gercel-Taylor C. MicroRNA signatures of tumor-derived exosomes as diagnostic biomarkers of ovarian cancer. *Gynecologic oncology*. 2008;110:13-21.

145. Mackie AR, Klyachko E, Thorne T, Schultz KM, Millay M, Ito A, Kamide CE, Liu T, Gupta R, Sahoo S, Misener S, Kishore R and Losordo DW. Sonic hedgehog-

modified human CD34+ cells preserve cardiac function after acute myocardial infarction. *Circulation research*. 2012;111:312-21.

146. Zhang H, Xiang M, Meng D, Sun N and Chen S. Inhibition of Myocardial Ischemia/Reperfusion Injury by Exosomes Secreted from Mesenchymal Stem Cells. *Stem cells international*. 2016;2016:4328362.

147. Feng Y, Huang W, Meng W, Jegga AG, Wang Y, Cai W, Kim HW, Pasha Z, Wen Z, Rao F, Modi RM, Yu X and Ashraf M. Heat shock improves Sca-1+ stem cell survival and directs ischemic cardiomyocytes toward a prosurvival phenotype via exosomal transfer: a critical role for HSF1/miR-34a/HSP70 pathway. *Stem cells*. 2014;32:462-72.

148. Wang Y, Zhang L, Li Y, Chen L, Wang X, Guo W, Zhang X, Qin G, He SH, Zimmerman A, Liu Y, Kim IM, Weintraub NL and Tang Y. Exosomes/microvesicles from induced pluripotent stem cells deliver cardioprotective miRNAs and prevent cardiomyocyte apoptosis in the ischemic myocardium. *International journal of cardiology*. 2015;192:61-9.

149. Kishore R and Khan M. More Than Tiny Sacks: Stem Cell Exosomes as Cell-Free Modality for Cardiac Repair. *Circulation research*. 2016;118:330-43.

150. Arslan F, Lai RC, Smeets MB, Akeroyd L, Choo A, Aguor EN, Timmers L, van Rijen HV, Doevendans PA, Pasterkamp G, Lim SK and de Kleijn DP. Mesenchymal stem cell-derived exosomes increase ATP levels, decrease oxidative stress and activate PI3K/Akt pathway to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. *Stem cell research*. 2013;10:301-12.

151. Ibrahim AG, Cheng K and Marban E. Exosomes as critical agents of cardiac regeneration triggered by cell therapy. *Stem cell reports*. 2014;2:606-19.

152. Sluijter JP and van Rooij E. Exosomal microRNA clusters are important for the therapeutic effect of cardiac progenitor cells. *Circulation research*. 2015;116:219-21.

153. Pang T, Chen W, Lu ZM, Luo TH, Zhou H, Xue XC, Bi JW and Fang GE. Endothelial progenitor cells are influenced by serum of patients with systemic inflammatory response syndrome or multiple organ dysfunction. *European review for medical and pharmacological sciences*. 2013;17:3169-77.

154. Lin CP, Lin FY, Huang PH, Chen YL, Chen WC, Chen HY, Huang YC, Liao WL, Huang HC, Liu PL and Chen YH. Endothelial progenitor cell dysfunction in cardiovascular diseases: role of reactive oxygen species and inflammation. *BioMed research international*. 2013;2013:845037.

155. Hong Y, Eleftheriou D, Klein NJ and Brogan PA. Impaired function of endothelial progenitor cells in children with primary systemic vasculitis. *Arthritis research & therapy*. 2015;17:292.

156. Thery C, Amigorena S, Raposo G and Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Current protocols in cell biology*. 2006;Chapter 3:Unit 3 22.

157. Verma SK, Garikipati VNS, Krishnamurthy P, Schumacher SM, Grisanti LA, Cimini M, Cheng Z, Khan M, Yue Y, Benedict C, Truongcao MM, Rabinowitz JE, Goukassian DA, Tilley D, Koch WJ and Kishore R. Interleukin-10 Inhibits Bone Marrow Fibroblast Progenitor Cell-Mediated Cardiac Fibrosis in Pressure-Overloaded Myocardium. *Circulation*. 2017;136:940-953.

