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

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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 diseases[5,6] and animal models of heart failure.[7,8] Despite these promising results, poor survival and low retention of the donated stem cell population[9,10] remain a significant limitation prompting research into new alternative remedies.

Editorial, see p 7
In This Issue, see p 2

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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Nonstandard Abbreviations and Acronyms	
CPCs	cardiac progenitor cells
GFP	green fluorescent protein
iPS	induced pluripotent stem
MI	myocardial infarction
MEF Ex	mouse embryonic fibroblast–derived exosomes
mES Ex	mouse embryonic stem cell–derived exosomes

failure, however, this also has been reported to enhance arrhythmogenic response.[12,13] In spite of their impressive cardiac repair ability, teratoma formation has been observed[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,[16] capable of instigating cell analogous response in target cells may provide a promising alternative for cardiac protection and regeneration.[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 (MEFs) 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 were cultured in cardiac stem cell media and were differentiated as previously described[20] with 10^{-8} mol/L dexamethasone treatment for 7 days. Additional details are available in Online Data Supplement.

Exosome Isolation and Labeling

mES and MEF cells were cultured for 40 hours followed by collection and purification by ultracentrifugation of exosomes as described previously.[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.[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.[21] 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[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[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.

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 (25 nmol/L, Invitrogen, CA) using Lipofectamine RNAiMAX (Invitrogen) for 24 hours as per manufacturer's instructions.[25] Total RNA from CPCs and the heart is extracted using the miRNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. Real-time reactions were performed in triplicate on a 7500FAST Real-Time PCR system (Applied Biosystems, CA). Ct values were averaged and normalized to snoRNA236. Relative expression was determined by the $^{\Delta\Delta}$Ct comparative threshold method. Detailed methods are provided in the Online Data Supplement.

Oxygen Consumption Rates

A Seahorse Bioscience XF96 extracellular flux analyzer was used to measure oxygen consumption rates in CPCs± exosomes with modification of a previously reported protocol (detailed in Online Data Supplement).

Animal Studies

All mice (male 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 MI

Mice underwent surgery to ligate the left anterior descending coronary artery as reported previously[24] 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.[23,24] 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).[16] 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.

