



Original Article

Allogeneic Mesenchymal Stem Cells Restore Endothelial Function in Heart Failure by Stimulating Endothelial Progenitor Cells



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ABSTRACT

Background: Endothelial dysfunction, characterized by diminished endothelial progenitor cell (EPC) function and flow-mediated vasodilation (FMD), is a clinically significant feature of heart failure (HF). Mesenchymal stem cells (MSCs), which have pro-angiogenic properties, have the potential to restore endothelial function. Accordingly, we tested the hypothesis that MSCs increase EPC function and restore flow-mediated vasodilation (FMD).

Methods: Idiopathic dilated and ischemic cardiomyopathy patients were randomly assigned to receive autologous (n = 7) or allogeneic (n = 15) MSCs. We assessed EPC-colony forming units (EPC-CFUs), FMD, and circulating levels of vascular endothelial growth factor (VEGF) in patients before and three months after MSC transcatheter injection (n = 22) and in healthy controls (n = 10).

Findings: EPC-colony forming units (CFUs) were markedly reduced in HF compared to healthy controls (4 ± 3 vs. 25 ± 16 CFUs, $P < 0.0001$). Similarly, FMD% was impaired in HF ($5.6 \pm 3.2\%$ vs. $9.0 \pm 3.3\%$, $P = 0.01$). Allogeneic, but not autologous, MSCs improved endothelial function three months after treatment ($\Delta 10 \pm 5$ vs. $\Delta 1 \pm 3$ CFUs, $P = 0.0067$; $\Delta 3.7 \pm 3\%$ vs. $\Delta -0.46 \pm 3\%$ FMD, $P = 0.005$). Patients who received allogeneic MSCs had a reduction in serum VEGF levels three months after treatment, while patients who received autologous MSCs had an increase ($P = 0.0012$), and these changes correlated with the change in EPC-CFUs ($P < 0.0001$). Lastly, human umbilical vein endothelial cells (HUVECs) with impaired vasculogenesis due to pharmacologic nitric oxide synthase inhibition, were rescued by allogeneic MSC conditioned medium ($P = 0.006$).

Interpretation: These findings reveal a novel mechanism whereby allogeneic, but not autologous, MSC administration results in the proliferation of functional EPCs and improvement in vascular reactivity, which in turn restores endothelial function towards normal in patients with HF. These findings have significant clinical and biological implications for the use of MSCs in HF and other disorders associated with endothelial dysfunction.

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1. Introduction

Heart failure (HF) remains a leading cause of morbidity and mortality in the United States and worldwide (Go et al., 2014; Bui et al., 2011). The failing circulation is characterized not only by depressed cardiac function, but also by endothelial dysfunction (Blum, 2009). Endothelial dysfunction—defined by impaired flow-mediated vasodilation (FMD) and endothelial progenitor cell (EPC) function—produces increased systemic vascular resistance, which augments stress on the failing heart,

and contributes to HF symptomology (Marti et al., 2012). Endothelial dysfunction is also a crucial component of the pathophysiology of numerous cardiovascular (CV) disorders and manifests in patients with CV risk factors such as atherosclerosis, hypertension, and diabetes mellitus (Schulman et al., 2006). Moreover, EPCs regulate the health of the vasculature by incorporating into the endothelium, replacing injured endothelial cells, and secreting angiogenic factors that activate mature endothelial cells (Zampetaki et al., 2008). Notably, HF patients have decreased circulating EPC levels and bioactivity (Schmidt-Lucke et al., 2005).

Mesenchymal stem cells (MSCs), under evaluation as a regenerative therapeutic approach for HF (Hare et al., 2012; Heldman et al., 2014; Karantalis and Hare, in press), have the potential for clinical benefit in CV disease by virtue of their antifibrotic, anti-inflammatory, and pro-angiogenic properties (Williams and Hare, 2011; Cao et al., 2015), and

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their ability to stimulate endogenous progenitor cells (Hatzistergos et al., 2010; Chen et al., 2008). Given this capacity of MSCs and the role of impaired EPCs in human HF (Werner et al., 2005; Shantsila et al., 2007a), we hypothesized that MSCs would stimulate the bioactivity of circulating EPCs and improve endothelial function in the failing circulation. Accordingly, we tested the hypothesis that MSCs stimulate EPC function and augment vascular relaxation in patients with HF due to either idiopathic dilated cardiomyopathy (DCM) or ischemic cardiomyopathy (ICM). Our results show that allogeneic, but not autologous, MSCs improve EPC bioactivity and endothelial function in HF patients, regardless of the etiology. These findings demonstrate a novel clinical beneficial effect of allogeneic MSCs transplantation in patients with HF and have implications for all disorders associated with endothelial dysfunction.

2. Materials and Methods

2.1. Experimental Design

The objective of this study was to analyze the change in endothelial function measured by EPC-CFUs and FMD% after either allogeneic or autologous MSC administration. Patients enrolled in ongoing clinical trials with both ischemic and non-ischemic cardiomyopathy were recruited to this endothelial trial. Power calculations were performed for both our primary endpoint, FMD%, and our secondary endpoint, EPC-CFUs. In order to study the response from autologous versus allogeneic patients for both endpoints, we needed five autologous and five allogeneic subjects to be able to reject the null hypothesis. Data was collected at baseline and 3 months post-treatment, and treatment group was blinded.

2.2. Study Population

This study entitled “Studying Endothelial Function and Endothelial Progenitor Stem Cells’ Colonies Before and After Heart Mesenchymal Stem Cell Transplantation” is a University of Miami Institutional Review Board approved endothelial trial (#20110543). The HF patients recruited for this study are enrolled in POSEIDON-DCM (NCT01392625), “A Phase I/II, Randomized Pilot Study of the Comparative Safety and Efficacy of Transendocardial Injection of Autologous Mesenchymal Stem Cells Versus Allogeneic Mesenchymal Stem Cells in Patients with Nonischemic Dilated Cardiomyopathy” (Mushtaq et al., 2014) and in TRIDENT (NCT02013674), “The Transendocardial Stem Cell Injection Delivery Effects on Neomyogenesis Study”. In POSEIDON-DCM, patients were randomized to receive by transendocardial delivery either 100 million autologous or allogeneic MSCs. Autologous MSCs were derived from the patient’s bone marrow (iliac crest aspiration) 4–6 weeks before cardiac catheterization. In the TRIDENT study, ICM patients were randomized to receive either 20 or 100 million allogeneic MSCs transendocardially. Allogeneic MSCs were manufactured by the University of Miami Cell Manufacturing Program (Da Silva and Hare, 2013). Healthy subjects (n = 10) were enrolled ranging in ages from 22–58 years and both genders. All subjects provided written informed consent.

2.3. Cell Characterization

Both autologous and allogeneic MSCs were manufactured by the Foundation for Accreditation of Cellular Therapy (FACT)-accredited Good Manufacturing Practice (GMP) Cell Production Facility at the Interdisciplinary Stem Cell Institute, University of Miami Miller School of Medicine, as previously described (Hare et al., 2012; Da Silva and Hare, 2013). Cells were released for patient administration after meeting the following criteria: negative for mycoplasma via polymerase chain reaction, $\geq 70\%$ cell viability, growth assay via colony forming unit-fibroblasts assay, positive for CD105 ($>80\%$) and negative for

CD45 by flow cytometry, and no growth of bacteria. On average, autologous MSCs were $92.9 \pm 3.2\%$ CD105+/CD45–, and the allogeneic MSCs were $96.4 \pm 0.04\%$ CD105+/CD45– (Supplemental Fig. 1).

