Embryonic Stem Cell–Derived Exosomes Promote Endogenous Repair Mechanisms and Enhance Cardiac Function Following Myocardial Infarction


Rationale: Embryonic stem cells (ESCs) hold great promise for cardiac regeneration but are susceptible to various concerns. Recently, salutary effects of stem cells have been connected to exosome secretion. ESCs have the ability to produce exosomes, however, their effect in the context of the heart is unknown.

Objective: Determine the effect of ESC-derived exosome for the repair of ischemic myocardium and whether c-kit+ cardiac progenitor cells (CPCs) function can be enhanced with ESC exosomes.

Methods and Results: This study demonstrates that mouse ESC-derived exosomes (mES Ex) possess ability to augment function in infarcted hearts. mES Ex enhanced neovascularization, cardiomyocyte survival, and reduced fibrosis post infarction consistent with resurgence of cardiac proliferative response. Importantly, mES Ex augmented CPC survival, proliferation, and cardiac commitment concurrent with increased c-kit+ CPCs in vivo 8 weeks after in vivo transfer along with formation of bonafide new cardiomyocytes in the ischemic heart. miRNA array revealed significant enrichment of miR290-295 cluster and particularly miR-294 in ESC exosomes. The underlying basis for the beneficial effect of mES Ex was tied to delivery of ESC specific miR-294 to CPCs promoting increased survival, cell cycle progression, and proliferation.

Conclusions: mES Ex provide a novel cell–free system that uses the immense regenerative power of ES cells while avoiding the risks associated with direct ES or ES-derived cell transplantation and risk of teratomas. ESC exosomes possess cardiac regeneration ability and modulate both cardiomyocyte and CPC-based repair programs in the heart. (Circ Res. 2015;117:52-64. DOI: 10.1161/CIRCRESAHA.117.305990.)

Key Words: embryonic stem cells ▪ exosomes ▪ microRNAs

Endogenous myocardial repair in response to injury has been reported to involve limited self-division of preexisting cardiomyocytes and the activation and differentiation of resident cardiac stem cells.1-4 However, the insufficiency of these responses to meaningful repair paved the way for administration of exogenous stem cell–based therapies. Adoptive transfer of different cell types has been associated with enhanced cardiac function in patients with cardiovascular diseases3,6 and animal models of heart failure.7,8 Despite these promising results, poor survival and low retention of the donated stem cell population7,10 remain a significant limitation prompting research into new alternative remedies.

Pluripotent stem cells, including both embryonic stem cells (ESCs) and induced pluripotent stem (iPS) cells, hold immense promise for cardiac regeneration because they possess unparalleled differentiation ability.11 Although, cardiomyocytes derived from ESCs have been shown to improve cardiac regeneration and function in animal models of heart

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failure, however, this also has been reported to enhance arrhythmic response.\textsuperscript{12,13} In spite of their impressive cardiac repair ability, teratoma formation has been observed\textsuperscript{14,15} after transplantation of an unpurified ESC-derived cardiomyocyte population. Derivation of induced pluripotent cells has solved the issues with the availability of autologous ES cells, however, ES- or iPS-derived cells may still suffer the same difficulties in cell retention, coupling, and survival in ischemic myocardium because is noted for adult stem cells. Thus, there is a critical need for exploiting the powerful regenerative capacity of pluripotent cells while avoiding the problems associated with cell transplantation.

Discovery of cell-free components, such as exosomes,\textsuperscript{16} capable of instigating cell analogous response in target cells may provide a promising alternative for cardiac protection and regeneration.\textsuperscript{17–19} Novel, nontraditional use of cell-free components of ESC/iPS, such as exosomes, which carry ES-specific miRs and proteins may still allow for harnessing the regenerative power of these cells to augment and modulate endogenous repair mechanisms.

In this article, we report that mouse embryonic stem cell (mES)-exosome delivery in the heart after myocardial infarction (MI) stimulates and augments cardiac progenitor cell (CPC) and cardiomyocyte proliferation--based endogenous myocardial repair, which in part involves transfer of ES-specific microRNA-294. Our data suggest that ESC/iPS-derived exosomes represent a novel cell-free system for enhancing endogenous cardiac repair after pathological injury and bypass limitations of adoptive cell transplantation.

**Methods**

**Cell Culture and Differentiation**

mES isolated from C57Bl/6 were obtained from ATCC and cultured in Dulbecco’s modified eagle medium (DMEM; high glucose) with 15% fetal bovine serum and supplemented with β-mercaptoethanol (100 μmol/L), nonessential amino acids (100 μmol/L), Leukemia inhibitory factor (1000 U/mL), and penicillin/streptomycin (50 μg/mL each). Mouse embryonic fibroblasts (MEPs) were cultured in DMEM with 10% fetal bovine serum, nonessential amino acids (100 μmol/L), and penicillin/streptomycin (50 μg/mL each). H9c2 myoblasts and human umbilical vein endothelial cells were maintained in their respective culture mediums. CPCs from syngeneic male FVB mice and penicillin/streptomycin (50 μg/mL each). H9c2 myoblasts and human umbilical vein endothelial cells were maintained in their respective culture mediums. CPCs from syngeneic male FVB mice were cultured in cardiac stem cell media and were differentiated as previously described\textsuperscript{20} with additional information in Online Data Supplement.

**Exosome Isolation and Labeling**

mES and MEP cells were cultured for 40 hours followed by collection and purification by ultracentrifugation of exosomes as described previously.\textsuperscript{21} The purified exosome fraction was resuspended in saline for use. Purified exosomes were labeled with PKH26 Red Fluorescent Cell Linker Kit for in vitro studies according to the manufacturer’s protocol. Additional details are available in Online Data Supplement.

**Dynamic Light Scattering**

Exosome size analysis was performed by dynamic light scattering measurement as described previously.\textsuperscript{22} Briefly, exosomes were suspended in phosphate-buffered saline containing 2 mmol/L ethylenediaminetetraacetic acid; then, dynamic light scattering measurements were performed with a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, United Kingdom). Additional details are available in Online Data Supplement.

**Electron Microscopy**

Cells were fixed with 4% paraformaldehyde and processed, contrasted, and embedded as described previously.\textsuperscript{23} Transmission electron microscopy images were obtained with an FEI (Hillsboro, OR) Tecnai Spirit G2 transmission electron microscope operating at 120 kV. Additional details are available in Online Data Supplement.

**Immunoblot**

Immunoblot analysis was performed as described previously\textsuperscript{23} with additional detail in Online Data Supplement.

**Immunohistochemistry**

Immunocytochemistry, terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling assays, and immunohistochemistry were performed as previously described\textsuperscript{22–24} with additional information in Online Data Supplement and a list of antibodies in Online Table II.