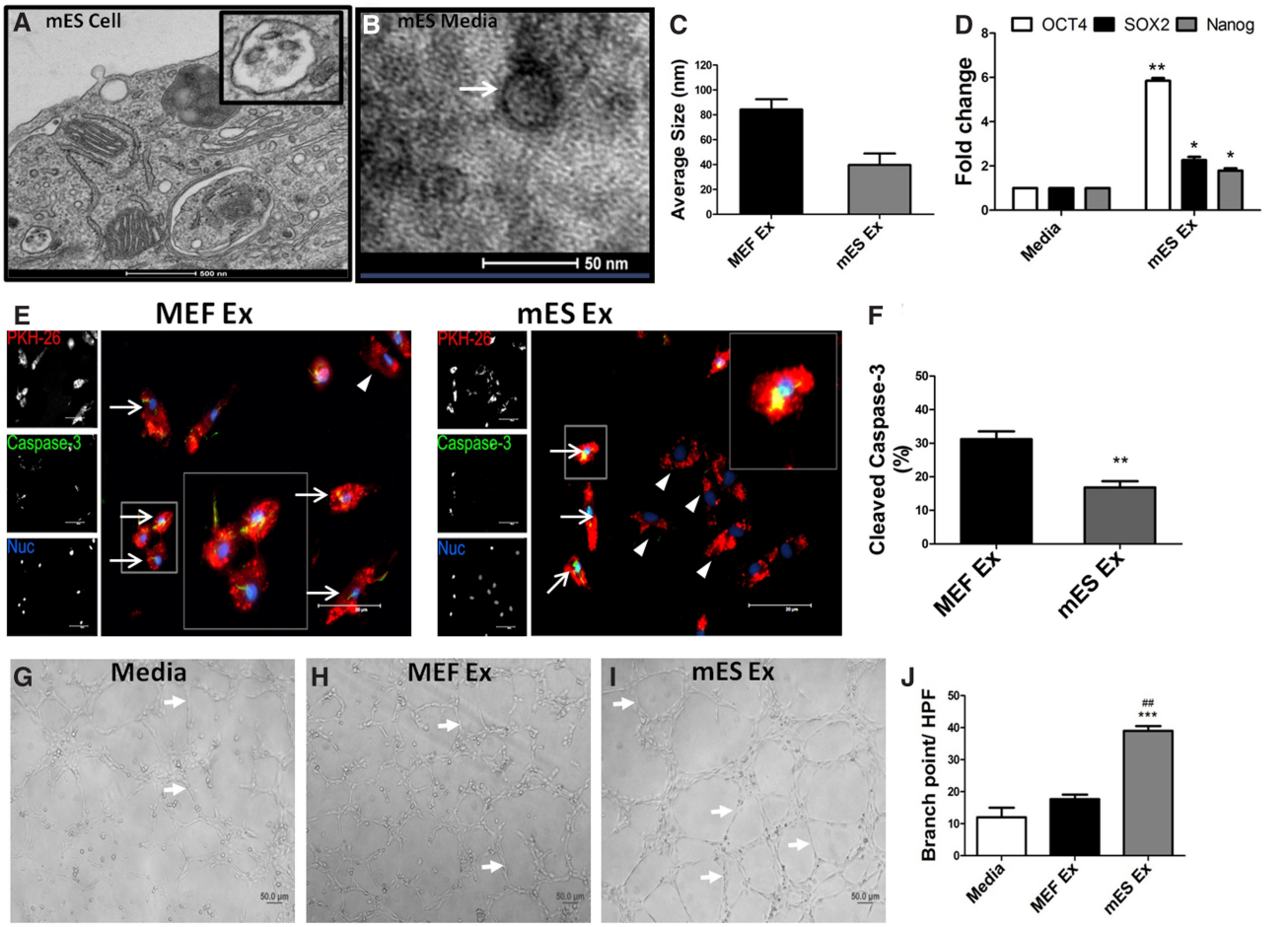

Figure 1. Characterization and functional validation of exosomes derived from embryonic stem cells (ESCs). A, Exosome secretion from a mouse embryonic stem cell (mES) as evidenced by electron microscopy, scale bar=500 nm; inset shows higher magnification of an ESC exosome. **B**, ESC culture medium shows 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 capsase3 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_2O_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_2O_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.

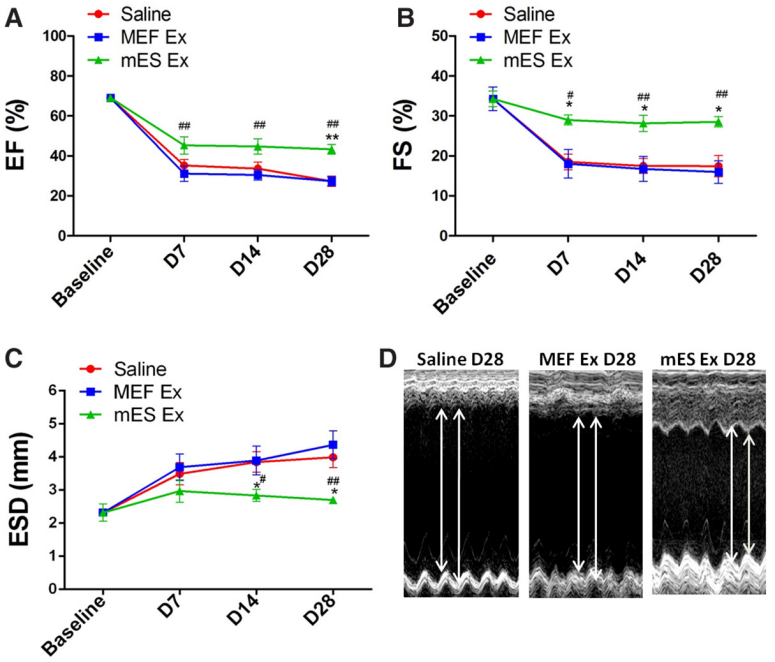

Figure 2. Enhanced cardiac function after myocardial infarction in mice transplanted with mouse embryonic stem cell (mES) exosome. **A**, Increased ejection fraction (EF) and (**B**) fractional shortening (FS) in mice transplanted with mES exosomes (n=6) compared with mice with MEF exosomes (n=6) and saline (n=6) treated animals after 4 weeks after infarction. mES-derived exosome (mES Ex), mouse embryonic fibroblast–derived exosome (MEF Ex), and saline were administered to animals at the time of infarction. **C**, Reduced left ventricular end-systolic diameter (LVESD) after mES Ex treatment compared with saline treatment. **D**, Increased wall motion in mice treated with mES exosomes after 4 weeks as evidenced by M-mode recordings by echocardiography. 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.