2.4. Endothelial Colony Forming Units (EPC-CFUs)

Peripheral blood samples were obtained from patients before and three months after MSC injection. EPCs were isolated from samples using Ficoll-Paque and five million cells were seeded on 6-well fibronectin-coated dishes (BD biosciences) in CFU-Hill medium (stem cell technologies, cat#05900) (Hill et al., 2003; Solomon et al., 2012). The non-adherent cells were collected 48 h later and one million cells were seeded on 24-well fibronectin-coated dishes. On day five, EPC-CFUs were counted in five wells and the average was obtained.

2.5. Immunofluorescence

Colonies were directly fixed on fibronectin-coated dishes using 4% PFA. Cells were blocked in 10% normal donkey serum/0.3% Triton X-100/PBS for 1 h and then incubated in anti-CD31 and anti-VEGFR overnight at 4* (DAKO #235218, Cell Signaling #55B1R). Next, cells were incubated in Alexa Flour 564 anti-mouse and Alexa Flour 488 anti-rabbit for 45 min at room temperature. Lastly, wells were covered with Vectashield plus DAPI. Images were obtained using immunofluorescent microscopy.

2.6. Flow-Mediated Vasodilation (FMD)

Brachial artery diameter measurements and FMD% were performed in the morning, after an overnight fast. The subjects’ right arm was immobilized in an extended position, and the brachial artery was scanned via ultrasound 5–10 cm above the antecubital fossa (Hill et al., 2003; Corretti et al., 2002). A brachial cuff was then inflated to a supra-systolic pressure (40 to 50 mm Hg above systolic pressure) for 5 min. Subsequently, the cuff was deflated and the brachial artery diameter was recorded for 3 min.

2.7. VEGF ELISA

Serum vascular endothelium growth factor (VEGF) levels (Invitrogen #KHG0111) were measured in DCM patients at baseline and 6 months after allogeneic (n = 6) or autologous (n = 5) MSC treatment. In ICM patients (n = 4), VEGF was measured at baseline and three months after allogeneic MSC treatment. DCM and ICM patients who received allogeneic MSCs were combined and compared to patients who received autologous. Lastly, VEGF was measured in healthy controls (n = 9), which provided written informed consent.

2.8. Matrigel Assays

Human umbilical vein endothelial cells (HUVECs) were grown to passage seven in EGM-2 medium (LONZA). Autologous (n = 7) and allogeneic (n = 5) donor MSCs were grown to 70% confluence and the conditioned medium was obtained. Briefly, MSCs were starved in MEM alpha for 24 h at 5%, the supernatant was collected, centrifuged at 2000 g for 10 min, and stored at -20° until use. 50,000 HUVECs were plated on Matrigel (BD Biosciences) in 24-well plates and pre-treated with 15 μ M L-NAME (Cayman Chemical #80210) dissolved in alpha-MEM (GIBCO) for 45 min. 80% of either MSC conditioned medium (MSC-CM) or plain MEM alpha was added to respective treatment wells, and L-NAME was kept in the medium. After 6 h, six pictures per well were taken and Image J was used to analyze vascular index (tube length \times tube number).

2.9. Statistical Analysis

To assess the difference between autologous and allogeneic groups, an unpaired, two-tailed *t*-test was used. To measure the difference before and after treatment in each group, both a paired, two-tailed *t*-test and a one-way ANOVA was utilized. Correlations were measured using Pearson correlation, assuming a Gaussian distribution. Data are presented as mean and standard deviation of the mean. Both D'Agostino-Pearson omnibus normality test and Shapiro–Wilk normality tests were run to measure within-group variability on all data (only significant differences were reported as D'Agostino-Pearson). Lastly, differences between groups regarding gender, race/ethnicity, history of smoking, and medications were analyzed using a Fisher exact test.

3. Results

3.1. Baseline Characteristics

A total of 22 patients were analyzed for this study. Allogeneic (n = 15) and autologous (n = 7) MSCs were administered transendocardially. Baseline characteristics of the study subjects are summarized in Table 1. Patients with DCM were evenly distributed for both age and sex (P = NS, ANOVA). Additionally, there was no difference in age between ICM and DCM patients receiving allogeneic MSCs (P = NS, ANOVA); however patients with ICM were older than patients with DCM receiving autologous MSCs (P < 0.01, ANOVA). There were more White/Hispanic patients with DCM compared to all other treatment groups (P = 0.022, Fisher exact test). Additionally, patients with DCM who received allogeneic MSCs had higher cholesterol than patients with ICM who received allogeneic MSCs (P < 0.05, ANOVA). As expected, there was a significant difference

between groups regarding coronary artery disease (CAD); specifically, all patients with ICM had CAD (P = 0.0058).

3.2. EPC-Colony Forming Units (CFUs) and Flow-Mediated Vasodilation (FMD) in Heart Failure Patients and Healthy Subjects

Patients with ischemic (n = 6) as well as non-ischemic (n = 16) cardiomyopathy had endothelial dysfunction at baseline, characterized by a reduced ability to form EPC-CFUs and an impaired FMD response. Specifically, patients had decreased EPC-CFU counts compared to healthy controls (4 ± 3 vs. 25 ± 16, respectively, P < 0.0001, *t*-test; Fig. 1A) as well as diminished FMD% (5.6 ± 3.2 vs. 9.0 ± 3.3, respectively, P = 0.01, *t*-test; Fig. 1B).

Patients were further analyzed by disease etiology. There was no difference in EPC-CFUs or FMD% comparing patients with DCM (n = 16) versus ICM (n = 6) at baseline (3 ± 3 vs. 6 ± 1 CFUs, respectively; 5.9 ± 3.6 vs. 5.1 ± 2.0%, respectively; Figs. S2A and S3A). Additionally, there was no difference at baseline in EPC-CFUs comparing DCM patients who received allogeneic MSCs (n = 9) to DCM patients who received autologous MSCs (n = 7) (3 ± 2 vs. 5 ± 4, respectively; Fig. S2B). However, DCM patients receiving autologous MSCs had a higher FMD% at baseline compared to DCM patients receiving allogeneic MSCs (8.3 ± 3.5 vs. 4 ± 2.4, P = 0.011, *t*-test; Fig. S3B).

Moreover, we did a sub-analysis of the patients that received allogeneic MSCs. At baseline, patients with ICM (n = 6) had more EPC-CFUs than patients with DCM (n = 9) (6 ± 1 vs. 3 ± 2, P = 0.0011, *t*-test), and there was no difference in FMD% (5.1 ± 2 vs. 2.4 ± 5.7, P = 0.3742, *t*-test). Despite ICM patients having more EPC-CFUs at baseline, the improvement from baseline to 3 months post-injection was the same (11 ± 8 vs. 10 ± 4 DCM, P = 0.6195, *t*-test). Comparably, there was no difference in the change in FMD % from baseline to 3 months

Table 1

Baseline characteristics of patients (n = 22) and healthy controls (n = 10). Patient data are broken down by etiology and cell treatment: Dilated cardiomyopathy (DCM) patients receiving allogeneic mesenchymal stem cells (MSCs) (n = 9), DCM patients receiving autologous MSCs (n = 7) and ischemic cardiomyopathy (ICM) patients receiving allogeneic MSCs (n = 6). *Asterisks next to P values indicate significant differences between groups. *Asterisks next to values indicate significant difference between noted groups (ANOVA). † indicates that healthy controls were left out of Fisher Exact test.