**TaqMan Array MicroRNA**

Single-stranded cDNA is synthesized from all samples using the TaqMan MicroRNA Reverse Transcription Kit (Part Number 4366593) and the Megaplex RT Primers, Rodent Pool Set v3.0 (Part Number 4444746) as described in the Applied Biosystems protocol Megaplex Pools for microRNA Expression Analysis (Part Number 4399721 Rev. C). The reverse transcription product is preamplified using Megaplex PreAmp Primers, Rodent Pool B v3.0 (4444308). The preamplified product is used to run real-time polymerase chain reaction (PCR) using TaqMan Universal PCR Master Mix, No AmpErase UNG (Part Number 4324018) on a TaqMan Array Rodent MicroRNA A+B Cards set v3.0 (Part Number 4444909). The array cards are run on a 7900HT system.
Induction of Acute MI
Mice underwent surgery to ligate the left anterior descending coronary artery as reported previously followed by administration of exosomes from mES (n=6) and MEF (n=6) cells suspended in saline intramyocardially into the left ventricular wall (border zone) at 2 different locations immediately after left anterior descending ligation. The saline group underwent the same surgery but received saline without exosomes (n=6). Tissue was harvested at 5 or 14 days and 8 weeks after acute MI for histological analysis.

Echocardiography
Transthoracic 2-dimensional M-mode echocardiography was performed using the Vevo770 (VisualSonics, Toronto, ON, Canada) equipped with a 30-MHz transducer as described previously. Additional details are available in Online Data Supplement.

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 5.0 software.

Results
Embryonic Stem Cells Secrete Physiologically Functional Exosomes
Electron microscopy and dynamic light scattering analysis of mouse embryonic stem cells (mES) and MEF showed that both cell types secrete exosomes of typical size range (Figure 1A–1C; Online Figure IA–IF). In addition, exosomes from both cells expressed exosomal marker protein, flotillin-1 indicating their cytoplasmic origin and were negative for Lamin B, a nuclear protein (Online Figure IG). Expression of ES-specific transcripts was exclusively detected in mES-derived exosomes (mES Ex; Online Figure IH) confirming the embryonic stem cell origin of the mES Ex.

Figure 1. Characterization and functional validation of exosomes derived from embryonic stem cells (ESCs). A, Exosome secretion from a mouse embryonic stem cell (mES) as evidenced by electron microscopy, scale bar=500 nm; inset shows higher magnification of an ESC exosome. B, ESC culture medium shows exosome by electron microscopy, scale bar=50 nm. C, Measurement of exosome size in mES and mouse embryonic fibroblast (MEF) cells by dynamic light scattering (DLS) analysis shows that mES exosome are 39.7 nm in size compared with MEF-derived exosome (MEF Ex, 84.3 nm; n=4). D, Increased mRNA expression of pluripotent markers OCT4, SOX2, and Nanog in MEF cells treated with mES exosomes after 24 hours in comparison with control cells (n=3). Media vs mES Ex *P<0.05, **P<0.01, and ***P<0.001. E, Reduction in caspase3+ H9c2 cells treated with PKH-26 labeled mES Ex compared with MEF Ex–treated cells along with corresponding quantification in F (n=3). Arrows indicate caspase3 expressing cells, whereas arrowhead shows H9c2 cells negative for caspase3 expression while inset show higher magnification. PKH-26 (red), Caspase3 (green), and nuclei (blue). MEF Ex vs mES Ex **P<0.05, ***P<0.01, and ****P<0.001. G–I, Enhanced tube formation in human umbilical vein endothelial cells (HUVECs) treated with mES Ex in comparison with MEF Ex and media-treated control HUVECs. J, Quantification of branch points in HUVECs given different treatments. Media vs mES Ex *P<0.05, **P<0.01, and ***P<0.001; MEF Ex vs mES Ex, ##P<0.01.
Ability of mES Ex to modulate cellular function was assessed in vitro using different cell types. mES Ex enhanced expression of pluripotent markers OCT-4, SOX-2, and Nanog in MEF cells 24 hours after treatment indicating efficient delivery of exosomal cargo to target cells (Figure 1D). Cell survival after exosomal uptake was determined by labeling mES Ex and MEF-derived exosomes (MEF Ex) with PKH26 followed by administration to H9c2 myoblasts under challenge from \( H_{2}O_{2} \)-induced stress. A significant reduction in cleaved caspase-3 expression was observed in H9c2 myoblasts treated with mES Ex (16.8%) compared with MEF Ex–treated cells (31.8%) in response to 16 hours of \( H_{2}O_{2} \) challenge (Figure 1E and 1F). Finally, human umbilical vein endothelial cells were treated with mES Ex and MEF Ex and cultured on matrigel to assess whether mES Ex can enhance in vitro tube formation. Human umbilical vein endothelial cell tube formation was significantly increased exclusively after mES Ex treatment (Figure 1G–1J). Collectively, results showed that mES Ex are readily uptaken by target cells and modulate target cell function, including cell survival.

Intramyocardial Delivery of mES Ex Improved Post MI Cardiac Function
To assess their therapeutic efficacy in postinfarct myocardium, mES Ex were intramyocardially administered in mice at the time of MI, whereas MEF Ex and saline served as controls. Left ventricular contractility and function were consistently increased with mES Ex treatment as evidenced by significantly improved ejection fraction (EF; Figure 2A) and fractional shortening (FS; Figure 2B) measurements 4 weeks after infarction. Similarly, significant reduction in left ventricular end-systolic diameter (ESD, Figure 2C) was observed in mES Ex–treated animals compared with control groups in conjunction with significantly improved wall motion in the mES Ex–treated animals (Figure 2D) at 4 weeks.

Histological analysis of the heart 4 weeks post infarction indicated decreased infarct size in mES Ex transplanted mice (20.8%) compared with MEF Ex (33.1%) and saline (32.1%) administered animals (Online Figure IIA–IID). Interestingly, no tumor formation was observed in the hearts of mice transplanted with mES Ex 4 weeks after administration (Online Figure IIIA–IIIC). Together these results provide evidence for a therapeutic role of mES Ex in augmenting cardiac function after MI.

mES Exosomes Augment Neovascularization, Myocyte Proliferation, and Survival After MI
Immunohistochemical analysis of the hearts isolated from various treatment groups was performed to determine whether mES Ex induce morphometric changes in the heart. Capillary density was significantly increased in mES Ex transplanted hearts (border zone) as evidenced by lectin staining (Figure 3A–3D) together with decreased apoptosis (Figure 3E–3H) compared with MEF Ex and saline groups 4 weeks after infarction. Next, analysis of heart sections in animals that were administered with BrdU 24 hours before terminal experiments, revealed that BrdU+ cardiomyocytes were significantly increased in mES Ex hearts (5.8-fold) compared with saline 28 days after infarction (Online Figure IVA–IVD) coupled with increased mRNA levels of cyclins (A2, D1, D2, E1; Online Figure IVE) and decreased expression of cyclin inhibitors (p16, p19, p21, p53; Online Figure IVF) when analyzed at day 5 after MI. Moreover, a significant increase in pH3+ cardiomyocytes in hearts treated with mES Ex compared with control hearts further supporting evidence toward myocyte cycling (Figure 3I–3L). Collectively, these results indicate that mES Ex lead to induction of cardiac protective response and promote myocyte proliferative and survival response that in turn contribute to the endogenous repair process.
mES Ex augment Resident c-Kit+ CPCs in Infarcted Myocardium

Resident CPCs within the heart capable of regulating cardiac homeostasis form an integral part of the endogenous cardiac repair response to injury. Because mES Ex enable functional augmentation after myocardial damage, effect of mES Ex on CPC number, survival, and proliferation was assessed in vivo. Compared with controls, the number of resident c-kit+ CPCs in the myocardium after mES Ex treatment significantly increased (Figure 4A–4D). Additional characterization of c-kit+ CPCs was performed by colabeling with GATA binding protein 4 (GATA-4) that revealed a corroborating increase in c-kit+/GATA-4 CPCs in the heart-treated mES Ex compared with MEF Ex and saline-treated animals 28 days after infarction. Corresponding quantification is shown in L. Scale bar=40 μm. Saline vs mES Ex *P<0.05, **P<0.01, and MEF Ex vs mES Ex ##P<0.05, ###P<0.01.

