Embryonic Stem Cell-derived Exosomes promote Endogenous Repair Mechanisms and Enhance Cardiac Function following Myocardial Infarction

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Khan: Cardiac reparative potential of ESC exosomes

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Abstract
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+ 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 cardiac progenitor cell (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 utilizes 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.

Keywords: Embryonic stem cells, exosomes, miRNA, cardiac repair, cardiac progenitor cells,
**List of Non-standard Abbreviations**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>mES Ex</td>
<td>Mouse embryonic stem cell derived exosomes</td>
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<td>MEF Ex</td>
<td>Mouse embryonic fibroblast derived exosomes</td>
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<tr>
<td>CPCs</td>
<td>Cardiac progenitor cells</td>
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<tr>
<td>GFP</td>
<td>Green fluorescent protein</td>
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<tr>
<td>TUNEL</td>
<td>Terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling</td>
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<td>mES Ex-CPC</td>
<td>Mouse embryonic stem cell derived exosome pretreated cardiac progenitor cells</td>
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<tr>
<td>MEF Ex-CPC</td>
<td>Mouse embryonic fibroblast derived exosome pretreated cardiac progenitor cells</td>
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<tr>
<td>HUVEC</td>
<td>Human umbilical vein endothelial cells</td>
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<tr>
<td>NRCM</td>
<td>Neonatal rat cardiomyocytes</td>
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<tr>
<td>EF</td>
<td>Ejection fraction</td>
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<tr>
<td>FS</td>
<td>Fractional shortening</td>
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<tr>
<td>ESD</td>
<td>End-systolic diameter</td>
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Introduction
Endogenous myocardial repair in response to injury has been reported to involve limited self-division of pre-existing cardiomyocytes and the activation and differentiation of resident cardiac stem cells (CSC)\(^1\)\(^-\)\(^4\). 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\(^9\)\(^-\)\(^10\) remains a significant limitation prompting research into new alternative remedies.

Pluripotent stem cells including both embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) hold immense promise for cardiac regeneration since 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 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 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.

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, non-traditional 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 manuscript, we report that mES-exosome delivery in the heart after myocardial infarction stimulates and augments CPC and cardiomyocyte proliferation based endogenous myocardial repair, which in part involves transfer of ES-specific microRNA-294. Our data suggests 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**

Mouse embryonic stem cells (mES) isolated from C57Bl/6 were obtained from ATCC and cultured in DMEM (high glucose) with 15% FBS and supplemented with β-mercaptoethanol (100μM), Non-essential amino acids (100 μM), Leukemia inhibitory factor (LIF; 1000U/ml) and penicillin/streptomycin (50ug/ml each). Mouse embryonic fibroblasts (MEF) were cultured in DMEM with 10% FBS, Non-essential amino acids, (100 μM) and penicillin/streptomycin (50ug/ml each). H9c2 myoblasts and Human umbilical vein endothelial cells (HUVECs) 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 with \(10^{-8}\) mol/L dexamethasone treatment for 7 days. Additional detail in online 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. The purified exosome fraction was re-suspended 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 detail in online supplement.

**Dynamic light scattering**

Exosome size analysis was carried out by dynamic light scattering measurement as described previously. Briefly, exosomes were suspended in phosphate-buffered saline (PBS) containing 2 mM ethylenediaminetetraacetic acid (EDTA); then, dynamic light-scattering measurements were performed with a Zetasizer Nano ZS (Malvern Instruments Ltd, Worcestershire, UK). Additional detail in online supplement.

**Electron microscopy**

Cells were fixed with 4% paraformaldehyde and processed, contrasted and embedded as described previously. Transmission electron microscopy images were obtained with an FEI (Hillsboro, OR, USA) Tecnai Spirit G2 transmission electron microscope operating at 120 kV. Additional detail in online supplement.

**Immunoblot**

Immunoblot analysis was performed as described previously with additional detail in online supplement.