Characteristic	DCM allogeneic (n = 9, 27%)	DCM autologous (n = 7, 21%)	ICM allogeneic (n = 6, 18%)	Healthy controls (n = 10, 33%)	P-values
<i>Age – yr.</i>					
Median	60	55*	71*	44	*<0.01
Range	45–70	48–73	65–75	22–58	
Male sex – no.	8 (89%)	6 (86%)	6 (100%)	7 (70%)	0.419
<i>Race/ethnicity</i>					
White	8 (89%)	3 (43%)	6 (100%)	7 (70%)	0.114
White/Hispanic	0 (0%)	4 (57%)	0 (0%)	3 (30%)	*0.022
Black	1 (11%)	0 (0%)	0 (0%)	0 (0%)	0.451
<i>History of CVD</i>					
CAD	1 (11%)	0 (0%)	6 (100%)	0 (0%)	**0.0058
History of smoking†	4 (44%)	4 (57%)	4 (67%)	0 (0%)	0.689
<i>Blood pressure-mm Hg (systolic/diastolic)</i>					
Median	126/77	108/65	113/70	118/67	0.979
Range	108–141/54–91	99–142/59–84	85–134/53–76	111–130/59–90	
<i>Cholesterol (mg/dL)</i>					
Median	202*	175	147*	N/A	* < 0.05
Range	129–282	112–207	129–178	N/A	
<i>C-reactive protein (mg/mL)</i>					
Median	0.3	0.3			
3	0.15	N/A	0.923		
Range	0.2–0.4	0.2–0.5	0.1–0.6	N/A	
<i>Medications</i>					
ASA/NSAIDS†	5 (56%)	4 (57%)	5 (83%)	0 (0%)	0.5
Beta-blockers†	7 (78%)	7 (100%)	6 (100%)	0 (0%)	0.204
ACE† inhibitors/ARBs	6 (67%)	7 (100%)	6 (100%)	1 (10%)	0.166
Diuretics†	8 (89%)	6 (86%)	4 (67%)	0 (0%)	0.0814
Statins†	3 (33%)	3 (43%)	6 (100%)	1 (10%)	*0.03
Antiplatelet†	4 (44%)	2 (29%)	3 (50%)	0 (0%)	0.707
Other†	9 (100%)	7 (100%)	6 (100%)	0 (0%)	NS

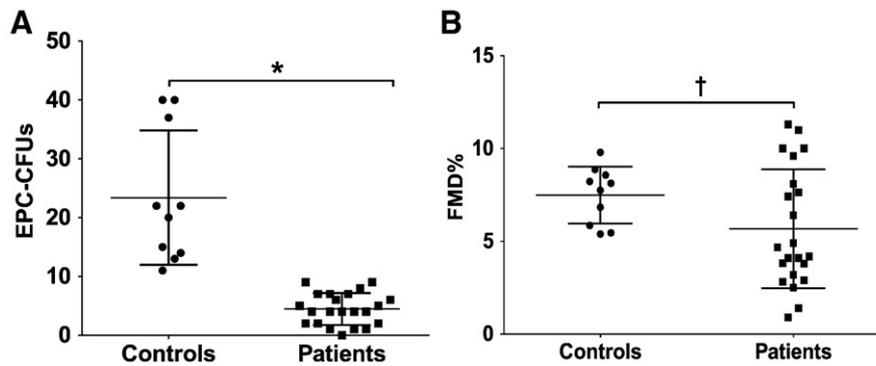


Fig. 1. Endothelial function in patients with heart failure, including dilated and ischemic cardiomyopathy. (A) Patients ($n = 22$) have impaired endothelial progenitor cell-colony forming units (EPC-CFUs) compared to healthy controls ($n = 10$, $*P < 0.0001$, t -test). (B) Patients ($n = 22$) have reduced flow-mediated vasodilation (FMD%) compared to healthy controls ($n = 10$, $†P = 0.01$, t -test).

post-injection comparing DCM vs. ICM patients that received allogeneic MSCs (3.4 ± 2.86 vs. 4.18 vs. 3.16 , $P = 0.6280$, t -test).

3.3. EPC-CFUs in Heart Failure Patients Treated With MSCs

Patients were assessed for EPC-CFUs before and 3 months after MSC treatment. Patients who received allogeneic MSCs had a significant improvement in the number of EPC-CFUs post-treatment ($\Delta 10 \pm 5$, $P < 0.0001$, t -test; Fig. 2A). On the other hand, patients who received autologous MSCs showed no improvement ($\Delta 1 \pm 3$, $P = \text{NS}$, t -test; Fig. 2B). Moreover, we compared allogeneic versus autologous MSC treatment, and allogeneic MSCs were superior in stimulating EPC colony formation ($P = 0.0067$, t -test).

We also compared allogeneic treatment versus autologous treatment in DCM patients. 3 months post-injection, DCM patients who received allogeneic MSCs had significantly higher EPC-CFUs compared to patients who received autologous MSCs (12 ± 4 vs. 6 ± 5 , $P = 0.015$, t -test; Fig. S2C). Accordingly, DCM patients who received allogeneic MSCs had a significantly greater positive change in EPC-CFUs from baseline to 3 months post-injection compared to DCM patients who received autologous MSCs (10 ± 4 vs. 1 ± 3 , respectively, $P < 0.0001$, t -test; Fig. S2D).

EPC-CFUs were examined for morphology. EPCs from HF patients had disorganized and incomplete colony formation (Fig. 2C and D), resulting in clusters that failed to form functional colonies. Three months after allogeneic MSC treatment, patient colonies were organized and healthy in appearance (Fig. 2E and F). Importantly, we found that these colonies were positive for the endothelial cell markers CD31/PECAM and VEGFR (Fig. 2G). Together, these findings suggest that transcatheter MSC therapy stimulates EPC bioactivity in patients with HF of both ischemic and non-ischemic etiology.

3.4. FMD in Heart Failure Patients Treated With MSCs

All patients were evaluated using brachial artery FMD before and 3 months after MSC injection. Patients who received allogeneic MSCs had a dramatic improvement in FMD% ($\Delta 3.7 \pm 3\%$, $P = 0.0002$, t -test; Fig. 3A). In contrast, patients who received autologous MSCs had no improvement and the majority of patients worsened 3 months post-treatment ($\Delta -0.46 \pm 3\%$, Fig. 3B).

We next analyzed the difference between treatment with autologous MSCs and allogeneic MSCs. There was a striking difference between the two cell types ($P = 0.005$, t -test), suggesting that autologous MSCs do not restore endothelial function in this patient population. We also assessed the correlation between EPC-CFUs and FMD% in all patients and found a highly significant correlation between $\Delta \text{FMD}\%$ and $\Delta \text{EPC-CFUs}$ ($P = 0.0004$, $R = 0.68$, Pearson correlation; Fig. 3C).

Lastly, we analyzed DCM patients only, comparing autologous vs. allogeneic treatment. While patients who received allogeneic MSCs had similar FMD% compared to patients who received autologous MSCs 3 months after treatment (7.4 ± 4.7 vs. 7.9 ± 3.1 ; Fig. S2C), patients who received allogeneic MSCs had a greater change from baseline to 3 months post-injection compared to patients who received autologous MSCs (3.8 ± 2.6 vs. 0.7 ± 2.9 , $P = 0.042$, t -test; Fig. S3D). Notably, the majority of patients receiving autologous MSCs had no improvement or worsened, while all allogeneic patients improved ($P = \text{NS}$ vs. $P < 0.0001$, t -test).