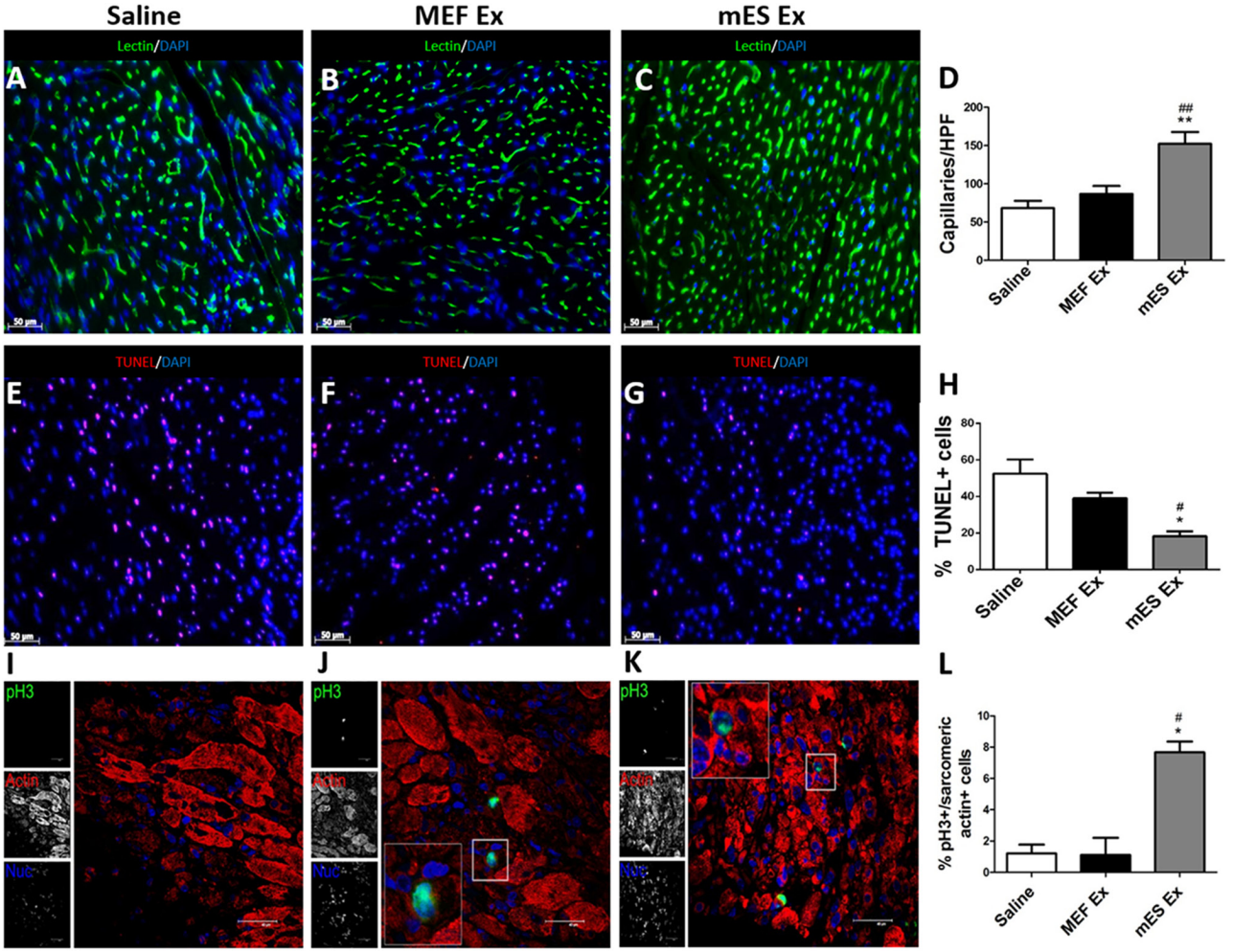

Figure 3. Increased capillary density and reduced apoptosis in the hearts after myocardial infarction. A–C, Increased capillary density after mouse embryonic stem cell–derived exosome (mES Ex) treatment in mice 4 weeks after myocardial infarction along with corresponding quantification (**D**). Lectin (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.

mES Ex augment Resident c-Kit$^+$ CPCs in Infarcted Myocardium

Resident CPCs within the heart capable of regulating cardiac homeostasis[3,4] form an integral part of the endogenous cardiac repair response to injury.[26] 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 heart (Figure 4I–4K). Similarly, CPC proliferation, measured by c-kit$^+$/pH3$^+$ cells, increased by 4.1-fold (Online Figure VA–VD) in conjunction with a 3.8-fold decrease in c-kit$^+$/terminal deoxynucleotidyl transferase–mediated dUTP nick end labeling$^+$ apoptotic 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.

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 treated with mES Ex showed enhanced survival as evidenced