**Immunohistochemistry**

Immunocytochemistry, TUNEL assays, and immunohistochemistry were performed as previously described with additional information in online supplement and a list of antibodies in Supplementary 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 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\(^\text{25}\). 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 USA). Ct values were averaged and normalized to snoRNA236. Relative expression was determined by the \(^{\Delta\Delta}C_t\) comparative threshold method. Detailed methods are provided in the online supplemental information.

**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 myocardial infarction**

Mice underwent surgery to ligate the left anterior descending coronary artery as reported previously\(^\text{24}\) 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 or 14 days and 8 weeks after AMI for histological analysis.

**Echocardiography**

Transthoracic two-dimensional M-mode echocardiography was performed using the Vevo770 (VisualSonics, Toronto, ON, Canada) equipped with a 30-MHz transducer as described previously\(^\text{23, 24}\). Additional details in online 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 murine embryonic stem cells (mES) and embryonic fibroblasts (MEF) showed that both cell types secrete exosomes of typical size range\(^1\)\(^\text{16}\) (Figure 1A-C). Additionally, exosomes from both cells expressed exosomal marker protein, flotillin-1 indicating their cytoplasmic origin and were negative for Lamin B, a nuclear protein (Figure 1D). Expression of ES-specific transcripts was exclusively detected in mES Ex (data not shown) confirming the embryonic stem cell origin of the mES Ex.

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 hrs after treatment indicating efficient delivery of exosomal cargo to target cells (Figure 1E). Cell survival after exosomal uptake was determined by labeling mES Ex and 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 to MEF Ex treated cells (31.8%) in response to 16hrs of H\(_2\)O\(_2\) challenge (Figure 1H-K). Finally, human umbilical vein endothelial cells (HUVECs) were treated with mES Ex and MEF Ex and cultured on matrigel to assess whether mES Ex can enhance in vitro tube formation. HUVEC tube formation was significantly increased exclusively after mES Ex treatment (Figure 1G-J). Collectively, results showed that mES Ex are readily up-taken by target cells and modulate target cell function including cell survival.

Intramycocardial delivery of mES Ex improved post-MI cardiac function. In order to assess their therapeutic efficacy in post-infarct myocardium, mES Ex were intramyocardially administered in mice at the time of myocardial infarction while 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 to control groups at 4 weeks. Histological analysis of the heart 4 weeks post infarction indicated decreased infarct size in mES Ex transplanted mice (20.8%) compared to MEF Ex (33.1%) and saline (32.1%) administered animals (data not shown). Together these results provide evidence for a therapeutic role of mES Ex in augmenting cardiac function after myocardial infarction.

mES Exosomes augment neo-vascularization, myocyte proliferation and survival after MI. Immunohistochemical analysis of the hearts isolated from various treatment groups was carried out 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 2D) together with decreased apoptosis (Figure 2E) compared to MEF Ex and saline groups 4 weeks after infarction. A significant increase in pH3+ cardiomyocytes in hearts treated with mES Ex compared to control hearts supported evidence towards myocyte cycling (Figure 2F). 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 cardiac progenitor cells (CPCs) within the heart capable of regulating cardiac homeostasis\(^3\)\(^,\)\(^4\) form an integral part of the endogenous cardiac repair response to injury\(^2\)\(^6\). Since, mES Ex enable functional augmentation following myocardial damage, effect of mES Ex on CPC number, survival and proliferation was assessed in vivo. Compared to controls, the number of resident c-
kit+ CPCs in the myocardium after mES Ex treatment significantly increased (Figure 3A-D). Additional characterization of c-kit+ CPCs was done by colabeling with GATA-4 that revealed a corroborating increase in c-kit+/GATA-4 CPCs in the heart treated mES Ex compared to MEF Ex heart (Figure 3I-K). Similarly, CPC proliferation, measured by c-kit+/ph3+ double positive cells, increased by 4.1 fold (Figure 3E-H) in conjunction with a 3.8 fold decrease in c-kit+/TUNEL+ apoptotic CPCs (data not shown). 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 by decreased annexin V+ cells (8.6%) compared to MEF Ex (20.2%) and non-treated CPCs (18.8%) (Figure 4A) 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) (data not shown) suggesting that mES Ex were predominantly responsible for the observed survival response in CPCs with minimal or no contribution from serum exosomes. Additionally, mES Ex treatment of CPCs also resulted in significantly enhanced CPC proliferation (Figure 4B). 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 towards cardiac lineages. mRNA expression of cardiomyocyte and endothelial cell markers (Figure 4C-D) was increased in CPCs treated with mES Ex compared to MEF Ex under stimulation with dexamethasone for 7days. Independent experiment on CPC tube formation ability on matrigel corroborated increased endothelial differentiation in response to mES Ex (Figure 4E-H).