158. Edlinger C, Schreiber C, Wernly B, Anker A, Ruzicka K, Jung C, Hoppe UC and Lichtenauer M. Stem Cell Therapy for Myocardial Infarction 2001-2013 Revisited. *Stem cell reviews*. 2015;11:743-51.

159. Desouza CV, Hamel FG, Bidasee K and O'Connell K. Role of inflammation and insulin resistance in endothelial progenitor cell dysfunction. *Diabetes*. 2011;60:1286-94.

160. Gallet R, Dawkins J, Valle J, Simsolo E, de Couto G, Middleton R, Tseliou E, Luthringer D, Kreke M, Smith RR, Marban L, Ghaleh B and Marban E. Exosomes secreted by cardiosphere-derived cells reduce scarring, attenuate adverse remodelling, and improve function in acute and chronic porcine myocardial infarction. *European heart journal*. 2017;38:201-211.

161. Sharma S, Mishra R, Bigham GE, Wehman B, Khan MM, Xu H, Saha P, Goo YA, Datla SR, Chen L, Tulapurkar ME, Taylor BS, Yang P, Karathanasis SK, Goodlett DR and Kaushal S. A Deep Proteome Analysis Identifies the Complete Secretome as the Functional Unit of Human Cardiac Progenitor Cells. *Circulation research*. 2016.

162. Garikipati VNS and Kishore R. Induced Pluripotent Stem Cells Derived Extracellular Vesicles: A Potential Therapy for Cardiac Repair. *Circ Res*. 2018;122:197-198.

163. Sun X, Shan A, Wei Z and Xu B. Intravenous mesenchymal stem cell-derived exosomes ameliorate myocardial inflammation in the dilated cardiomyopathy. *Biochem Biophys Res Commun*. 2018;503:2611-2618.

164. Wang Y, Ding N, Guan G, Liu G, Huo D, Li Y, Wei K, Yang J, Cheng P and Zhu C. Rapid Delivery of Hsa-miR-590-3p Using Targeted Exosomes to Treat Acute Myocardial Infarction Through Regulation of the Cell Cycle. *J Biomed Nanotechnol*. 2018;14:968-977.

165. Ye W, Tang X, Yang Z, Liu C, Zhang X, Jin J and Lyu J. Plasma-derived exosomes contribute to inflammation via the TLR9-NF-kappaB pathway in chronic heart failure patients. *Mol Immunol*. 2017;87:114-121.

166. Huang X, Ding J, Li Y, Liu W, Ji J, Wang H and Wang X. Exosomes derived from PEDF modified adipose-derived mesenchymal stem cells ameliorate cerebral

ischemia-reperfusion injury by regulation of autophagy and apoptosis. *Exp Cell Res*. 2018;371:269-277.

167. Hassanpour M, Cheraghi O, Brazvan B, Hiradfar A, Aghamohammadzadeh N, Rahbarghazi R and Nouri M. Chronic Exposure of Human Endothelial Progenitor Cells to Diabetic Condition Abolished the Regulated Kinetics Activity of Exosomes. *Iran J Pharm Res*. 2018;17:1068-1080.

168. Luther KM, Haar L, McGuinness M, Wang Y, Lynch Iv TL, Phan A, Song Y, Shen Z, Gardner G, Kuffel G, Ren X, Zilliox MJ and Jones WK. Exosomal miR-21a-5p mediates cardioprotection by mesenchymal stem cells. *J Mol Cell Cardiol*. 2018;119:125-137.

169. Lu H, Fedak PW, Dai X, Du C, Zhou YQ, Henkelman M, Mongroo PS, Lau A, Yamabi H, Hinek A, Husain M, Hannigan G and Coles JG. Integrin-linked kinase expression is elevated in human cardiac hypertrophy and induces hypertrophy in transgenic mice. *Circulation*. 2006;114:2271-9.

170. Bettink SI, Werner C, Chen CH, Muller P, Schirmer SH, Walenta KL, Bohm M, Laufs U and Friedrich EB. Integrin-linked kinase is a central mediator in angiotensin II type 1- and chemokine receptor CXCR4 signaling in myocardial hypertrophy. *Biochem Biophys Res Commun*. 2010;397:208-13.

171. Chen X, Li Z, Feng Z, Wang J, Ouyang C, Liu W, Fu B, Cai G, Wu C, Wei R, Wu D and Hong Q. Integrin-linked kinase induces both senescence-associated alterations and extracellular fibronectin assembly in aging cardiac fibroblasts. *J Gerontol A Biol Sci Med Sci*. 2006;61:1232-45.

