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Circulation. 2014;130:S70-S76
doi: 10.1161/CIRCULATIONAHA.113.007908
Circulation is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231

Print ISSN: 0009-7322. Online ISSN: 1524-4539

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Background—The impact of diabetes mellitus on the cardiac regenerative potential of cardiac stem cells (CSCs) is unknown yet critical, given that individuals with diabetes mellitus may well require CSC therapy in the future. Using human and murine CSCs from diabetic cardiac tissue, we tested the hypothesis that hyperglycemic conditions impair CSC function.

Methods and Results—CSCs cultured from the cardiac biopsies of patients with diabetes mellitus (hemoglobin A1c, 10±2%) demonstrated reduced overall cell numbers compared with nondiabetic sourced biopsies (P=0.04). When injected into the infarct border zone of immunodeficient mice 1 week after myocardial infarction, CSCs from patients with diabetes mellitus demonstrated reduced cardiac repair compared with nondiabetic patients. Conditioned medium from CSCs of patients with diabetes mellitus displayed a reduced ability to promote in vitro blood vessel formation (P=0.02). Similarly, conditioned medium from CSCs cultured from the cardiac biopsies of streptozotocin-induced diabetic mice displayed impaired angiogenic capacity (P=0.0008). Somatic gene transfer of the methylglyoxal detoxification enzyme, glyoxalase-1, restored the angiogenic capacity of diabetic CSCs (diabetic transgenic versus nondiabetic transgenic; P=0.8). Culture of nondiabetic murine cardiac biopsies under high (25 mmol/L) glucose conditions reduced CSC yield (P=0.003), impaired angiogenic (P=0.02) and chemotactic (P=0.003) response, and reduced CSC-mediated cardiac repair (P<0.05).

Conclusions—Diabetes mellitus reduces the ability of CSCs to repair injured myocardium. Both diabetes mellitus and preconditioning CSCs in high glucose attenuated the proangiogenic capacity of CSCs. Increased expression of glyoxalase-1 restored the proangiogenic capacity of diabetic CSCs, suggesting a means of reversing diabetic CSC dysfunction by interfering with the accumulation of reactive dicarbonyls. **(*Circulation*. 2014;130[suppl 1]:S70-S76.)**

Key Words: adult stem cells ■ diabetes mellitus ■ myocardial infarction ■ oxidative stress ■ regeneration ■ stem cells

Ischemic heart disease remains the most prevalent cause of death worldwide. Cellular cardiomyoplasty using cardiac stem cells (CSCs) holds great promise as a treatment after acute myocardial infarction.[1,2] Although preclinical studies have established the beneficial effects of CSC therapy, the impact of patient comorbidities is only beginning to emerge.[3–5] Specifically, the impact of diabetes mellitus and hyperglycemia on CSC function remains poorly defined.[6]

It has long been established that patients with type 2 diabetes mellitus are at an elevated risk of heart disease and demonstrate worse clinical outcomes after acute myocardial infarction.[7] Diabetes mellitus adversely affects the function of several adult stem cell populations from noncardiac tissues.[8–11] Resident CSC abundance has been shown to negatively correlate with type 2 diabetes mellitus.[12]

Studies on diabetic bone marrow–derived angiogenic cells have revealed that increased dicarbonyl stress may be causally linked to diabetes mellitus–associated cellular dysfunction.[13] A hyperglycemic environment enhances glucose flux through the glycolytic pathway increasing dihydroxyacetone-phosphate and glyceraldehyde-3-phosphate intermediates, which are nonenzymatically degraded into the reactive aldehyde, methylglyoxal. Excessive methylglyoxal accumulation, as seen in the circulation of patients with diabetes mellitus, results in dicarbonyl stress and the formation of advanced glycation end products (AGE).[14,15] Excess methylglyoxal has been linked to oxidative stress in a variety of cultured cell types, as well as diabetic retinopathy, neuropathy, and cardiovascular disease.[16–18] Methylglyoxal is eliminated through the sequential activities of glyoxalase 1 (GLO-1) and glyoxalase

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Guest Editor for this manuscript was John Ikonomidis, MD, PhD.

Presented at the 2013 American Heart Association meeting in Dallas, TX, November 16–20, 2013.

The online-only Data Supplement is available with this article at http://circ.ahajournals.org/lookup/suppl/doi:10.1161/CIRCULATIONAHA.113.007908/-/DC1.

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Circulation **is available at http://circ.ahajournals.org** **DOI: 10.1161/CIRCULATIONAHA.113.007908**

2. Overexpression of GLO-1 has recently been shown to restore function to diabetic bone marrow–derived stem cells.[13]

In this study, we have investigated the effects of diabetes mellitus and a hyperglycemic environment on the function of ex vivo proliferated human and murine CSCs. Furthermore, we assessed the ability of GLO-1 overexpression to prevent and reverse hyperglycemia-induced CSC dysfunction.

Methods

Detailed experimental methods are available in the online-only Data Supplement.

CSC Isolation and Culture

Human CSCs were obtained from left atrial appendages donated by patients (aged 18–80 years) undergoing clinically indicated heart surgery after informed consent. Murine CSCs were obtained from cardiac tissue of wild-type C57Bl/6, C57Bl/6-cKit-EGFP, or C57Bl/6-PEP8-hGlo-1 transgenic mice (aged 2 to 12 months) under isoflurane sedation. CSCs were cultured as described previously.[19,20] Hyperglycemia was induced in C57BL/6 or PEP8-hGlo-1 mice by intraperitoneal injection of streptozotocin (50 mg/kg for 5 days) in 0.05 mol/L sodium citrate. Nondiabetic control mice received equal volumes of 0.05 mol/L sodium citrate. Fasting blood glucose measurements were obtained 10 to 14 days after the fifth streptozotocin injection and again before euthanasia. Mean fasting blood glucose at the time of euthanasia was 29.0±2.3 mmol/L for streptozotocin-injected animals versus 5.6±0.1 mmol/L for controls (P=0.0005).

Diabetic Cardiac Explant GLO-1 and Green Fluorescent Protein Overexpression

Three days after explant plating, streptozotocin-induced diabetic cardiac explants were incubated with 5×10^9 lentiviral particles containing human GLO-1 or green fluorescent protein (control), both under the control of the cytomegalovirus promoter. Explants were preincubated for 5 minutes with 1 μg/mL polybrene. Cultures were transduced for 72 hours in growth medium containing no antibiotics. After transduction, the medium was replaced with complete growth medium, and explants were cultured as above. All protocols were approved by the University of Ottawa Heart Institution Research Ethics Board.

Statistical Analysis

All data are presented as mean±SEM. To determine whether differences existed within groups, data were analyzed by a 1-way ANOVA or 2-way ANOVA, as appropriate. If such differences existed, Bonferroni corrected t test was used to determine the groups with the differences (Prism 5.00; GraphPad Software, Inc). Differences in categorical measures were analyzed using a χ^2 test. A final value of $P\leq0.05$ was considered significant for all analyses.