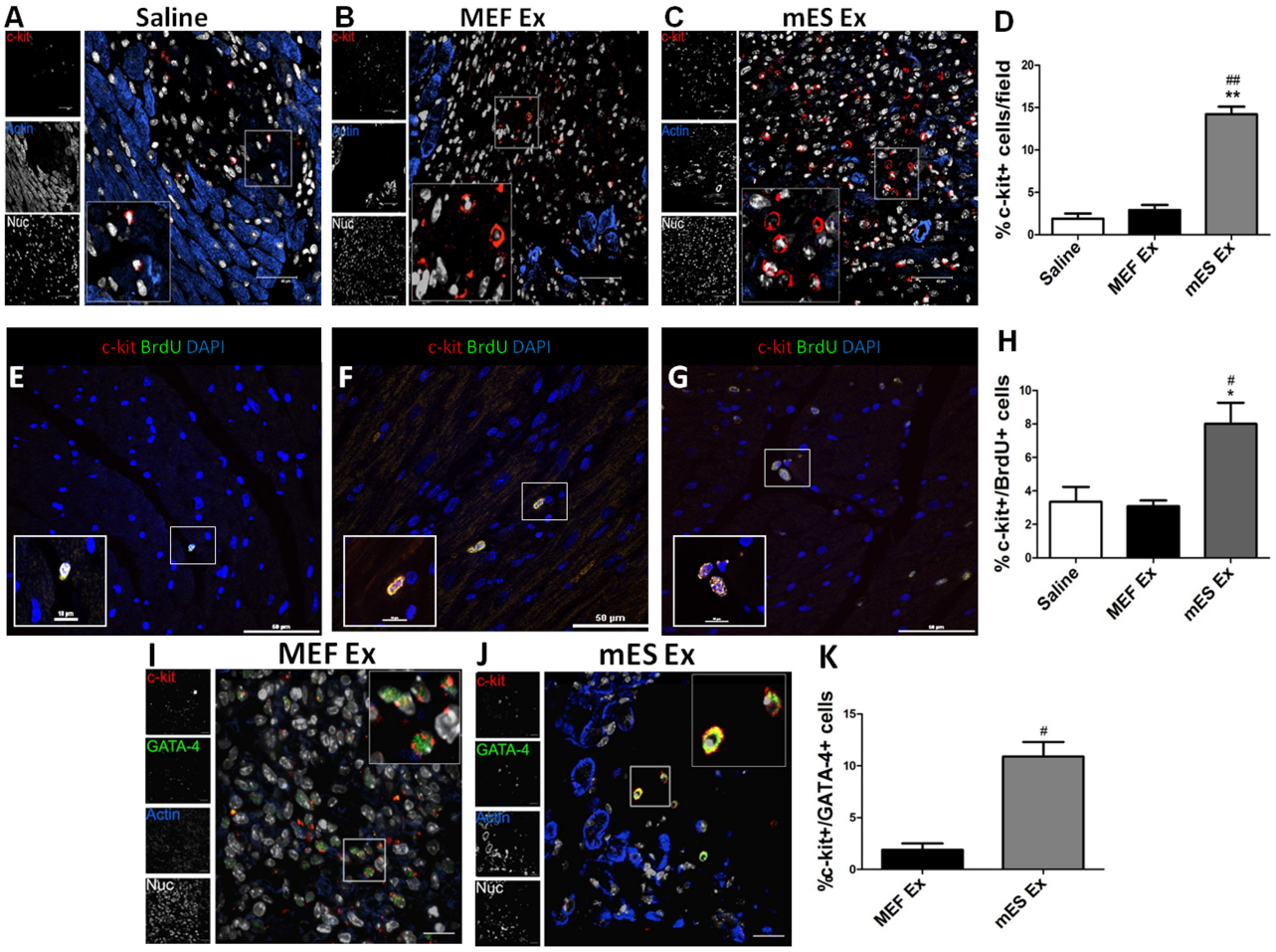

Figure 4. Mouse embryonic stem cell–derived exosome (mES Ex) promote cardiac progenitor cell (CPC) numbers and proliferation in hearts after infarction. A–C, Increased number of c-kit⁺ CPCs in hearts 5 days after mES Ex transplantation compared with mouse embryonic fibroblast–derived exosome (MEF Ex) 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.

by decreased annexin V⁺ cells (8.6%) compared with MEF Ex (20.2%) and nontreated CPCs (18.8%; Figure 5A) in response to H_2O_2 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 pretreated 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 pretreated 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