mES Ex Enhance CPC Survival and Function Both In Vitro and In Vivo

CPC survival, proliferation, and ability for cardiac commitment in response to mES Ex treatment was assessed in vitro to corroborate findings in injured hearts receiving mES Ex. CPCs (Online Figure VE–VH). An important aspect of mES Ex administration is whether early CPC proliferation and survival translates into long-term enhancement of CPC numbers in the heart. In this respect, mES Ex administration led to ≈2.5-fold increase in c-kit+/BrdU+ CPCs compared with control hearts 4 weeks after infarction (Figure 4E–4H) indicating that mES Ex have the ability to sustain long-term CPC proliferation in the heart. Therefore, these results support the postulate that mES Ex promotes CPC survival and proliferation in hearts after infarction that may be in part responsible for augmented cardiac function.

Figure 3. Increased capillary density and reduced apoptosis in the hearts after myocardial infarction. A–C. Increased capillary density after mouse embryonic stem cell–derived exosome (mES Ex) treatment in mice 4 weeks after myocardial infarction along with corresponding quantification (D). Lectin (green) and nuclei (blue; n=6). E–G, Reduced terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling (TUNEL)+ nuclei in hearts transplanted with mES Ex compared with mouse embryonic fibroblast–derived exosome (MEF Ex) and saline-treated hearts. Quantification of TUNEL+ cells in H. TUNEL (magenta) and nuclei (blue; n=6). Scale bar=50 μm. I–K, Enhanced cardiomyocyte cycling as evidenced by pH3+/sarcomeric actin+ cells in hearts treated with mES Ex compared with MEF Ex and saline-treated animals 28 days after infarction. Corresponding quantification is shown in L. Scale bar=40 μm. Saline vs mES Ex *P<0.05, **P<0.01, and MEF Ex vs mES Ex #P<0.05, ###P<0.01.
by decreased annexin V+ cells (8.6%) compared with MEF Ex (20.2%) and nontreated CPCs (18.8%; Figure 5A) in response to H2O2 challenge. Importantly, no significant change in CPC survival was observed after treatment with equal amount of mES media, MEF media, mES exosome–free media (mES Ex free), and MEF exosome–free media (MEF Ex free; Online Figure VIA), suggesting that mES Ex were predominantly responsible for the observed survival response in CPCs with minimal or no contribution from serum exosomes. In addition, mES Ex treatment of CPCs also resulted in significantly enhanced CPC proliferation (Figure 5B) and metabolic activity as measured by MTT assay (Figure 5C) and confirmed by Seahorse assay for oxygen consumption rates (Online Figure IXA–IXE). The ability of CPCs to commit to cardiac lineages is an important aspect of cardiac regenerative response, and it was hypothesized that mES Ex may enhance CPC commitment toward cardiac lineages. mRNA expression of cardiomyocyte and endothelial cell markers (Figure 5D–5E) was increased in CPCs treated with mES Ex compared with MEF Ex under stimulation with dexamethasone for 7 days. Independent experiment on CPC tube formation ability on matrigel corroborated increased endothelial differentiation in response to mES Ex (Figure 5F–5I).

To elucidate whether mES Ex enhance CPC survival and function in vivo, green fluorescent protein (GFP)-CPCs pre-treated with mES Ex and MEF Ex were transplanted after induction of MI (Figure 6A). Long-term follow-up studies (8 weeks after MI) showed consistently improved left ventricular function in mice receiving mES Ex–treated CPCs compared with MEF Ex–treated CPCs (Figure 6B and 6C). Moreover, significant reduction in fibrosis was observed in mES Ex pre-treated CPC hearts compared with controls (Figure 6D–6G). The enhanced function was attributed to increased ability of GFP+ mES Ex pretreated CPC to survive in the injured hearts observed mainly in the border zone, infarcted region, and in close proximity to blood vessels 14 days after infarction parallel with their de novo differentiation to small myocytes (Online Figure VIB–VID). Furthermore, GFP colocalized

![Figure 4. Mouse embryonic stem cell–derived exosome (mES Ex) promote cardiac progenitor cell (CPC) numbers and proliferation in hearts after infarction. A–C, Increased number of c-kit+ CPCs in hearts 5 days after mES Ex transplantation compared with mouse embryonic fibroblast–derived exosome (MEF Ex) and saline-treated animals (n=4). Quantification of c-kit+ cells is shown in D. c-kit (red), sarcomeric actin (blue), and nuclei (white). Scale bar=40 μm. E–G, Enhanced c-kit+/BrdU+ cells in the heart 28 days after mES Ex transplantation along with corresponding quantification (n=6). c-kit (red), BrdU (green), and nuclei (blue). Scale bar=50 μm. H, Quantification of c-kit+/BrdU+ CPCs in all animal groups. I and J, Identification of CPCs by colocalization of c-kit with GATA-4 in hearts treated with mES Ex and MEF Ex along with corresponding quantification in K. c-kit (red), GATA-4 (green), sarcomeric actin (blue), and nuclei (white). Saline vs mES Ex *P<0.05, **P<0.01, and MEF Ex vs mES Ex #P<0.05, ##P<0.01.


with c-kit+ CPCs confirming the identity of the adoptively transferred CPCs 5 days after infarction (Figure 7A and 7B). Pretreatment with mES Ex also enhanced the proliferation of the transplanted CPCs (Figure 7C–7E) along with reduction in terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling+ GFP cells (Online Figure VIIA–VIIC). Interestingly, persistence of GFP+ CPCs and new GFP + myocytes in mES Ex pretreated CPC transplanted hearts was still evident even after 8 weeks of transplantation (Figure 7F–7I) concurrent with increased contribution of GFP + CPC to new blood vessel formation (Online Figure VIIF). Therefore, salutary effects of mES Ex on CPC survival and proliferation in infarcted hearts effectively translate into significant modulation of CPC function in vivo, suggesting mES Ex as a novel regimen for enhancing CPC function and survival.

mES Exosomes Are Highly Enriched for ES Cell-Specific MiRNAs

Exosomes carry cell-specific proteins or mRNA/miRNA that mediate the functional effect of exosomes. Global miRNA profiling of mES Ex and MEF Ex demonstrated 59 miRs upregulated (>2-fold) in mES Ex compared with MEF Ex, whereas 169 showed no change (Figure 8A; Online Figure VIIIA). However, members of the ES-specific miR-290 family, including miR-291, miR-294, and miR-295, demonstrated >104-fold expression in mES Ex compared with MEF Ex (Figure 8B), confirming ESC-specific origin of mES exosomes and the ability to carry ESC miRs. Previously, it has been shown that miR-290 family is exclusively expressed in ESCs and forms 70% of the known miRNAs produced by ESCs. Furthermore, members of the miR-290 family are