Results

Diabetes Mellitus Reduces CSC Yield, Proangiogenic Capacity, and CSC-Mediated Cardiac Repair

CSCs were cultured from atrial appendages donated by diabetic and nondiabetic patients undergoing cardiac surgery (hemoglobin A1c, 10.3±1.7% versus 5.8±0.2%, respectively; P=0.03). With regard to other clinical variables known to influence CSC function,[4,5,21–23] diabetic and nondiabetic patients did not differ in terms of age (66±1.8 versus 68±3.5 years, respectively; P=0.63), male sex (66% versus 66%, respectively; χ^2 value, 1.00; P=1.00 versus the expected frequency of female patients), or a history of heart failure (33% versus 17%, respectively; χ^2 value, 0.32; P=0.57 versus the expected frequency of patients with a history of congestive heart failure). With the exception of diabetic status, no other clinical variable shown to influence stem cell function differed between the 2 groups, with analysis including history of hypertension (56%), ongoing smoking (11%), and surgical indication (22% valve surgery alone). Interestingly, despite the markedly elevated preoperative hemoglobin A1c, patients with diabetes mellitus had a preoperative fasting glucose similar to the nondiabetic patients (8.3±0.7 versus 7.5±0.8 mmol/L, respectively; P=0.58), which may reflect the appropriate use of oral hypoglycemic agents (100% of patients with diabetes mellitus) and insulin (66% of patients with diabetes mellitus) immediately before surgery.

Diabetic cardiac biopsies yielded significantly fewer CSCs than nondiabetic controls ($0.5\times10^6\pm1.6\times10^5$ versus $1.2\times10^6\pm1.9\times10^5$; P=0.04; Figure 1A). To test whether diabetes mellitus affects the capacity of CSCs to promote cardiac repair, human CSCs from diabetic and nondiabetic cardiac biopsies were injected into the infarct border zone of nonobese diabetic/severe combined immunodeficiency mice 1 week after left anterior descending artery ligation. Transplantation of nondiabetic CSCs resulted in an 8.1±1.1% increase in the left ventricular ejection fraction (LVEF) of infarcted mice 3 weeks after transplantation. This improvement in LVEF was reduced when diabetic sourced CSCs were transplanted (3.6±2.1% increase in the LVEF 3 weeks after transplantation; P=0.04 versus nondiabetic transplantation; Figure 1B; Table I in the online-only Data Supplement).

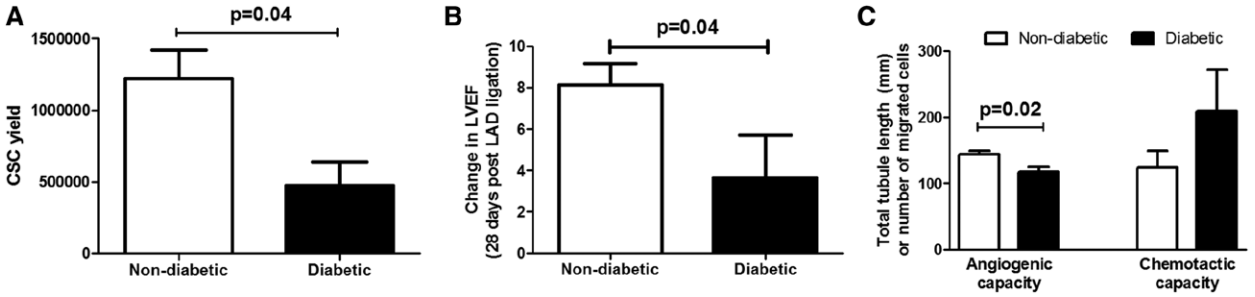

Figure 1. Diabetes mellitus impairs human cardiac stem cell (CSC) function. **A**, CSC yield from the second harvest of human atrial appendage explant cultures obtained from nondiabetic (n=20) or diabetic (n=7) patients. Cell number was normalized with the number of explant culture dishes generated from each patient sample. **B**, Improvement in left ventricular ejection fraction (LVEF) 3 weeks after transplantation of human CSCs collected from nondiabetic (n=4) or diabetic (n=3) atrial appendage explant cultures. **C**, Cumulative tubule length of human umbilical vein endothelial cells seeded on matrigel and exposed to CSC-conditioned medium from nondiabetic (n=10) or diabetic (n=3) patient CSCs for 18 hours (**left**). Total number of circulating angiogenic cells that migrated through a transwell filter after an 18-hour incubation with CSC-conditioned medium from nondiabetic (n=12) or diabetic (n=3) patient CSCs (**right**). LAD indicates left anterior descending.

Given that indirect paracrine-mediated repair plays a pivotal role in CSC treatment outcomes,[24] we examined the effect of diabetes mellitus on in vitro measures of CSC performance by comparing the ability of diabetic CSC-conditioned media to promote blood vessel formation and recruitment of circulating progenitor cells. Although the chemotactic capacity of CSCs was not significantly altered by the presence of diabetes mellitus, diabetes mellitus significantly impaired the proangiogenic capacity of CSCs (144.1±4.9 versus 117.5±7.3 mm cumulative tubule length; P=0.02; Figure 1C). In a manner consistent with these findings, animals treated with diabetic CSCs demonstrated a significantly lower capillary density within the peri-infarct region compared with nondiabetic CSC-treated animals (13.2±0.8% versus 24±0.1%, respectively; $P \leq 0.01$).

Compromised CSC Function Is Reproduced in a Murine Model of Hyperglycemia

To investigate the mechanism of diabetes mellitus–associated CSC dysfunction further, we generated hyperglycemic mice by streptozotocin injection. As observed in diabetic humans, we found that diabetic murine cardiac tissue produced fewer CSCs (−38±14% of nondiabetic control; P=0.049) while significantly compromising the proangiogenic capacity of CSC-conditioned media (wild-type mice 59.1±4.6 versus 37.1±3.5 mm; P=0.0008; Figure 2A). Quantitative polymerase chain reaction demonstrated that the presence of hyperglycemia tended to reduce c-Kit+ expression (0.57±0.18-fold of non-streptozotocin control; P=0.07; n=3) while increasing the expression of CD90+ (5.4±1.9-fold of non-streptozotocin control; P=0.09; n=3). Reminiscent of the human disease state, the chemotactic capacity of CSC-conditioned media was unaltered (Figure 2B). Measurement of secreted cytokines revealed a significant increase in the release of interleukin-6 (P<0.01; Figure 2C) from diabetic CSCs, with no change in the production of proangiogenic vascular endothelial growth factor.