172. Valen G, Yan ZQ and Hansson GK. Nuclear factor kappa-B and the heart. *J Am Coll Cardiol*. 2001;38:307-14.

173. Abdullah M, Berthiaume JM and Willis MS. Tumor necrosis factor receptor-associated factor 6 as a nuclear factor kappa B-modulating therapeutic target in cardiovascular diseases: at the heart of it all. *Transl Res*. 2018;195:48-61.

174. Valen G. Signal transduction through nuclear factor kappa B in ischemia-reperfusion and heart failure. *Basic Res Cardiol*. 2004;99:1-7.

175. Kumar R, Yong QC and Thomas CM. Do multiple nuclear factor kappa B activation mechanisms explain its varied effects in the heart? *Ochsner J*. 2013;13:157-65.

APPENDICES

A TABLE 1 LIST OF PRIMERS.

primer list		
primer		sequence (5'-3')
ICAM-1	Forward	GTGATGCTCAGGTATCCATCCA
	Reverse	CACAGTTCTCAAAGCACAGCG
VCAM-1	Forward	AGTTGGGGATTCGGTTGTTCT
	Reverse	CCCCTCATTCCTTACCACCC
P-selectin	Forward	GAAAGGGCTGATTGTGACCCC
	Reverse	AGTAGTTCCGCACTGGGTACA
E-selectin	Forward	ATGAAGCCAGTGCATACTGTC
	Reverse	CGGTGAATGTTTCAGATTGGAGT
mGAPDH	Forward	TCAGGAGAGTGTTTCCTCGTC
	Reverse	CCCTGTTGCTGTAGCCGTAT
hGAPDH	Forward	GAAAGCCTGCCGGTGACTAA
	Reverse	TTCCCGTTCTCAGCCTTGAC
mIL-10	Forward	CAGCCGGGAAGACAATAACTG
	Reverse	CCGCAGCTCTAGGAGCATGT
m 18S	Forward	ACGAGACTCTGGCATGCTAACTAG
	Reverse	CGCCACTTGTCCCTCTAAGAA
mTNFa	Forward	CTGAGTTCTGCAAAGGGAGAG
	Reverse	CCTCAGGGAAGAATCTGGAAAG
mIL-1b	Forward	CCTCACAAGCAGAGCACAA
	Reverse	CCAGCCCATACTTTAGGAAGAC
mILK	Forward	ACTACTTGGGGTGCCTTGGA
	Reverse	CAAACCCGTCTCTGCGATCTCT
mVEGFR	Forward	TGCTGAAAGAGGGCGCTACT
	Reverse	TGCCGATGTGAATTAGGATCTTG
mMCP-1	Forward	CTTCCTCCACCACCATGCA
	Reverse	CCAGCCGGCAACTGTGA

B TABLE 2 LIST OF REAGENTS

list of reagents	
Item	Cat #

Amicon Ultra-15 Centrifugal Filter Units	UFC910008
anti-ILK antibody	ab76468
beta-actin antibody	sc-47778
CD63 Antibody (H-193)	sc-15363
control siRNA	sc-37007
EGMTM-2 Endothelial Cell Growth Medium-2 BulletKit	cc-3162
Ethylenediaminetetraacetic Acid (0.5M Solution/pH 8.0)	BP2482100
Exosome depleted FBS	A2720801
flotilin-1 antibody	sc-74566
Gelatin from bovine skin	G9391
Gibco™ (Phosphate Buffered Saline) Solution, pH 7.4	10010023
HEPES (1 M)	15630080
HSC 70 Antibody (B-6)	sc-7298
HUVEC cell line	ATCC® CRL-1730™
ILK siRNA (m)	sc-35667
in situ cell death detection kit, TMR red	12156792910
Lipofectamine® RNAiMAX Transfection Reagent	13778150
MCEC cell line	CLU510
NF-κB p65 (L8F6) Mouse mAb	# 6956S
Opti-MEM® I Reduced Serum Medium	31985070
Penicillin-Streptomycin (10,000 U/mL)	15140122
Phospho-NF-κB p65 (Ser536) (93H1) Rabbit mAb	#3033
Pierce™ BCA Protein Assay Kit	23225
Recombinant Mouse TNF-alpha (aa 80-235) Protein, CF	410-MT-010/CF
Standard Corning Matrigel Matrix	356234
SVEC4-10 cell line	ATCC® CRL-2181™