3.5. Vascular Endothelial Growth Factor (VEGF) in Patients Receiving Autologous and Allogeneic MSCs

We measured circulating VEGF levels in patients and healthy control serum. At baseline, patients had profoundly elevated circulating VEGF compared to controls (1130.3 ± 803.3 vs. 2.0 ± 5.9 pg/mL, $P = 0.0009$, t -test; within-group, $P = 0.21$, $P < 0.001$ respectively, D'Agostino-Pearson omnibus normality test; Fig. 4A). Allogeneic MSCs reduced VEGF levels (-547.5 ± 350.8 pg/mL, $P = 0.0015$, t -test; within-group $P = 0.96$, D'Agostino-Pearson omnibus normality test), while patients who received autologous MSCs had an increase in VEGF levels (814.1 ± 875.8 pg/mL, Fig. 4B). Furthermore, there was a significant difference between patients who received allogeneic MSCs versus patients who received autologous MSCs ($P = 0.0012$, t -test; Fig. 4B).

VEGF levels correlated with EPC-CFUs ($P = 0.026$, $R = -0.421$, Pearson correlation; Fig. 4C). Even more striking, there was a significant correlation between the change in VEGF and the change in EPC-CFUs from baseline to 3 months post-treatment ($R = 0.863$, $P < 0.0001$, Pearson correlation; Fig. 4D). Notably, high levels of VEGF correlated with low levels of EPCs, evidenced by the autologous group. Conversely, lower levels of VEGF correlated with high levels of EPC-CFUs, illustrated by the allogeneic group. Taken together, these data demonstrate that allogeneic MSCs stimulate EPC mobilization and suppress compensatory elevations in circulating VEGF concentrations.

3.6. Autologous and Allogeneic MSC Paracrine Effect on Endothelial Cells

Human umbilical vein endothelial cells (HUVECs), pre-treated with L-NAME to block endogenous nitric oxide (NO) synthesis, were subsequently treated with autologous or allogeneic MSC-conditioned media (CM), and vasculogenesis was examined in Matrigel assays (Fig. 5A–D). HUVECs treated with L-NAME exhibited severely impaired vasculogenesis (Fig. 5B), evident by their depressed vascular index (305.2 ± 196.8 vs. 1170.9 ± 352.6 , $P = 0.02$, t -test; Fig. 5E). Only the addition of allogeneic MSC-CM prevented the L-NAME-induced impairment in vasculogenesis (vascular index 305.2 ± 196.8 L-NAME alone vs. 1113.4 ± 296.2 L-NAME + Allogeneic

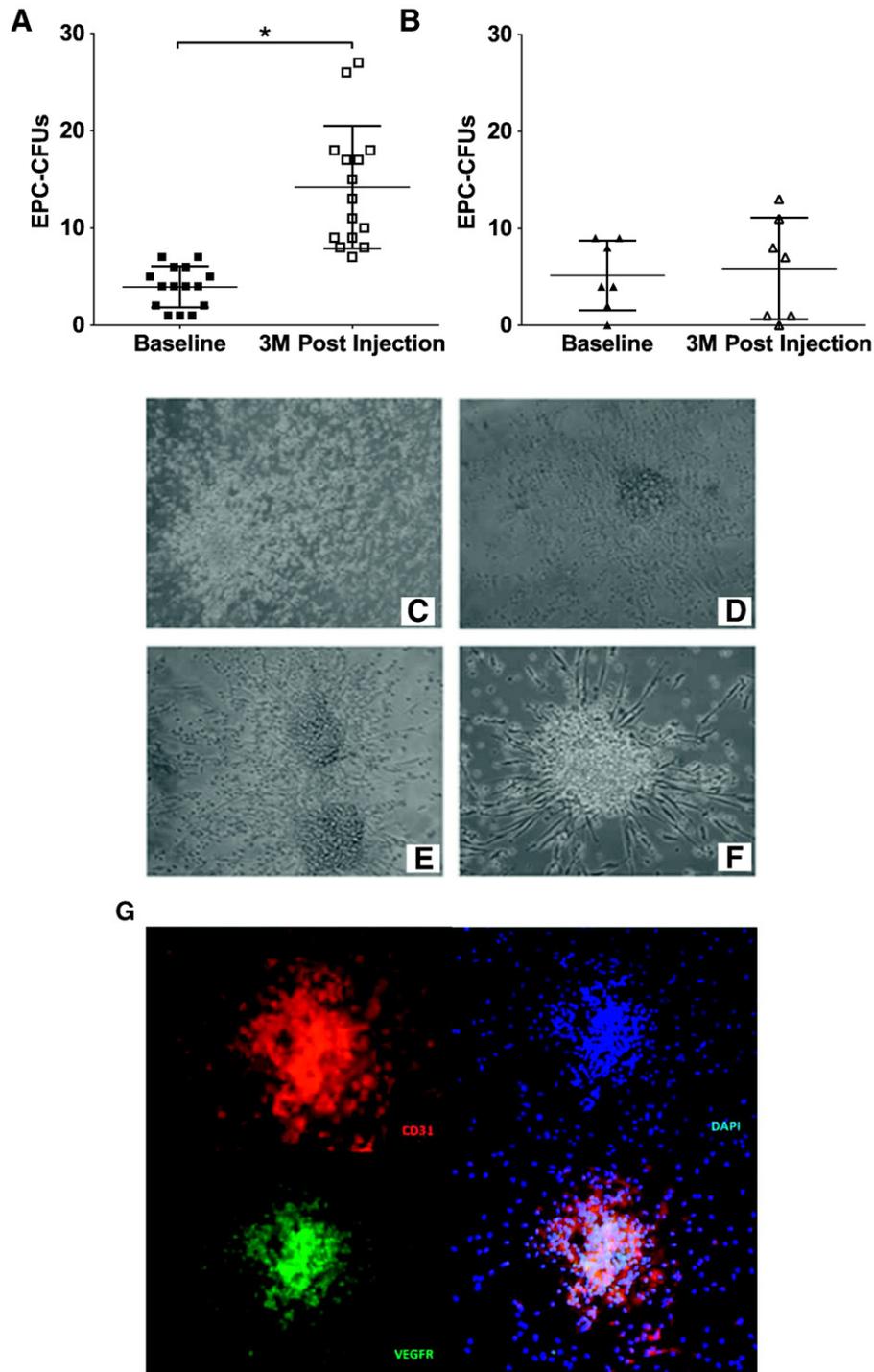


Fig. 2. Endothelial colony forming units in heart failure patients treated with either allogeneic or autologous mesenchymal stem cells (MSCs). (A) Patients treated with allogeneic MSCs had a significant improvement in endothelial progenitor cell-colony forming units (EPC-CFUs) 3 months post-treatment ($n = 15$, $*P < 0.0001$, t -test). (B) Patients treated with autologous MSCs had no change in EPC-CFUs post-treatment ($n = 7$, $P = NS$, t -test). (C–F) Representative EPC-CFUs plated on fibronectin for 5 days before (C, D) and after (E, F) MSC administration (magnification 20×). (G) Colonies are positive for the endothelial cell markers CD31 (red) and VEGFR (green) (magnification 20×).