Figure 5. Modulation of cardiac progenitor cell (CPC) function by mouse embryonic stem cell (mES) exosome administration. A, Increased survival of CPCs after treatment with mES exosomes in comparison with mouse embryonic fibroblast (MEF) exosomes under H2O2 challenge (n=3). B, Increased CPC proliferation at day 1, 3, and 5 after mES exosome treatment compared with MEF exosomes and nontreated CPCs as evidenced by CyQuant assay (n=3). Nontreated (NT) vs mES Ex *P<0.05, **P<0.01, ***P<0.001 and MEF Ex vs mES Ex #P<0.05, ##P<0.01, ###P<0.001. C, Increased metabolic activity in CPCs at day 3 and 5 after mES-derived exosome (mES Ex) treatment compared with MEF-derived exosome (MEF Ex) and nontreated CPCs as measured by MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay (n=3). D and E, Enhanced mRNA levels of cardiac markers (cTnT, MEF2c, and GATA-6) and endothelial markers (α-SMA, Ang1, CD31, and VE-cadherin) in CPCs treated with mES Ex in the presence of dexamethasone compared with MEF Ex dex–treated CPCs and nontreated controls as evidenced by quantitative real-time polymerase chain reaction (n=3). NT vs mES Ex +dex *P<0.05, **P<0.01, ***P<0.001 and MEF Ex +dex vs mES Ex +dex #P<0.05, ##P<0.01, ###P<0.001. F–H, Tube formation is increased in CPCs treated with mES Ex cultured on matrigel compared with MEF Ex and nontreated CPCs after 24 hours (n=3) along with corresponding quantification I.
involved in the maintenance of the unique ESC cell cycle regulating G1/S transition. Therefore, it was hypothesized that mES Ex enriched with members of the miR-290 cluster deliver these miRs to target cells. Indeed, de novo expression of miR-291 and miR-294 was detected in mES Ex hearts, whereas no expression of these miRs was detected in saline-treated animals 5 days after infarction (Online Figure VIIIB). Concurrently, elevated levels of miR-291 (6.7-fold), miR-294 (6.4-fold), and miR-295 (2.8-fold) were detected in CPCs treated with mES Ex compared with MEF Ex–treated CPCs (Figure 8C). This data demonstrate that mES exosomes are highly enriched for miR-290 family, including miR-291, miR-294, and miR-295, and efficiently deliver these miRs to target cells.

**miR-294 Mimics mES Exosome Effects on CPCs**

To provide evidence toward a central role played by miR-290 cluster in mediating the effects of mES Ex on CPC function, miR-294 gain of function studies were performed in CPCs. Recent evidence shows that miR-291-3p, miR-294-3p, and miR-295 form the predominantly active core group of the miR-290 cluster. CPCs were treated with miRNA mimics for miR-291-5p, miR-294-3p, and miR-295-3p to characterize the effect on cell cycle progression. A significant shift in the number of CPCs in S-phase of the cell cycle was observed after treatment with miR-290 mimics, however, miR-294-3p treatment enhanced accumulation of CPCs in S-phase (45.6%) together with significant reduction of the G1-phase (27.4%) compared with nontreated CPCs (G1-phase, 71.0%; S-phase, 8.2%; Figure 8D). miR-291 treatment also enhanced increased S-phase transition albeit at lower magnitude (S-phase, 19.2%; G1, 58.0%) in CPCs. Interestingly, treatment with miR-291 and miR-294 mimics together did not lead to an additive effect on S-phase cell number compared with miR-294 alone suggesting a critical role for miR-294 in cell cycle modulation of CPCs. Similarly, mRNA expression of cyclins (E1, A2, and D1) was increased in CPCs treated with miR-294-3p mimic compared with miR-291-5p mimic and nontreated control CPCs (Figure 8E). In parallel, neonatal rat cardiomyocyte treated with miR-mimic for miR-294-3p showed a similar increase in mRNA levels of proliferative markers (Cyclin E1, Cyclin A2, and Cdk2; Online Figure VIIIC) compared with miR-291-5p and nontreated neonatal rat cardiomyocytes.
Next, underlying molecular signaling was assessed after miR-mimic treatment in CPCs. AKT phosphorylation was increased in CPCs treated with miR-294-3p mimic concomitant with elevated expression of nucleostatin, a marker for multipotency for CPCs31 and LIN28, a miR-binding protein that has been shown to be involved in regulating pluripotency by miR-29432 compared with miR-291-5p mimic and nontreated controls (Figure 8F). In addition, mRNA expression of c-myc and Klf4 were increased in miR-294-3p treated CPCs compared with nontreated cells (Online Figure VIIID). A significant increase in proliferation and survival was also evident in miR-294-3p mimic-treated CPCs after H2O2 stress (Figure 8G and 8H, respectively). Therefore, miR-294 plays a central role in regulating CPC cell cycle in association with promoting proliferation, survival, and largely mimics the effect of mES Ex.

**Discussion**

Discovery of cell-free components, such as exosomes,16 capable of instigating cell analogous response in target cells may provide a promising alternative for cardiac regeneration and allow utilization of benefits associated with adoptive stem cell therapies. Recent reports suggest that exosomes derived from various stem cells enhance myocardial viability and prevent adverse remodeling of the pathological heart because of reduction in oxidative stress and AKT activation in a MI model.15 Similarly, exosomes secreted by CPCs were reported to stimulate migration of endothelial cells18 and protect ischemic myocardium from ischemia/reperfusion injury19 validating that exosome derived from stem cells recapitulate cardiac regeneration representative of adoptively transferred stem cells. However, mechanism of exosome-mediated cardiac protection remains unclear as either exosomes used in these studies were characteristic of stem cells with paracrine abilities or unable to activate endogenous repair processes in the heart after injury.

However, all stem cell–derived exosomes are not created equal. Because exosomes largely pack small RNAs and protein representative of parent stem cell phenotype, the choice of stem cells becomes critical. Embryonic stem cells with
their unique microRNA and protein content, as well as signature cell cycle activity, represent an attractive source of exosomes for augmentation of endogenous cardiomyocyte/CPC proliferative and survival/differentiation responses after myocardial injury. This study demonstrates that mES Ex augment post MI physiological and anatomic myocardial repair in cell autonomous manner that strongly suggests cardiac therapeutic potential of mES Ex in augmenting endogenous repair mechanisms. Importantly, data presented in this article suggest that our findings can be easily translated to autologous iPS cells thereby paving way for iPS-exosomes for potential clinical trials. Thus, proposed studies represent a novel cell–free system that recapitulates ESC regenerative power for cardiac repair and circumvents concerns and limitations associated with direct cell administration.

Evidence from literature suggests that cardiomyocytes are capable of limited cell division, whereas CPCs regulate cardiac homeostasis forming a critical axis for endogenous repair potential of ESC derived exosomes.
myocardial repair. Recently, however, the relative contribution of the endogenous c-kit\(^+\) CPCs to cardiomyogenesis has come into question.\(^{33}\) Despite the low occurrence of cardiomyocytes originating from endogenous CPCs observed in the above report using lineage tracing technology, existence of c-kit\(^+\) CPCs in the heart together with their ability to form cardiomyocytes, albeit few, is remarkably clear. Ideal strategies for cardiac repair would bank on not only increasing CPC function but also promoting cardiomyocyte replenishment in failing hearts. Indeed, our results point toward significant activation of cardiomyocyte and CPC-based repair and regenerative programs in heart receiving mES-Ex. Importantly, our data provide evidence that CPCs when pretreated with mES Ex before transplantation to ischemic myocardium survive for long term (≤8 weeks of experimental window) and support the possibility for high engraftment and de novo cardiomyocyte differentiation. Thus, our findings may represent a novel strategy to enhance CPC contribution to cardiomyogenesis.