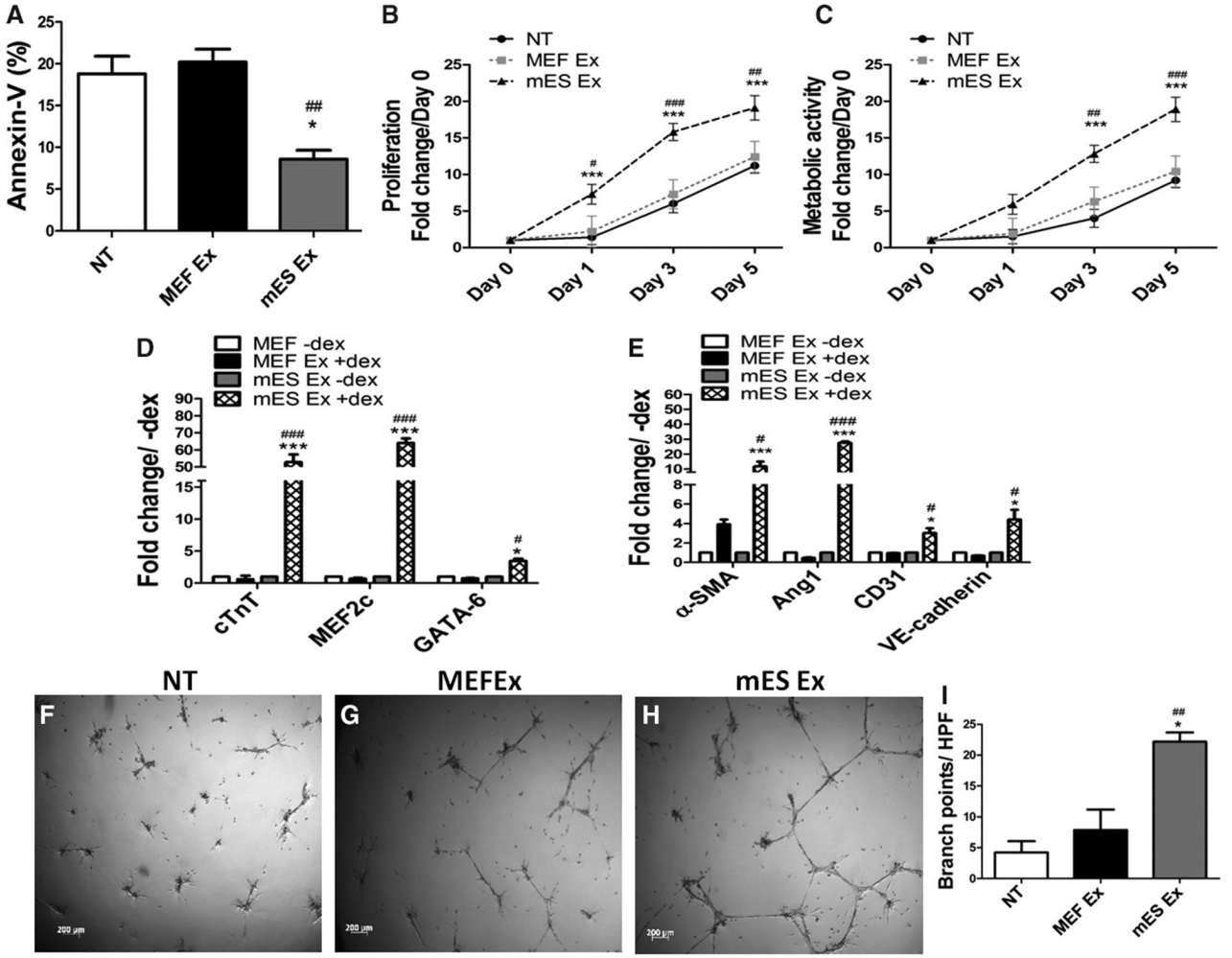

Figure 5. Modulation of cardiac progenitor cell (CPC) function by mouse embryonic stem cell (mES) exosome administration. **A**, Increased survival of CPCs after treatment with mES exosomes in comparison with mouse embryonic fibroblast (MEF) exosomes under H_2O_2 challenge (n=3). **B**, Increased CPC 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**.

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.[22] 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 >10^4-fold expression in mES Ex compared with MEF exosomes (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.[27,28] Furthermore, members of the miR-290 family are