C TABLE 3 NGS ANALYSIS COMPARING WT-EPC-EXO AND IL-10KO-EPC-EXO (P<0.05)

Gene_ID	description	WT-EPC-Exo abundance	IL-10KO-EPC-Exo abundance	WT/IL-10KO fold change	P value
chr1.tRNA706-GlyGCC	tRNA	146240.85	51893.02	-1.495	0.0245
chr1.tRNA704-GlyGCC	tRNA	146240.85	51893.02	-1.495	0.0245

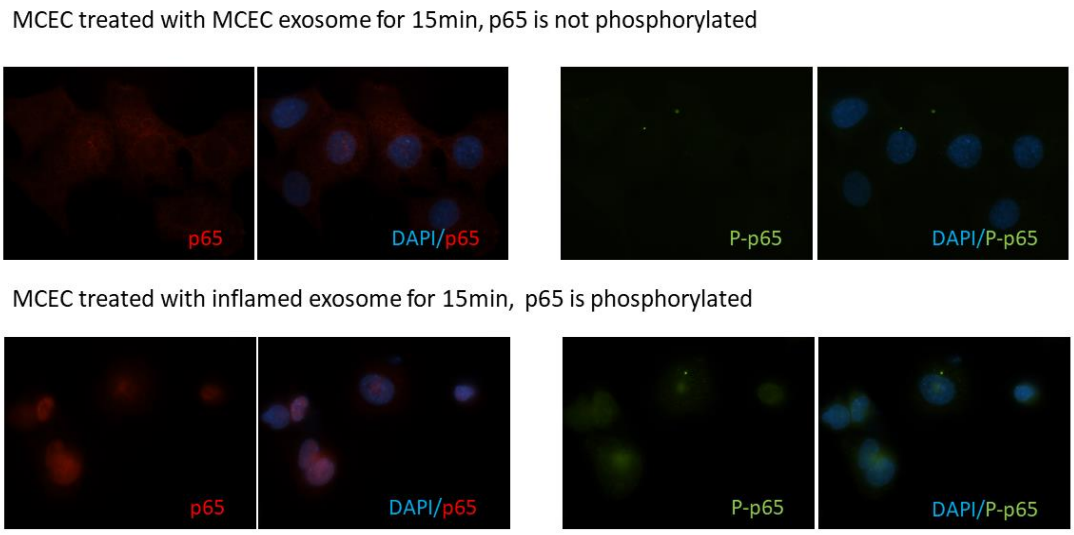
chr1.tRNA702-GlyGCC	tRNA	146240.85	51893.02	-1.495	0.0245
tRNA-Val-GTG_copy12	tRNA_like	3720.23	1306.55	-1.510	0.0451
chr13.tRNA986-ValAAC	tRNA	10385.55	3544.86	-1.551	0.0208
Dleu2	other_ncRNA	40687	13640.19	-1.577	0.0213
Smc4	RefSeq	39739.27	13139.19	-1.597	0.018
mmu-mir-16-1	miRNA	38719.14	12611.49	-1.618	0.0145
mmu-mir-16-2	miRNA	38729.63	12574.46	-1.623	0.0141
mmu-miR-16-5p	miRNA	38630.68	12541.73	-1.623	0.0141
chr13.tRNA974-ValAAC	tRNA	2782.89	890.89	-1.643	0.0138
chr13.tRNA93-ValAAC	tRNA	2782.89	890.5	-1.644	0.0138
mmu-mir-181a-2	miRNA	13090.94	4183.74	-1.646	0.0138
mmu-miR-181a-5p	miRNA	13067.53	4170.54	-1.648	0.0138
mmu-mir-181a-1	miRNA	13148.3	4180.4	-1.653	0.0138
chr13.tRNA97-ValCAC	tRNA	2799.11	867.68	-1.690	0.0103
chr13.tRNA977-ValAAC	tRNA	2754.59	853.32	-1.691	0.0103
Tbc1d91	RefSeq_antisense	1618.46	492.02	-1.718	0.0174
chr3.tRNA287-GlyCCC	tRNA	3114.46	944.45	-1.721	0.0077
chr3.tRNA755-GlyCCC	tRNA	3114.46	944.45	-1.721	0.0077
mmu-mir-328	miRNA	773.28	228.51	-1.759	0.0342
mmu-miR-328-3p	miRNA	773.28	228.51	-1.759	0.0342
Hhipl1	RefSeq	1383.37	408.67	-1.759	0.0173
Grn1	RefSeq_antisense	1215.19	352.82	-1.784	0.0141
Samd4b1	RefSeq_antisense	2060.51	500.77	-2.041	0.0006
Mtch1	RefSeq	1134.55	273.3	-2.054	0.002
mmu-miR-222-3p	miRNA	3381.87	806.02	-2.069	0.0001
Fam19a5: copy3	other_ncRNA	523.59	124.3	-2.075	0.0103
mmu-mir-222	miRNA	3403.78	806.02	-2.078	0.0001

