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Transplanted Induced Pluripotent Stem Cells Improve Cardiac Function and Induce Neovascularization in the Infarcted Hearts of db/db Mice

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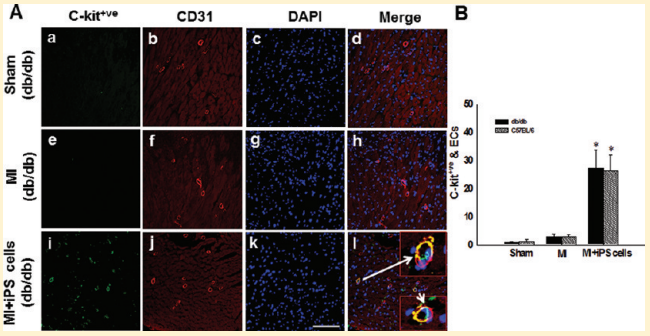
Supporting Information

ABSTRACT: Recently, we proclaimed that induced pluripotent stem (iPS) cells generated from H9c2 cells, following transplantation into infarcted nondiabetic mice, can inhibit apoptosis and differentiate into cardiac myocytes. iPS cells can be an ideal candidate to expand regenerative medicine to the clinic. Therefore, examining the wide range of their potential to differentiate into neovascular cell types remains a major interest. We hypothesized that transplanted iPS cells in the infarcted diabtetic db/db and nondiabetic mice can differentiate into vascular smooth muscle (VSM) and endothelial cells (ECs) as well as activate endogenous c-kit progenitor cells to enhance neovascularization along with improved cardiac function. We transplanted intramyocardially 50,000 iPS cells in the peri-infarct zone of infarcted db/db and C57BL/6 mice and hearts were examined at D14 post-MI. Cardiac function was examined using echocardiography. Our data implies that there was a significant ($p < 0.001$) increase in VSM and ECs in the infarcted heart following iPS cell transplantation compared with MI and sham groups in both db/db and C57BL/6 animals. Furthermore, the MI+iPS cell transplanted group also displayed a significant ($p < 0.001$) increase in c-kit^{+ve} activated VSM and ECs confirmed with combined stainings of c-kit and cell specific markers, compared with respective controls. Next, our histology data in the MI+iPS cell group also establishes a significant ($p < 0.05$) increase in coronary artery vessels compared with MI, suggesting neovascularization. Furthermore, our data demonstrates significant improved cardiac function following iPS cell transplantation compared with MI. Overall increased neovascularization in the infarcted db/db and C57BL/6 mice is associated with improved cardiac function following iPS cell transplantation.



KEYWORDS: neovascularization, endothelial cells, vascular smooth muscle cells, induced pluripotent stem cells, heart

INTRODUCTION

Diabetes, characterized by increased blood-glucose levels, results from decreased insulin production and or resistance.[1] There are two major types of diabetes: type I (insulin-dependent dependent diabetes mellitus) and type II (non-insulin dependent diabetes mellitus, NIDDM).[1] Myocardial infarction (MI) linked with type II diabetes is a serious problem which is associated with increased morbidity and mortality in cardiovascular diseases contrasted with nondiabetic counterparts.[2] Diabetes mellitus (type II) integrated with MI leads to severe cardiac and vascular remodeling characterized as a complex, dynamic and time-dependent process portrayed by (a) death of cardiac myocytes; (b) cardiac fibrosis; (c) hypertrophy; and (d) death of vascular cell types such as vascular smooth muscle (VSM) and endothelial cell (EC) types.[2] Augmented vascular cell death may contribute significantly in the abnormality of myocardial angiogenesis in diabetes.[3,4] Altered angiogenesis also displays an imbalance of angiogenesis growth factors and cytokines such as vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1).[3,4] Gene therapy using the Ang-1 gene displayed significant improvement in the maturation of the imperfect vasculature in type II diabetic (db/db) mice.[3,4] However, attenuation of impaired angiogenesis in type II diabetic individuals and animals associated with MI remains a big challenge.

Cell therapy used to manage injured myocardium is considered a viable option.[5−7] Reported studies suggest that transplanted embryonic and adult stem cells in the infarcted myocardium can engraft and differentiate into cardiac myocytes as well as VSM and ECs.[5−7] Induced pluripotent stem (iPS) cells generated from fibroblasts or other somatic stem cells also demonstrate their ability to differentiate into cardiac myocytes following transplantation in the nondiabetic infarcted heart.[8−10] In fact, we recently for the first time published that iPS cells transduced from H9c2 cells differentiate into cardiac myocytes in the cell culture system as well

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as repair and regenerate nondiabetic infarcted myocardium.[9] However, whether transduced H9c2 cells into iPS cells can generate neovascularization in the infarcted diabetic db/db mice remains unknown. Therefore, we hypothesized that transplanted iPS cells in infarcted db/db mice can differentiate into neovascular cell types, activate endogenous c-kit^{+ve} cells for their differentiation into VSM and ECs, which contributes to the neovascularization, and ultimately, improve cardiac function.

MATERIALS AND METHODS

iPS Cell Culture. We recently reported the generation of iPS cells via transduction of H9c2 cells (cardiomyoblasts).[9] H9c2 cells are specialized cell types isolated from ventricular tissue of rat embryonic heart as reported previously. Generated iPS cells were cultured in growth medium (GM) containing 15% fetal bovine serum, conditioned medium (CM) prepared from mouse embryonic fibroblasts (MEF) and embryonic stem cell-growth medium (Dulbecco's modified Eagle's medium). This medium contains additional additives including antibiotics (streptomycin and penicillin), 0.1 mM β-mercaptoethanol, 2 mM L-glutamine, 0.1 mM MEM nonessential amino acids, 0.1% leukemia inhibitory factor (LIF), glutamine, bFGF and activin A.