In order to elucidate whether mES Ex enhance CPC survival and function in vivo, GFP-CPCs pretreated with mES Ex and MEF Ex were transplanted after induction of myocardial infarction. Long-term follow-up studies (8 weeks after MI) showed consistently improved LV function in mice receiving mES Ex treated CPCs compared to MEF Ex treated CPCs (Figure 4l-J). Moreover, significant reduction in fibrosis was observed in mES Ex-CPC hearts compared to controls (Figure 4K-L). The enhanced function was attributed to increased ability of GFP+ mES Ex-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 (data not shown). Furthermore, GFP colocalized with c-kit+ CPCs confirming the identity of the adoptively transferred CPCs 5 days after infarction (Figure 5A-B). Pre-treatment with mES Ex also enhanced the proliferation of the transplanted CPCs (Figure 5C-E) along with reduction in TUNEL+ GFP cells (Figure 5F-H). Interestingly, persistence of GFP+ CPCs and new GFP+ myocytes in mES Ex-CPC transplanted hearts was still evident even after 8 weeks of transplantation (Figure 6A-D) concurrent with increased contribution of GFP+ CPC to new blood vessel formation (Figure 6E-G). Therefore, salutary effects of mES Ex on CPC survival and proliferation in infarcted hearts effectively translates 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 (>2fold) in mES Ex compared to MEF Ex while 169 showed no change (Figure 7A). However, members of the ES-specific miR-290 family including miR-291, miR-294 and miR-295 demonstrated >105 fold
expression in mES Ex compared to MEF exosomes (Figure 7B) 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 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 while no expression of these miRs was detected in saline treated animals 5 days after infarction (data not shown). 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 to MEF Ex treated CPCs (Figure 7C). This data demonstrates 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. In order to provide evidence towards 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 carried out 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 in order 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 to non-treated CPCs (G1-phase 71.0%, S-phase 8.2%) (Figure 7D). 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 to 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 to miR-291-5p mimic and non-treated control CPCs (Figure 7E).

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 multi-potency for CPCs, and LIN28, a miR-binding protein that has been shown to be involved in regulating pluripotency by miR-294 compared to miR-291-5p mimic and non-treated controls (Figure 7F). A significant increase in proliferation and survival was also evident in miR-294-3p mimic treated CPCs after H2O2 stress (Figure 7G and H 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 due to reduction in oxidative stress and AKT activation in a myocardial infarction model\(^ {17}\). Similarly, exosomes secreted by cardiac progenitor cells 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 utilized 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. Since 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. The present study demonstrates mouse embryonic stem cell (ESC) derived exosomes (mES) augment post-MI physiological and anatomical 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 manuscript suggests that our findings can be easily translated to autologous induced pluripotent stem cells (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 while cardiac progenitor cells (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 promote cardiomyocyte replenishment in failing hearts. Indeed, our results point towards significant activation of cardiomyocyte and CPC based repair and regenerative programs in heart receiving mES-Ex. Importantly, our data provides evidence that CPCs when pre-treated with mES Ex before transplantation to ischemic myocardium survive for long-term (up to 8 weeks of experimental window) and supports 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 embryonic stem cells (ESC) 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\(^ {13}\). Moreover, ethical, technical and regulatory issues as well as unavailability of autologous human ESC for cell therapy applications limit the potential therapeutic utility of ESC in humans. The remarkable discovery by Yamanaka and colleagues\(^ {34}\) towards the derivation of induced pluripotent cells (iPS) has 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 towards generating and extensively characterizing “clinical grade” iPS cells before human cell replacement therapies can be attempted \(^\text{35}\). 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 appears 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 \(^\text{22, 36}\). 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 very 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) \(^\text{30}\), bear a common seed sequence (AAAGUGC), are functionally dominant miRNAs in ES cells and comprise of approximately 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 \(^\text{37}\) with corresponding effect on proliferation and differentiation \(^\text{29, 38}\). Indeed, overexpression of miR-294 mimics both in CPCs in vitro and treatment of mice with miR294 mimics in vivo after MI, 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 \(^\text{39}\), proliferation \(^\text{37}\) and survival \(^\text{40}\).