LSU-rRNA_Hsa_copy150	rRNA	73050.12	16489.82	-2.147	0
Rbfox31	RefSeq_antisense	5554.93	1178.67	-2.237	0
Fxyd51	RefSeq_antisense	624.29	128.49	-2.281	0.0011
Ccnyl1	RefSeq	4389.07	869.48	-2.336	0
Adcyap1r11	RefSeq_antisense	1405.84	276.28	-2.347	0.0267
LSU-rRNA_Hsa_copy349	rRNA	615.01	118.24	-2.379	0.0138
mmu-miR-221-3p	miRNA	18469.32	3429.15	-2.429	0
mmu-mir-221	miRNA	18469.32	3429.15	-2.429	0
Gm76161	RefSeq_antisense	9055.78	1650.52	-2.456	0
mmu-miR-24-3p	miRNA	21310.79	3478.42	-2.615	0
mmu-mir-24-1	miRNA	21318.42	3478.42	-2.616	0
mmu-mir-24-2	miRNA	21431.19	3495.08	-2.616	0
Maf	RefSeq	181.55	24.89	-2.867	0.0141
mmu-miR-34a-5p	miRNA	1396.7	177.41	-2.977	0
mmu-mir-34a	miRNA	1402.11	177.41	-2.982	0
Bmp10	RefSeq	142.85	15.37	-3.216	0.0141
2310005E17Rik: antisense	other_ncRNA_antisense	4582.86	485.87	-3.238	0
1700106J16Rik	RefSeq	215.11	20.71	-3.377	0.0002
Fga1	RefSeq_antisense	134.79	11.93	-3.498	0.0061
mmu-mir-511	miRNA	106.91	4.11	-4.701	0.0006
mmu-miR-511-3p	miRNA	99.77	1.64	-5.927	0
mmu-mir-338	miRNA	53.94	0.41	-7.040	0.0108