GLO-1 Overexpression Prevents and Restores the Proangiogenic Activity of Diabetic CSCs

Increased methyglyoxal production occurs under hyperglycemic conditions and results in AGE formation with resultant pathological changes to diabetic tissues. Methylglyoxal levels are endogenously reduced by GLO-1 and glyoxalase 2. We tested the ability of GLO-1 overexpression to prevent CSC dysfunction in diabetic mice. PEP8-Glo-1 transgenic mice were made diabetic by streptozotocin injection. CSCs prepared from the cardiac biopsies of diabetic GLO-1 transgenic mice demonstrated a greater proangiogenic capacity compared with CSCs from diabetic wild-type mice (55.0±4.3 versus 37.1±3.5 mm; P=0.003; Figure 2A). The proangiogenic capacity of diabetic GLO-1 transgenic CSCs was comparable with both nondiabetic transgenic (P=0.78) and wild-type (P=0.55) CSCs. Interestingly, overexpression of GLO-1 reversed the effects of streptozotocin treatment on interleukin-6 production by reducing the levels of this proinflammatory cytokine to baseline (Figure 3C).

To test whether direct overexpression of GLO-1 in diabetic CSCs could restore function to these cells, cardiac biopsies from diabetic (streptozotocin injected) or nondiabetic mice were transduced with a lentivirus containing GLO-1 or green fluorescent protein as control. Direct GLO-1 overexpression in diabetic cardiac biopsies enhanced CSC proangiogenic capacity (1.49±0.11-fold increase versus nontransduced diabetic CSCs; P=0.02; Figure 2D) to a level comparable with nontransduced nondiabetic CSCs (P=0.38).

High-Glucose Culture Reduces CSC Angiogenic and Chemotactic Capacity

To further evaluate the impact of high glucose in the promotion of diabetes mellitus–associated CSC dysfunction, we cultured cardiac biopsies from nondiabetic mice in growth medium containing physiological (5 mmol/L) or high (25 mmol/L) glucose. During 4 weeks of culture, high glucose levels reduced CSC yield ($6.7\times10^5\pm8.8\times10^4$ versus $1.2\times10^6\pm3.8\times10^5$ cells;

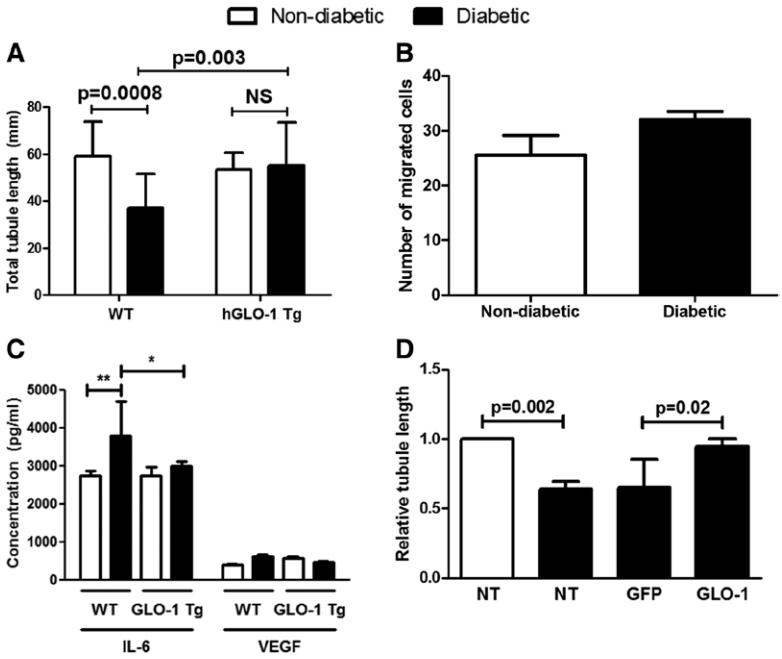

Figure 2. Glyoxalase-1 (GLO-1) overexpression restores proangiogenic capacity to diabetic murine cardiac stem cells (CSCs). **A**, Cumulative tubule length of human umbilical vein endothelial cells (HUVECs) seeded on matrigel and exposed for 18 hours to conditioned medium prepared from CSCs derived from cardiac explant cultures of streptozotocin (STZ)-induced diabetic or nondiabetic wild-type (WT) and hGlo-1 transgenic (Tg) mice. **B**, Total number of bone marrow mononuclear cells that migrated through a transwell filter after an 18-hour incubation with CSC-conditioned medium from STZ-induced diabetic (n=3) or nondiabetic (n=5) WT mice. **C**, Interleukin-6 (IL-6) and vascular endothelial growth factor (VEGF) protein content in conditioned medium prepared from CSCs derived from cardiac explant cultures of STZ-induced diabetic or nondiabetic WT and hGlo-1 transgenic mice (n=3). **D**, HUVEC cells were cultured as in **A**. Conditioned medium was prepared from CSCs derived from STZ-induced diabetic cardiac explants transduced with a lentivirus containing hGlo-1 or green fluorescent protein (GFP) control. Data are expressed relative to the nontransduced nondiabetic CSC-conditioned medium control (n=3). NT indicates nontransduced.