The inherent plasticity of ESCs is argued to be an advantage for their potential application in regenerative medicine. ESCs have been used in animal studies of cardiac repair,\(^{12,15}\) and transplantation of human ES-derived cardiomyocyte in primate models has recently been associated with arrhythmogenic response, despite myocardial regeneration.\(^{33}\) Moreover, ethical, technical, and regulatory issues, as well as unavailability of autologous human ESC, for cell therapy applications limit the potential therapeutic use of ESC in humans. The remarkable discovery by Takahashi and Yamanaka\(^{34}\) toward the derivation of iPSCs have solved the issue of availability of autologous pluripotent cells and despite rapid research on iPSC-derived cardiac lineage cell, these cells also present some of the same burden that is associated with ESC cells. Although iPSC-derived cardiac cells provide a fantastic tool for disease modeling and drug screening, further work needs to be done toward generating and extensively characterizing clinical grade iPSC cells before human cell replacement therapies can be attempted.\(^{35}\) Beyond these concerns, ES/iPS derivative cells, when used as cell replacement therapy, may still suffer the same difficulties in cell retention and survival in ischemic myocardium as is noted for adult stem cells. Thus, there is a critical need for exploiting the powerful regenerative capacity of pluripotent cells while avoiding the problems associated with cell transplantation and exosomes derived from pluripotent cells may provide such therapeutic tool.

The underlying molecular basis for cardioprotection observed by exosome in published studies remains unclear although it seems that exosomes directly communicate with the target cells and deliver the specific microRNAs, proteins, and other small RNAs representative of their parental cell of origin.\(^{22,36}\) Therefore, we postulated that ESC-specific miRs involved in regulation of pluripotency, proliferation, and the distinctive ESC cell cycle are consigned within exosomes derived from ESCs and are delivered to target cells. Indeed, analysis of miR expression in ES exosome revealed high expression of ES-specific miRs especially that of miR-290 family. Elevated levels of miR-291, miR-294, and miR-295 were observed in the heart and CPCs after treatment with mES Ex suggested not only mES exosome as their source (these miRs are not expressed in adult cells or organs) but also a possible role for members of the miR-290 family in mediating the effect of mES Ex. This miRNA family comprises 14 miRNA (290–295),\(^{30}\) bear a common seed sequence (AAAGUGC), are functionally dominant miRNAs in ES cells and comprise ≈70% of all ES miR contents. In particular, miR-291, miR-294, and miR-295 encoded in the 290 cluster are expressed exclusively during early development and ES cells and regulate ES cell cycle and self-renewal\(^{17}\) with corresponding effect on proliferation and differentiation.\(^{29,38}\) Indeed, over-expression of miR-294 mimics both in CPCs in vitro, recapitulated some of the similar effects as were observed by exosome treatment suggesting a direct role of ES-specific miRs in the augmentation of post-MI cardiac repair. These results are in concordance with studies that document the multifaceted role played by miR-294 in modulating cellular reprogramming,\(^{39}\) proliferation,\(^{37}\) and survival.\(^{40}\) In contrast, inhibition of miR-294 is an important aspect of the study and would have enabled us to compare the effect of miR-294 enriched exosomes to miR-294 alone. However, because miR-294 critically regulates various ESCs characteristics, including pluripotency, cell cycle, and proliferation as described above, altering miR294 levels leads to a complete loss in cellular properties including their survival and proliferation and in turn changing both the yield and characteristics of exosomes.

In summary, the beneficial effect of mES Ex in the heart after injury in our study suggests that cardiomyocyte survival and cell cycle entry, enhanced neovascularization and potentiation of CPC expansion, differentiation and survival are mediated by miR-294 delivered via ESC exosomes to the heart (Figure 8I). Recent studies conform to these findings and demonstrate efficiency of cardiac repair after restoration of endogenous repair processes by ex vivo delivery of therapeutic agents.\(^{7,8}\) Furthermore, enhanced neovascularization by mES Ex maybe caused by increased activation and cycling of endothelial cells in the heart. Synergistic CPC adoptive transfer combined with exosome delivery or engineering of CPC with ES-specific microRNAs may provide for a potential powerful therapeutic regimen preserving adoptively transferred cells and at the same time revitalizing endogenous myocardial repair processes.

Acknowledgments

We thank all members of the Kishore laboratory for their valuable discussions and Dr Muniswamy Madesh for their help with confocal microscopy.

Sources of Funding

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Disclosures

None.
Cardiac Reparative Potential of ESC Exosomes

References


17. Khan et al.
Circulation Research  June 19, 2015


What Is Known?

• Embryonic stem cells are a promising source of cardiac myocytes, yet their use remains controversial because of ethical concerns and methodological limitations.
• Stem cell–derived exosomes are able to recapitulate regenerative potential of parent cells of their origin.

What New Information Does This Article Contribute?

• Mouse embryonic stem cells–derived exosomes (mES Ex) modulate cellular processes in target cells.
• mES Ex augment cardiac function after myocardial infarction.
• Hearts receiving mES Ex show enhanced activation and contribution of cardiac progenitor cells toward cardiac repair.
• Salutary effects of mES Ex are mediated by the transfer of miR-294 to the heart and cardiac progenitor cell promoting their survival and proliferation.

Novelty and Significance

ESCs possess the ability to form functional cardiomyocytes, but their use remains controversial. Recent identification of small vesicles called exosomes in the stem cell secretome carries significant implications for cardiac regeneration. ESCs have the ability to secrete exosomes yet their role in cardiac repair is not well defined. Here, we report that mES Ex have the ability to modulate molecular signaling, survival, and tube formation in target cells. Delivery of mES Ex in the heart after myocardial infarction leads to significant augmentation of cardiac function in conjunction with enhanced cardiac proliferative response. Moreover, mES Ex promote cardiac progenitor cell survival, proliferation, persistence, and contribution toward repair processes in the heart after myocardial infarction. The beneficial effects of mES Ex are mediated by miR-294 transfer to the heart and cardiac progenitor cells promoting survival and proliferation. Cardiac repair potential of mES Ex represents a novel cell–free strategy that harnesses the regenerative power of ESC. Understanding the mechanisms underlying the stem cell exosome-mediated cardiac repair and regeneration may be beneficial in developing an alternate cell-free strategy for the treatment of cardiac diseases.
Embryonic Stem Cell–Derived Exosomes Promote Endogenous Repair Mechanisms and Enhance Cardiac Function Following Myocardial Infarction


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Supplemental Material

Online Methods

Cell Culture
Mouse embryonic stem cells are cultured in DMEM (high glucose) with 15% FBS and supplemented with β-mercaptoethanol (100 μM), non-essential amino acids (100 μM), leukemia inhibitory factor (LIF; 1000U/ml) and penicillin/streptomycin (50μg/ml each). Mouse embryonic fibroblasts (MEF) are cultured in DMEM with 10% FBS, Non-essential amino acids, (100 μM) and penicillin/streptomycin (50μg/ml each). H9c2 myoblasts are maintained in DMEM supplemented with 10% FBS and pen/strep. Human umbilical vein endothelial cells (HUVECs) are maintained in EGM-2 Bullet Kit (CC-3162) contains one 500 ml bottle of Endothelial Cell Basal Medium-2 and the following growth supplements: Hydrocortisone, hFGF-B, VEGF, R3-IGF-1, Ascorbic Acid, Heparin, FBS, hEGF, GA-1000 with is 2% FBS. CPCs from syngeneic male FVB mice are cultured in cardiac stem cell media 3 comprising of DMEM/F12 supplemented with 10% FBS, basic fibroblast growth factor (bFGF), leukemia inhibitory factor (LIF), epidermal growth factor (EGF) and penicillin/streptomycin/glutamine solution. CPCs were differentiated as previously described2 with 10^-8 mol/L dexamethasone treatment for 7 days.