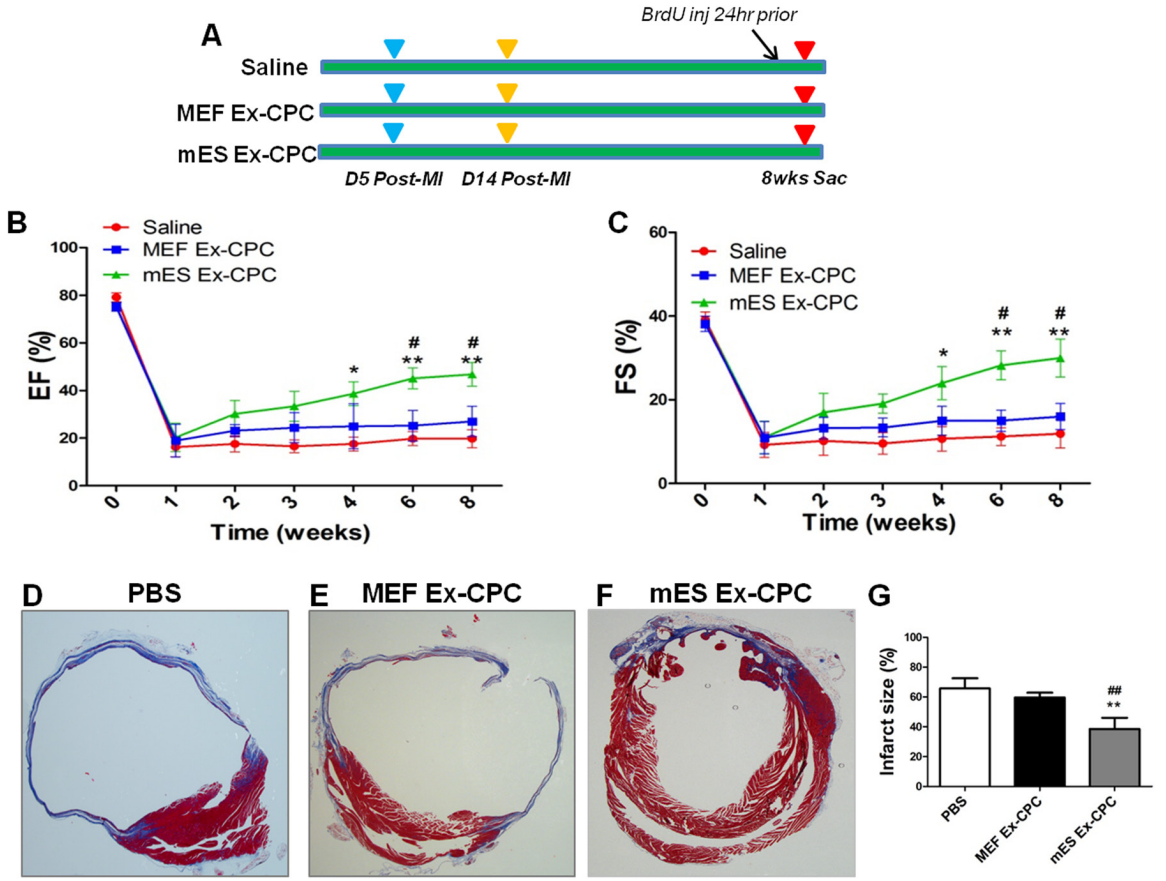

Figure 6. mouse embryonic stem cell–derived exosome (mES Ex) pretreatment of cardiac progenitor cells (CPCs) enhance their potential to augment function and reduce fibrosis. A, Representation of experimental design showing mES Ex pretreated CPC (mES Ex-CPCs), mouse embryonic fibroblast–derived exosome pretreated cardiac progenitor cells (MEF Ex-CPCs), and phosphate buffered saline (PBS) administration in mice after infarction. Animals were followed till 8 weeks and received injections of BrdU before sac. Increased cardiac function ejection fraction (EF; B) and fractional shortening (FS; C) in mES Ex-CPC hearts compared with MEF Ex-CPC and PBS transplanted hearts 8 weeks after infarction. PBS vs mES Ex-CPC **P*<0.05, ***P*<0.01, ****P*<0.001 and MEF Ex-CPC vs mES Ex-CPC #*P*<0.05, ##*P*<0.01, ###*P*<0.001. **D**–**G**, Decreased infarct size in mES Ex-CPC hearts compared with MEF Ex-CPC and PBS transplanted hearts 8 weeks after injury. PBS vs mES Ex-CPC **P*<0.05, ***P*<0.01, and MEF Ex-CPC vs mES Ex-CPC #*P*<0.05, ##*P*<0.01. MI indicates myocardial infarction.

involved in the maintenance of the unique ESC cell cycle regulating G1/S transition.[29] 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.[30] 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.