Immunostaining To Detect VSM and ECs in the Cell Culture. Neovascular cell type differentiation from iPS cells was determined using a hanging drop method to generate embryoid bodies (EBs). This method has been published by us.[9,11] Briefly, iPS cells growing in cell culture growth medium containing LIF and MEF-CM were centrifuged and the pellet was resuspended in differentiating medium without LIF and MEF-CM. Single cell suspension was generated by repeated gentle pipetting at a concentration of 2.5×10^4 cells/mL. Using a pipet a small aliquot of 20 μL containing around 500−600 cells/drop was placed on the lid of a Petri dish. After this procedure, the Petri dish lid was flipped back onto the bottom portion of the Petri dish which contained 10 mL of 1× PBS. After 48 h of incubation, EBs were transferred to another ultralow adherence Petri dish containing differentiation medium. After incubation, EBs were transferred into gelatin coated 100 mm^2 cell culture plates and maintained for 16−17 days for the determination of neovascularization.

Next, beating EBs were identified, pipetted to dissociate the large areas and transferred into gelatinized 8 well plates. After 24 h incubation, EBs were fixed in methanol:acetone (7:3) and blocked with 10% normal goat serum (NGS, Vector) for nonspecific binding. EB containing cells were incubated at room temperature (RT) for 1 h with the primary antibodies anti-smooth muscle (SM)-α-actin, (1:50); anti-red fluorescence protein (RFP), (1:500), anti-CD31 (1:200) and anti-von Willebrand factor VIII (vWF VIII, 1:40) containing 10% NGS in phosphate buffered saline (PBS). Sections were incubated for 1 h with the secondary antibodies Alexa 488, 568, or 660 goat anti-mouse IgG (1:100, Invitrogen). Sections were washed with PBS, air-dried, and mounted with antifade Vectashield medium containing 4, 6-diamidino-2-phenylindole (DAPI; Vector Laboratories) to stain nuclei. Sections were examined to count the number of differentiated cells and to prepare photomicrographs uisng Leica TCS SP2 laser scanning confocal microscope. Data was obtained from a set of 4 different experiments.

iPS Cell Transplantation. MI was induced in diabetic db/db and C57BL/6 mice as approved by the institutional animal care committee at the University of Central Florida. A total of 48 animals were divided into six groups (three for diabetic db/db and three for C57BL/6): sham (n = 8), MI (n = 8), and MI+iPS cells (n = 8). Coronary artery ligation was performed to induce MI as we reported.[9,12,13] Briefly, animals were sedated with isofluorane, chest was shaved, left thoracotomy surgery was practiced and mid left anterior descending coronary artery was ligated. We delivered either 50,000 iPS cells in 20 μL of medium or 20 μL of cell culture medium following MI using two intramyocardial injections at the peri-infarct region. Sham group animals also experienced complete surgery protocol. However, the coronary artery was not ligated in the sham group. Animals were sacrificed at D14 post-MI, and hearts were removed and used for further analysis.

Immunohistochemistry To Identify VSM and ECs. Heart tissue was paraffin embedded. 5 μm sections were cut at the mid-papillary level, deparaffinized in xylene and rehydrated using various combinations of absolute alcohol as we reported recently.[9,12,13] Sections were washed in distilled water followed by phosphate-buffered saline (1× PBS) and covered with NGS to avoid any nonspecific binding. After nonspecific blocking, sections were incubated with primary mouse monoclonal antibodies anti-CD31, (1:20, Santa Cruz) to identify ECs and anti-SM α-actin (1:15, Sigma) to identify VSM cells. Sections were costained with anti-RFP (1:1000 Evrogen/axxora) mouse monoclonal antibody, to identify transplanted iPS cells and to determine whether these cells differentiated into VSM and ECs.

To determine endogenous activation of c-kit^{+ve} progenitor cells and their differentiation into neovascular cell types, heart sections were incubated with mouse monoclonal primary antibody anti-c-kit and costaining was performed with anti-CD31 and anti-SM α-actin. After one hour with primary antibodies, sections were incubated with Alexa Fluor 568 or 488 labeled anti-mouse IgG antibodies obtained from Invitrogen. Sections were stained with DAPI present in the Vectashield mounting medium (Vector Laboratories) to visualize all nuclei. Immunostained sections were examined under a confocal microscope to obtain photomicrographs and quantitative analysis. For quantitative analysis, each heart section from 6 to 8 different animals was used to obtain the average number of cells. Moreover, newly differentiated cells in the heart are generated from rat H9c2 cells, therefore we state that our antibodies used in the present study SM α-actin and anti-CD31 had cross reactivity with rat antigens.

Determination of Angiogenesis Using Histology. Heart sections were stained with Masson's trichrome using our standard protocol.[9,12,13] Stained sections were used to identify total number of vessels by measuring the cross sectional area of the lumen using a grid (1−100μ, Hunt Optics, Pittsburgh, PA) inserted into the Olympus microscope eyepiece. Heart vessels present in the sections were examined under 20×. Most of the heart vessels in these sections varied in size from 1 to 300 μm^2 with the exception of a few that were larger than 300 μm^2. Total numbers of vessels from all sizes were combined and plotted in the graphs with n = 6−8 animals in each group.