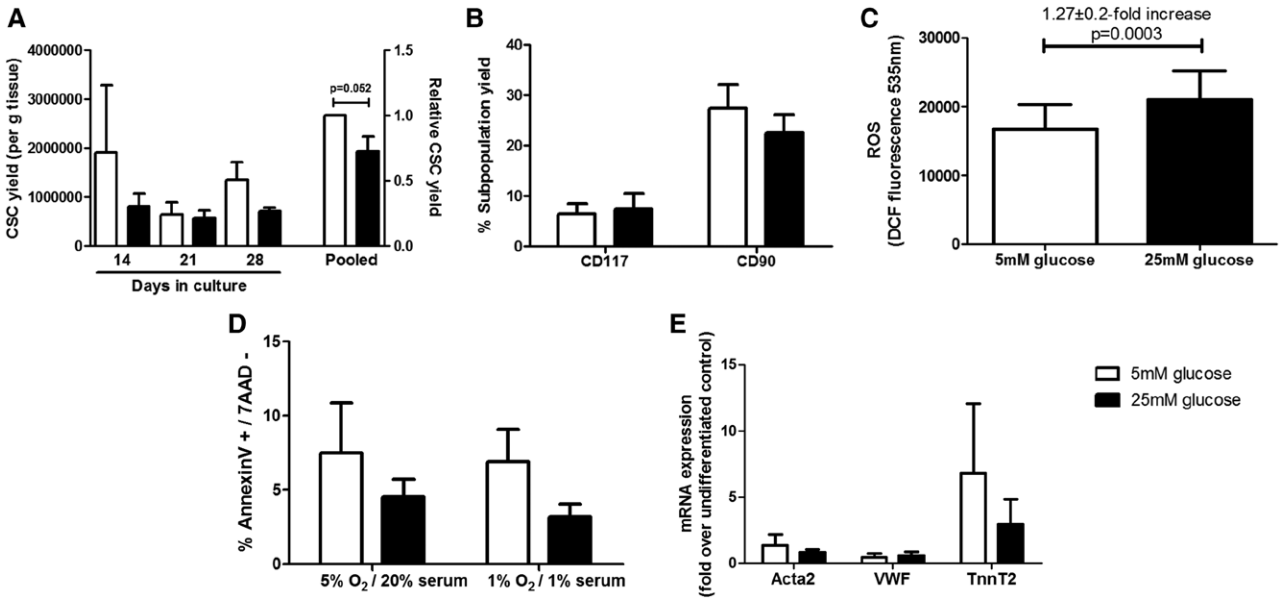

Figure 3. High-glucose culture reduces cardiac stem cell (CSC) yield and increases CSC reactive oxygen species (ROS) production. **A**, CSC yield from murine cardiac explants cultured in 5 mmol/L or 25 mmol/L glucose for the indicated number of days (n=2, **left**). The relative mean yield after 14, 21, and 28 days of explant culture is shown as pooled data (n=7, **right**). **B**, The percentage of CD117- and CD90-positive cells were quantified by immunomagnetic cell sorting of the total heterogeneous CSC population after explant culture in 5 mmol/L or 25 mmol/L glucose (n=7). **C**, Basal ROS levels were measured in CSCs derived from cardiac explants cultured in 5 mmol/L or 25 mmol/L glucose. Data are expressed as the 2', 7'–dichlorofluorescein (DCF) fluorescence intensity after incubation of CSCs with 2',7'–dichlorofluorescein diacetate (n=3). **D**, Apoptotic susceptibility of CSCs cultured in 5 mmol/L or 25 mmol/L glucose was measured by flow cytometric quantification of the percentage of annexinV+/7-aminoactinomycin D– CSCs after 48-hour incubation in a low-serum/low-oxygen environment (n=3). **E**, The cardiogenic potential of CSCs from cardiac explants cultured in 5 mmol/L or 25 mmol/L glucose was measured by quantifying the expression of Acta2 (smooth muscle aortic alpha-actin; smooth muscle cell), TnnT2 (cardiac troponin T; cardiomyocyte), and VWF (von Willebrand factor; endothelial cell) gene expression after 7 days of culture in cardiogenic growth medium (n=3).

P=0.05; Figure 3A), without altering the CD117 or CD90 content within the heterogeneous CSC outgrowth (CD117 6.5±2.1% versus 7.4±3.1%, *P*=0.68; CD90 27.4±4.7% versus 22.7±3.6%, *P*=0.18; Figure 3B). High-glucose culture increased CSC reactive oxygen species (ROS) production by 26.8±2.4% (*P*=0.0003; Figure 3C). No effect of high-glucose culture was observed on CSC apoptotic susceptibility or in vitro cardiogenic differentiation (Figure 3D and 3E).

High-glucose culture significantly affected CSC proangiogenic capacity, inhibiting tube formation by 29±10% (42±2 versus 29±3 mm tubule length; *P*=0.02; Figure 4A). In addition, we noted a significant impairment in the ability of high-glucose CSC-conditioned media to recruit bone marrow–derived progenitor cells in a transwell migration assay (36.1±5.7% inhibition in chemotactic capacity versus 5 mmol/L glucose-cultured CSC-conditioned media; *P*=0.0002; Figure 4A).

We profiled proangiogenic cytokine production using a broad 53 cytokine antibody array and noted no significant changes in any of the profiled cytokines (Figure 4C). ELISA measurements of CSC-conditioned medium revealed a slight increase in the production of interleukin-6 from high glucose–cultured CSCs (*P*=0.02; Figure 4B) with no change in the levels of vascular endothelial growth factor, angiogenin, angiopoietin-1, and angiopoietin-2.

To inform on the effects of glucose culture content on human CSCs, nondiabetic human atrial appendage biopsies were cultured under high and physiological glucose conditions. In a manner consistent with the murine experiments, high glucose conditions reduced culture yields by 0.27±0.04-fold (*P*=0.03) without influencing cell product phenotype (CD117 5.8±1.4% versus 3.7±0.6%, *P*=0.26; CD90 29±2% versus 31±4%, *P*=0.53; n=6; Figure I in the online-only Data Supplement).

High-Glucose Culture Impairs CSC-Mediated Myocardial Repair

The ability of CSCs cultured under physiological or high glucose to promote myocardial repair was tested by injecting male CSCs into the infarct border zone of female mice 1 week after left anterior descending artery ligation. At baseline, all mice demonstrated comparably reduced cardiac function (33±2% versus 32±2% LVEF in the mice receiving 5 versus 25 mmol/L glucose-cultured CSCs, respectively). Three weeks after left anterior descending artery ligation, an improvement in the cardiac function of mice transplanted with either physiological or high glucose–cultured CSCs was observed (43±3% versus 36±2% LVEF, respectively), with physiological glucose–cultured CSCs promoting significantly greater cardiac repair (*P*≤0.05; Figure 5; Table I in the online-only Data Supplement). Scar formation and tissue viability within the infarct zone were reduced to a greater extent in ventricular sections sourced from mice transplanted with CSCs cultured under physiological glucose conditions compared with CSCs cultured under high glucose conditions (13±1% versus 18±1% fibrotic tissue in left ventricle, respectively; *P*=0.009). Although transplantation of CSCs cultured under physiological conditions did not promote greater long-term retention of