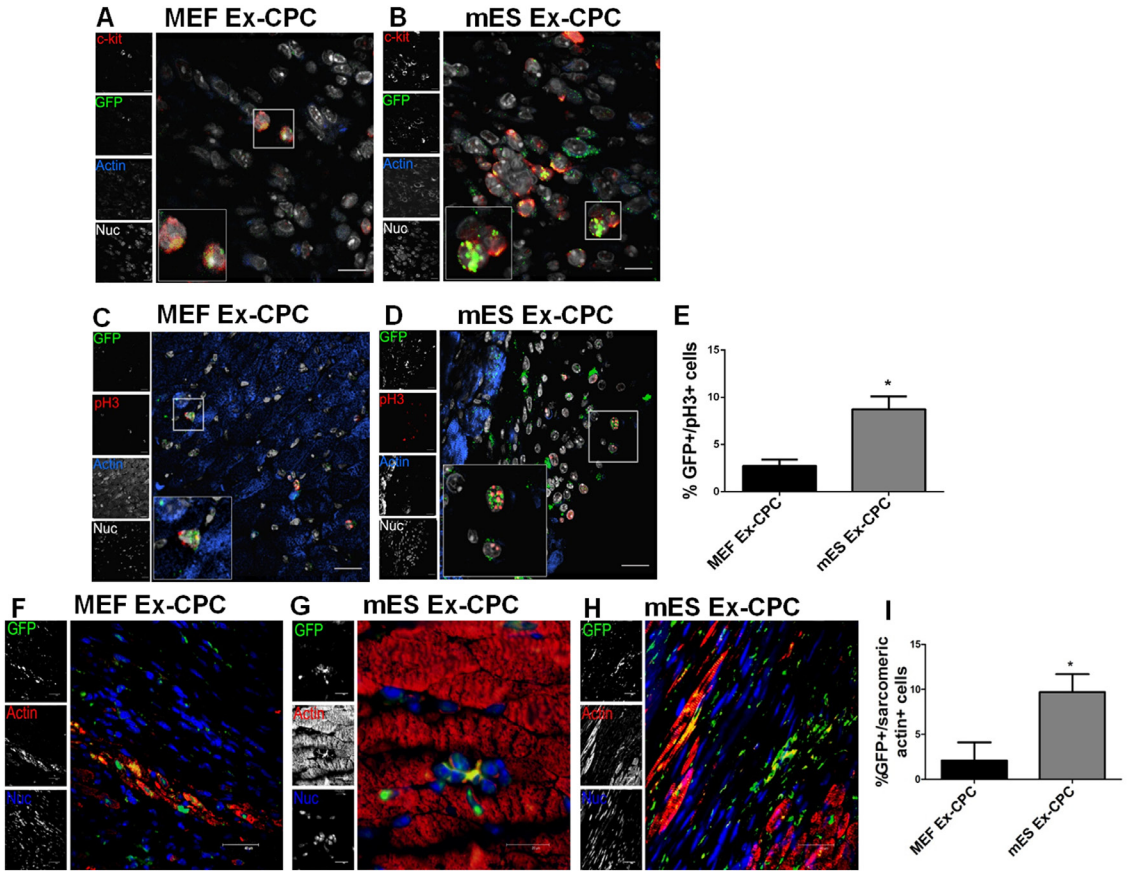

Figure 7. Enhanced persistence and ability to form myocytes of mouse embryonic stem cell–derived exosome pretreated cardiac progenitor cells (mES Ex-CPC). A and **B**, Colocalization of green fluorescent protein (GFP) with CPC marker c-kit in the heart transplanted with mES Ex and mouse embryonic fibroblast–derived exosome (MEF Ex) pretreated cells. c-kit (red), GFP (green), sarcomeric actin (blue), and nuclei (white). Scale bar=20 μm. **C** and **D**, Increased GFP⁺/pH3⁺ cells in mES Ex-CPC hearts 5 days after infarction along with corresponding quantification (**E**). GFP (green), pH3 (red), sarcomeric actin (blue), and nuclei (white). Scale bar=20 μm. mES Ex-CPC vs MEF Ex-CPC *P<0.05. **F**, Small GFP⁺ myocytes in MEF Ex-CPC hearts after 8 weeks of cell delivery. Persistence of mES Ex pretreated GFP⁺ myocytes (**G**) along with GFP⁺ myocyte formation (**H**) in hearts 8 weeks after injury corroborating with the augmented cardiac function. **I**, Quantification of GFP⁺ sarcomeric actin⁺ cells in the hearts transplanted with mES Ex-CPC and MEF Ex-CPC 8 weeks after infarction. GFP (green), sarcomeric actin (red), and nuclei (blue). Scale bar=40 μm. **E**, Scale bar=20 μm.

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 nucleostemin, a marker for multipotency for CPCs[31] and LIN28, a miR-binding protein that has been shown to be involved in regulating pluripotency by miR-294[32] 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 H_2O_2 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.[17] Similarly, exosomes secreted by CPCs were reported to stimulate migration of endothelial cells[18] and protect ischemic myocardium from ischemia/reperfusion injury[19] 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