Cardiac Function. Echocardiography was used to determine cardiac function in db/db and C57BL/6 mice at D14 post MI with and without iPS cell transplantation. We used a Phillips Sonos 5500 ultrasound system attached with a small animal heart function probe. Animals were anesthetized with isoflurane, chest hair was removed and animals were put in a supine position.[9] Imaging was recorded in a long axis projection with a 15-16L hockey stick transducer, and two-dimensional images were

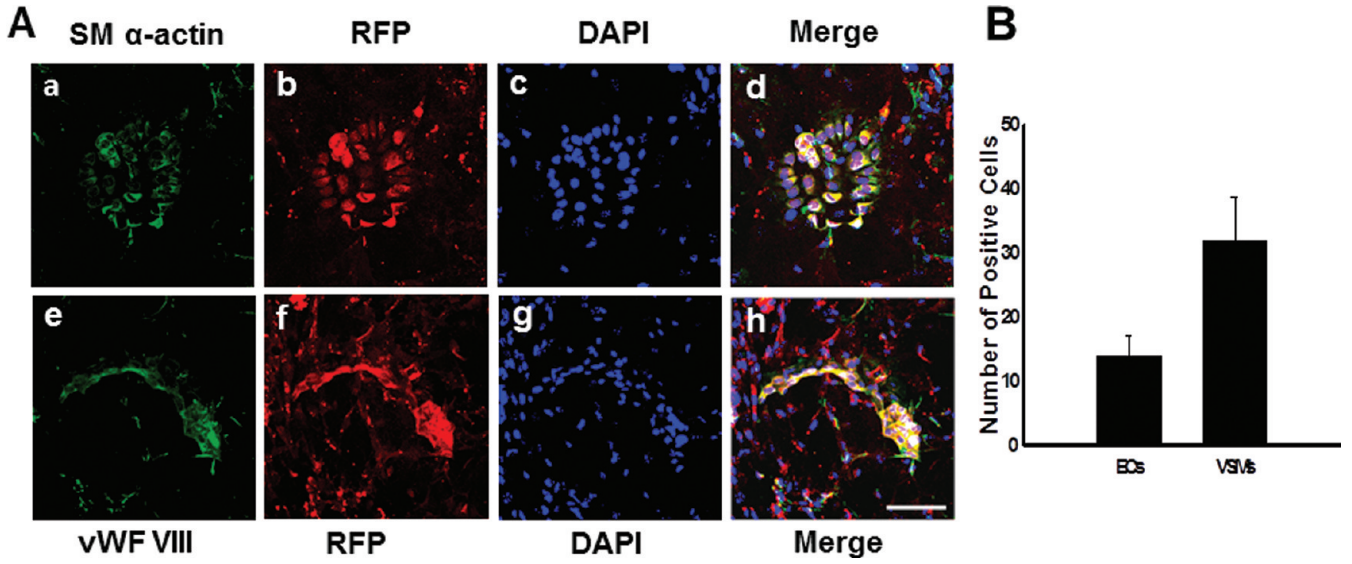

Figure 1. iPS cell derived embryoid bodies (EBs) differentiate into vascular smooth muscle (VSM) and endothelial cells (ECs). (A) Representative photomicrographs demonstrating SM α-actin identifying VSM cells (green, a), vWF VIII showing ECs (green, e), anti-RFP (red, b and f), and DAPI (blue, c and g) in iPS cell derived EBs. Merged images are shown in d and h. Scale bar = 100 μm. Panel B shows quantitative analysis of total number of VSM and ECs in EBs derived from iPS cells.

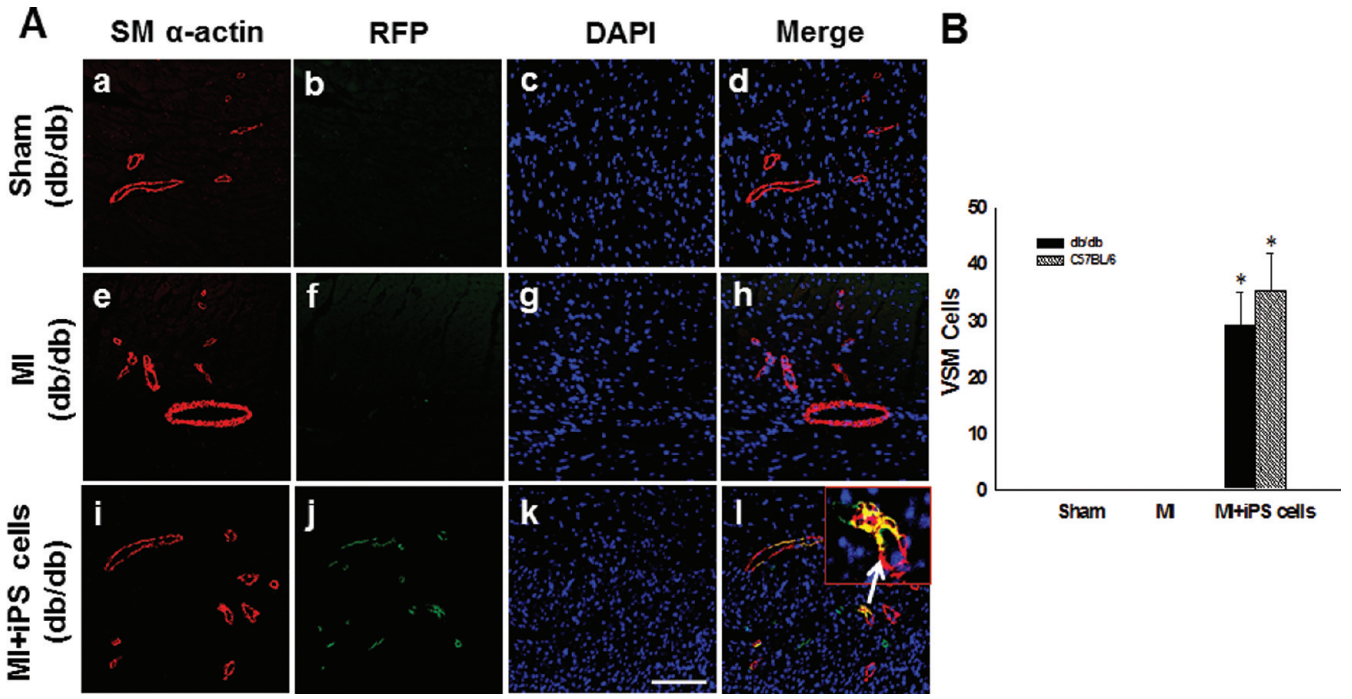

Figure 2. Transplanted iPS cells differentiate into smooth muscle cells post-MI. iPS cells were transplanted in db/db hearts post-MI and hearts were immunostained at D14 for anti-smooth muscle-α-actin in red (A, panels a, e, i), anti-RFP to identify donor cells in green (A, panels b, f, j), and DAPI in blue (A, panels c, g, k). Merged images are shown in A, panels d, h, i. Scale bar = 100 μm. Panel B shows quantitative analysis of iPS cell derived VSM cells in both C57BL/6 and db/db mouse hearts. *p < 0.001 vs MI.

obtained. M-mode recordings at the mid-ventricular level were used to measure internal dimensions, left ventricular fractional shortening and ejection fraction.

