

Research Article

ACE2 gene transfer ameliorates vasoreparative dysfunction in CD34⁺ cells derived from diabetic older adults

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Diabetes increases the risk for ischemic vascular diseases, which is further elevated in older adults. Bone marrow-derived hematopoietic CD34⁺ stem/progenitor cells have the potential of revascularization; however, diabetes attenuates vasoreparative functions. Angiotensin-converting enzyme 2 (ACE2) is the vasoprotective enzyme of renin–angiotensin system in contrast with the canonical angiotensin-converting enzyme (ACE). The present study tested the hypothesis that diabetic dysfunction is associated with ACE2/ACE imbalance in hematopoietic stem/progenitor cells (HSPCs) and that increasing ACE2 expression would restore reparative functions. Blood samples from male and female diabetic ($n=71$) or nondiabetic ($n=62$) individuals were obtained and CD34⁺ cells were enumerated by flow cytometry. ACE and ACE2 enzyme activities were determined in cell lysates. Lentiviral (LV) approach was used to increase the expression of soluble ACE2 protein. Cells from diabetic older adults (DB) or nondiabetic individuals (Control) were evaluated for their ability to stimulate revascularization in a mouse model of hindlimb ischemia (HLI). DB cells attenuated the recovery of blood flow to ischemic areas in nondiabetic mice compared with that observed with Control cells. Administration of DB cells modified with LV-ACE2 resulted in complete restoration of blood flow. HLI in diabetic mice resulted in poor recovery with amputations, which was not reversed by either Control or DB cells. LV-ACE2 modification of Control or DB cells resulted in blood flow recovery in diabetic mice. *In vitro* treatment with Ang-(1-7) modified paracrine profile in diabetic CD34⁺ cells. The present study suggests that vasoreparative dysfunction in CD34⁺ cells from diabetic older adults is associated with ACE2/ACE imbalance and that increased ACE2 expression enhances the revascularization potential.

Introduction

Angiotensin-converting enzyme (ACE), the most prominent enzyme of classical renin–angiotensin system (RAS), generates Angiotensin II (Ang II or Ang-(1-8)), from Ang I (Ang-(1-10)). Ang II excess largely produces cardiovascular detrimental effects including but not limited to vasoconstriction, oxidative stress, mitochondrial dysfunction, inflammation and fibrosis, by acting on the angiotensin II type 1 receptor (AT1R) thus increasing risk for cardiovascular diseases [1]. The alternative ACE, angiotensin-converting enzyme-2 (ACE2), which was discovered by two independent groups [2,3], converts Ang II into Ang-(1-7) [4,5]. Physiological effects produced by Ang-(1-7) are counter-regulatory to the actions of Ang II, and mediated by Mas receptor (MasR). Ang-(1-7) promotes vasodilation, antihypertensive, antifibrotic, antithrombotic and antihypertrophic effects in experimental models of cardiovascular disorders [6].

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A subpopulation of bone marrow (BM)-derived hematopoietic stem/progenitor cells (HSPCs) has been identified to have the potential to stimulate re-endothelialization and vascular regeneration [7]. Circulating HSPCs are therefore believed to be responsible for maintaining vascular homeostasis. This population of cells was originally described as endothelial progenitor cells but the vasoreparative functions are largely accomplished by paracrine release of cytokines and growth factors that act on the peri-ischemic endothelium to stimulate angiogenesis but not by direct differentiation to endothelial cells [8,9]. Ischemic injury stimulates mobilization of BM-resident cells into the bloodstream and homing of cells to areas of ischemia. This is largely by generation of SDF and VEGF from the injured tissue. The accumulated literature defines this subpopulation in humans by using different combinations of hematopoietic and endothelial markers, CD34, CD133, CD31, VEGFR2, VEGFR1, and Tie-2 [10]. Clinical studies strongly supported that reduced number of BM-derived circulating angiogenic cells (CACs) are predictors of severe cardiovascular events [11,12]. These findings collectively lead to rapid transition to clinical trials to determine the therapeutic potential of autologous cells for the treatment of ischemic cardiovascular diseases. Several clinical trials were carried out and mixed therapeutic outcomes were reported. A recent meta-analysis of randomized clinical trials indicate transient beneficial effects of cell-based therapies for acute myocardial ischemia [13]. However, in the setting of chronic conditions such as ischemic heart disease or heart failure, cell therapies have showed consistent and significant clinical outcomes [14,15.]

Patients with diabetes that are at high risk for ischemic vascular disease have reduced number of vasoreparative cells characterized by $\text{Lin}^- \text{CD34}^+ \text{CD45}^{\text{low}}$ cells and other immunophenotypic markers [16]. In addition, vascular repair-relevant functions of these cells *in vitro*, migration, proliferation, transmigration and endothelial tube formation, and *in vivo*, restoring blood flow to areas of ischemia and revascularization, are impaired [17,18]. Aging with diabetes accelerates the development of vascular complications by both decreasing the BM reserve of regenerative cells as well as by attenuating the reparative functions [19,20]. Decreased number of HSPC phenotypes in the circulation is an independent determinant of all-cause mortality in diabetic older adults [12,21]. Therefore, increased mortality in diabetic older adults is largely due to pathological involvement of BM stem cell mobilopathy resulting in decreased overall regenerative capacity leading to widespread pathology. Collectively these reports indicate that feasibility and success of the approach of cell-based therapies for the treatment of vascular disorders in diabetic older individuals is challenging.

Recent studies have shown evidence for a protective role of ACE2/Ang-(1-7)/MasR axis of RAS in stimulating vasoreparative functions of HSPCs. Lentiviral (LV) expression of Ang-(1-7) transgene restored migration of diabetic CD34^+ HSPCs *in vitro* and *in vivo* [22]. Activation of MasR restored mobilization of HSPCs from BM into the bloodstream that was impaired in mouse models of diabetes, and accelerated revascularization of ischemic areas [23,24]. This study tested the hypothesis that vasoreparative dysfunction of HSPCs is associated with relative decrease in ACE2 and that the dysfunction can be reversed by ACE2 gene transfer. Specifically, we have demonstrated decreased number of circulating CD34^+ HSPCs diabetic individuals is associated with ACE2/ACE imbalance. Then, we overexpressed ACE2 in diabetic cells, and tested their ability to restore blood flow to ischemic areas in diabetic mice experiencing vascular injury.

Materials and methods

Characteristics of subjects

Research involving human subjects has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) and approved by Institutional Review Board and the Institutional Biosafety Committee of North Dakota State University. All subjects have provided written informed consent. Either male or female nondiabetic ($n=62$) or diabetic ($n=71$), both type 1 and type 2, individuals of age 21 years or older have participated in the study. Characteristics of participants are listed in the Table 1. Whole blood samples were obtained by phlebotomy into vacutainer tubes (Museum District Eye Clinic, Houston, Texas; Sanford Health, Fargo, North Dakota) and leukocyte samples were obtained from leukoreduction chambers following apheresis by using TrimaAccel system (80440) (Vitalant, previously United Blood Services, Fargo, North Dakota).