Exosome purification
mES and MEF cells are cultured for 40 hours, and then exosomes were collected and ultrapurified as described previously 3. Briefly, the cells and conditioned media are separated by centrifugation (800 g for 5 minutes); the conditioned media is clarified by centrifugation (14,000 g for 20 minutes) and the exosomes are collected by ultracentrifugation (100,000 g for 1 hour) on a 30% sucrose-D2O solution (density ~1.127 g/cm3), washed in PBS and pelleted. The purified exosome fraction is re-suspended in PBS for use.

Electron microscopy
Cells are fixed with 4% paraformaldehyde and 1% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) (Electron Microscopy Sciences, Hatfield, PA, USA) for 3 hours at room temperature, washed with cacodylate buffer, post fixed in 1% osmium tetroxide, progressively dehydrated in a graded ethanol series (50–100%), and embedded in Epon. Thin (1-mm) and ultrathin (70- to 80-nm) sections were cut from the polymer with a Reichert (Depew, NY, USA) Ultracut S microtome, placed on copper grids, and briefly stained with uranyl acetate and lead citrate. Exosomes are fixed with 2% paraformaldehyde, loaded on 300-mesh formvar/carboncoated electron microscopy grids (Electron Microscopy Sciences, PA, USA), post-fixed in 1% glutaraldehyde, and then contrasted and embedded as described previously 3. Transmission electron microscopy images are obtained with an FEI (Hillsboro, OR, USA) Tecnai Spirit G2 transmission electron microscope operating at 120 kV.

CyQuant and Metabolic assay
CyQuant and 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide assay of CPCs are performed by plating cells in quadruplicate (2000 cells/well) in 96-well plates, followed by incubation with CyQuant (Invitrogen, CA, USA) or 3-(4,5-Dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide reagent (Sigma, Mo, USA) as previously described 4.
**Matrigel tube formation assay**
CPCs treated with exosomes and miRNA mimics are starved overnight prior to the Matrigel assay in CPC medium. Starved CPCs were then seeded in 48-well plate coated with growth factor-reduced Matrigel (BD Biosciences, CA, USA). Tube formation was examined by phase-contrast microscopy 6hrs and 24 hrs later.

**Dynamic light scattering**
Exosomes are suspended in phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA); then, dynamic light-scattering measurements are performed with a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK) as described previously. Intensity, volume, and distribution data for each sample are collected on a continuous basis for 4 minutes in sets of three. At least three different measurements from three different samples are performed for each exosome population.

**Western blotting**
Western blot analysis is carried out as previously described. Briefly, Cells or purified exosomes are lysed with 0.1M Tris, 0.3 M NaCl, 0.1% SDS, 0.5% sodium deoxycholate, and 1% Triton X-100 in a cocktail of antiproteases (Sigma-Aldrich Corporation, St. Louis, MO, USA); then, the nuclei and membranes are cleared by centrifugation (15,000g for 10 minutes). Protein extracts are separated on an 8% SDS-PAGE gel, blotted on Immobilon (Millipore, Billerica, MA, USA) with TSG101 (4A10; Abcam Inc.), and visualized with enhanced chemoluminescence substrate (Thermo Fisher Scientific, Rockford, IL, USA). Images are acquired with a Chemidoc XRS (Kodak, Rochester, NY, USA).

**Real-Time Quantitative reverse transcription Polymerase chain reaction**
Total RNA is isolated from frozen heart or cultured cells using RNA-stat and reverse-transcribed into cDNA using iScript cDNA Synthesis kit (Bio-Rad, CA, USA). Quantitative reverse transcriptase polymerase chain reaction is performed on all samples in triplicate using iQ SYBR Green (Bio-Rad, CA, USA) according to the manufacturer’s instructions. Primer sequences are provided in Online Table I.

**Flow Cytometry**
Cell death is measured by Annexin V staining (BD Biosciences, CA, USA) according to the manufacturer’s instructions. Cell cycle analysis with propidium iodide (PI, BD Biosciences, CA, USA) is performed by fixing the cells in cold ethanol for 15min followed by labeling with PI for 40min at 37°C. Cytometry is performed using a BD LSRII Flow Cytometer (BD Biosciences, CA, USA).

**TaqMan® MicroRNA Array**
Single-stranded cDNA is synthesized from all samples using the TaqMan® MicroRNA Reverse Transcription Kit (Part Number 4366593) and the Megaplex™ RT Primers, Rodent Pool Set v3.0 (Part Number 4444746) as described in the Applied Biosystems protocol “Megaplex™ Pools for microRNA Expression Analysis (Part Number 4399721 Rev. C). The reverse transcription product is pre-amplified using Megaplex™ PreAmp Primers, Rodent Pool B v3.0 (4444308). The pre-amplified product is used to run real time PCR reactions using TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Part Number 4324018) on a TaqMan® Array Rodent MicroRNA A+B Cards set v3.0 (Part Number 4444909). The array cards are run on a 7900HT system.
MicroRNA treatment and quantification
Cells are transfected with mouse miR-291a-5p, miR-294-3p, miR-295-3p (mimics) or negative control mimics. CPCs are grown in DMEM/F12 media without antibiotics and transfected with either miRNA mimics or controls (25nM, Invitrogen, CA, USA) using Lipofectamine RNAiMAX (Invitrogen, CA, USA) for 24 hrs as per manufacturer instructions. Total RNA from the CPCs and heart tissue is extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer’s protocol including a DNase step. RNA concentrations are verified on the NanoDrop Spectrophotometer (NanoDrop, Thermo Scientific, DE, USA). Equal amount of RNA (5ng) is reverse transcribed using the Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, CA, USA) using a specific miRNA primer to generate cDNA for use with individual Taqman MicroRNA Assays (Applied Biosystems, CA, USA). Real time Reactions are performed in triplicate on a 7500FAST Real-Time PCR system (Applied Biosystems, CA, USA). Ct values are averaged and normalized to snoRNA234. Relative expression is determined by ∆∆Ct comparative threshold method.

Neonatal Rat Cardiomyocyte Isolation
Neonatal rat cardiomyocytes (NRCM) are prepared by enzymatic digestion of hearts obtained from newborn (0–2 day old) Sprague–Dawley rat pups using percoll gradient centrifugation and plated on six-well cell culture grade plates (coated with collagen IV) at a density of 0.85 × 106 cells/well in DMEM/M199 medium and maintained at 37°C in humid air with 5% CO27.

Animal Studies
All mice (C57BL/6; 8-12 weeks old) used in this study were obtained from The Jackson Laboratories (Bar Harbor, ME). All surgical procedures and animal care protocols were approved by the Temple University Animal Care and Use Committee.