Statistical Analysis. Group data was presented as means ± SE. One way ANOVA followed by Tukey test was performed to assess the significant difference between groups. Statistical significance was considered at the level of $p < 0.05$.

RESULTS

Cell Culture Model of VSM and ECs Differentiation from iPS Cells. We used the iPS induced EB system to determine whether iPS cells can differentiate into neovascular cell types as well as retain RFP after differentiation in the cell culture system. Our data suggests that generated EBs were positive for the VSM

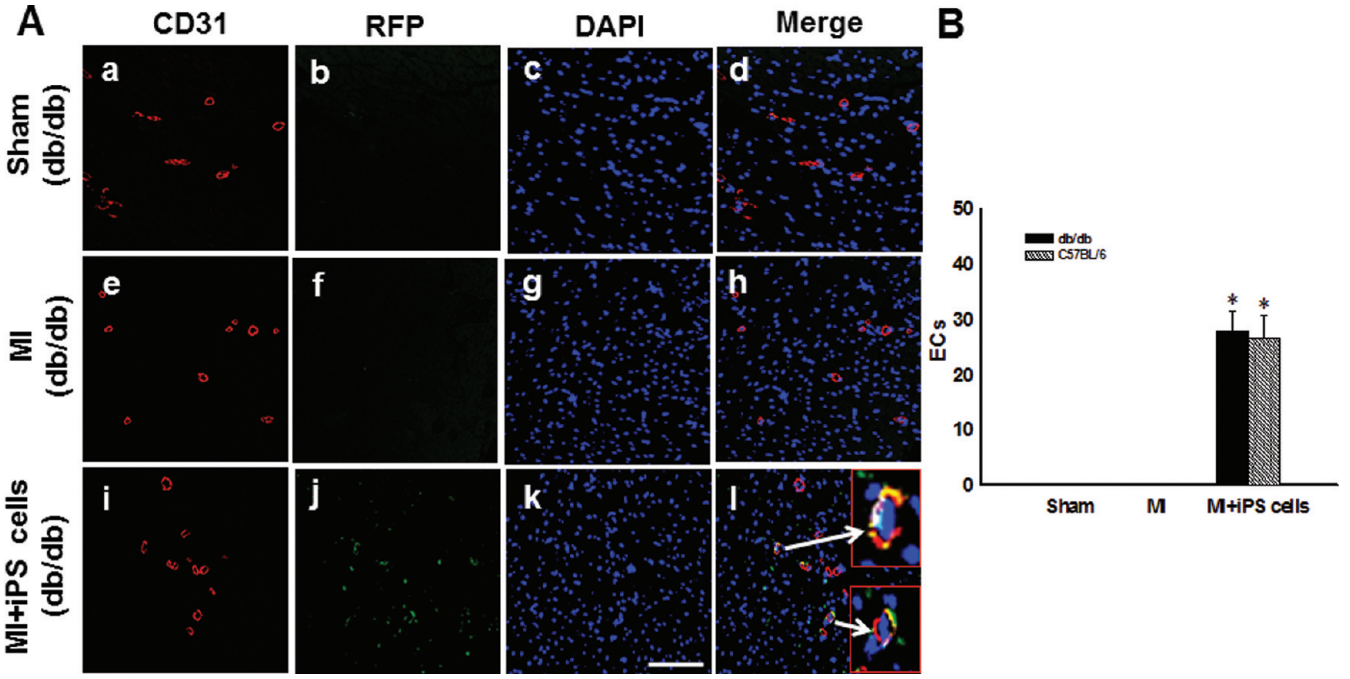

Figure 3. Effect of transplanted iPS cells on EC Differentiation post MI. Representative photomicrographs of db/db immunostained heart sections demonstrating anti-CD-31 in red (A, panels a, e, i), RFP in green (A, panels b, f, j) and total nuclei stained with DAPI in blue (A, panels c, g, k). Merged images are shown in A, panels d, h, i. Scale bar = 100 μm. Panel B shows quantitative analysis of total EC generation from transplanted iPS cells in both C57BL/6 and db/db mouse hearts two weeks post-MI, $^*p < 0.001$ vs MI.

cell specific marker SM α-actin and EC specific marker CD31 or vWF VIII, suggesting iPS cells have the potential to differentiate into neovascular cell types in the cell culture (Figure 1). Next, we stained the EBs with anti-RFP as RFP is present in the undifferentiated iPS cells. Our data suggests that iPS cells, which differentiated into neovascular cell types, were positive with RFP which suggests, upon differentiation, can detect donor cells (Figure 1). Our quantitative data suggests that a number of VSM and ECs were present in the each EB. Interestingly, many of the individual cell type stainings, when merged, suggest the formation of coronary artery vessels in the cell culture system (Figure 1). Therefore, we confirm that iPS cells have the capacity to differentiate into neovascular cell types and, following differentiation, these cells retain the reporter protein RFP.