Isolation of HSPCs

Peripheral blood mononuclear cells (MNCs) were separated by gradient centrifugation ($800 \times g$, 30 min) using Ficoll (Ficoll-Paque; GE Healthcare Biosciences). MNCs were either used for flow cytometry analysis of lineage-negative (Lin^-) or $\text{Lin}^- \text{CD45}^{\text{low}} \text{CD34}^+$ HSPC populations, or for the enrichment of CD34^+ HSPCs. A negative selection kit (Stemcell Technologies) was used for the enrichment of Lin^- cells by following supplier's instructions, which involves depletion of cells expressing CD3, CD14, CD16, CD19, CD20, and CD56. Lin^- cells were further enriched for CD34^+

Table 1 Characteristics of study participants

	Nondiabetic [62]	Diabetic [71]
Males/Females	33/29	39/32
Age (years)	28–78 (54 ± 3)	34–76 (59 ± 2) NS
Ethnicity	Caucasians/Hispanics (46/16)	Caucasians/Hispanics (42/29) NS
Blood glucose (mg/dl)	91–116 (101 ± 5)	160–240 (208 ± 6, $P < 0.0001$)*
HbA1C	4.6–5.7 (4.8 ± 0.2)	6.5–11 (7.7 ± 0.4, $P < 0.0001$)
Type 1	None	12
Type 2	None	59
Vascular complications	None	[†] Hypertension [32], neuropathy [21], nephropathy [26], retinopathy (proliferative or nonproliferative diabetic retinopathy) [34], myocardial ischemia [13], and erectile dysfunction [9]
Medications	None	[‡] Insulin [12], sulfonylureas [31], statins [29], calcium channel blockers [31], metformin [46], clopidogrel [11], β -blockers [24], gabapentin/pregabalin [7] and gabapentin [11]

Sulfonylureas—Glyburide, glimepiride or glipizide; Statins—Pravastatin, Atorvastatin or rosuvastatin or xxx; Calcium channel blockers—Nifedipine or amlodipine; β -blockers—Metoprolol, carvedilol or timolol.

*Compared with nondiabetic group.

[†]Shown in parenthesis is the number of individuals.

NS, not significant compared with nondiabetic. Ages were compared by 't' test. Prevalence of ethnicity in groups was compared by Chi-square test.

cells by positive-selection by using immunomagnetic separation kit (Stemcell Technologies) as per manufacturer's instructions. Cells were either used for experiments *in vitro* or processed for *in vivo* testing in mice.

Flow cytometry

Flow cytometry was carried out by suspending MNCs in the stain buffer and treated first with FcR blocking reagent (1:100, Miltenyi Biotech) followed by incubation with the following fluorescent-conjugated antibodies (Biolegend), allophycocyanin (APC) anti-human lineage cocktail (1:500, Biolegend), phycoerythrin (PE) anti-human CD45 (1:500) and Fluorescein isothiocyanate (FITC) anti-human CD34 antibodies (1 in 250) or isotype control antibodies (1 in 500) (Biolegend, San Diego, CA, U.S.A.) for 45 min at 4°C. Dead cells were excluded using 7-AAD viability staining solution. Flow cytometry was carried out by using C6 Accuri cytometer (BD).

ACE and ACE2 activity assays

ACE and ACE2 activities were determined in the cell lysates or plasma and cell supernatants by using enzyme-specific fluorogenic substrates (ES005 and ES007 for ACE and ACE2, respectively, R&D Systems), as reported before [25]. Enzyme-specific inhibitors, captopril or MLN-4760, were used to define ACE- and ACE2-specific activity. The inhibitor-sensitive fluorescence was expressed as arbitrary fluorescence units/ μ g of protein/hour.

Western blotting

Western blotting of ACE2 was carried out in the cell lysates of CD34⁺ cells as described before [26]. Cells were lysed in a radioimmuno precipitation assay (RIPA) buffer (Tris 10 mM pH 7.4, containing 140 mM NaCl, 1 mM EDTA, 1 mM NaF, 0.10% SDS, 0.50% sodium deoxycholate 0.1% NP-40, 1% Triton X-100) in the presence of protease inhibitors (Thermo Fisher). Protein concentration in cell lysates and cell supernatants was determined using bicinchoninic acid with bovine serum albumin as a standard (Thermo Fisher). Equal amounts of protein (30 μ g) were loaded and separated by SDS-PAGE using SurePage 10% pre-casted gels (Genescript). Proteins were electro-blotted onto nitrocellulose membranes (Bio-Rad). The blots were blocked using 5% (w/v) milk in Tris-buffered saline containing 0.5% (v/v) Tween-20. The membranes were then incubated with ACE2 antibody, ab87436 or with a β -actin antibody (mab8929; R&D Systems). Molecular weight marker, Protein Kaleidoscope (Biorad) was used to identify protein bands. HRP-conjugated goat anti-mouse (Biolegend) or donkey anti-rabbit (406-401; Biolegend) secondary antibodies were used at 1:20,000 dilution. Enhanced chemiluminescence reagent (ECL, K15045-D50; Advansta) was used to visualize bands and developed on X-ray films (Phoenix research products). ImageJ (NIH) was used for quantification of band intensities.

LV transduction of CD34⁺ cells

LV particles packaged with gene encoding human soluble ACE2 with eGFP reporter under EF1a promoter were custom-made from GeneCopoeia (U.S.A.). Presence of furin cleavage site results in the secretion of soluble ACE2. This construct was previously used in animal models of cardiopulmonary disorders previously [27,28]. LV particles made of a custom transfer vector pseudotyped with VSV-G protein, were generated by using highly purified plasmids and EndoFectin-Lenti and TiterBoost reagents (GeneCopoeia). Cells were transduced with viral particles by using spinoculation technique as described before [29]. Briefly, this technique involves preparing a cell suspension in Dulbecco's modified Eagle's medium (high glucose) with viral particles at 50 MOI and polybrene (10 µg/ml) at a density of 5×10^4 cells/ml. Then, the cell suspension was plated in aliquots in a U-bottom 96-well plate and centrifuged at $150 \times g$ and at the temperature 23°C for 2 h.

Proliferation

Proliferation of CD34 cells was determined as described before by using cell proliferation BrdU assay kit (Roche Bioscience) [30]. The assay was performed by using 10000 cells per treatment group for 48 or 72 h. Proliferation was evaluated by measuring absorbance at 370 nm (Spectramax plate reader) and expressed as fold increase as compared with mitomycin (1 µM), which is known to inhibit cell proliferation.

Animal models

All animal studies were approved by the Institutional Animal Care and Use Committee at North Dakota State University and all experiments were carried out at this institute. Male athymic nude mice (Foxn1^{nu} mice) of age 6–8 weeks, were obtained from Envigo (Indianapolis, IN). Foxn1^{nu} mice are T cell-deficient due to dysfunctional rudimentary thymus and do not show graft versus host response therefore suitable for evaluating reparative functions of human cells. Mice were maintained on a 12-h light–dark cycle with food and water *ad libitum*. Where applicable, mice were treated with A779 by using subcutaneous osmotic pumps (Alzet) at a perfusion rate of 1 µg/kg/min for 4 weeks.

Hindlimb ischemia

Hindlimb ischemia (HLI) was induced in mice by ligation and excision of femoral artery was performed as described by Niyama et al. [31] under isoflurane anesthesia. Blood perfusion of the limbs was measured by imaging the flux (blood \times area⁻¹ \times time⁻¹) by using Laser Doppler imaging system (Moor Instruments Inc.) under isoflurane anesthesia, which was expressed relative to the mean blood flux in the contralateral non-ischemic limb.