Induction of Acute Myocardial Infarction and Injections
Myocardial infarction. Mice underwent surgery to ligate the left anterior descending coronary artery as reported previously 8 followed by administration of exosomes from mES cells (n=6) and MEF (n=6) cells suspended in saline intramyocardially into the left ventricular wall (border zone) at two different locations immediately after left anterior descending ligation. The saline group underwent the same surgery but received saline without exosomes (n=6). Tissue was harvested at 5 days, 2, 4 and 8weeks after AMI for histological analysis. Acute myocardial infarction (AMI) was induced as described previously 7, 8. Briefly, mice are anesthetized, orally intubated and placed in a supine position. Respiration is controlled by mechanical ventilation using a rodent ventilator (Nemi Scientific, Inc., Framingham, MA) with tidal volume set to 0.4 ml at a rate of 110 strokes/min. The chest is then shaved, cleaned free of hair and sterilized. Under a dissecting microscope, a left thoracotomy is performed in the fourth intercostal space. After displacing the pericardium, an 8-0 monofilament nylon suture on a curved tapered needle is passed under the left anterior descending coronary artery (LAD) 4 mm below the left atrium and permanently tied to eliminate blood flow distal to the suture. Following verification of induced ischemia via epicardial blanching, exosome re-suspended in sterile PBS are injected into the infarct border zone as two separate 10 μl injections (one on either side of the ligation). Pericardium is re-draped over heart, and the chest was then closed following the injection. A 22 gauge syringe was used to re-establish negative pressure within the chest cavity prior to extubation. Animals received post-surgical pain management with buprenorphine and surgical inflammation control with meloxicam. Animals were recovered until freely mobile on a heating pad at which point they were then placed into a clean cage and housed for the duration of the experiment.
Echocardiography

Transthoracic two-dimensional M-mode echocardiography was performed using the Vevo770 (VisualSonics, Toronto, ON, Canada) equipped with a 30-MHz transducer. Mice were anesthetized for analysis with a mixture of 1.5% isoflurane and oxygen (1 L/min) before AMI (baseline) at 1, 2, 3, 4, 6 and 8 weeks after AMI. M-mode tracings were used to measure left ventricular wall thickness and left ventricular inner diameter in systole and diastole. The mean value of three measurements was determined for each sample. Percentage fractional shortening and percentage ejection fraction were calculated as described previously. 7, 8.

Histology and Staining

Immunostaining of CPCs is performed on cells grown on permanox or glass chamber slides. Cells are fixed by 4% paraformaldehyde (PFA), permeabilized in PBS supplemented by 0.2% Triton-X for 10 min, and blocked in PBS supplemented with 10% horse serum for 1 hr. Primary antibodies diluted are applied overnight at 4°C after blocking in PBS with 10% horse serum. The next day, cells are washed with PBS and incubated for 1 h at room temperature with secondary antibodies (Jackson Laboratories, USA) diluted in blocking solution. Sytox Blue or To-Pro (Molecular Probes, USA) is diluted in Vectashield (Vector Labs, CA, USA) mounting media at 1:500 vol/vol and used as nuclear staining. Paraffin heart sections are deparaffinized in xylene and rehydrated through graded alcohols to distilled water. Antigen retrieval is achieved by boiling the slides in 10 mmol/L citrate pH 6.0 for 12–15 min. Slides are washed several times with distilled water and once with TN buffer (100 mmol/L Tris, 150 mmol/L NaCl). Endogenous tissue peroxidase activity is quenched with TN buffer supplemented with 3% H2O2 for 20 min whenever necessary. Slides are then washed in TN buffer and blocked in TNB buffer (TSATM kit from Perkin-Elmer) at room temperature for at least 30 min. Primary antibodies are applied overnight at 4°C in TNB buffer. The next day, samples are washed in TN buffer and incubated with secondary antibodies at room temperature in the dark for 1 hr. When amplification of signal is needed, slides are washed in TN buffer and incubated with streptavidin horseradish peroxidase conjugated diluted 1:100 vol/vol in TNB buffer for 30 min at room temperature, and signals are developed using Tyramide substrate diluted 1:50 vol/vol in Amplification Diluent (Perkin-Elmer) for 10 min. Slides are washed in TN buffer and coverslipped using Vectashield in the presence of DNA staining. List of primary and secondary antibodies is reported in OnlineTable II.

Infarct Size Determination

All fixed hearts were sectioned starting from the height of the ligating suture and then sequentially at 250-μm distances below the suture as far as effective sectioning would permit. Infarct size was evaluated on Masson's trichrome-stained heart sections cut 500 μm below the ligation point with ImageJ (National Institutes of Health), and the transmural, fibrotic infarct perimeter was then assessed as a percentage of the entire LV chamber perimeter.

Capillary Density Analysis

Capillaries were identified by injecting mice with BS-1 lectin (Vector Laboratories, Burlingame, CA) 10 min prior to sacrifice. Subsequent staining of sections included a goat anti-lectin primary antibody (Vector Laboratories) and FITC-conjugated donkey anti-goat IgG secondary antibody. Slides were imaged using fluorescent microscopy (Zeiss), and capillary density was evaluated by counting positively stained tubular structures within the infarct border zone in sections 500 μm below the ligation point in all hearts. Three high-power visual fields (×20) were analyzed from three independent mouse sections.
Oxygen consumption rate (OCR) measurement

A Seahorse Bioscience XF96 Extracellular Flux Analyzer was utilized to measure oxygen consumption rates (OCR) in cardiac progenitor cells (CPCs) using a protocol similar to that previously reported. CPCs were plated at 3,000 cells per well in XF media supplemented with 1 mM pyruvate, 2 mM glutamine, 10 mM glucose. Three independent OCR measurements were acquired for each condition: baseline, following the addition of 3 μM oligomycin, 2 μM FCCP, and 0.5 μM Antimycin A and 0.5 μM Rotenone. At the conclusion of each experiment, cells were lysed in RIPA buffer and protein concentration was determined for each well using a standard Bradford assay. All calculations for assessment of OCR were evaluated from the third reading in each condition and all values reported as mean +/- SEM, (pmoles O2/min/mg).
Online Figure IV
Online Figure V

A: Saline
B: MEF Ex
C: mES Ex

D: % c-kit+/pH3+ cells

E
F
G

H: % c-kit+/TUNEL+ cells
Online Figure VI

A

Annexin-V (%)

mES media  MEF media  mES Ex-free  MEF Ex-free

B  mES Ex-CPCs  C  mES Ex-CPCs  D  MEF Ex-CPC

GFP  Actin  GFP  Actin  GFP

SD
Online Figure VII

A  MEF Ex-CPC  B  mES Ex-CPCs

C

% GFP+TUNEL+ cells  

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D  E

F

% GFP+SM22+ cells  

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Online Figure VIII

A. miRNAs Significantly Expressed in mES Exosomes and Differential Expression with MEF Exosomes
- Not Significantly Expressed: 430
- >2 Fold Increase Expression: 338
- <2 Fold Decrease Expression: 110
- Same expression: 59

B. miR expression levels for snoRNA234
- Saline expression
- miES Ex expression

C. NRCMs
- Fold change/NT for Cyclin E1, Cyclin A2, and Cdk2 across NT, miR-291, and miR-294

D. CPCs
- Fold change/NT for c-myc and Kit4 across NT and miR-294
Online Figure IX

A

B

C

D

E

Time (min)

Baseline

Oligo

FCCP

Rot/AA

NT

MEF Ex

mES Ex

Basal Resp.