In sum, 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 is mediated by miR-294 delivered via ESC exosomes to the heart (Figure 7I). 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 \(^\text{7, 8}\). 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
There is no conflict of interest. We thank all members of the Kishore lab for their valuable discussions especially Rana Saber and Dr Harris Perlman for their assistance in Flow Cytometry and Drs Nicholas Hoffman and Muniswamy Madesh for their help with confocal microscopy.

Funding sources
This work was supported in part by funding from the National Institute of Health grants HL091983, HL105597, HL095874, HL053354, HL108795 and HL108806. Mohsin Khan is supported by American Heart Association Scientific development grant 15SDG22680018.

Disclosure
None
References


to enhance myocardial viability and prevent adverse remodeling after myocardial ischemia/reperfusion injury. Stem Cell Res. 2013;10:301-312


34. Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblast cultures by defined factors. *Cell.* 2006;126:663-676
Figure 1: Characterization and functional validation of exosomes derived from embryonic stem cells (ESCs). A) Exosome secretion from a mouse embryonic stem cell (ESC) as evidenced by electron microscopy Scale bar = 500nm, inset shows higher magnification of an ESC exosome. B) ESC culture medium shows exosome by electron microscopy, Scale bar = 50nm. C) Measurement of exosome size in mES and MEF cells by dynamic light scattering (DLS) analysis shows that mES exosome are 39.7nm in size compared to MEF ex (84.3nm) (n=4). D) 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. E) Increased mRNA expression of pluripotent markers OCT4, SOX2 and Nanog in MEF cells treated with mES exosomes after 24 hrs in comparison to control cells. (n=3) media vs. mES Ex *p < 0.05, **p < 0.01, ***p < 0.001. F) Reduction in caspase3+ H9c2 cells treated with PKH-26 labeled mES ex compared to MEF ex treated cells along with corresponding quantification in G) (n=3). Arrows indicate caspase3 expressing cells while 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, ***p < 0.001. H-J) Enhanced tube formation in HUVECs treated with mES Ex in comparison to MEF Ex and media treated control HUVECs. K) Quantification of branch points in HUVECs given different treatments. Media 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 2: Enhanced cardiac function and augmented myocardial environment after infarction in mice transplanted with mES exosome. A) Increased ejection fraction (EF) and B) fractional shortening (FS) in mice transplanted with mES exosomes (n=6) compared to mice with MEF exosomes (n=6) and saline (n=6) treated animals after 4 weeks after infarction. mES Ex, MEF Ex and saline was administered to animals at the time of infarction. C) Reduced left ventricular end-systolic diameter (LVSED) after mES Ex treatment compared to saline treatment. D) Increased capillary density after mES Ex treatment in mice 4 weeks after myocardial infarction along with corresponding quantification. Lectin (green) and nuclei (blue) (n=6). E) Reduced TUNEL+ nuclei in hearts transplanted with mES Ex compared to MEF Ex and saline treated hearts with quantification of TUNEL+ cells. TUNEL (magenta) and nuclei (blue) (n=6). Scale bar=50um. F) Enhanced cardiomyocyte cycling as evidenced by pH3+/sacromeric actin+ cells in hearts treated with mES Ex compared to MEF Ex and saline treated animals 28 days after infarction with corresponding quantification. Scale bar=40um. 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: mES Ex promote 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 to 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=40µm. E-G) 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 H) (n=4). c-kit (red), pH3 (green), sarcomeric actin (blue) and nuclei (white). I-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, ***p < 0.001 and MEF Ex vs mES Ex p < 0.05, ##p < 0.01, ###p < 0.001.