MSC-CM, $P < 0.05$, ANOVA; Fig. 5E). Overall, these results suggest that allogeneic MSCs are able to restore the vascular potential of endothelial cells.

4. Discussion

Patients with cardiomyopathy of ischemic or non-ischemic etiology manifest endothelial dysfunction, characterized by reduced EPC colony

formation, impaired FMD, and elevated VEGF levels. The major new finding of this study is that MSC administration to these patients stimulates EPC bioactivity and restores flow-mediated vasodilation towards normal. Importantly, the ability of allogeneic MSCs greatly exceeded that of autologous MSCs in restoring endothelial function, enhancing EPC colony formation, and suppressing VEGF levels. Together these findings offer major new clinical insights into the bioactivity of MSCs, suggest a novel therapeutic principle for disorders characterized by

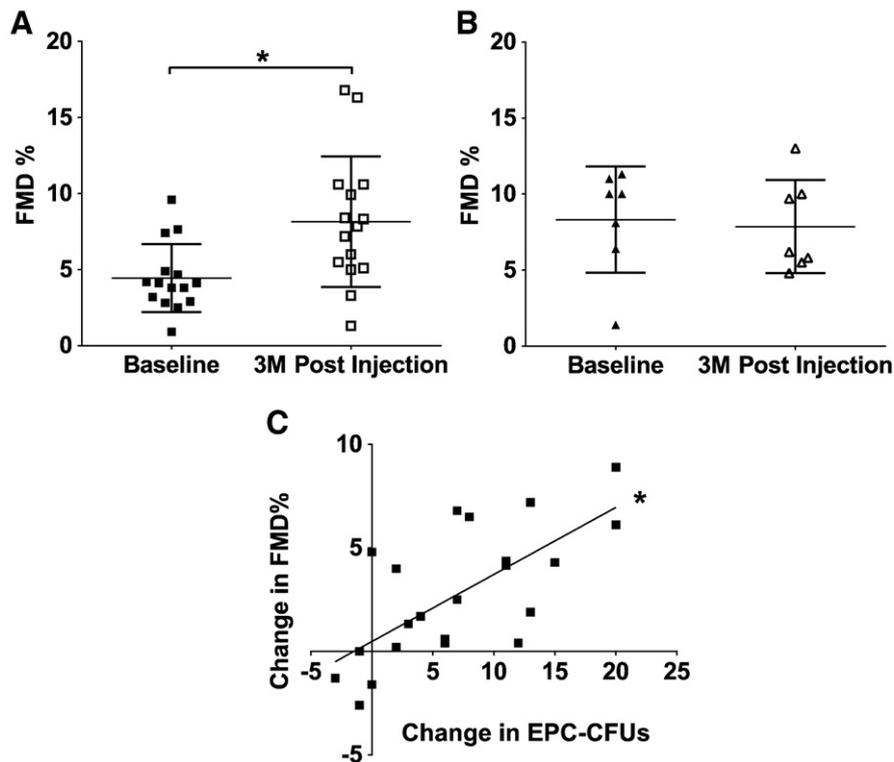


Fig. 3. Flow-mediated vasodilation (FMD) measurements before and after mesenchymal stem cell (MSC) treatment. (A) Patients treated with allogeneic MSCs had an increase in FMD% 3 months post-injection ($n = 15$, $*P = 0.0002$, t -test). (B) Patients treated with autologous MSCs had no significant difference in FMD% 3 months post-injection ($n = 7$, $P = \text{NS}$, t -test). (C) There is a strong correlation between the absolute change in FMD% and the absolute change in endothelial progenitor cell-colony forming units (EPC-CFUs) from baseline to 3 months post-MSC injection in all patients ($*P = 0.0004$, $R = 0.68$, Pearson correlation).

endothelial dysfunction, and have implications for the choice of allogeneic vs. autologous MSC cell therapy.

There is an increasing awareness of the central role endothelial dysfunction plays in CV disorders. Low numbers of EPC-CFUs strongly correlate with endothelial dysfunction (Werner et al., 2007) and are associated with a high Framingham risk score for adverse CV health outcomes (Hill et al., 2003). Furthermore, circulating EPC levels predict CV events—specifically in patients with coronary artery disease, heart failure, and angina (Schmidt-Lucke et al., 2005; Hill et al., 2003; Shantsila et al., 2007b). In addition, elevated levels of circulating VEGF are linked to endothelial dysfunction and HF (Chin et al., 2002; Tsai et al., 2005). In this regard, Eleuteri et al. demonstrated that elevated levels of VEGF correlated with HF disease progression (Eleuteri et al., 2011). Moreover, Wei et al. investigated circulating EPCs and VEGF levels in patients with cerebral aneurysm and found that decreased levels of circulating EPCs and increased levels of plasma VEGF were associated with chronic inflammation in the vascular walls of cerebral arteries and the development of cerebrovascular abnormalities leading to aneurysm formation and rupture (Wei et al., 2011). Thus, endothelial dysfunction is a central feature of CV disease, and may represent a powerful surrogate marker in the development of new treatments for CV disease.

MSCs are adult stem cells that are prototypically found in bone marrow and have the capacity to differentiate into multiple cell types (Williams and Hare, 2011). Importantly, they stimulate the proliferation and differentiation of endogenous precursor cells and play a crucial role in maintaining stem cell niches (Williams and Hare, 2011). In addition, MSCs secrete paracrine factors that participate in angiogenesis, cardiomyogenesis, neovascularization, stimulation of other endogenous stem cells, and regulation of the immune system (Gomes et al., 2013; Gnechchi et al., 2008). While MSCs are known to stimulate cardiac precursor cells and cell cycle activity in the heart (Hatzistergos et al., 2010), their role in stimulating other endogenous precursor populations has heretofore been unknown. Here we report that MSCs stimulated

endogenous EPC activation, increasing the number and quality of functional EPCs. These findings suggest that augmentation of EPCs may represent a novel mechanism of action by which MSCs exert favorable biological effects.

Over the last decade, there has been an emerging interest in the use of MSCs in CV disorders (Karantalis and Hare, in press; Telukuntla et al., 2013). Clinical trials have demonstrated a major safety profile for MSC administration, and suggested efficacy in patients with HF (Hare et al., 2012; Telukuntla et al., 2013); however, underlying mechanism(s) of action continue to be vigorously debated. Our finding that allogeneic MSC injections in patients with both ischemic and non-ischemic HF results in an improvement in endothelial function, specifically by restoring EPC function and FMD and reducing VEGF levels towards normal, offers a major new insight into the mechanisms of action of MSCs. In the study population, increased serum VEGF correlated with diminished EPC-CFUs, consistent with the idea that VEGF plays a compensatory role, a finding also reported in patients with cerebral aneurysm (Wei et al., 2011). This is also supported by the study of Vasa et al. which showed a diminished response of EPCs to VEGF in patients with CAD (Vasa et al., 2001). Moreover, Alber et al. found that a key beneficial effect of atorvastatin therapy is reducing the levels of plasma VEGF in patients with CAD (Franz Alber et al., 2002). This coincides with our study using MSCs, rather than a pharmacological intervention, to decrease pathologic VEGF and increase endothelial function. Thus our findings establish a previously unappreciated therapeutic principle whereby allogeneic MSCs can be employed to stimulate EPC bioactivity, improve arterial physiologic vasodilatory responses, and decrease unfavorable cytokine mobilization in patients with CV disease and other disorders associated with endothelial dysfunction.