ATP Production

Max Resp.

Spare Capacity

OCR (pmol/min/mg)

NT

MEF Ex

mES Ex

NT

MEF Ex

mES Ex

NT

MEF Ex

mES Ex

NT

MEF Ex

mES Ex
Online Figure legends

**Online Figure I: Characterization exosomes derived from embryonic stem cells (ESC).** A-C) Exosomes secreted by MEF cells with higher magnification of the exosome in adjacent panels compared to mES exosomes (D-F) as confirmed by electron microscopy Panel A, D scale bar=5µm, Panel B, E scale bar=1µm, Panel C, F scale bar=100nm. G) Exosomes from MEF and mES cells express exosome marker Flotillin-1 and are negative for nuclear marker Lamin B as confirmed by immunoblot analysis. Protein from MEF and mES cells was used as controls. H) Increased mRNA levels of pluripotency markers OCT-4, SOX2, Nanog in mES exosomes compared to MEF cells and media alone confirming the ESC origin of exosomes. mES cells were used as positive control and showed significantly high expression of the pluripotency markers.

**Online Figure II: Reduced fibrosis in the heart after mES Ex treatment.** A-C) Decreased fibrosis in mice receiving mES Ex compared to MEF Ex and saline treated animals at 4 weeks after infarction as evidenced by Masson's trichrome staining with corresponding quantification of infarct size (D) (n=6). Scale bar =20µm. Saline vs. mES Ex \#p < 0.05, \##p < 0.01, \###p < 0.001 and MEF Ex vs mES Ex \#p < 0.05, \##p < 0.01, \###p < 0.001.

**Online Figure III: Assessment of tumor formation in mice receiving mES Ex.** Hematoxylin and Eosin staining of the heart samples showed no tumor in the heart of animals receiving saline A), MEF Ex B) and mES Ex C).

**Online Figure IV: mES Ex modulate expression of markers of proliferation.** A-C) Increased BrdU+ cardiomyocytes in mES exosomes treated hearts compared to MEF exosomes and saline treated hearts along with corresponding quantification. D) BrdU (green), nuclei (blue) and sarcomeric actin (red). E) Increased mRNA expression of cyclinA2, D1, D2 and E1 after treatment with mES Ex compared to saline and MEF Ex in hearts after myocardial infarction by qRT-PCR (n=5). F) Reduced expression of cell cycle inhibitors p16, p19, p21 and p53 after mES exosome treatment 2 days after infarction (n=4). Saline vs. mES Ex \*p < 0.05, \**p < 0.01, \***p < 0.001 and MEF Ex vs mES Ex \#p < 0.05, \##p < 0.01, \###p < 0.001.

**Online Figure V: Enhanced CPC proliferation and survival in heart after mES Ex administration.** A-C) Increase in number of c-kit+/pH3+ CPCs in the heart 5 days after mES Ex administration compared to MEF Ex and saline treated animals along with corresponding quantification in (D) (n=4). c-kit (red), pH3 (green), sarcomeric actin (blue) and nuclei (white). E-G) Reduction in c-kit+/TUNEL+ CPCs in mES Ex administered hearts after 5 days compared to MEF Ex and saline treated animals. H) Quantification of c-kit+/TUNEL+ cells in all treatment groups (n=4). c-kit (red), TUNEL (green), sarcomeric actin (blue) and nuclei (white). Scale bar=40µm. Saline vs. mES Ex \*p < 0.05, \**p < 0.01, \***p < 0.001 and MEF Ex vs mES Ex \#p < 0.05, \##p < 0.01, \###p < 0.001.

**Online Figure VI: A) Characterization of cell survival in CPCs treated with exosome free media, MEF exosome free media and mES and MEF growth media shows no significant effect on the number of Annexin-V+ cells as measured by FACS based cell death assay.** B) mES Ex pretreated CPCs are observed in the heart 14 days after infarction. C) GFP+ mES Ex-CPCs are observed in close proximity to blood vessels along with formation of a few small myocytes. D) MEF Ex-CPCs form rare GFP+ myocytes in the heart after 14 days. Scale bar=40µm.

**Online Figure VII: Reduced apoptosis and increased blood vessel formation by GFP+ mES Ex-CPCs.** A-B) Reduced GFP+/TUNEL+ cells in mES Ex-CPC hearts compared to MEF Ex-CPC animals 5 days after infarction along with corresponding quantification (C). GFP
(green), TUNEL (red), sarcomeric actin (blue) and nuclei (white). Scale bar=20µm. D-E) Increased GFP+/SM22+ cells in the heart transplanted with mES Ex-CPC compared to controls at 8 weeks after infarction with quantification (F). GFP (green), SM22 (red), sarcomeric actin (blue) and nuclei (white). Scale bar=20µm. mES Ex-CPC vs. MEF Ex-CPC *p < 0.05, **p < 0.01, ***p < 0.001.

Online Figure VIII: A) Differential expression analysis of 338 miRs in mES Ex and MEF Ex shown on the right pie. The left pie indicates total number of miRs with no change in expression (430) and with significant change (338). 59 miRs had significantly high expression in mES exosomes including certain ES-specific miRs (miRs-291, 294, 295). Total identified indicate significant expression of 338 miRs in ES exosomes (left pie). B) Increased expression of miR-291 and miR-294 in hearts transplanted with mES Ex while no expression was detected in saline and MEF Ex transplanted animals (n=6). Saline vs. mES Ex *p < 0.05, **p < 0.01, ***p < 0.001. C) Enhanced expression of cell cycle markers (Cyclin E1, A2 and Cdk2) in neonatal rat cardiomyocytes 24 hrs after treatment with mimics for miR-291 (50nM) and miR-294 (50nM) as measured by miRNA qRT-PCR (n=3). Expression of miRs was normalized to endogenous control snoRNA234 while fold expression was calculated against non-treated group that represents cells treated with miR negative control (50nM). D) Increased levels of pluripotent markers (c-myc, Klf4) in CPCs treated with miR-294 (n=3). NT vs. miR-294 *p < 0.05, **p < 0.01, ***p < 0.001.

Online Figure IX: mES Ex increase CPC spare-respiratory capacity. CPCs treated with either MEF Ex or mES Ex were examined for oxidative phosphorylation function by measuring oxygen consumption rates (OCR) in a Seahorse XF96 Analyzer. OCR was evaluated at baseline, after the addition of Oligomycin, FCCP, and Rotenone + Antimycin A. A) Mean oxygen consumption rates for all experimental conditions for control NT (black line), MEF Ex (grey line), and mES Ex (red line). B) Basal respiration rate, calculated as (baseline OCR - Rot/AA OCR), Rot/AA OCR = non-mitochondrial respiration. C) ATP production, calculated as (baseline corr. OCR – Oligo corr. OCR). D) Maximal respiration, calculated as (FCCP OCR – Rot/AA OCR). E) Spare respiratory capacity, calculated as (Max Resp. - Basal Resp.). (n=28 for all groups, data corrected to protein and shown as mean +/- SEM, *p<0.05, **p<0.01)
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Supplemental references