Figure 4: Modulation of CPC function in vitro and in vivo by mES Exosome administration. A) Increased survival of CPCs after treatment with mES exosomes in comparison to MEF exosomes under H2O2 challenge (n=3). B) Increased CPC proliferation at day 1, 3 and 5 after mES exosome treatment compared to MEF exosomes and non-treated CPCs as evidenced by CyQuant assay (n=3). 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-D) Enhanced mRNA levels of cardiac markers such (cTnT, MEF2c and GATA-6) and endothelial markers (α-SMA, Ang1, CD31, VE-cadherin) in CPCs treated with mES Ex in the presence of dexamethasone compared to MEF Ex dex treated CPCs and non-treated controls as evidenced by qRT-PCR (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. E-G) Tube formation is increased in CPCs treated with mES Ex cultured on matrigel compared to MEF Ex and non-treated CPCs after 24hrs (n=3) along with corresponding quantification H). Increased cardiac function EF I) and FS J) in mES Ex-CPC hearts compared to 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. K-L) Decreased infarct size in mES Ex-CPC hearts compared to MEF Ex-CPC and PBS transplanted hearts 8 weeks after injury. 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.
Figure 5: Enhanced persistence, proliferation and survival of mES derived exosomes pretreated CPCs. A-B) Colocalization of GFP with CPC marker c-kit in the heart transplanted with mES Ex and MEF Ex pretreated cells. c-kit (red), GFP (green), sarcomeric actin (blue) and nuclei (white). Scale bar=20µm. C-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=20µm. F-G) Reduced GFP+/TUNEL+ cells in mES Ex-CPC hearts compared to MEF Ex-CPC animals 5 days after infarction along with corresponding quantification H). GFP (green), TUNEL (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.
Figure 6: Increased ability to form myocyte and blood vessels of mES derived exosomes pretreated CPCs. A) Small GFP+ myocytes in MEF Ex-CPC hearts after 8 weeks of cell delivery. Persistence of mES Ex pretreated GFP+ CPCs B) along with GFP+ myocyte formation C) in hearts 8 weeks after injury corroborating with the augmented cardiac function. D) 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=40µm. Panel E Scale bar=20µm. E-F) Increased GFP+/SM22+ cells in the heart transplanted with mES Ex-CPC compared to controls at 8 weeks after infarction with quantification G). 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.
Figure 7: miRNA profiling of mES derived exosomes. A) Comparative analysis of miRs in mES Ex, 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 to MEF cells and MEF Ex groups. C) CPCs treated with mES Ex have enhanced expression of miR-291, miR-294 and miR-295 as confirmed by miRNA qRT-PCR (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 (25nM) compared to miR-291 (25nM) and miR291 (25nM)/miR294 (25nM) and non-treated control cells as analyzed by FACS based cell cycle assay (n=4). E) Enhanced mRNA expression of cyclins (E1, A2 and D1) in CPCs treated with miR-294 (25nM) compared to miR-291 (25nM) and non-treated controls (n=3). F) Increase phosphorylation of AKT in association with elevated levels of nucleostemin and LIN28 in miR-294 (25nM) treated CPCs compared to miR-291 and non-treated controls (n=3). G) Increased CPC proliferation at day 3 and 5 after miR-294 treatment compared to miR-291 and non-treated 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 to miR-291 and non-treated 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.
BIOGRAPHICAL SKETCH

Provide the following information for the key personnel and other significant contributors in the order listed on Form Page 2. Follow this format for each person. DO NOT EXCEED FOUR PAGES.