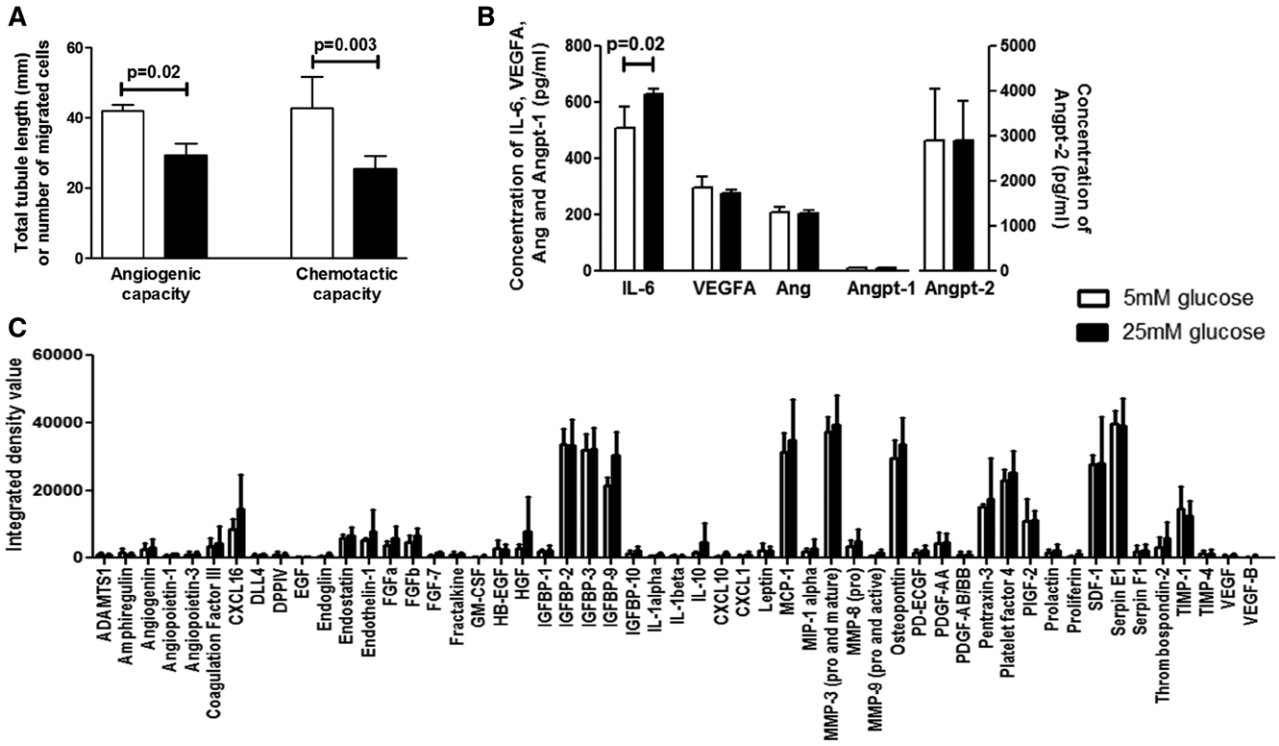

Figure 4. High-glucose culture reduces the angiogenic and chemotactic capacity of cardiac stem cells (CSCs). **A**, Cumulative tubule length of human umbilical vein endothelial cells seeded on matrigel and exposed to CSC-conditioned medium from 5 mmol/L and 25 mmol/L cardiac explant cultures for 18 hours (n=4, **left**). Total number of circulating angiogenic cells that migrated through a transwell filter after an 18-hour incubation with CSC-conditioned medium from 5 mmol/L and 25 mmol/l cardiac explant cultures (n=5, **right**). **B**, Interleukin-6 (IL-6), vascular endothelial growth factor (VEGF), angiogenin (Ang), angiopoietin (Angpt) 1, and Angpt-2 protein content in conditioned medium prepared from CSCs derived from 5 mmol/L and 25 mmol/L cardiac explant cultures (n=4). **C**, Fifty-three angiogenesis-related cytokines were assayed in conditioned medium from CSCs derived from 5 mmol/L and 25 mmol/L cardiac explant cultures. Data are expressed as the mean integrated density after densitometric analysis of each individual cytokine (n=3). ADAMTS1 indicates ADAM metallopeptidase with thrombospondin type 1 motif; CXCL16, chemokine (C-X-C motif) ligand 16; DLL4, delta-like ligand 4; DPPIV, dipeptidyl peptidase-4; EGF, epidermal growth factor; FGF-a, acidic fibroblast growth factor; FGF-b, basic fibroblast growth factor; FGF-7, fibroblast growth factor 7; GM-CSF, granulocyte-macrophage colony-stimulating factor; HB-EGF, heparin-binding EGF-like growth factor; HGF, hepatocyte growth factor; IGFBP, insulin-like growth factor-binding protein; MCP-1, monocyte chemotactic protein 1; MMP, matrix metalloproteinase; PD-ECGF, platelet-derived endothelial cell growth factor; PDGF, platelet-derived growth factor; PlGF, placental growth factor; SDF, stromal cell-derived factor; and TIMP, tissue inhibitor of metalloproteinase.

transplanted cells (825±638 versus 860±526 cells; P=0.97), transplant of CSCs cultured under physiological glucose conditions demonstrated a significantly greater capillary density within the peri-infarct region compared with animals treated with CSCs cultured under high glucose conditions (42.3±8.6 versus 15±2.1 isolectin B4–positive cells per nuclei, respectively; P=0.028).

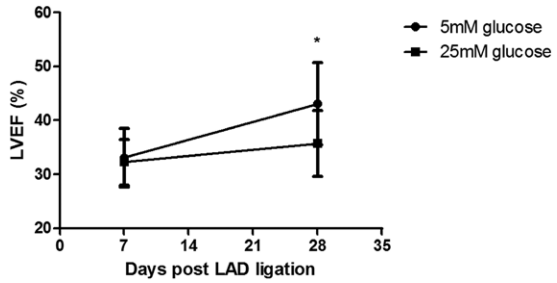

Figure 5. High-glucose culture impairs cardiac stem cell (CSC)–mediated cardiac repair. Change in left ventricular ejection fraction (LVEF) after transplantation of 5 mmol/L or 25 mmol/L glucose-cultured CSCs into mice 7 days after experimental myocardial infarction induced by permanent left anterior descending (LAD) artery ligation (n=8; *P<0.05).

Discussion

This study reveals the impact of diabetes mellitus and hyperglycemia on the performance of ex vivo proliferated CSCs sourced from diabetic mice and humans undergoing cardiac surgery. Furthermore, we have identified a molecular target (GLO-1) that is capable of preventing and restoring function to these cells. To our knowledge, this is the first such report demonstrating impaired cardiac regenerative potential of transplanted diabetic human CSCs.[6]

The beneficial effects of current CSC therapies in phase II clinical trials rely heavily on paracrine-mediated proangiogenic and endogenous stem cell recruitment responses to repair damaged myocardium. The severely reduced capacity of CSCs sourced from patients with type 2 diabetes mellitus to repair damaged myocardium correlated with a significant reduction in their in vitro proangiogenic capacity. Here, we have used CSCs sourced from the cellular outgrowth of cardiac explant cultures. These cells have previously been characterized to represent a heterogeneous population of cardiogenic cells capable of promoting cardiac repair to a degree equivalent to cells under clinical investigation.[19,20] Furthermore, similar heterogeneous CSC products have been reported to be

functionally superior to antigenically selected c-Kit+ CSCs with respect to cardiac repair and paracrine profile.[25]

Diabetes mellitus exerts profound pathophysiological effects on the heart, including impaired myocardial function. These effects are linked to a state of persistent hyperglycemia and may manifest as a result of endogenous CSC dysfunction. Previous studies have revealed impaired c-Kit+ and Sca1+ cell abundance and proliferation in the hearts of streptozotocin-induced diabetic mice.[26,27] Our results further extend these observations because we noted reduced numbers of complimentary cardiac (c-Kit+), endothelial (CD34+), and mesenchymal (CD90+) progenitor cell yields from the hearts of both diabetic mice and patients with type 2 diabetes mellitus.