Neovascularization in the Infarcted db/db Mice Following iPS Cell Transplantation. The following procedure was carried out to determine whether transplanted iPS cells differentiated into neovascular cell types such as VSM and ECs in the infarcted db/db and control C57BL/6 mice. Heart sections were stained with SM α-actin for VSM cells and with CD31 for ECs. Figure 2A, panels a, e and i, show the presence of VSM cells in the sham, MI and MI+iPS cell transplanted hearts of db/db mice as heart sections stained positive for the VSM cell specific protein SM α-actin. The same sections were stained with anti-RFP and were positive in the MI+iPS cell transplanted group, suggesting transplanted stem cells differentiated into new VSMs (Figure 2A, panel j). However, sham and MI heart sections were negative for the anti-RFP antibody (Figure 2A, panels b and f). Figure 2A, panels c, g and k, shows total nuclei, and merged images appear in panels d, h and l. A comparable pattern of immunostainings was observed in C57BL/6 infarcted hearts with and without iPS cell transplantation (see Supplemental Figure 1 in the Supporting Information). Next, we determined a 28- and 35-fold increase in newly formed VSM cells in MI+iPS cell transplanted db/db and C57BL/6 mice, respectively, compared with MI and sham groups (Figure 2B). However, newly formed VSM cells in MI+iPS cell groups were not statistically significant between db/db and C57BL/6 mice (Figure 2B). Similarly, ECs were identified by staining with CD31 in sham, MI and MI +iPS cell groups (Figure 3A, panels a, e and i). CD31 and RFP combined staining was evident in the MI+iPS cell group (Figure 3A, panel j) whereas sham and MI groups did not demonstrate the presence of a combined immunostaining in db/db mice (Figure 3A, panels b and f) and C57BL/6 mice (Supplemental Figure 2 in the Supporting Information), suggesting transplanted iPS cells differentiated into ECs in db/db mice and C57BL/6 mice. Moreover, differentiated ECs were significantly increased ($p < 0.05$, around 30-fold) in the MI+iPS cell group compared with MI and sham groups of infarcted db/db and C57BL/6 mice (Figure 3B). This data suggests that transplanted iPS cells enhance neovascularization in the infarcted db/db and C57BL/6 mice.

The effect of transplanted iPS cells on cardiac endogenous c-kit progenitor cells and their contribution in the differentiation into VSM and ECs were examined. Our c-kit cell specific staining combined with SM α-actin and CD31 data confirms that, following iPS cell transplantation, VSM cells and ECs were significantly increased compared with MI and sham groups, suggesting transplanted iPS cells may have activated endogenous c-kit^{+ve} progenitor cells and their differentiation into mature VSM (Figure 4A) and ECs (Figure 5A) in db/db mice. Activated c-kit progenitor cell differentiation into VSM and ECs was significantly ($p < 0.05$) increased in the MI+iPS cell group in C57BL/6 compared with MI and sham groups (Supplemental Figures 3 and 4 in the Supporting Information).

Histology Analysis of Vessel Formation Following iPS Cell Transplantation in db/db Mice. To determine the effect of transplanted iPS cells on coronary artery formation, we counted

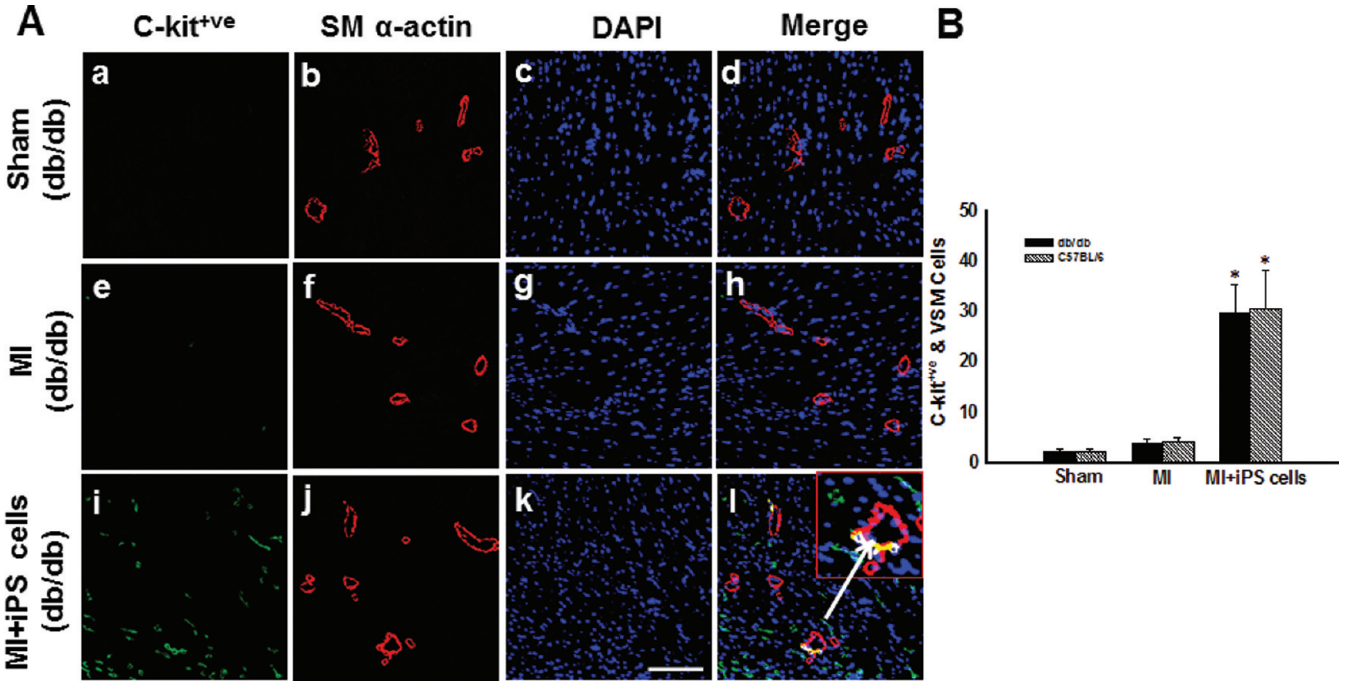

Figure 4. Effect of transplanted iPS cells on c-kit activation and VSM cell generation in C57BL/6 and db/db mouse infarcted hearts. Representative photomicrographs from db/db mouse hearts illustrating c-kit^{+ve} in green (A, panels a, e, i) anti-SM-α-actin in red (A, panels b, f, j), and total nuclei stained with DAPI in blue (A, panels c, g, k). Merged images are shown in A, panels d, h, I, Scale bar = 100 μm. Panel B shows significant activation of c-kit^{+ve} cells in both C57BL/6 and db/db MI + iPS cell groups. *p < 0.001 vs MI.