NAME
Mohsin Khan

POSITION TITLE
Associate Scientist

eRA COMMONS USER NAME (credential, e.g., agency login)

EDUCATION/TRAINING  (Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable.)

<table>
<thead>
<tr>
<th>INSTITUTION AND LOCATION</th>
<th>DEGREE (if applicable)</th>
<th>YEAR(s)</th>
<th>FIELD OF STUDY</th>
</tr>
</thead>
<tbody>
<tr>
<td>Forman Christian College, Lahore, Pakistan</td>
<td>BS</td>
<td>2000</td>
<td>Biology</td>
</tr>
<tr>
<td>Government College University, Lahore, Pakistan</td>
<td>MS</td>
<td>2002</td>
<td>Zoology</td>
</tr>
<tr>
<td>University of the Punjab, Lahore, Pakistan</td>
<td>PhD</td>
<td>2008</td>
<td>Molecular Biology</td>
</tr>
</tbody>
</table>

A. Personal Statement

I have been working in the field of cardiac regeneration for the past several years. The focus of my research has been to develop and understand stem cell based therapies for cardiac repair. In particular, I have developed strategies to utilize mesenchymal stem cells and cardiac stem cells for cardiac regeneration after myocardial infarction and understand the impact of environmental changes such as β-adrenergic signaling, ageing and diabetes on stem cell function as highlighted by my recent publications. Presently, I am working on pluripotent stem cell derived exosomes and their effect on heart disease. Utilization of cell-free components such as exosomes for cardiac repair will provide a novel way harness stem cell regenerative power circumventing some of the adverse effects.

B. Positions and Honors (chronological order)

4/14-present  Associate Scientist, Center for Translational Medicine, Temple University

3/13-3/14  Postdoctoral Fellow, Dr. Raj Kishore, PhD, Feinberg cardiovascular research institute, Northwestern University

1/10-3/13  Postdoctoral Fellow, Dr. Mark Sussman, PhD, Department of Biology, SDSU Research Foundation, San Diego, CA

1/09-1/10  Assistant Professor, University of the Punjab, Lahore, Pakistan.

1/07-12/08  Senior Research Officer, University of the Punjab, Lahore, Pakistan.

Honors

- Scientific Development Grant – American Heart Association, National Center, 2015
- 1st Prize – Poster Competition at Third Annual Temple Translational Science Symposium, Temple University, Philadelphia, 2014
- 2nd Prize – Poster Competition at American Heart Association Metro Chicago Research Network Symposium, 2013
- Abstract top 10% - Selected for “Best of AHA Specialty Conferences at Scientific Sessions 2012”
- Postdoctoral Fellowship – American Heart Association WSA Spring 2011 (11POST7370097) 2011 - 2012

Professional Memberships

Member, American Heart Association (AHA)
Member, International Society for Heart Research (ISHR)
Member, Temple Institute of Regenerative Medicine and Engineering (TIME)
**Professional publications (editor, reviewer)**

**Reviewer** – *Journal of Biological Chemistry, PLoS One, BBA-Molecular Basis of Disease*

**Guest Editor** – Special Issue on Stem cells for Cardiac Repair”, *Stem cell International*

**Editor** – *Autoimmune diseases and therapeutic approaches*

**C. Selected peer-reviewed publications or manuscripts in press (in chronological order).**


*both authors contributed equally

C. Research Support

Ongoing Research Support

15SG22680018 (PI: Khan) Dates: 01/01/2015 - 12/31/2018
Agency: American Heart Association, National Center, Scientific Development Grant
Title: Pluripotent stem cell derived exosome therapy for myocardial repair