Biochemical assays

Quantitative analysis of selected cytokines and growth factors in the conditioned medium (CM) was performed by AssayGate Inc., by using the Luminex Bead-based immunoassay platform. CM was prepared by suspension culture of cells in a basal medium (Lonza), for 18 h in round bottom 96-well plates at a density of 20000 cells/150 µl/well. Cell supernatant was collected by centrifugation at $440 \times g$ for 10 min and concentrated 10 \times by using CentriPrep filtering units (Amicon Ultra-15 Millipore centrifugal filter units, 3 kDa cut-off) at 4°C and snap-frozen in liquid nitrogen and preserved at –80°C. Basal medium was concentrated and preserved following similar protocol for CM.

Data analysis and statistical comparison

Data were presented as mean \pm SEM with 'n', the number of experiments equals the number of subjects used for the respective treatment group. Where applicable data were presented either as box plots or as scatter plots. Datasets were tested for statistical significance by carrying out Nonparametric Mann–Whitney test, paired or unpaired 't' test, one sample *t* test or by two-way ANOVA with multiple comparisons, where applicable. Correlation of two different variables was tested by using Spearman correlation. Datasets were considered significantly different if $P < 0.05$.

Results

Diabetic individuals have decreased number of CD34⁺ HSPCs in the circulation compared with nondiabetic individuals

The number of circulating Lin⁻CD45^{low}CD34⁺ HSPCs in the peripheral blood samples from nondiabetic or diabetic individuals (HbA1c 6.5 or higher) was enumerated by flow cytometry. Either Lin⁻ or Lin⁻CD45^{low}CD34⁺ cells were lower in diabetic individuals ($n=33$) compared with nondiabetic individuals ($n=23$) (Lin⁻ cells $P < 0.01$; Lin⁻CD45^{low}CD34⁺ cells $P < 0.001$) (Figure 1A and B).

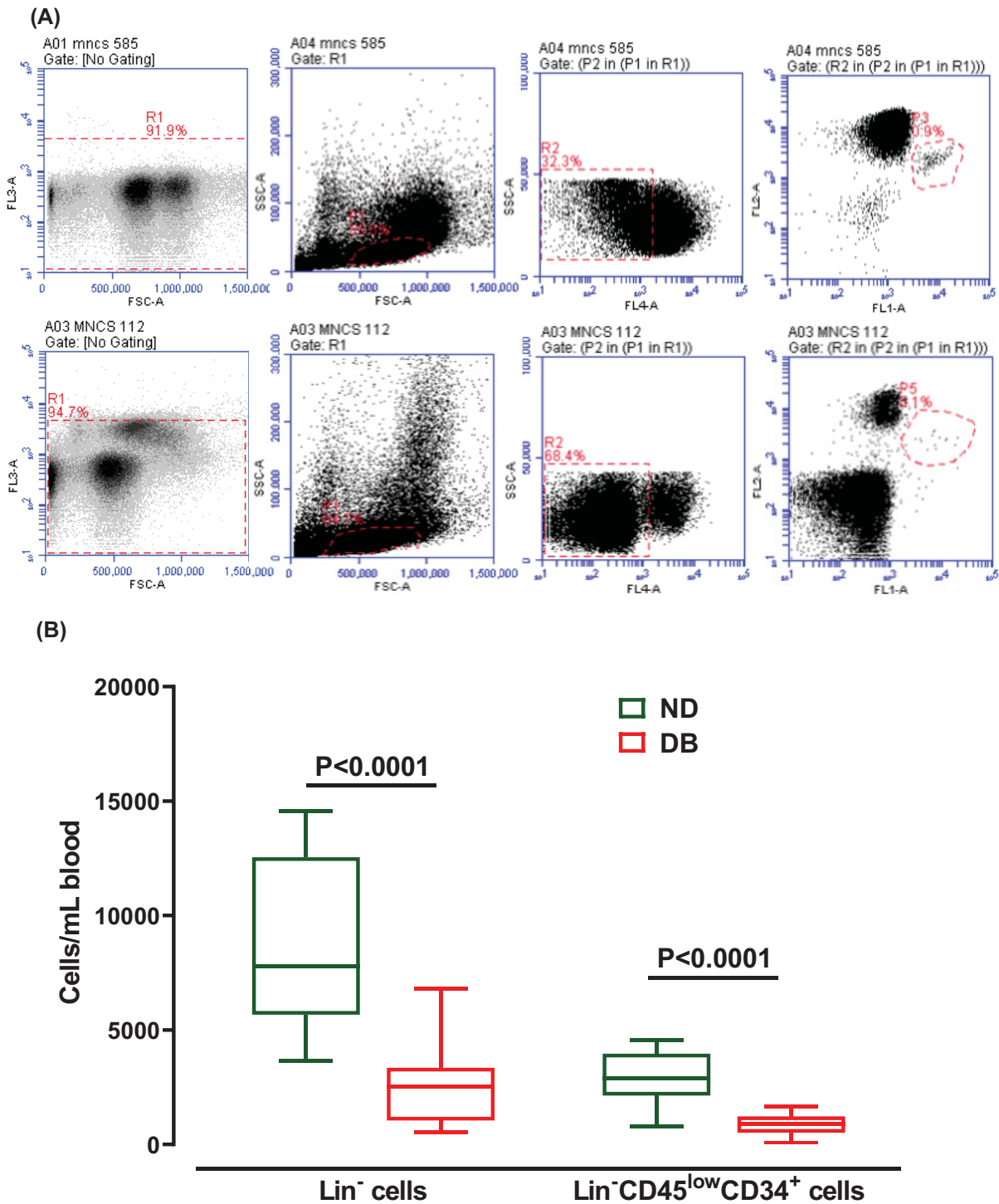


Figure 1. Circulating CD34⁺ cells are decreased in diabetic individuals compared with nondiabetic subjects

(A) Representative dot plots of flow cytometric enumeration of Lin⁻ and Lin⁻CD45^{low}CD34⁺ cell populations. Top panel – nondiabetic and bottom panel – diabetic. Shown were dot plots in order from left to right depicting the selection of live cells, selection of monocyte-lymphocyte population, selection of Lin⁻ population and selection of Lin⁻CD45^{low}CD34⁺ population. (B) Summary of circulating Lin⁻ and Lin⁻CD45^{low}CD34⁺ cell populations in nondiabetic (ND) or diabetic (DB) individuals. Both populations are decreased in DB ($P < 0.0001$, $n = 33$) compared with ND individuals ($n = 23$).