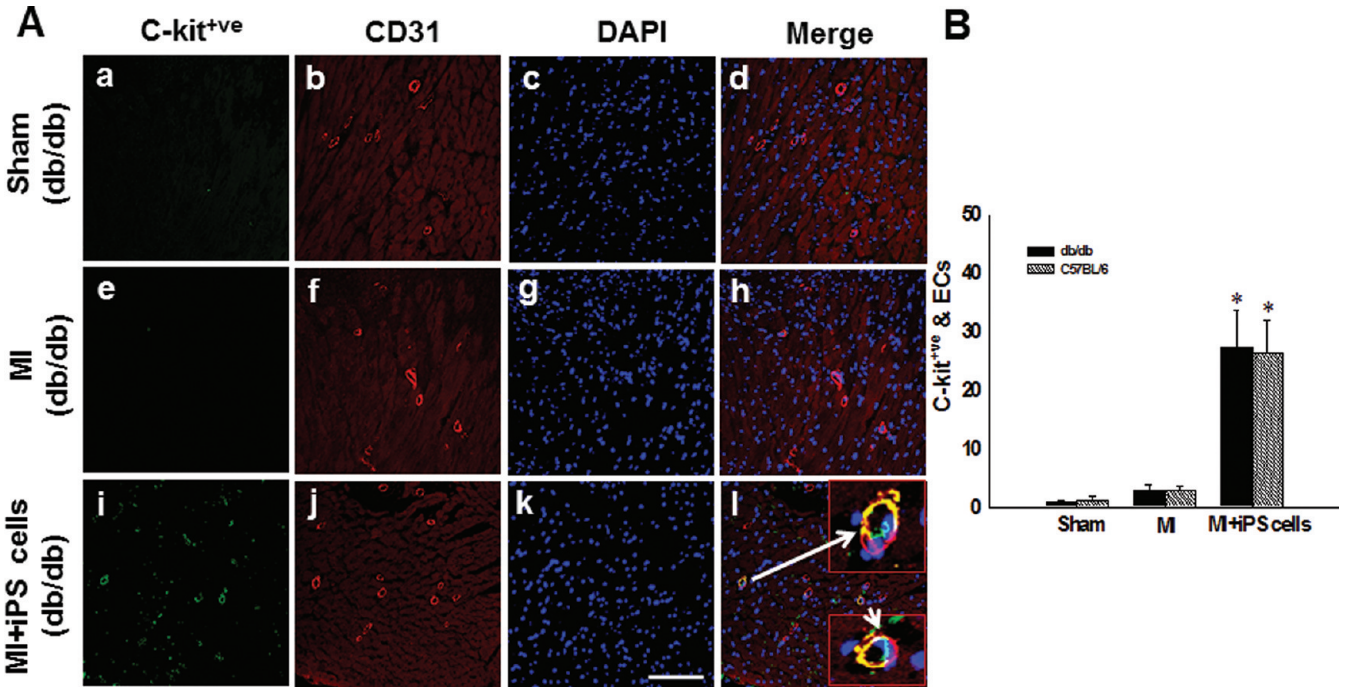

Figure 5. Transplanted iPS cells activate c-kit^{+ve} cells which contribute to EC generation in the infarcted heart. Representative photomicrographs show c-kit in green (A, panels a, e, i) anti-CD-31 in red (A, panels b, f, j), and total nuclei stained with DAPI in blue (A, panels c, g, k). Merged images are shown in A, panels d, h, I. Scale bar = 100 μm. Panel B shows quantitative analysis of total positive c-kit and CD-31 cells in both C57BL/6 and db/db mice hearts. *p < 0.001 vs MI.

the total number of small, medium and large vessels in Masson's trichrome stained sections in addition to CD31 and SM α-actin stainings described above. Our data suggests that there was a 58% decrease in coronary artery vessels in the MI group compared with sham in db/db and C57BL/6 mice (Figure 6). However, following iPS cell transplantation there were 146 and 141% newly

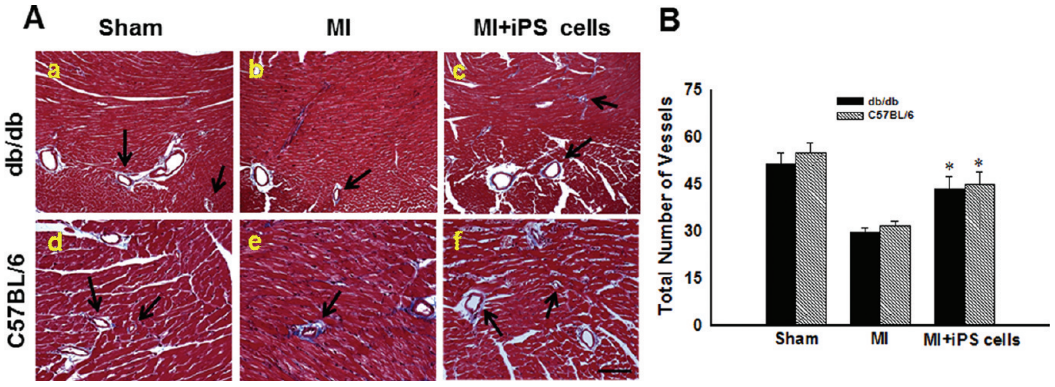

Figure 6. Effects of transplanted iPS cells on coronary artery formation following MI in C57BL/6 and db/db mice hearts. Representative photomicrographs from db/db heart sections stained with Masson's trichrome at D14 post MI surgery (A, panels a−c) and C57BL/6 (A, panels d−f). Scale bar = 100 μm. Panel B shows significant increase in vessel formation in both C57BL/6 and db/db transplanted with iPS cells compared with MI. $^{*}p < 0.001$ vs MI.