The fundamental mechanism underlying diabetes mellitus–induced CSC dysfunction is not clear. Hyperglycemia can induce the production of ROS, which we observed within high glucose–cultured CSCs. This notion finds traction in literature from a study by Rota et al[27] who demonstrated that diabetic mice deficient for the critical redox protein $p66^{shc}$ had less oxidative stress and increased the numbers of c-Kit+ cells found in the native diabetic hearts. The reduction in oxidative stress and increase in c-kit+ cell number correlated with improved ventricular function in the diabetic heart.[27] Although previous studies have demonstrated an increase in ROS and apoptosis in high glucose–cultured c-Kit+ and Sca1+ murine CSCs,[26,27] we failed to observe an increase in annexinV+/7-aminoactinomycin D– cells after high-glucose culture, despite increased ROS production in these cells. This may reflect a reduced apoptotic susceptibility of our heterogeneous CSC population compared with antigenically selected cells for prolonged culture to experimentally relevant numbers. Future work directed toward exploring the effects of modified redox capacity on ventricular function, matrix remodeling, and angiogenesis after in vivo implantation in a cardiac ischemic model will help to fully define the functional consequences of increased ROS production in high glucose–cultured CSCs.

Given that oxidative stress is linked to dicarbonyl stress and AGE formation,[28] it is unsurprising that an increase in AGE production has been reported in Sca1+ murine diabetic CSCs.[26] Methylglyoxal is a major glycating agent in diabetic tissues through its covalent modification of lysine and arginine residues.[29] Methylglyoxal levels are reduced by enzymatic conversion to d-lactic acid by GLO-1 and glyoxalase 2. In the presence of glutathione, methylglyoxal forms a hemithioacetal that is irreversibly converted to lactoylglutathione by GLO-1. Lactoylglutathione then forms the substrate for glyoxalase 2, which produces d-lactic acid. This pathway is the principal means to detoxify excess methylglyoxal and prevent AGE formation. In support for a role of protein glycation, we observed that somatic gene transfer of GLO-1 restored the angiogenic capacity of diabetic CSCs. Interestingly, GLO-1 overexpression also returned production of the proinflammatory cytokine interleukin-6 to nondiabetic levels, which may suggest a critical role in CCS-mediated repair.

Ultimately, the significant impact of poorly controlled diabetes mellitus and hyperglycemia on ex vivo proliferated CSCs reported here threatens to limit prospect of autologous CSC therapy for patients with type 2 diabetes mellitus. This study should prompt careful review of the entry criteria for newly started phase 1/2 studies to avoid metabolic preconditioning, limiting the interpretation of CSC therapeutic outcomes. The extent to which this extends to well-controlled patients with diabetes mellitus (ie, hemoglobin A1c <7%) is not clear and, although beyond the scope of these data, merits future study. Furthermore, it should be noted that this study design highlights the persistent effects of hyperglycemia on ex vivo proliferated CSCs without extension to cell performance after transplantation back into a hyperglycemic host. Based on our findings and relevant literature, one could assume that CSC function would be further impaired, but this important point deserves further study.[30,31]

Most importantly, current CSC culture standard operating procedures in clinical testing invariably subject cells to 25 mmol/L glucose culture conditions because this represents the industry standard for mammalian cell culture. The origin of this nonphysiological glucose content is based on the use of commercially sourced basal media formulations (Iscove's Modified Dulbecco's Medium), which early reports found enhanced hematopoietic cell proliferation.[32] Our observation that high-glucose culture reduced the angiogenic capacity and cardiac repair potential of CSCs highlights an immediate low-cost means of enhancing CSC performance. This report confirms this notion and provides direct evidence that diabetes mellitus and hyperglycemia have profound effects on one of the leading preclinical agents for myocardial repair when applied soon after myocardial infarction.[1,2]

Acknowledgments

We thank the following individuals: (1) Nicholas Latham, Glenn Hay, and Megan Fitzpatrick who assisted with data collection; (2) Richard Seymour who assisted with animal surgery; and (3) Dr Buu-Khanh Lam who supplied surgical human atrial appendages for cardiac stem cell culture.

Sources of Funding

This work was supported by the Canadian Institutes of Health Research (Operating Grant 229694) and the Heart and Stroke Foundation of Canada (Grant in Aid G-14-0006113). Dr Molgat is supported by the University of Ottawa Heart Institute's Vered-Beanlands Endowed Research Scholarship. Dr Davis is funded by the Canadian Institutes of Health Research (Clinician Scientist Award).

Disclosures

None.

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SUPPLEMENTAL MATERIAL

Detailed Methods

Cardiac stem cell isolation and culture

Human left atrial appendages were obtained from patients undergoing clinically indicated heart surgery after informed consent. All protocols were approved by the University of Ottawa Heart Institution Research Ethics Board. Inclusion criteria for tissue donors consisted of patients between the ages of 18 and 80 who required cardiac surgery for coronary artery bypass grafting and/or valve surgery. Exclusion criteria included chronic infectious diseases (HIV, hepatitis), pregnant women or active sepsis.

Murine cardiac tissue was obtained from wild-type C57Bl/6, C57Bl/6-cKit-EGFP, or C57Bl/6-PEP8-Glo-1[1] transgenic mice, under isoflurane sedation in accordance with University of Ottawa Heart Institute guidelines (mean age was 5.1±3.0 months; range from 2 to 12 months).

CSCs were cultured as described previously.[2, 3] Briefly, cardiac tissue was minced, enzymatically digested with collagenase (1mg/mL; Invitrogen) and plated as cardiac explants on fibronectin coated dishes. Cardiac explants were cultured at 37C, 5% O_2 (murine explants), or 20% O_2 (human explants) in Iscove's Modified Dulbecco's Medium containing 25 mmol/l D-glucose (Invitrogen) or custom formulated glucose-free Iscove's Modified Dulbecco's Medium (Invitrogen), supplemented with 25 mmol/l D-glucose (high glucose) or 5 mmol/l D-Glucose (physiological glucose) plus 20 mmol/l D-mannitol, 20% fetal bovine serum (FBS) (Invitrogen), 100 U/ml penicillin G, 100 ug/ml streptomycin (Invitrogen), 2 mmol/l L-glutamine (Invitrogen), and 0.1 mmol/l 2-mercaptoethanol (Invitrogen). The heterogeneous population of cellular cardiac outgrowth emigrating from the plated tissue was harvested using mild trypsinization

(0.05% trypsin; Invitrogen) and enumerated with a Neubauer hemocytometer every 7 days. Human CSCs used for these studies were obtained exclusively from the second harvest.

STZ induced diabetes

Hyperglycemia was induced in C57BL/6 or PEP8-Glo-1 mice by intraperitoneal injection of STZ (50mg/kg for 5 days) in 0.05M sodium citrate. Non-diabetic control mice received equal volumes of 0.05M sodium citrate. Fasting blood glucose (FBG) measurements were obtained 10-14 days after the fifth STZ injection and again prior to sacrifice. Mean FBG at time of sacrifice was 29.0±2.3 mmol/L for STZ injected animals, vs. 5.6±0.1 mmol/L for controls ($p=0.0005$).