We found that allogeneic MSCs restored endothelial function in patients to a degree greatly exceeding that of autologous MSCs. One possible explanation for this may be the age of the cells. Recent studies highlight that MSC's therapeutic function declines as a result of aging

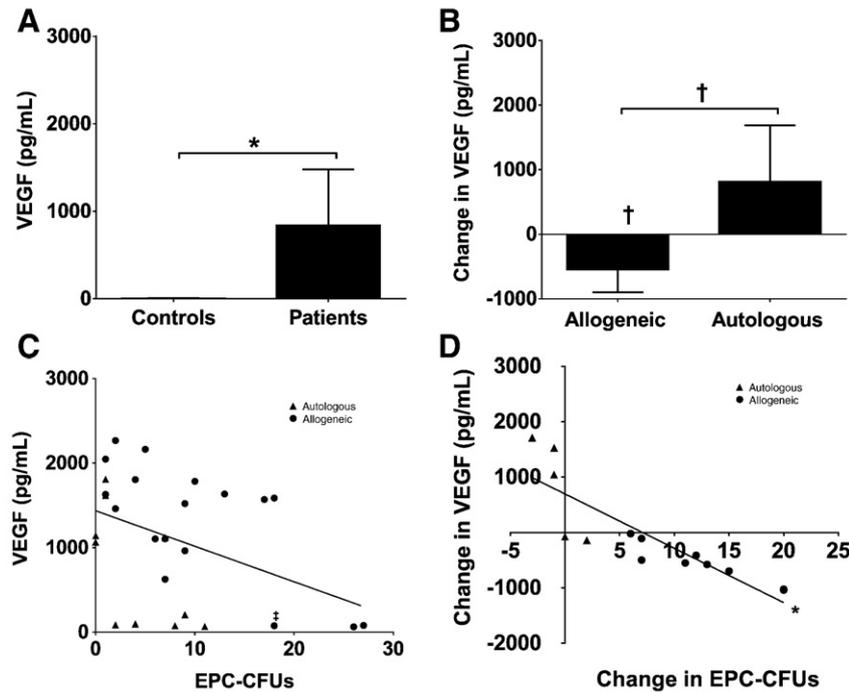


Fig. 4. Serum vascular endothelium growth factor (VEGF) concentration in patients and controls. (A) Patients ($n = 14$) have a higher level of circulating VEGF compared to controls ($n = 9$) at baseline ($*P = 0.0009$, t -test; within-group, $P = 0.21$, $P < 0.001$, respectively, D'Agostino-Pearson omnibus normality test). (B) Patients who received allogeneic MSCs ($n = 9$) had a decrease in VEGF serum levels post-injection ($\Delta -547.5 \pm 350.8$ pg/mL, $^{\dagger}P = 0.0015$, t -test; within-group $P = 0.96$, D'Agostino-Pearson omnibus normality test), while patients who received autologous MSCs ($n = 5$) had an increase in serum VEGF post-injection ($\Delta 814.1 \pm 875.8$), and there was a difference between the groups ($^{\dagger}P = 0.0012$, t -test). (C) There is a correlation between endothelial progenitor cell-colony forming units (EPC-CFUs) and serum VEGF in patients at both baseline and 3 months post-MSC treatment ($R = -0.421$, $^{\ddagger}P = 0.026$, Pearson correlation). (D) The change in EPC-CFUs from baseline to 3 months post-treatment strongly correlated with the change in VEGF ($R = -0.863$, $*P < 0.0001$, Pearson correlation).

(Efimenko et al., 2013; Asumda, 2013). Efimenko et al. showed that adipose-derived MSCs from aged patients with coronary artery disease have impaired angiogenic potential (Efimenko et al., 2013). Similarly, Kasper et al. demonstrated that MSC function is altered and diminished with age, specifically showing lower actin turnover and therefore decreased motility, decreased antioxidant power, decreased responsiveness to chemical and mechanical signaling, and increased senescence (Kasper et al., 2009). Stolzing et al. also reported a decline in “fitness” as a result of aging, as evidenced by a decline in colony-forming unit-fibroblasts and increase in reactive oxygen species levels and oxidative stress (Stolzing et al., 2008). In our study, all allogeneic stem cell donors were healthy, young donors between the ages of 20 and 35. Patients receiving their own stem cells not only had underlying chronic diseases, but also were older (between the ages of 45 and 75). MSC aging may impair the survival, differentiation, and ability to recruit EPCs to areas of damage, ultimately reducing their therapeutic efficacy (Asumda, 2013). Additionally, due to underlying patient comorbidities, the autologous MSC microenvironment may be negatively altered due to systemic inflammation. Consistent with this notion, Teraa et al. showed that systemic inflammation affects the bone marrow microenvironment, disturbing EPC function (Teraa et al., 2013). Although more studies are necessary to validate that the advantage evident here is due to the health and age of MSCs, this study supports the encouraging idea of using “off the shelf” allogeneic MSCs over autologous MSCs.

In this study, we report positive systemic effects from local, cardiac transendocardial MSC injections. We have previously shown that MSC engraftment after intramyocardial injection is approximately 10 to 20%, suggesting that these cells migrate and circulate systemically (Quevedo et al., 2009). MSCs are known to secrete anti-inflammatory factors and cytokines (such as IL-2, TGF- β 1, hepatocyte growth factor, NO, prostaglandin 2, and stromal cell derived factor-1), which can modulate the mobilization of EPCs from bone marrow (Williams and Hare,

2011; Iyer and Rojas, 2008). Additionally, MSCs have been shown to secrete paracrine factors that stimulate resident cells (Williams and Hare, 2011). Thus, we propose a potential mechanism whereby allogeneic MSCs injected into cardiac tissue respond to local microenvironment cues, thereby secreting anti-inflammatory and EPC mobilizing factors that ultimately improve endothelial function alleviating cardiac stress.

There are several limitations of our study. All ICM patients received allogeneic MSCs, therefore we were unable to study the effect of autologous MSCs in this specific HF population. Additionally, patients who received autologous MSCs had higher FMD% at baseline compared to patients who received allogeneic MSCs. Notably, however, all patients receiving autologous MSCs had lower FMD% post-treatment, highlighting the allogeneic advantage. There was also a significantly higher White/Hispanic population in the DCM autologous group compared to other treatment groups. Despite this, there was no difference in baseline or treatment response comparing White/Hispanic patients to White patients (Supplementary Table 1). Furthermore, patients with ICM received different total number of cells (either 20 or 100 million cells). Regardless, all patients had an improvement in endothelial function and there was no intergroup variability. Lastly, there was variability within our control group for circulating VEGF levels. The majority of our controls had too low levels of circulating VEGF to detect, ultimately highlighting the elevated levels of VEGF evident in HF patients. Despite these limitations, we are confident our results provide novel insights into the positive endothelial function effect of allogeneic MSCs in patients with HF.