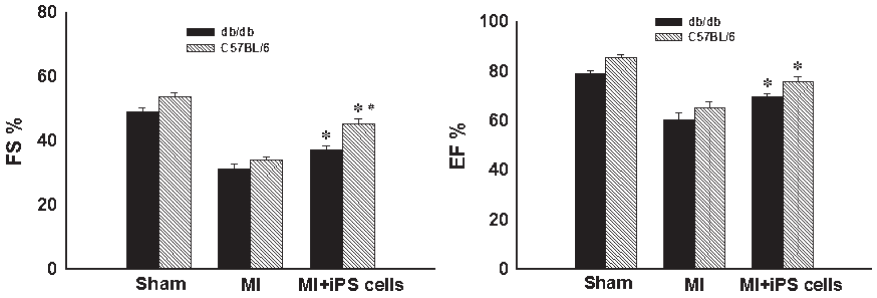

Figure 7. Effects of transplanted iPS cells two weeks following MI on cardiac function in C57BL/6 and db/db mice. Left panel shows that, 2 weeks following MI, C57BL/6 and db/db mice transplanted with iPS cells had a significant improvement in fractional shortening compared with MI controls; right panel, histogram demonstrates left ventricular ejection fraction in both C57BL/6 and db/db mice hearts two weeks post-MI. $^{*}p < 0.05$ vs MI. $^{\#}p < 0.05$ vs db/db.

formed vessels in db/db and C57BL/6 mice post-MI respectively compared with the MI group (Figure 6). However, MI+iPS cell group shows number of coronary vessels is lower when compared with sham animals in db/db and C57BL/6 mice groups.

Echocardiographic Evidence of Improvement in LV Function. Cardiac function was determined 2 weeks after the coronary artery ligation. Our data suggests a significant improvement in cardiac fractional shortening (FS) following iPS cell transplantation in db/db mice ($p < 0.05$, MI+iPS cells; 37 ± 1.4 vs MI; 31 ± 1.6) and C57BL/6 mice ($p < 0.05$, MI+iPS cells; 45 ± 1.4 vs MI; 34 ± 1, Figure 7, left panel). However, improved function following iPS cell transplantation was lower than sham in both db/db (48.94 ± 1.42) and C57BL/6 (53.67 ± 1.26) groups. We also determined that left ventricular ejection fraction (EF) was significantly increased in the MI+iPS cell group (69.8 ± 1) compared with MI (60.5 ± 3) in db/db mice (Figure 7, right panel). Moreover, significantly increased ejection fraction was also observed in the MI+iPS cell group (75.7 ± 2.1) compared with MI (65.33 ± 2.4) in C57BL/6 mice (Figure 7, right panel). An improved ejection fraction value in MI+ iPS cells was lower than normal controls (db/db; 78.80 ± 1.22 and C57BL/6 85.39 ± 1.08).

DISCUSSION

Type II diabetes provokes various cardiovascular related complications such as coronary artery disease, stroke and peripheral artery disease, which is associated with impairment in angiogenesis.[14] Impaired angiogenesis is well observed in db/db mice which is partially corrected with Ang-1 gene therapy.[3,4] Following ES cells, bone marrow stem cells, or endothelial progenitor cell (EPCs) transplantation in the nondiabetic infarcted animals a significant increase in neovascularization is demonstrated.[15−17] Generation of iPS cells is a major breakthrough in the current field of regenerative medicine as any major issue of ethical concerns associated with pluripotent human and mouse ES cells is circumvented. With this hope, various investigators have generated normal, patient disease specific and rodent iPS cell lines from fibroblasts.[8−10,18,19] Therefore, whether diverse iPS cell lines will be favorable over each other and have different regeneration potential remains unclear. We recently proclaimed for the first time our unique iPS cells derived from H9c2 cardiomyoblast cells.[9] We also reported the potential of these cells to differentiate into cardiac myocytes in vitro, inhibit apoptosis, differentiate into cardiac myocytes and form gap junction proteins in the infarcted mouse heart following transplantation.[9] However, there are no published reports on whether H9c2 cell induced iPS cells demonstrate their differentiation potential into VSM and ECs in the cell culture.

To achieve this target we generated iPS cell derived beating EBs as previously reported.[9] In this study we examined beating EBs for the presence of neovascular cell types as based on positive staining with the VSM cell specific marker SM α-actin, and EC specific markers CD31 and vWF VIII. Our data is well in accordance with the data published using ES cell differentiation into VSM and ECs in vitro.[20,21]

Next, we investigated whether transplanted iPS cells have the capacity to differentiate into VSM and ECs and enhance neovascularization in the infarcted diabetic db/db, and nondiabetic C57BL/6 mice. In the present study, we provide proof that following iPS cell transplantation in the infarcted diabetic and nondiabetic mice, differentiation into VSM cells and ECs was manifested by positively stained heart sections with cell specific markers combined with an anti-RFP antibody used to determine the donor cell marker (Figure 1 and 2 and Supplemental Figures 1 and 2 in the Supporting Information). However, such combined stainings were not observed in the MI and sham groups. Moreover, our quantitative data suggests that there was an increase in VSM and EC differentiation in the iPS cell groups compared with respective controls. Recently, murine iPS cell derived Flk-1 positive cells have been demonstrated to vascularize the infarcted nondiabetic mouse heart.[22] Moreover, various other studies suggest that transplanted stem cells or EPCs increase capillary density.[6,16,22] These studies are well in compliance with our current report that iPS cells have the potential to enhance neovascularization in the infarcted nondiabetic myocardium. In the present study, our data on neovascularization following iPS cell transplantation in the infarcted db/db mice is reported for the first time.