Diabetic cardiac explant Glo-1 and GFP overexpression

Three days after explant plating, STZ-induced diabetic cardiac explants were incubated with $5x10^9$ lentiviral particles containing human Glo-1 or GFP (control), both under the control of the CMV promoter. Explants were pre-incubated for 5 min with 1ug/ml polybrene. Cultures were transduced over 72 h in growth medium containing no antibiotics. Following transduction, media was replaced with complete growth medium, and explants were cultured as above.

Cardiac stem cell outgrowth subpopulation distribution

CD117 (cKit-positive) and CD90-positive cells within total CSC outgrowth was quantified using Dynal bead separation (Invitrogen), flow cytometry (FACSAria I, BD Biosciences, USA) or qPCR, as indicated in the manuscript.

For magnetic separation, the total CSC outgrowth was first incubated with 1ug primary antibody against CD117 (rabbit polyclonal IgG; Santa Cruz, sc-168) followed by a secondary incubation with sheep-anti rabbit IgG dynabeads (Invitrogen, 112.03D/04D). The CD117-

negative population was subsequently incubated with 1ug primary antibody against CD90 (rat polyclonal IgG; SouthernBiotech, 1740-01) followed by sheep-anti rat IgG dynabeads (Invitrogen, 11035). All incubations were for 1 h at 4C. CD117- and CD90-positive cells were enumerated with a Neubauer hemocytometer.

For flow cytometry, monoclonal antibodies and similarly conjugated isotype-matched control monoclonal antibodies for CD90 (555596, BD Biosciences) and c-Kit (FAB332A, RD Systems) were used. A minimum of 100,000 events were collected after performing fluorescent compensation using single labelled controls. Positive cells were defined as the percent of the population falling above the 99th percentile of the isotype control (FlowJo v. 10, TreeStar Inc., USA).

For qPCR, RNA was extracted (70022, Qiagen), and cDNA was synthesized (Roche). Gene-specific dual labeled qPCR primers and probes were designed for qPCR using a Roche LightCycler 480II thermocycler.

Preparation and analysis of CSC-conditioned medium

Conditioned medium was prepared from confluent cultures of CSCs following 48 h of hypoxic culture (1% O_2) in physiological or high glucose medium containing 1% FBS, 100 U/ml penicillin G, 100 ug/ml streptomycin, 2 mmol/l L-glutamine, and 0.1 mmol/l 2-mercaptoethanol. The conditioned medium was centrifuged at 1100 rpm for 5 min at 4C to remove cellular debris and stored at -80C. The concentration of cytokines (VEGFA, interleukin-6, Angiogenin, Angiopoeitin-1, and Angiopoietin-2) were measured by ELISA (RnD systems). The angiogenic cytokine profile of CSC-conditioned medium was screened with the Proteome Profiler Mouse Angiogenesis Array, as per manufacturer's directions (ARY015, R&D Systems). Results were

corrected to account for differences in proliferation and seeding using the protein content of the cell lysate which was determined using standard Bradford techniques (BioRad, Canada).

Assay of CSC pro-angiogenic and pro-migratory capacity

The capacity of CSCs to stimulate angiogenic growth was assessed using a matrigel assay (ECM625, Millipore), as previous described.[3] Briefly, human umbilical vein endothelial cells (HUVECs) were seeded on matrigel and incubated in CSC-conditioned media for 18 h at 37C, 5%-20% O_2. Fields were analyzed using phase microscopy. Cumulative tubular growth was determined using Image J software plug-in, NeuronJ (National Institutes of Health (NIH); http://rsb.info.nih.gov/ij).

The capacity of CSC conditioned media to promote stem cell migration, was assessed using fibronectin coated transwell plates (24 wells, 3.0 μm pores; Corning). 6.0 x 10^4 human circulating angiogenic cells (for human CSC-conditioned medium) or bone-marrow derived mononuclear cells (for murine CSC-conditioned medium) were seeded into the upper well in 100 μl serum-free IMDM) and incubated for 18 h with 600 μl CSC-conditioned media (placed in the bottom well). Cells that successfully migrated through the polycarbonate membrane were fixed (4% paraformaldehyde) and stained with DAPI (Sigma-Aldrich). The total number of cells were quantified in five random fields.

In vitro cardiogenic differentiation

The relative capacity of CSCs to undergo cardiac differentiation was assessed as previously described.[3] Briefly, CSCs were cultured in cardiogenic media (CGM; DMEM-LG, 40% MCDB-201, 0.75% dimethylsulfoxide, 0.1% 10 mmol/l L-ascorbic acid, 0.01% ITS liquid media supplement, 0.01% linoleic acid-albumin, 0.01% Pen-Strep, 0.0002% 0.25 mmol/l dexamethasone, 0.001% 2-mercaptoethanol, 10 ng/ml recombinant mouse fibroblast growth

factor 8b, 100 ng/ml fibroblast growth factor 4, 10 ng/ml recombinant human protein rhDKK-1 and 10 ng/ml recombinant human bone morphogenetic protein 2) for 7 days, with 50% of the media being replaced every 2 days. Control cells were cultured in the absence of cardiogenic factors (DMEM-LG, 40% MCDB-201, 0.75% dimethylsulfoxide, 0.1% 10 mmol/l L-ascorbic acid, 0.01% ITS liquid media supplement, 0.01% linoleic acid-albumin, 0.01% Pen-Strep) On day 7, RNA was isolated cells and cardiac identity was assessed by quantitative RT-PCR, as described.

Measurement of reactive oxygen species

The content of ROS within confluent cultures of CSCs was measured using the 2',7-dichlorofluorescein diacetate (DCFDA) fluorimetric assay (Abcam), as per the manufacturer's directions. Briefly, adherent CSCs, cultured in 5mM or 25mM glucose medium were incubated with 20mM DCFDA for 45 min at 37C. Cells were subsequently washed and the fluorescence emission was measured at 535nm following excitation at 485nm (BioTek Instruments, Inc.).

Measurement of apoptotic resistance

To assess the ability of high glucose cultured CSCs to resist cell death induced by an ischemic-like environment, murine CSCs cultured in 5mM or 25mM glucose were incubated for 48 h with either complete growth medium (20% FBS), or serum-reduced growth medium (1% FBS) in a 37C incubator containing either 5% or 1% O_2. Following incubation, floating and adherent cells were collected by trypsinization and stained for 15 min with Annexin V-phycoerythrin and 7AAD. The percentage of apoptotic cells (AnnexinV+/7AAD-) was quantified by flow cytometry.