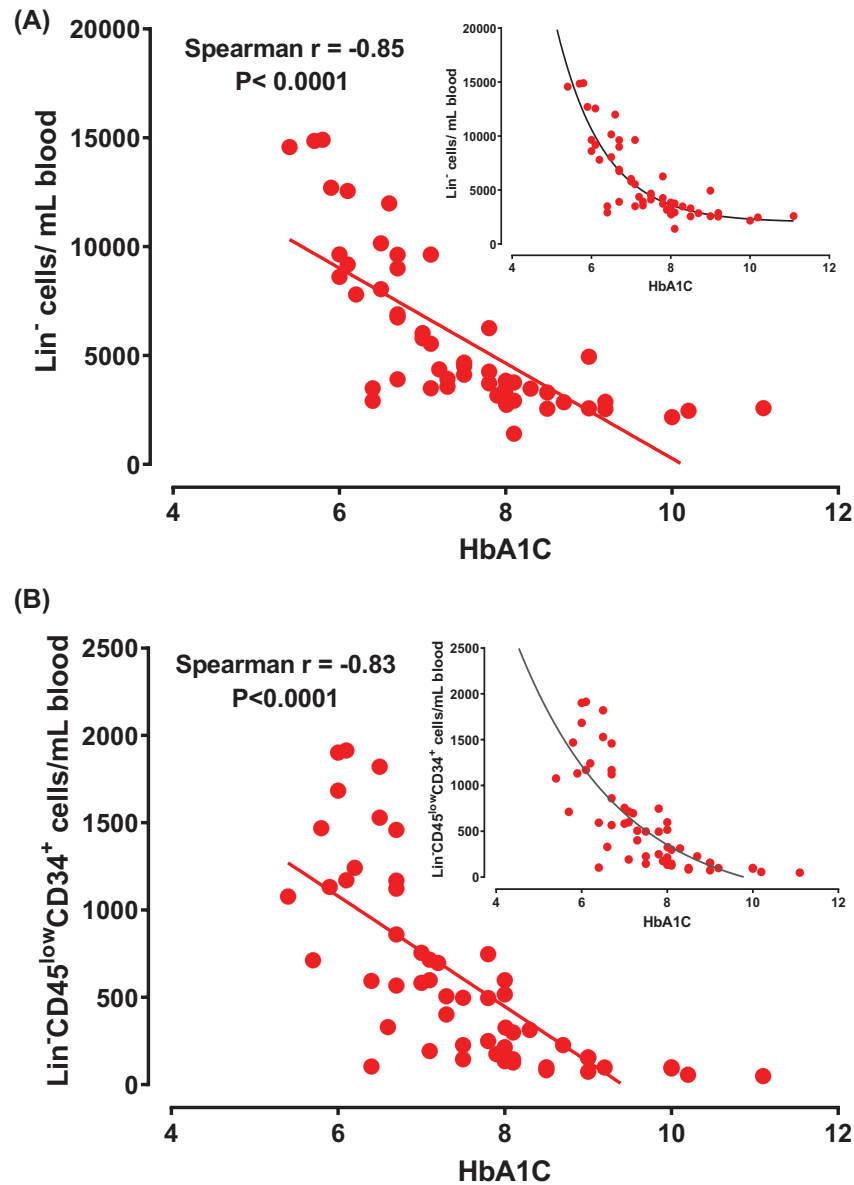


Figure 2. Circulating CD34⁺ cells are inversely proportional to HbA1C in diabetic individuals

Both Lin⁻ (A) and Lin⁻CD45^{low}CD34⁺ (B) cell populations are inversely proportional to HbA1C in the range of 5.5 up to 11.2 with a Spearman correlation 'r' -0.85 and -0.83, respectively. Shown in inset were nonlinear regression analysis of the respective data with goodness of fit 0.74 and 0.67, respectively.

In another set of analysis, we have correlated the circulating number of cells with HbA1c ranging from 5.5 to highest in the cohort. Both Lin⁻ or Lin⁻CD45^{low}CD34⁺ cell populations in the circulation were inversely proportional to the HbA1c (Figure 2A,B). Spearman correlation analysis detected significant negative correlation (Lin⁻ cells and Lin⁻CD45^{low}CD34⁺ cells (Spearman $r = -0.85$ and -0.83 , respectively, $P < 0.0001$, $n = 56$). Nonlinear regression analysis revealed that cell number vs HbA1c data follows nonlinear relationship with goodness of fit 0.74 and 0.67, respectively, for Lin⁻ or Lin⁻CD45^{low}CD34⁺ cell populations (inset in Figure 2A,B). These results are in agreement with previous reports that showed an inverse correlation between the number of BM-derived vascular reparative cells and presence of adverse cardiovascular risk score, cardiovascular dysfunction, cardiovascular morbidity and mortality, and diabetic complications [11,12].

Altered ACE and ACE2 enzyme activities in diabetic CD34⁺ HSPCs

Then, the ACE and ACE2 enzyme activities were determined in cell lysates derived from CD34⁺ HSPCs from non-diabetic or diabetic individuals. ACE activity is higher in diabetic CD34⁺ HSPCs ($n=20$) compared with that in cells derived from nondiabetic individuals ($n=23$) (Figure 3). In contrast, ACE2 activity is lower in CD34⁺ cells derived from diabetic individuals compared with nondiabetic cells (Figure 2). These simultaneous changes in ACE2 and ACE activities resulted in ACE2/ACE activity ratios that were inversely proportional to HbA1C (Spearman $r = -0.92$, $P < 0.0001$) (Figure 3).

CD34⁺ HSPCs derived from diabetic individuals have been shown to be dysfunctional in *in vitro* and *in vivo* experimental studies evaluating vascular repair-relevant functions [32,33]. Therefore, we sought to demonstrate this in a mouse model of HLI and then to test if increasing ACE2 expression in diabetic cells would reverse the vasoreparative dysfunction in this model. These studies were carried out in cells derived from individuals who have at least one or more cardiovascular complications due to diabetes (Table 1), which indicates impaired reparative potential of CD34⁺ cells. In the cohort of diabetic individuals that were studied, older adults aging 55 years and higher have HbA1C of 7.5 or higher and lower ACE2/ACE had one or more of the cardiovascular complications as listed in the Table 1 (Figure 3). Therefore following studies involving evaluation of *in vivo* vasoreparative functions were carried out in CD34⁺ cells derived from older diabetic individuals and age- and sex-matched nondiabetic individuals. Duration of diabetes ranged from 20 to 32 years.

LV ACE2 gene transfer in CD34⁺ HSPCs

Efficiency of LV transduction varied from 44 to 64% (Figure 4) in CD34⁺ HSPCs as detected by eGFP expression by flow cytometry (Figure 4). LV-ACE2-transduced cells have increased ACE2 enzyme activity in cell lysates (three-fold) and supernatants (five-fold) compared with that in the cells transduced with LV-control (Figure 4). Western blotting showed increased protein expression in ACE2-modified cells compared with LV-Control cells. Importantly, ACE2 protein was detected in higher concentrations in the supernatants of cells treated with LV-ACE2 compared with LV-Control (Figure 4). LV-transduction process did not affect the cell viability as tested by proliferation assay in either nondiabetic or diabetic cells for 72 h post-transduction (Figure 4). In consistence with our previous studies, proliferative potential is attenuated in diabetic cells compared with nondiabetic cells at either 48 or 72 h of proliferation ($P < 0.01$, $n=6$). Transduced cells suspended in 100 μ l PBS were injected into the mice 24 h following transduction protocol via intramuscular route in athymic mice undergoing HLI.

Effect of administration of CD34⁺ HSPCs in nondiabetic Foxn1^{nu} mice undergoing HLI

First, we have determined the reparative potential of HSPCs derived from nondiabetic and diabetic older individuals in control nondiabetic athymic mice by measuring blood flow recovery and by monitoring for toe and limb salvage. Following HLI, blood flow was completely restored by day 28 (91 ± 5 vs $11 \pm 2\%$ on day 1 of HLI, $n=5$) in control mice without any treatments (Figure 5). This trend was not altered following i.m administration of cells (3×10^5) that were derived from nondiabetic individuals ($98 \pm 6\%$, $n=6$). In contrast, administration of similar number of cells derived from older diabetic individuals, either with or without LV-Control transduction, resulted in significant reduction in the blood flow recovery ($65 \pm 7\%$ at day 28, $P < 0.01$ vs untreated or treated with nondiabetic cells, $n=6$). This is in agreement with previous studies showing that diabetic CD34⁺ cells were shown to impair vascular repair due to paracrine dysfunctions that were shown to be pro-inflammatory and antiangiogenic [32,34]. This dysfunction is reversed by overexpression of ACE2. Treatment with diabetic cells that were transduced with LV-ACE2 resulted in complete restoration of blood flow following HLI ($104 \pm 6\%$ at day 28, $P < 0.01$ vs LV-Control-cells, $n=8$) (Figure 5).