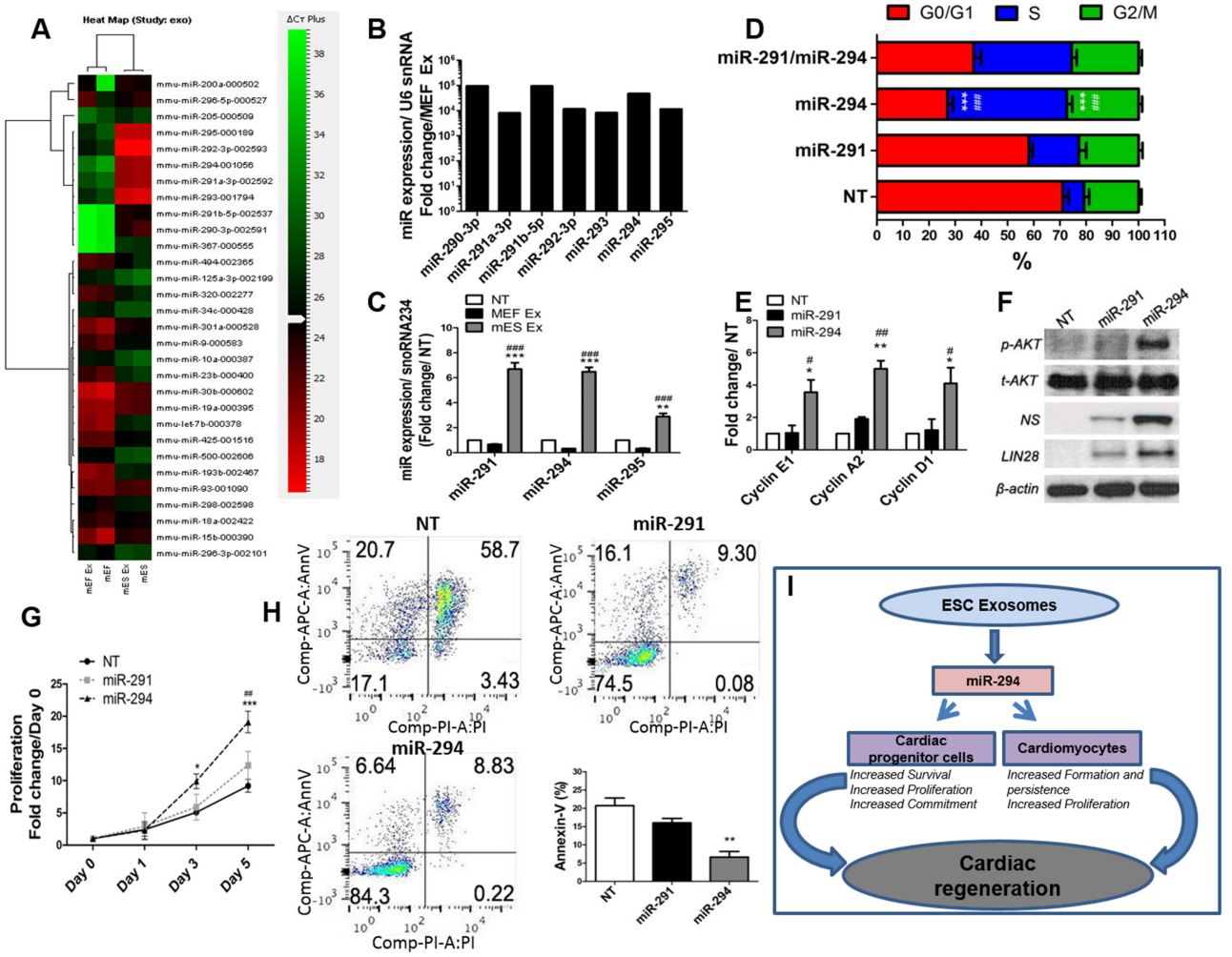

Figure 8. miRNA profiling of mouse embryonic stem cell–derived exosome (mES Ex). A, Comparative analysis of miRs in mES Ex, mouse embryonic fibroblast–derived exosome (MEF Ex) along with mES cells and MEF cells identified significantly high expression of miRNA-290 family in mES cells and mES Ex (>10^4-fold; **B**) compared with MEF cells and MEF Ex groups. **C**, Cardiac progenitor cells (CPCs) treated with mES Ex have enhanced expression of miR-291, miR-294, and miR-295 as confirmed by miRNA quantitative real-time polymerase chain reaction (n=3). Expression of miRs was normalized to snoRNA234. 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. **D**, Increased number of CPCs in G2-phase of the cells cycle after treatment with miR-294 (25 nmol/L) compared with miR-291 (25 nmol/L) and miR291 (25 nmol/L)/miR294 (25 nmol/L) and nontreated control cells as analyzed by fluorescent activated cell sorting (FACS)-based cell cycle assay (n=4). **E**, Enhanced mRNA expression of cyclins (E1, A2, and D1) in CPCs treated with miR-294 (25 nmol/L) compared with miR-291 (25 nmol/L) and nontreated controls (n=3). **F**, Increase phosphorylation of AKT in association with elevated levels of nucleostemin and LIN28 in miR-294 (25 nmol/L) treated CPCs compared with miR-291 and nontreated controls (n=3). **G**, Increased CPC proliferation at day 3 and 5 after miR-294 treatment compared with miR-291 and nontreated CPCs as evidenced by CyQuant assay (n=3). NT vs miR-294 **P*<0.05, ***P*<0.01, ****P*<0.001 and miR-291 vs miR-294 #*P*<0.05, ##*P*<0.01, ###*P*<0.001. **H**, miR-294 treated CPCs showed reduction in Annexin-V$^+$ cells compared with miR-291 and nontreated CPC in response to H_2O_2 challenge as evidenced by FACS-based cell cycle assay (n=3). **I**, Schematic representation of therapeutic effect of ESC-derived exosomes for cardiac repair after myocardial infarction. ESC exosome deliver miR-294 to the heart resulting in significant modulation of survival, proliferation, and cardiac commitment of cardiac progenitor cells. At the same time, enhanced cardiomyocyte survival and proliferation take place as a consequence of ESC exosome delivery that ultimately leads significant augmentation of cardiac regeneration in the heart after myocardial infarction.

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

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 arrythmogenic response, despite myocardial regeneration.[13] 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 iPS have solved the issue of availability of autologous pluripotent cells and despite rapid research on iPS-derived cardiac lineage cell, these cells also present some of the same burden that is associated with ES cells. Although iPS-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 iPS 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[37] with corresponding effect on proliferation and differentiation.[29,38] Indeed, overexpression 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.

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Disclosures

None.

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Novelty and Significance

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

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.