D TABLE 4 PROTEOMIC ANALYSIS KINASES ENRICHED IN IL-10 KO-EPC EXOSOMES

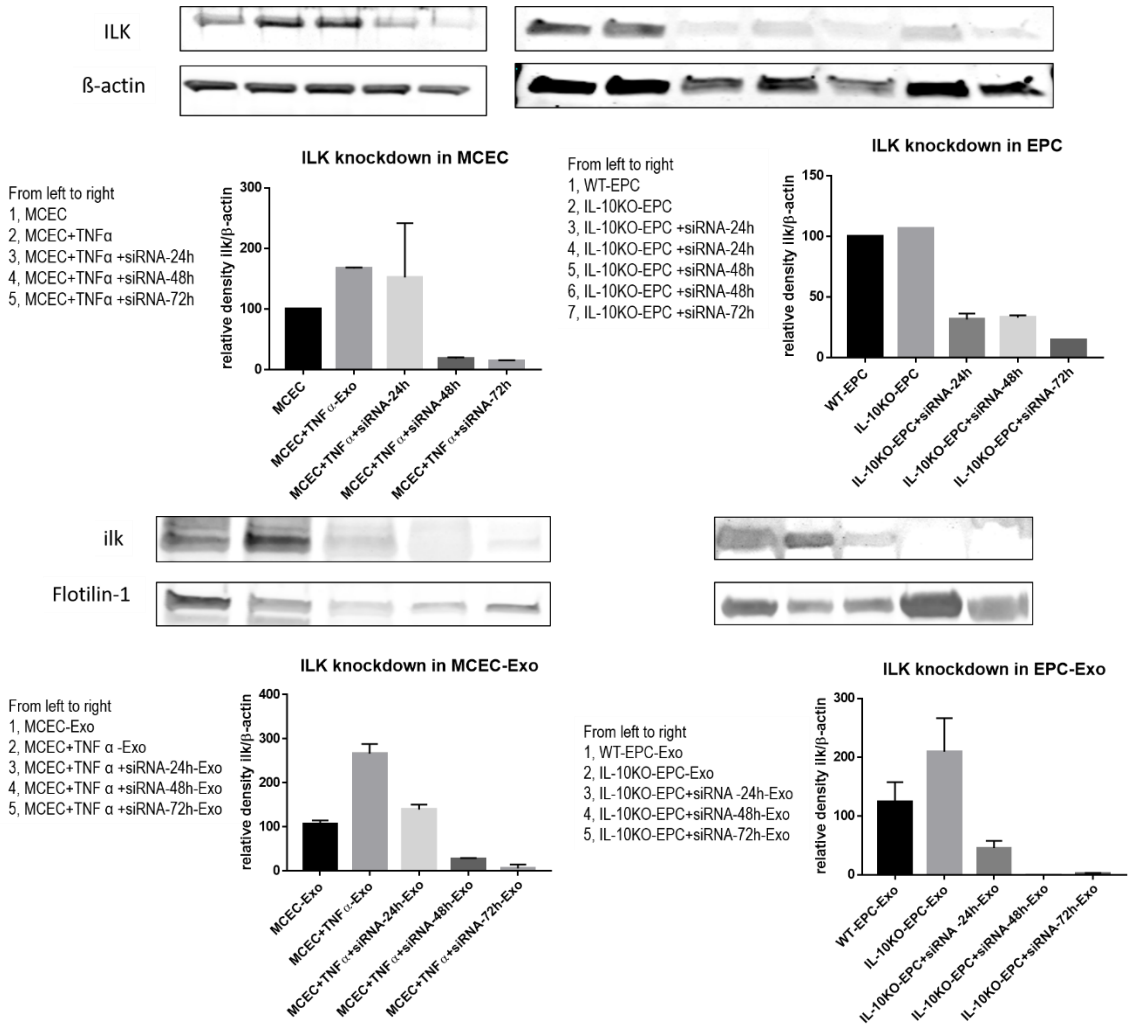
gene name	**LFQ-WT**	**LFQ-IL-10KO**
Syk	1.1E+08	4.3E+08
Lyn	3.5E+07	3.9E+07
Btk	0.0E+00	8.6E+07
Fyn; Src; Yes1;Lck	0.0E+00	4.5E+07
Hck	0.0E+00	1.0E+08
Fgr	0.0E+00	1.3E+08

Mapk1; Erk2; Mapk3	0.0E+00	5.6E+07
Mapk14	0.0E+00	1.3E+07
Adk	8.9E+07	3.4E+08
Ilk	2.0E+08	6.0E+08