Effect of administration of CD34⁺ HSPCs in diabetic Foxn1^{nu} mice undergoing HLI

Then, a similar study was carried out in athymic mice with STZ-induced type 1 diabetes. Blood flow recovery is not restored in diabetic mice following HLI with majority of mice (4/6) showing gangrene and tissue necrosis resulting in amputations of toes or foot between 5 and 7 days post-HLI. Administration of HSPCs derived from nondiabetic individuals (either at 3×10^5 or 1×10^6 cells/mouse) with or without modification with LV-Control did not enhance blood flow recovery or the occurrence of amputations (3/6). Cells transduced with LV-ACE2 have normalized the blood flow recovery and prevented amputations (84+4%, $n=8$). Administration of CD34⁺ cells (either at 3×10^5 or 1×10^6 cells/mouse) derived from diabetic older adults with or without LV-control transduction, did not restore blood flow to ischemic regions and resulted in 100% amputations ($n=5$). Diabetic CD34⁺ cells that were transduced

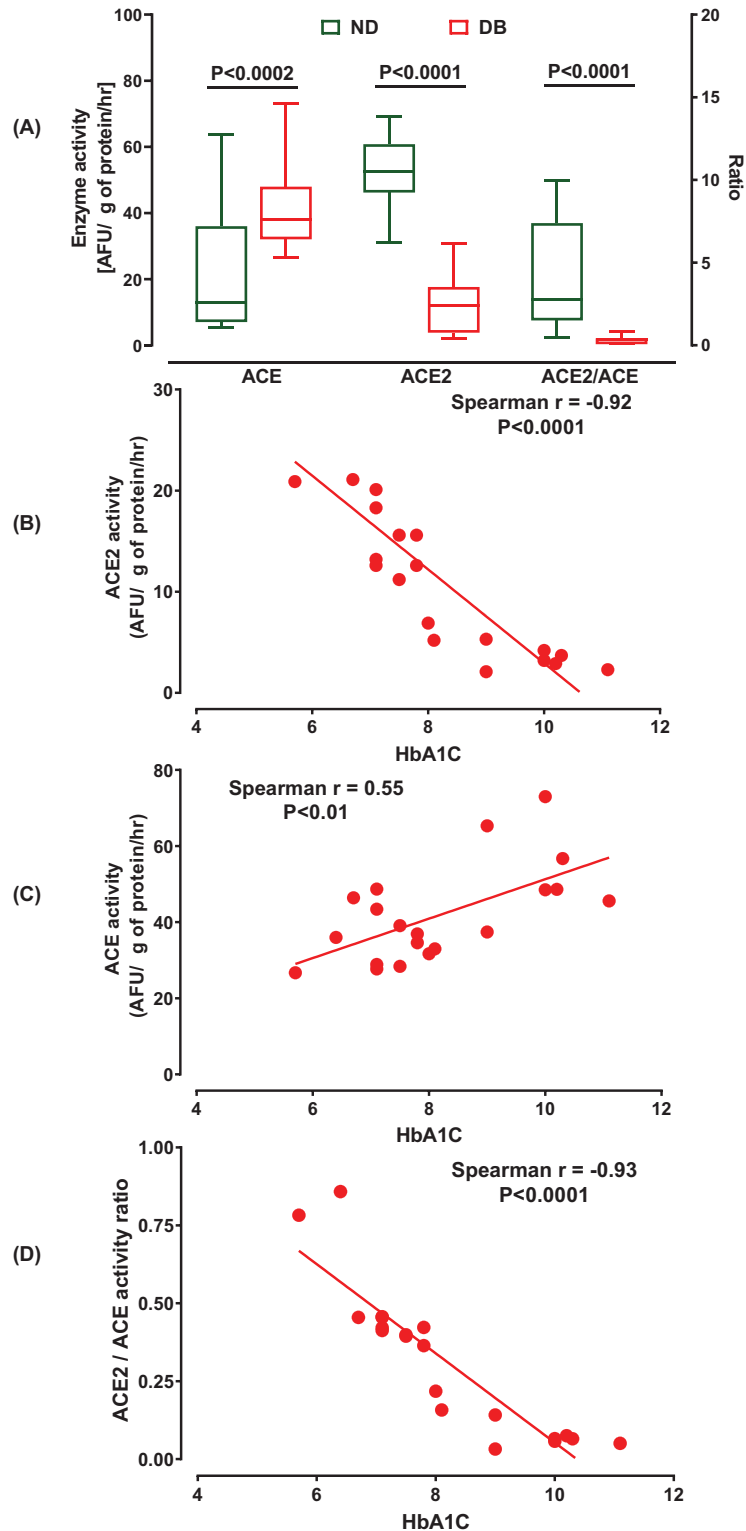


Figure 3. ACE and ACE2 enzyme activities are differentially altered in CD34⁺ cells by diabetes

(A) ACE activity is increased in diabetic (DB) CD34⁺ cells ($n=20$) compared with nondiabetic (ND) cells ($n=23$). ACE2 activity is decreased in DB cells compared with ND ($P < 0.0001$). These changes resulted in decreased ACE2/ACE ratio in DB cells. (B) ACE2 activity is inversely correlated with HbA1C in DB cells (spearman correlation 'r' -0.92). (C) ACE activity is directly correlated with HbA1C in DB cells (Spearman correlation 'r' -0.55) (D) ACE2/ACE ratio is inversely proportional to the HbA1C in DB cells with a Spearman correlation 'r' -0.93 .