In conclusion, this study demonstrates a potent and clinically relevant efficacy outcome of transendocardial therapy with MSCs in patients with advanced HF. Allogeneic MSCs restore flow mediated brachial artery dilatation, EPC bioactivity, and VEGF levels towards normal. As abnormalities in the vascular function of patients with CV disease is shown to be highly predictive of adverse outcomes and disease

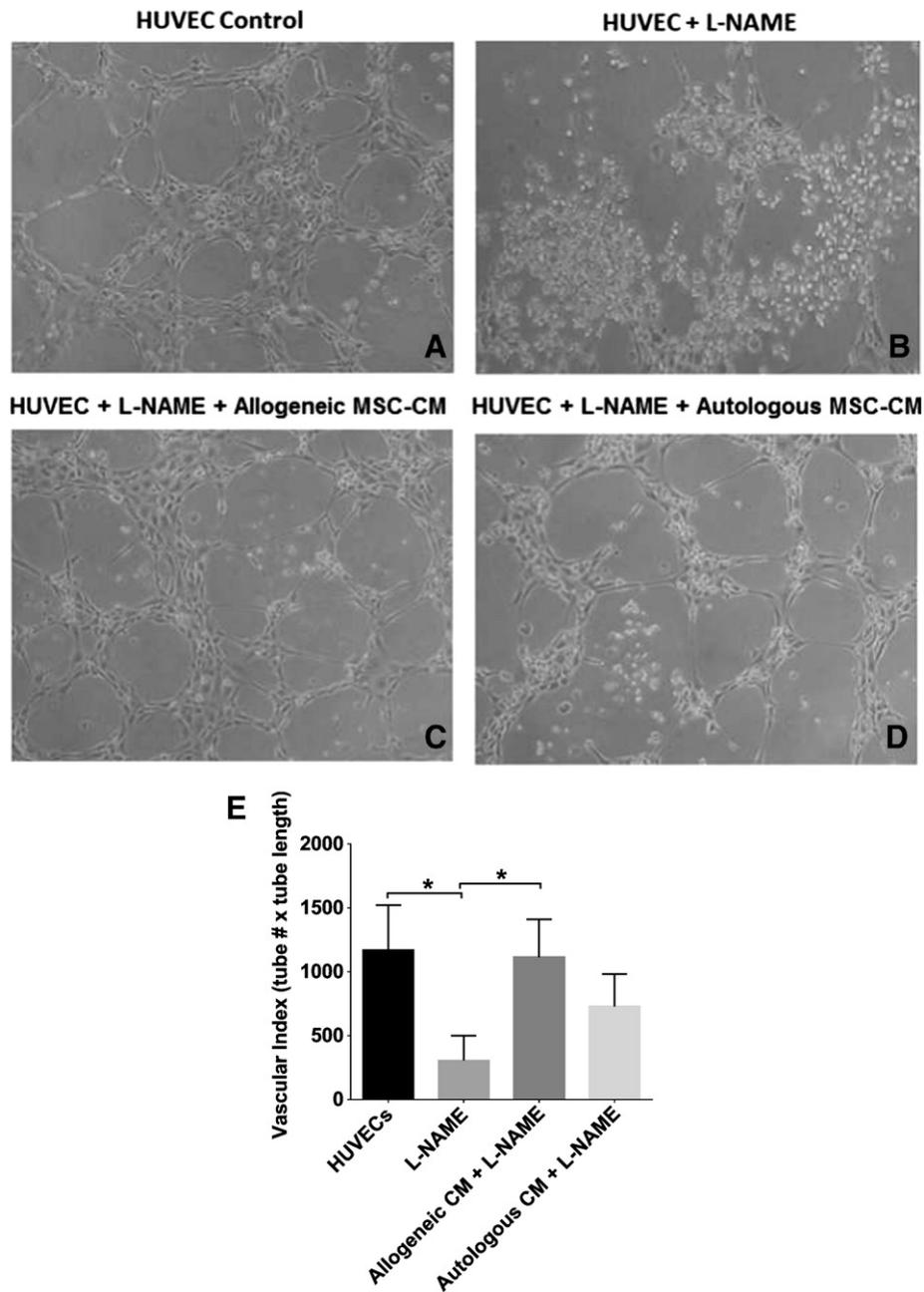


Fig. 5. Effect of autologous and allogeneic mesenchymal stem cell (MSC) treatment on vasculogenesis. (A–D) Representative pictures of human umbilical vein endothelial cells (HUVECs) alone ($n = 3$), HUVECs with the nitric oxide synthase inhibitor, L-N^G-Nitroarginine methyl ester (L-NAME) ($n = 3$), HUVECs with L-NAME and allogeneic MSC-conditioned media (CM) ($n = 5$), and HUVECs with L-NAME and autologous MSC-CM ($n = 7$) after 6 h on Matrigel (magnification 10 \times). (E) L-NAME greatly reduced vascular index ($*P < 0.05$, ANOVA), and only allogeneic-CM restored it ($*P < 0.05$, ANOVA).

progression, targeting endothelial function is a significant therapeutic strategy. Together, these findings offer a new mechanism of action underlying potentially clinically relevant responses to the use of allogeneic MSCs in CV disease.

Author Contributions

CP: study concept and design, EPC-CFUs, VEGF, and Matrigel experiments, data analysis, drafted manuscript; AB: study concept and design, drafted manuscript; MB: CFU assays, data analysis; IHS: study design, data analysis, manuscript editing; BH: FMD studies, data analysis; MP: FMD studies, data analysis; CD: Matrigel assay, VEGF assay, data analysis; WB: study design; DD: study design; AK: clinical cell manufacturing

and characterization; JMH: study design and concept, data analysis, manuscript drafting and editing.

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Role of Funding Sources

No funders played a role in study design, data collection, data analysis, interpretation, or writing of the report.

Conflict of Interest

Dr. Hare has a patent for cardiac cell-based therapy and reports equity interest and board membership in Vestion Inc.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <http://dx.doi.org/10.1016/j.ebiom.2015.03.020>.