It is becoming clearer that generation of neovascularization in the infarcted heart is a complex process which may involve a series of pro-angiogenic growth factors[23−25] such as a vascular endothelial growth factor (VEGF), growth colony stimulating factor (G-CSF), stem cell factor (SCF) and insulin growth factor (IGF-1) rather than solely direct differentiation of iPS cells into VSM and ECs required for neovascularization. Therefore, we also examined whether transplanted iPS cells have any effect on the c-kit progenitor cells present in the heart. To our astonishment we observed a significant increase in c-kit^{+ve} progenitor cells costained with CD31 identifying ECs, and SM α-actin demonstrating an increase in VSM cells following iPS cells transplantation. This suggests endogenous c-kit progenitor cells in the infarcted diabetic and nondiabetic hearts were activated and then differentiated into neovascular cell types. However, an increase in c-kit positive derived VSM and ECs was evident in the MI group compared with sham. In our previous studies, we have established that autocrine or paracrine factors released from ES cells enhance c-kit and FLK-1 positive cell differentiation into VSM and ECs, which contributes to the increase in neovascularization.[25] It remains to investigate what kind of autocrine or paracrine factors are being released that enhance neovascularization. Moreover, factors released from ES cells and certain growth factors such as G-CSF and stem cell factor (SCF) contribute to the increased neovscularization in the infarcted mice heart.[24,25] When tested in clinical settings, this hope was diminished as G-CSF could not demonstrate any significant difference in cardiac remodeling and infarct size compared with controls.[26] Moreover, isolated cardiac progenitor cells treated with IGF-1 and HGF, following transplantation into the infarcted heart, can differentiate into VSM and ECs, which contributes to the neovascularization.[27] Overall, our current study is consistent with these published reports that autocrine or paracrine factors enhance neovascularization in the infarcted heart. Therefore, we hypothesize that factors released from transplanted iPS cells may have activated endogenous c-kit positive cells and enhanced neovascularization. Moreover, it remains unclear and needs further investigation whether released factors from iPS cells are similar or distinct compared with ES cells or one among those factors listed above required for neovascularization.

Next, our Masson's trichrome data suggest that there was a significant increase in total number of vessels following iPS cell transplantation compared with MI and sham, suggesting increased vessels in this study are generated from direct differentiation from transplanted iPS cells as well as endogenous activation of c-kit positive cell differentiation. Furthermore, our echocardiography data demonstrates significant improved cardiac function in both groups.

To determine whether transplanted iPS cells induce teratoma formation following transplantation in the diabetic and nondiabetic infarcted hearts, we transplanted 50,000 iPS cells and observed no teratoma formation as we reported earlier.[9] Our study is in agreement with the studies published by us and other investigators that, if cell concentration of transplanted ES cells is less than 100,000 cells, then there is no risk of teratoma formation.[9,28] iPS cells are pluripotent stem cells which retain the potential to differentiate into all three embryonic germ layers including ectoderm, mesoderm and endoderm as their counterpart ES cells do.[28,29] Generation of all three germ layers may induce teratoma formation if injected in large quantities as reported.[28] Moreover, we have used 4F (Oct3/4, SOX2, Klf4, and c-myc) to induce iPS cells from H9c2 cells which may induce teratoma formation as the pluripotent gene SOX2[30] and c-myc[31] are oncogenic, which may limit the clinical applications. Therefore, we suggest that further studies are needed to generate iPS cells using 3F or 2F especially without the oncogenes c-myc and SOX2.

Moreover, iPS cells from various sources may vary in their epigenetic alterations.[32] A recently published paper in *Nature* suggests that iPS cells, generated from fibroblasts using retroviral and episomal approaches, are immunogenic in nature.[33] However, generated iPS cells by the episomal approach were less immunogenic compared with the cells generated by the retroviral approach.[33] We have generated iPS cells from rat H9c2 cells using plasmid transfection; whether they will be immunogenic is not well understood. Importantly in our method, the number of cells and location of injection are completely different compared to recent reports on the immunogenicity of iPS cells.[33] Moreover, ES cells transplanted in the sheep heart were compared with and without the immunosuppressant drug cyclosporin A,[34] suggesting there was engraftment and cardiac regeneration in both groups. Keeping all these reports in mind, we suggest that further studies are needed to understand the effect of H9c2 induced iPS cells on immunogenicity by comparing the results in db/db and C57BL/6 mice with and without the use of the immunosuppressant drug cyclosporin A.

In conclusion, we report for the first time that H9c2 cell induced iPS cells can differentiate into VSM and ECs in vitro, and enhance neovascularization via direct differentiation into neovascular cell types as well as mediated through the activation of endogenous c-kit progenitor cells that contributes to the improved cardiac function in both infarcted diabetic db/db and C57BL/6 mice.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Figures depicting differentiation of transplanted iPS cells in C57BL/6 mice into VSM cells and ECs post-MI, effect of transplanted iPS cells on c-kit activation and VSM cell generation in C57BL/6 mouse infarcted hearts, and effect of transplanted iPS cells on c-kit^{+ve} activation and EC generation in C57BL/6 mouse infarcted heart. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS USED

CM, conditioned medium; CC, cell culture; MI, myocardial infarction; FLK-1, fetal liver kinase; CPC, cardiac progenitor cells; ELISA, enzyme linked immunosorbent assay; IGF, insulin growth factor; HGF, hepatocyte growth factor; EPCs, endothelial progenitor cells; LVEDd, left ventricular end diastolic dimension; EC, endothelial cell; ECs, endothelial cells; LIF, leukemia inhibitory factor; DMEM, Dulbecco's minimum essential medium; VEGF, vascular endothelial growth factor; SCF, stem cell factor; EBs, embryoid bodies; VSM, vascular smooth muscle cells; Ang-1, angiopoietin; iPS cells, induced pluripotent stem cells

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