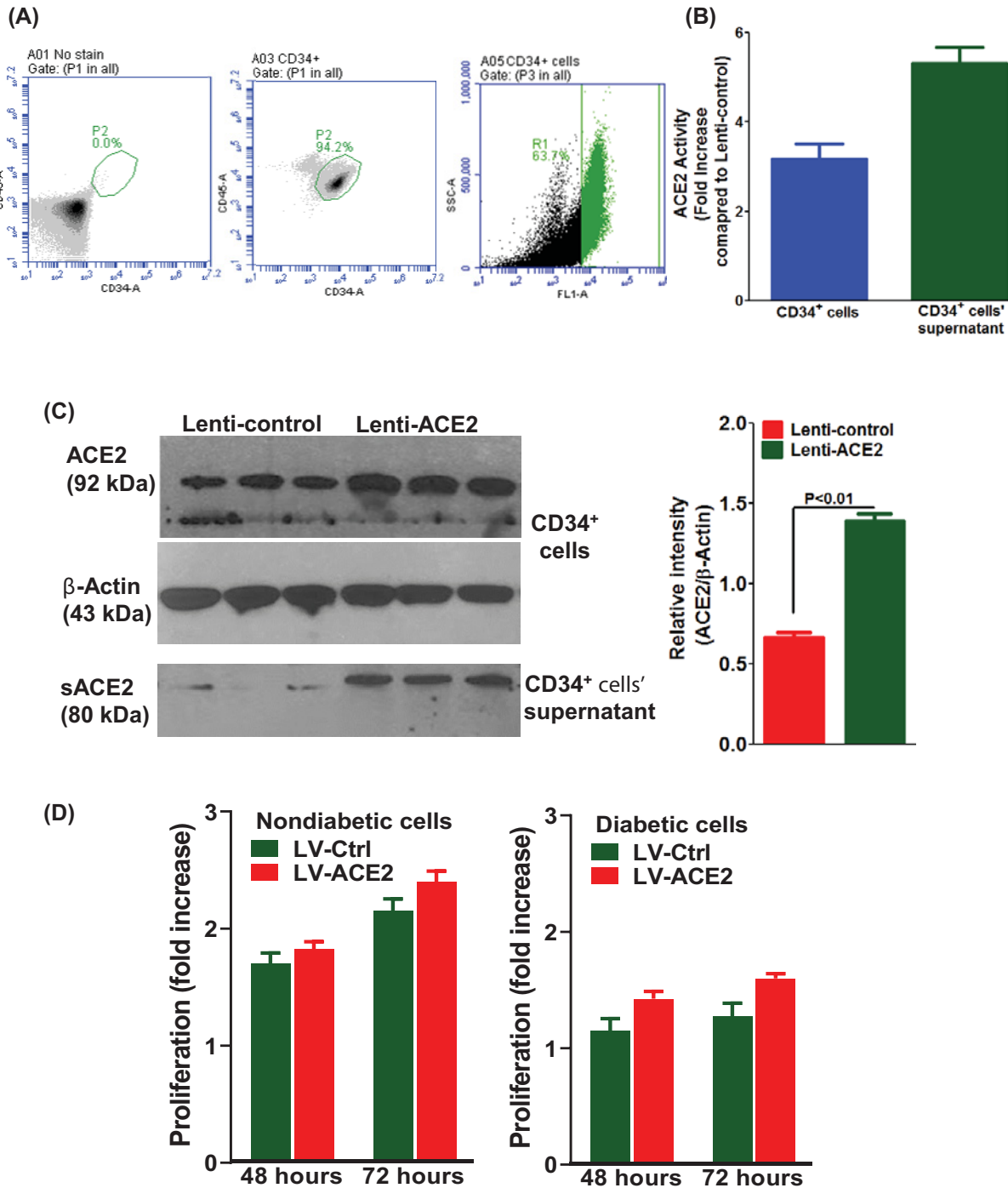


Figure 4. LV ACE2 gene transfer in CD34⁺ cells

(A) Representative flow cytometric analysis of eGFP⁺ cells following LV eGFP gene transfer in CD34⁺ cells ($n=5$). (B) ACE2 activity in cell lysates or cell supernatants in CD34⁺ cells following LV-ACE2 transfer relative to that observed in cells treated with LV-Control ($n=5$). Shown were fold-increase. (C) ACE2 protein in cell lysates and cell supernatants in cells with LV-Control or LV-ACE2 treatments. Representative Western blots and bar graph showing relative protein expression in cell lysates ($n=6$). Protein expression is higher in LV-ACE2 cells compared with LV-Control cells ($P<0.01$). Soluble ACE2 fragments were observed in the supernatants of LV-ACE2 cells but not in LV-Control cells. (D) Proliferation of cells, either nondiabetic or diabetic, is not affected by LV-transduction procedure with LV-Ctrl or LV-ACE2 treatments. Proliferation in diabetic cells is decreased compared with nondiabetic cells in both LV-Ctrl and LV-ACE2 groups ($P<0.01$ vs nondiabetic cells with respective LV-treatment, $n=6$).

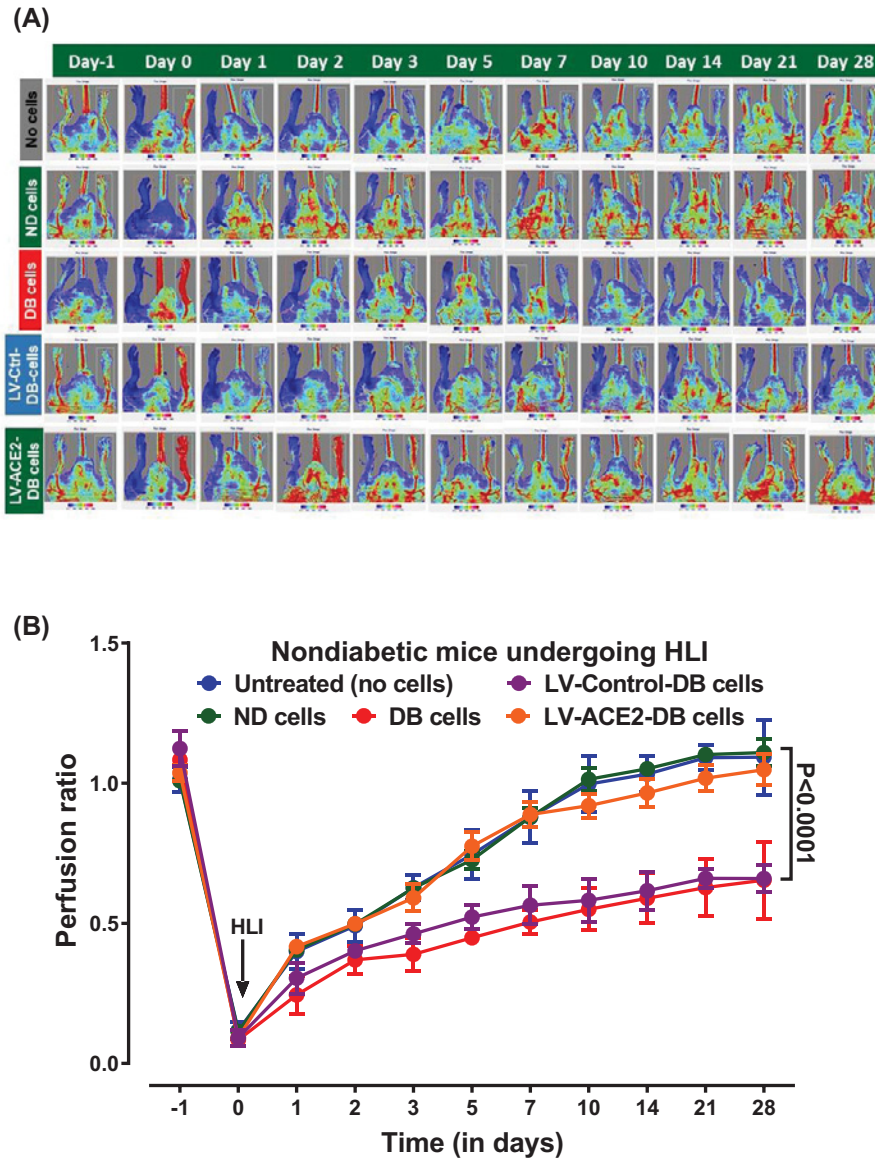


Figure 5. Blood flow recovery in nondiabetic mice undergoing HLI administered with CD34⁺ cells derived from nondiabetic or diabetic individuals

(A) Representative pseudocolor images of perfusion in hindlimbs of mice undergoing HLI and received different treatments. **(B)** Administration of ND cells have no effect on the blood flow restoration (green) compared with the untreated mice (blue). DB cells either with or without LV-Control treatment have decreased blood flow restoration compared with the untreated mice ($P < 0.0001$, two-way ANOVA). Multiple comparisons detected significant differences in the blood flow from Day 3 up to Day 28 ($P < 0.01$ to 0.0001). Blood flow recovery in mice administered with DB cells following ACE2 gene transfer (LV-ACE2-DB, orange) is significantly higher than that observed with LV-Ctrl-DB cells ($P < 0.0001$, two-way ANOVA). Multiple comparisons detected significant differences in the blood flow from Day 3 up to Day 28 ($P < 0.04$ to 0.0002).

with LV-ACE2 restored the blood flow recovery to the ischemic limbs (62 + 4%) pre-ischemic levels) and prevented amputations (Figure 6). Administration of A-779 for 4 weeks prior to HLI and administration of cells reversed the beneficial effects of ACE2-modified nondiabetic or diabetic cells ($n=3$, data not shown).