References

- Asumda, F.Z., 2013. Age-associated changes in the ecological niche: implications for mesenchymal stem cell aging. *Stem Cell Res. Ther.* 4 (3), 47.
- Blum, A., 2009. Heart failure—new insights. *Isr. Med. Assoc. J.* 11 (2), 105–111.
- Bui, A.L., Horwich, T.B., Fonarow, G.C., 2011. Epidemiology and risk profile of heart failure. *Nat. Rev. Cardiol.* 8 (1), 30–41.
- Cao, Y., Gomes, S., Rangel, E.B., et al., 2015. S-nitrosoglutathione reductase-dependent PPAR γ denitrosylation participates in adipogenesis and osteogenesis. *J. Clin. Investig.* 125 (4).
- Chen, L., Tredget, E.E., Wu, P.Y., Wu, Y., 2008. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* 3 (4), e1886.
- Chin, B.S.P., Chung, N.A.Y., Gibbs, C.R., Blann, A.D., Lip, G.Y.H., 2002. Vascular endothelial growth factor and soluble P-selectin in acute and chronic congestive heart failure. *Am. J. Cardiol.* 90 (11), 1258–1260.
- Corretti, M.C., Anderson, T.J., Benjamin, E.J., et al., 2002. Guidelines for the ultrasound assessment of endothelial-dependent flow-mediated vasodilation of the brachial artery: a report of the International Brachial Artery Reactivity Task Force. *J. Am. Coll. Cardiol.* 39 (2), 257–265.
- Da Silva, J.S., Hare, J.M., 2013. Cell-based therapies for myocardial repair: emerging role for bone marrow-derived mesenchymal stem cells (MSCs) in the treatment of the chronically injured heart. *Methods Mol. Biol.* 1037, 145–163.
- Efimenko, A., Dzhoyashvili, N., Kalinina, N., et al., 2014. Adipose-derived mesenchymal stromal cells from aged patients with coronary artery disease keep mesenchymal stromal cell properties but exhibit characteristics of aging and have impaired angiogenic potential. *Stem Cells Transl. Med.* 3 (1), 32–41.
- Eleuteri, E., Di Stefano, A., Tarro Genta, F., et al., 2011. Stepwise increase of angiotensin-2 serum levels is related to haemodynamic and functional impairment in stable chronic heart failure. *Eur. J. Cardiovasc. Prev. Rehabil.* 18 (4), 607–614.
- Franz Alber, H., Dulak, J., Frick, M., et al., 2002. Atorvastatin decreases vascular endothelial growth factor in patients with coronary artery disease. *J. Am. Coll. Cardiol.* 39 (12), 1951–1955.
- Gnecchi, M., Zhang, Z., Ni, A., Dzau, V.J., 2008. Paracrine mechanisms in adult stem cell signaling and therapy. *Circ. Res.* 103 (11), 1204–1219.
- Go, A.S., Mozaffarian, D., Roger, V.L., et al., 2014. Heart disease and stroke statistics—2014 update: a report from the American Heart Association. *Circulation* 129 (3), e28–e292.
- Gomes, S.A., Rangel, E.B., Premer, C., et al., 2013. S-nitrosoglutathione reductase (GSNOR) enhances vasculogenesis by mesenchymal stem cells. *Proc. Natl. Acad. Sci. U. S. A.* 110 (8), 2834–2839.
- Hare, J.M., Fishman, J.E., Gerstenblith, G., et al., 2012. Comparison of allogeneic vs autologous bone marrow-derived mesenchymal stem cells delivered by transendocardial injection in patients with ischemic cardiomyopathy: The POSEIDON randomized trial. *JAMA* 308 (22), 2369–2379.
- Hatzistergos, K.E., Quevedo, H., Oskoue, B.N., et al., 2010. Bone marrow mesenchymal stem cells stimulate cardiac stem cell proliferation and differentiation. *Circ. Res.* 107 (7), 913–922.
- Heldman, A.W., DiFede, D.L., Fishman, J.E., et al., 2014. Transendocardial mesenchymal stem cells and mononuclear bone marrow cells for ischemic cardiomyopathy: the TAC-HFT randomized trial. *JAMA* 311 (1), 62–73.
- Hill, J.M., Zalos, G., Halcox, J.P.J., et al., 2003. Circulating endothelial progenitor cells, vascular function, and cardiovascular risk. *N. Engl. J. Med.* 348 (7), 593–600.
- Iyer, S.S., Rojas, M., 2008. Anti-inflammatory effects of mesenchymal stem cells: novel concept for future therapies. *Expert. Opin. Biol. Ther.* 8 (5), 569–581.
- Karantalis, V., Hare, J.M., 2015. Use of mesenchymal stem cells for therapy of cardiac disease. *Circ. Res.* <http://dx.doi.org/10.1161/CIRCRESAHA.116.303614> (in Press).
- Kasper, G., Mao, L., Geissler, S., et al., 2009. Insights into mesenchymal stem cell aging: involvement of antioxidant defense and actin cytoskeleton. *Stem Cells* 27 (6), 1288–1297.
- Marti, C.N., Gheorghiad, M., Kalogeropoulos, A.P., Georgiopolou, V.V., Quyyumi, A.A., Butler, J., 2012. Endothelial dysfunction, arterial stiffness, and heart failure. *J. Am. Coll. Cardiol.* 60 (16), 1455–1469.
- Mushtaq, M., DiFede, D., Golpanian, S., et al., 2014. Rationale and design of the Percutaneous Stem Cell Injection Delivery Effects on Neomyogenesis in Dilated Cardiomyopathy (The POSEIDON-DCM Study). *J. Cardiovasc. Transl. Res.* 1–12.
- Quevedo, H.C., Hatzistergos, K.E., Oskoue, B.N., et al., 2009. Allogeneic mesenchymal stem cells restore cardiac function in chronic ischemic cardiomyopathy via trilineage differentiating capacity. *Proc. Natl. Acad. Sci. U. S. A.* 106 (33), 14022–14027.
- Schmidt-Lucke, C., Rössig, L., Fichtlscherer, S., et al., 2005. Reduced number of circulating endothelial progenitor cells predicts future cardiovascular events: proof of concept for the clinical importance of endogenous vascular repair. *Circulation* 111 (22), 2981–2987.
- Schulman, I.H., Zhou, M.S., Raji, L., 2006. Interaction between nitric oxide and angiotensin II in the endothelium: role in atherosclerosis and hypertension. *J. Hypertens. Suppl.* 24 (1), S45–S50.
- Shantsila, E., Watson, T., Lip, G.Y., 2007a. Endothelial progenitor cells in cardiovascular disorders. *J. Am. Coll. Cardiol.* 49 (7), 741–752.
- Shantsila, E., Watson, T., Lip, G.Y.H., 2007b. Endothelial progenitor cells in cardiovascular disorders. *J. Am. Coll. Cardiol.* 49 (7), 741–752.
- Solomon, A., Blum, A., Peleg, A., Lev, E.I., Leshem-Lev, D., Hasin, Y., 2012. Endothelial progenitor cells are suppressed in anemic patients with acute coronary syndrome. *Am. J. Med.* 125 (6), 604–611.
- Stolzing, A., Jones, E., McGonagle, D., Scutt, A., 2008. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech. Ageing Dev.* 129 (3), 163–173.
- Telukuntla, K.S., Suncion, V.Y., Schulman, I.H., Hare, J.M., 2013. The advancing field of cell-based therapy: insights and lessons from clinical trials. *J. Am. Heart Assoc.* 2 (5).
- Teraa, M., Sprengers, R.W., Westerweel, P.E., et al., 2013. Bone marrow alterations and lower endothelial progenitor cell numbers in critical limb ischemia patients. *PLoS ONE* 8 (1), e55592.
- Tsai, W.C., Li, Y.H., Huang, Y.Y., Lin, C.C., Chao, T.H., Chen, J.H., 2005. Plasma vascular endothelial growth factor as a marker for early vascular damage in hypertension. *Clin. Sci.* 109 (1), 39–43.
- Vasa, M., Fichtlscherer, S., Aicher, A., et al., 2001. Number and migratory activity of circulating endothelial progenitor cells inversely correlate with risk factors for coronary artery disease. *Circ. Res.* 89 (1), e1–e7.
- Wei, H., Mao, Q., Liu, L., et al., 2011. Changes and function of circulating endothelial progenitor cells in patients with cerebral aneurysm. *J. Neurosci. Res.* 89 (11), 1822–1828.
- Werner, N., Kosiol, S., Schiegl, T., et al., 2005. Circulating endothelial progenitor cells and cardiovascular outcomes. *N. Engl. J. Med.* 353 (10), 999–1007.
- Werner, N., Wassmann, S., Ahlers, P., et al., 2007. Endothelial progenitor cells correlate with endothelial function in patients with coronary artery disease. *Basic Res. Cardiol.* 102 (6), 565–571.
- Williams, A.R., Hare, J.M., 2011. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. *Circ. Res.* 109 (8), 923–940.
- Zampetaki, A., Kirton, J.P., Xu, Q., 2008. Vascular repair by endothelial progenitor cells. *Cardiovasc. Res.* 78 (3), 413–421.