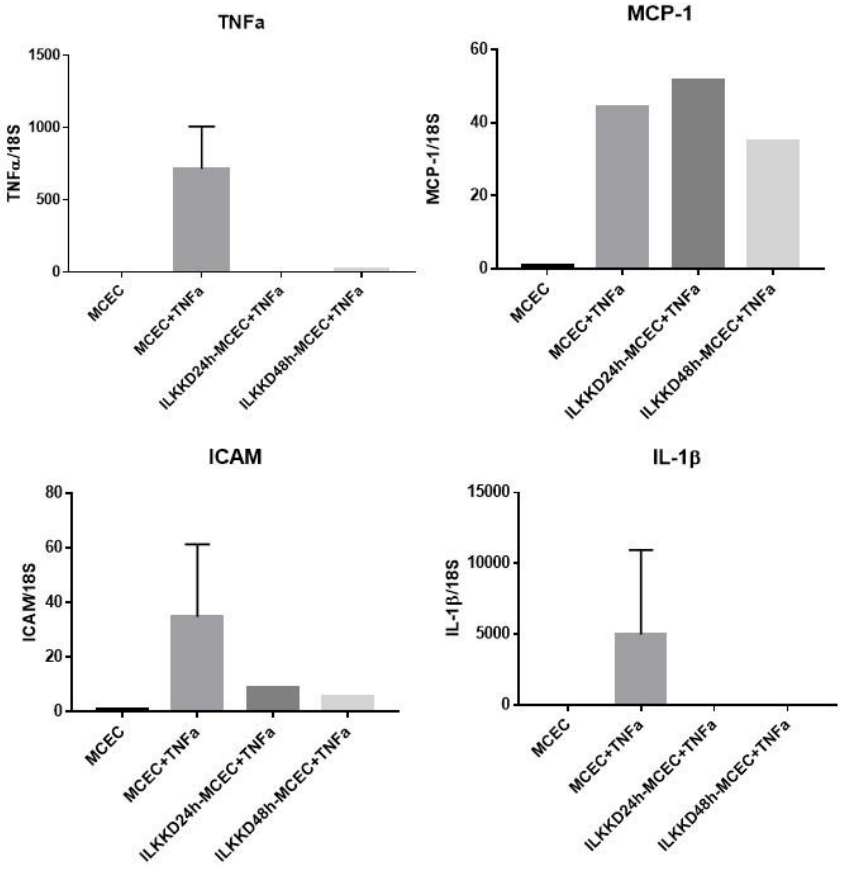
E SUPPLEMENTAL FIGURE 1 PHOSPHORYLATION OF NFκB P65.

7×10^4 MCEC were plated in 12-well plate with cover slips and treated with 7×10^6 exosomes (MCEC-Exo or MCEC+TNFα-Exo) for 15min, p65 and phosphor-65 were stained and observed. MCEC+TNFα-Exo short time stimulus activated p65 phosphorylation and nuclear translocation.



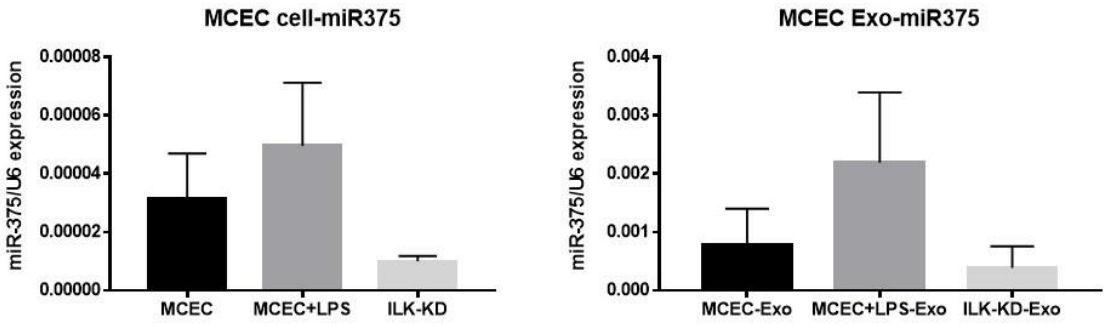
F SUPPLEMENTAL FIGURE 2 KNOCKDOWN EFFICIENCY OF SIRNA-ILK IN MCEC AND EPC (BOTH CELL AND EXOSOME).

The knockdown efficiency is around 70% to 90% in both types of cells and exosomes.



G SUPPLEMENTAL FIGURE 3 ILK KNOCKDOWN IN RECIPIENT CELLS BLOCKED IMMUNE RESPONSE.

MCEC cells were treated with siRNA against ILK for 24hours, 48hours, then stimulate with TNFα, qPCR result suggested that expression level of inflammation markers were decreased.



H SUPPLEMENTAL FIGURE 4 ILK KNOCKDOWN DECREASED ENRICHMENT OF MIR-375 IN BOTH CELLS AND EXOSOMES.

MCEC stimulated with LPS for 24 hours showed enrichment of miR-375 both in cells and exosomes. MCEC treated with siRNA-ILK for 24 hours then stimulated with LPS for another 24 hours showed even decreased miR-375 expression both in cells and exosomes. Results suggested ILK knockdown blocked other NFκB downstream miRs (such as miR-375) expression and delivery through exosomes.