***In vivo* cardiac repair**

The ability of CSCs to promote cardiac repair following permanent left anterior descending (LAD) coronary artery ligation was assessed as previously described.[2, 4, 5] For human CSC injections, male NOD-SCID recipient mice were used. Male murine CSCs were injected into female wild-type C57Bl/6 recipient mice. Briefly, 1 x 10^5 cells CSCs were transplanted into the infarct border zone via two echocardiographic guided injections of 0.5x10^5 cells. Twenty eight days after LAD ligation, the effect of cell therapy was evaluated from the left ventricular function (VisualSonics V1.3.8). After the final echocardiogram, the hearts were excised, fixed with 4% paraformaldehyde, embedded in OCT and sectioned. Tissue viability within the infarct zone was calculated from Masson's trichrome stained sections by tracing the infarct borders manually and then using ImageJ software to calculate the percent of viable myocardium within the overall infarcted area. To evaluate stem cell engraftment and differentiation, immunostaining for human nuclear antigen (HNA; SAB4500768, Sigma, Canada) was used to detect cells of human origin. Co-staining with non-specific α-SMA (ab125266; Abcam), cTnT (ab66133; Abcam) and vWF (11778-1-AP; Proteintech Group, USA) was used to identify cells that differentiated into functional cardiomyocytes. Capillary density within the infarct border zone was assessed by staining for non-specific isolectin B4 expression (B-1205; Vector Laboratories, Canada) in conjunction with DAPI (Sigma, Canada). The total number of nuclei within one image field of the border zone were counted and assessed for isolectin B4 expression. Myocardial retention of murine transplanted cells was assessed in a subset of mice using quantitative PCR for Rbmy.[6]

Statistical analysis

All data is presented as mean ± SEM. To determine if differences existed within groups, data was analyzed by a one-way ANOVA or two-way ANOVA, as appropriate. If such differences existed, Bonferroni's corrected t-test was used to determine the group(s) with the

difference(s) (Prism 5.00; GraphPad Software, Inc.). Differences in categorical measures were analyzed using a Chi Square test. A final value of $P \leq 0.05$ was considered significant for all analyses.

Supplemental References

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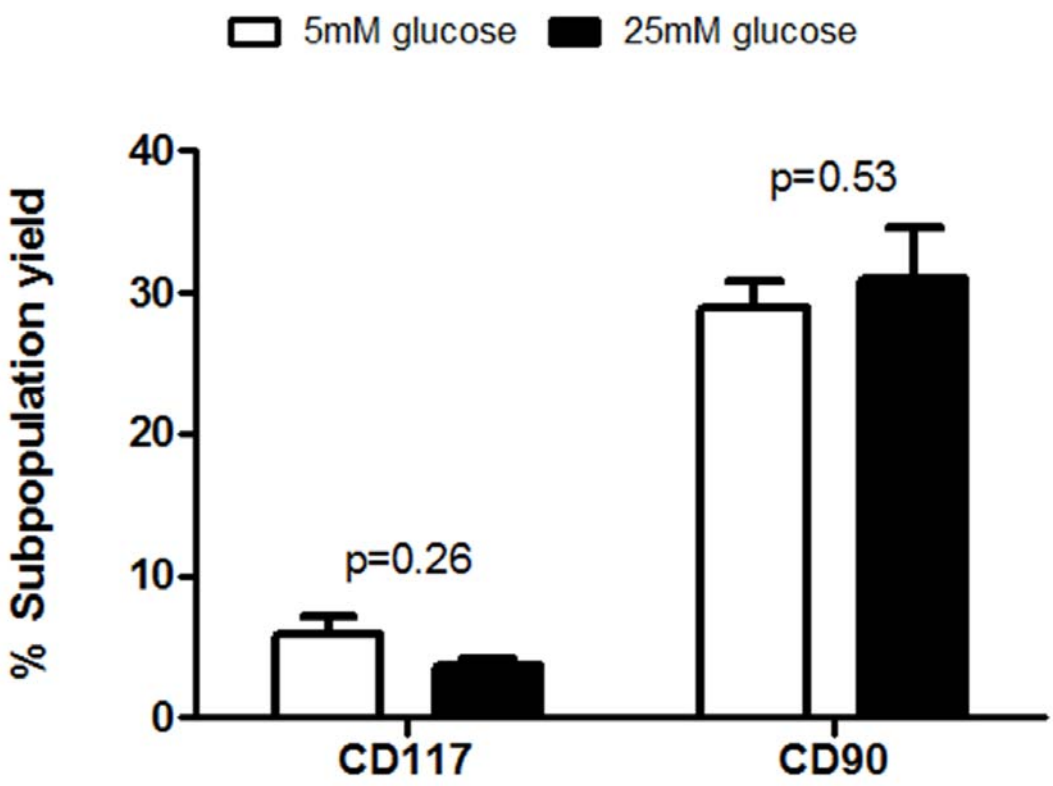
Supplemental Figure Legends

Supplement Figure S1. High glucose culture does not influence cellular phenotype. The percentage of CD117 and CD90 positive cells were quantified by flow cytometry of the total heterogenous CSC population following explant culture in 5mM or 25mM glucose. (n=3)



Supplemental Table and Table Legends

Weeks post MI	CSC source	EDV	ESV	SV	FAC	EF
		(μL)	(μL)	(μL)		(%)
Human CSCs injected into SCID mice						
1	Diabetic	72.4±3.9	51.7±2.8	22.5±0.7	19.6±1.5	27.8±0.4
	Non-diabetic	65.0±2.6	45.1±1.9	19.9±2.3	19.5±1.7	31.0±1.6
4	Diabetic	79.8±11.2	41.5±9.7	23.8±3.5	22.0±4.5	31.1±2.2
	Non-diabetic	74.3±7.4	57.7±6.6	30.7±3.7†	23.3±1.3	39.5±3.1*****†
Mouse CSCs cultured under physiologic (5 mM) or high (25 mM) glucose conditions injected into mice						
1	5 mM glucose	54.8±4.9	37.0±4.0	17.8±1.5	18.7±1.6	33.1±2.4
	25 mM glucose	58.2±3.0	39.4±2.6	19.0±0.7	18.6±0.9	33.1±1.2
4	5 mM glucose	67.9±8.2	39.5±5.9	28.5±2.9†	26.8±1.8*****†	43.0±2.7*****†
	25 mM glucose	72.2±7.9	47.1±6.3	25.2±2.1†	20.7±1.3†	35.7±2.1

Supplement Table S1. Echocardiographic measurements of left ventricular function over the 4 week follow-up period. *****$p \leq 0.05$ vs. non-diabetic status or 25 mM treatment 4 weeks post MI; $^{\dagger}p \leq 0.05$ vs. echo measurement 1 week post MI. EDV= end diastolic volume, ESV = end systolic volume, SV = stroke volume, FAC = fractional area shortening, EF = ejection fraction, SCID = severe combined immunodeficiency.