Completed Research Support

11POST7370097 (PI: Khan) Dates: 07/01/2011-06/30/2013
Agency: American Heart Association, Western Affiliates, Postdoctoral Fellowship
Title: Beta-adrenergic signaling inhibits myocardial repair by cardiac stem cell depletion and exhaustion

Pending Research Support

None
March 14th 2015

RE: Mohsin Khan, PhD; Candidate for Young Investigator Award 2015 for Senior Postdocs

Dear Young Investigator Award Committee,

This letter is in strong support of Mohsin Khan’s candidacy for the 2015 Young Investigator Award for senior postdocs, based on his work entitled “Embryonic Stem Cell-derived Exosomes promote Endogenous Repair Mechanisms and Enhance Cardiac Function following Myocardial Infarction”. I hope the committee will find Mohsin’s work worthy of consideration for this prestigious award. Manuscript detailing this work represents unpublished data and is not yet communicated for publication.

Mohsin has been working on this project for last one year or so and has done an exceptional job developing this line of investigation as his manuscript amply demonstrates. Alexander Mackie, Suresh Verma, Garikipati Srikanth, Prasanna Krishnamurthy, and Erin Vaughan provided valuable suggestions and minor experimental help throughout the execution of these studies. Emily Nickoloff, Jennifer Johnson, Cindy Benedict, Erin Lambers, Tatiana Abramova, Aiko Ito, Sol Misener and Veronica Ramirez provided technical support. Drs. Qin and Koch provided valuable discussion and suggestions. I supervised Mohsin and edited the final draft, but Mohsin conceptualized, designed, executed, and analyzed most of the data as well as wrote the manuscript. Considering these facts, it is clear that among all co-authors he is the most appropriate candidate for this award.

Data presented in this work may help develop a novel understanding of the therapeutic efficacy of pluripotent stem cell (embryonic and iPS)-derived exosomes in mediating the reparative processes in the injured myocardium and to determine the mechanisms involved in ES/iPS exosome-mediated myocardial repair. Establishing the therapeutic value of these exosomes would help develop a novel cell free system to enhance myocardial repair and would provide a new direction for the restoration and/or augmentation of endogenous myocardial repair process while overcoming the limitation of low cell engraftment and survival that compromises the full-functional benefit of adoptive cell transfer strategies.
Having dealt with the above formalities, I would like to add a few words on Mohsin’s behalf. Of the many young investigators I have been lucky enough to work with during my career; he is unequivocally one of the best. I say this because he brings to table an unusual combination of curiosity, intellect, work ethic, maturity and what separates him most from others is a tremendous imagination and knack for novelty and innovation. His personal characteristic also assure me that Mohsin will be an excellent researcher and leader of an independent laboratory in his future career. He is generous and patient teacher and collaborator. Indeed, he has already salvaged many projects that were not his own. He has done so by generously applying the same type of incisive thinking that he employs in designing his own studies, but has done so in a gentle, educational, and, therefore, persuasive fashion. This final quality is in my experience the rarest among investigators at any level and at the same time is the quality, that if present along with the other aforementioned characteristics, may be the best prognostic sign for a promising research career. This style will suit him well in the years to come as he strengthens his research career.

Mohsin’s background, training, native intellect and continued productivity as reflected by his past and current work makes him a formidable candidate for this prestigious award. He has selected an area of research with significant implications for the augmentation of endogenous myocardial repair process leading to the preservation of ventricular functions, post-infarction. Mohsin’s enthusiasm with an area of great potential impact is indeed very exciting. I am delighted to have this opportunity to support this young talent, in large part because Mohsin is a gentleman and such a pleasure to work with. I have great respect for his integrity and work ethics. His ability to provide crucial intellectual input to our entire lab and his ability to serve as a role model for new trainees is certainly an immense asset. I will certainly do everything possible to facilitate his career development here at Temple University and hope that this award may be the first step in his evolution to established Cardiovascular Research Scientist.

Thank you very much for your consideration of Mohsin Khan for the Young Investigator Award in ISHR North American Section.

__________________________
(Raj Kishore, PhD)