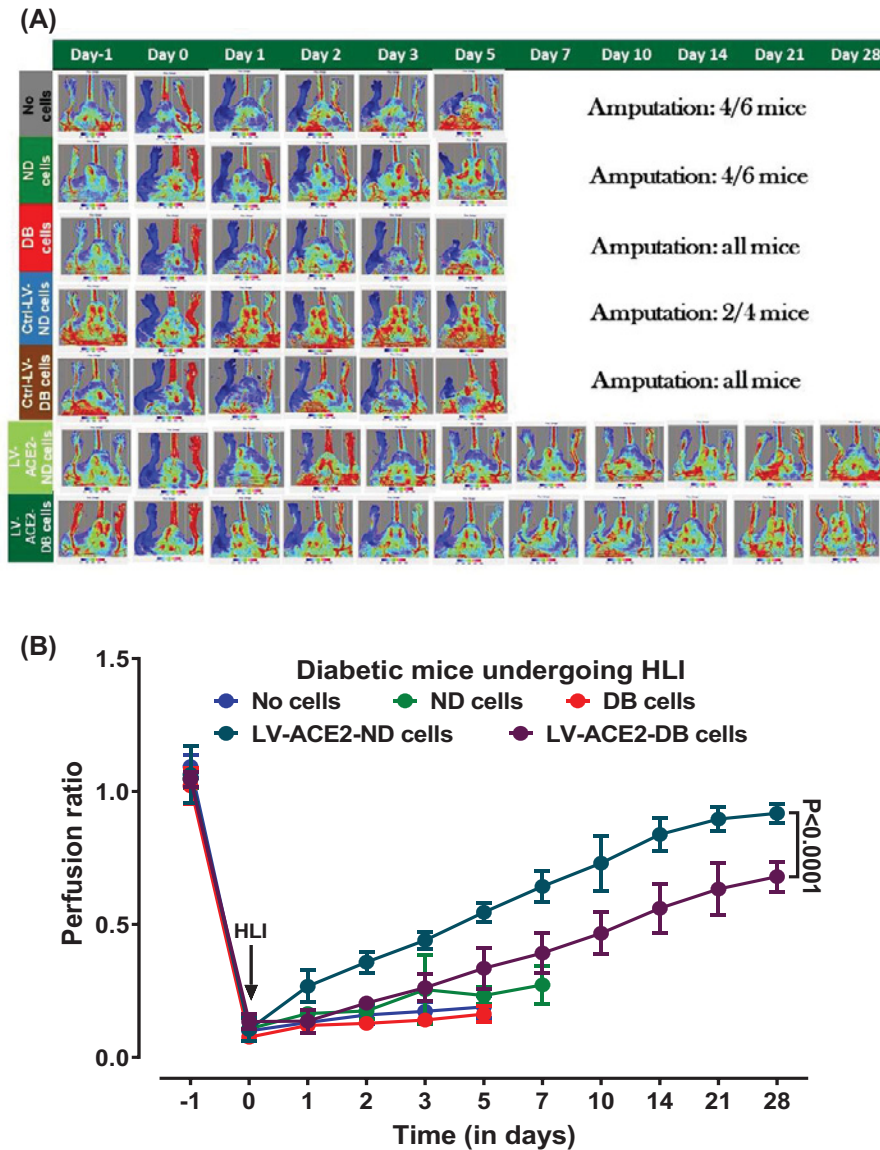


Figure 6. Blood flow recovery in diabetic mice undergoing HLI administered with CD34⁺ cells derived from nondiabetic or diabetic individuals

(A) Representative pseudocolor images of perfusion in hindlimbs of mice undergoing HLI and received different treatments. **(B)** Blood flow was not completely recovered, and toes or foot amputations are observed in diabetic mice that were either not treated or treated with ND or DB cells with or without LV-Control (LV-Ctrl) treatment. Administration of either ND or DB cells that were modified with LV-ACE2 resulted in the recovery of blood flow in diabetic mice by 84% ($n=8$) and 62% ($n=8$), respectively, blood flow recovery accomplished by LV-ACE2-DB cells is significantly lower than LV-ACE2-ND cells ($P < 0.0001$, Two-way ANOVA). Multiple comparisons detected significant differences in the blood flow from Day 2 up to Day 28 ($P < 0.01$ to 0.0001). Excluded from **(B)** are data from mice treated with ND or DB cells modified with LV-Control.

Ang-(1-7) modifies paracrine profile in CD34⁺ HSPCs

Our hypothesis predicts that ACE2-expressing cells metabolize Ang II and generate Ang-(1-7), which via acting on MasR in HSPCs or in the peri-ischemic endothelium will produce vasoprotective functions. Findings from *in vivo* studies as explained above imply that local increase in Ang-(1-7) is likely to increase angiocrine functions of diabetic CD34⁺ cells. Therefore, we tested if Ang-(1-7) alters paracrine profile in diabetic HSPCs in limited number of individuals. CM was obtained from cells that were untreated or Ang-(1-7) (100 nM)-treated for 15 h. Cells from the

Table 2 Changes in paracrine factors in the CM of diabetic CD34⁺ HSPCs relative to that in the media derived from nondiabetic cells

Paracrine factors	ND (μg/ml)	ND+Ang-(1-7) (μg/ml)	DB (μg/ml)	DB+Ang-(1-7) (μg/ml)
SCF	14420 ± 715	17321 ± 915	5417 ± 109*	7811 ± 356
Monocyte chemoattractant protein-1 (MCP1)	1220 ± 230	716 ± 167	1780 ± 410	1327 ± 288
G-CSF	12 ± 4	26 ± 8	42 ± 11 [†]	64 ± 11
GM-CSF	11 ± 3	39 ± 16	34 ± 8 [†]	25 ± 7
Tumor necrosis factor (TNF)-α	97 ± 14	121 ± 24	69 ± 12	125 ± 32
Decorin	328 ± 66	314 ± 76	207 ± 48 [†]	315 ± 61
IL-1β	24 ± 7	22 ± 64	67 ± 14 [†]	98 ± 29
Angiopoietin-1	897 ± 135	1273 ± 259	610 ± 107	1108 ± 217
IL-3	198 ± 28	247 ± 66	249 ± 35	418 ± 97
IL-8	1707 ± 351	2230 ± 581	4781 ± 785	7687 ± 914
IL-10	29 ± 6	51 ± 11	41 ± 11	62 ± 14

This profile was not altered by treatment with Angiotensin-(1-7). Abbreviations: DB, diabetic; ND, nondiabetic.
[†]*P*<0.05 and **P*<0.001 (compared with the ND group).

same individual, either nondiabetic or diabetic were used as untreated or treated group, which makes the observations paired. A total of sixteen factors were analyzed in cells derived from nondiabetic (*n*=5) and diabetic (*n*=5) individuals. Factors that were not affected by Ang-(1-7) treatment are shown in Table 2 and those that were altered by Ang-(1-7) are shown in Figure 7. Among factors that were not altered by Ang-(1-7), stem cell factor (SCF) was decreased and granulocyte-colony stimulating factor (G-CSF), granulocyte megakaryocyte-colony stimulating factor (GM-CSF) and interleukin (IL)-1β (IL-1β) were increased in CM from diabetic cells compared with that obtained from nondiabetic cells (Table 2). Hepatocyte growth factor (HGF) levels are decreased in the CM derived from diabetic cells compared with non-diabetic cells, this dysfunction was reversed by Ang-(1-7) treatment (Figure 7). Thrombopoietin (TPO) showed decreasing trend in diabetic CM compared with the nondiabetic, and it was increased significantly by Ang-(1-7) in both diabetic and nondiabetic CM (Figure 7). Transforming growth factor-β1 (TGF-β1) secretion is higher in diabetic CM compared with the nondiabetic that were significantly decreased by Ang-(1-7) but no effect was observed in nondiabetic CM. Along similar lines, IL6 levels were higher in diabetic CM compared with nondiabetic, that were decreased by Ang-(1-7), but no effect was observed in the nondiabetic. Further analysis by determining the change (Δ) between treated and untreated CM in ND or DB revealed inter-individual variation yet significant differences were detected by one sample *t* test against '0' (HGF *P*<0.05 both ND and DB; TPO *P*<0.01 both ND and DB; TGF-β1 *P*<0.05 DB; IL6 *P*<0.001 DB).

Discussion

This study for the first time reports ACE2/ACE imbalance in diabetic CD34⁺ HSPCs with decreased ACE2 and increased ACE. The number of CD34⁺ cells and ACE2/ACE ratio are inversely correlated with HbA1C. Physiological recovery of blood flow to areas of ischemia in nondiabetic mice is not altered by administration of cells derived from nondiabetic individuals however it was decreased by cells derived from diabetic older individuals. Cells from either nondiabetic or diabetic older individuals could not restore blood flow ischemic areas in diabetic mice. LV-ACE2 expression reversed this dysfunction and blood flow to ischemic area was restored by either nondiabetic or diabetic cells following ex vivo ACE2-modification. Beneficial effects of ACE2 gene transfer are at least in part due to the reversal of paracrine dysfunction in diabetic cells.

Widespread pathological involvement of the BM and the shortage of circulating stem/progenitor cells may be a more general feature associated with accelerated aging and excess mortality seen in diabetes [35]. Nondiabetic aging also increases risk for vascular diseases and may be associated with vasculogenic dysfunctions [19,20,36]. Aging with diabetes accelerate the vasoreparative dysfunctions and increases risk by many fold higher for ischemic vascular complications. Low levels of HSPC phenotypes CD34⁺ and CD34⁺CD133⁺ are independent determinants of all-cause mortality in different population of subjects, including older adults with diabetes or metabolic syndrome [37,38]. The cohort of individuals in the current study have nondiabetic subjects that are free from any cardiovascular complications or medications. On the other hand diabetic individuals of age 55 or higher have at least one or more of

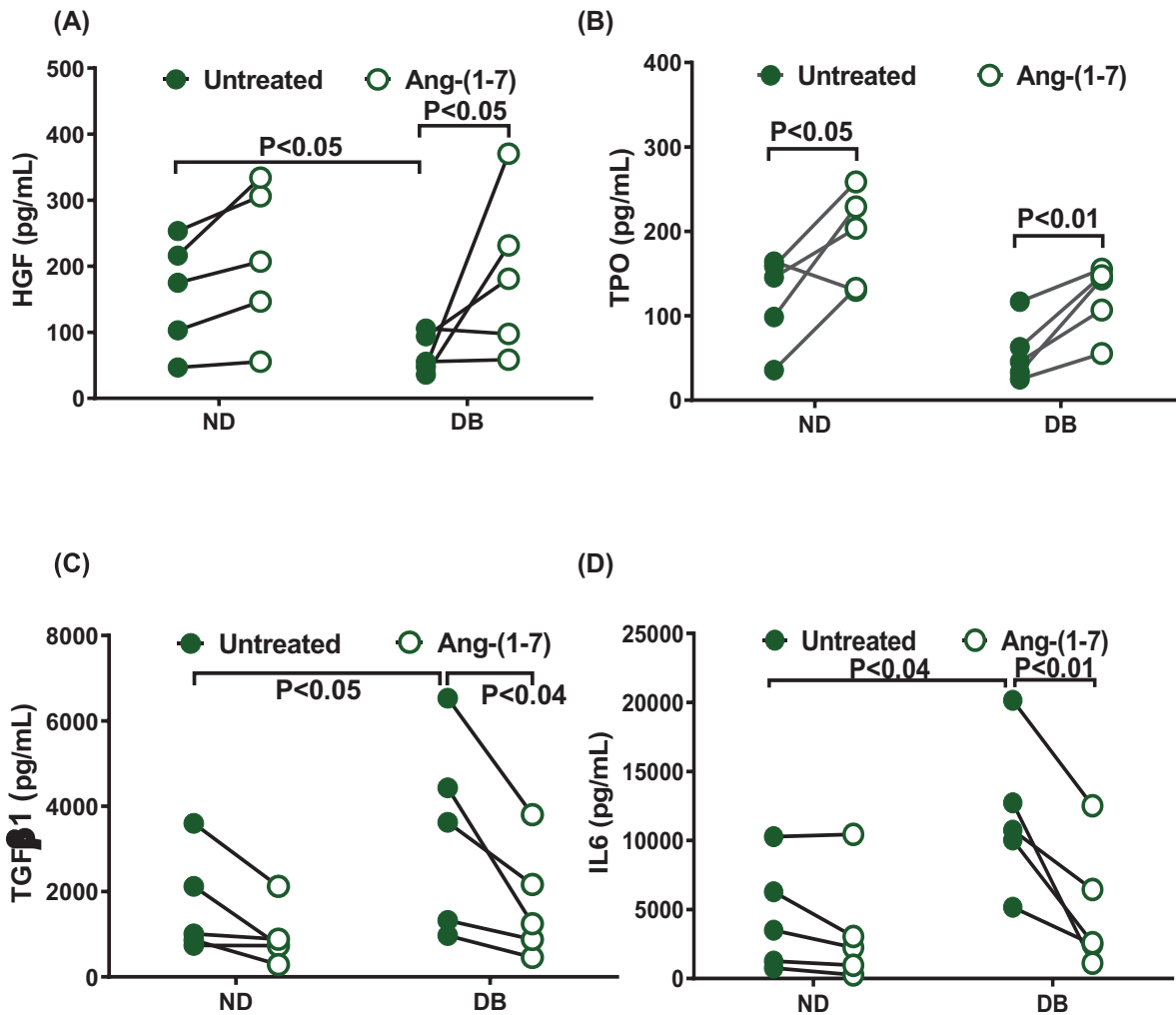


Figure 7. Angiotensin-(1-7) modifies paracrine profile in CD34⁺ cells

In vitro treatment with Ang-(1-7) altered six different factors by CD34⁺ cells. Shown were concentrations of paracrine factors, (A) HGF, (B) TPO, (C) TGF-β1, and (D) IL6, produced by either ND or DB cells with or without Ang-(1-7) (100 nM) in individual samples.

complications that are listed in Table 1 and younger diabetics have no ischemic vascular diseases except hypertension in a few.

Relevance of BM-derived adult stem/progenitor cells in human physiology and clinical therapies stems from studies in BM-transplanted individuals. Evidence was shown for the repopulation of donor cells in nonhematopoietic tissues, including myocardium, vasculature, lungs, liver, and kidneys [39–41]. However, it was the discovery of vasculogenic progenitor cells that provided strong impetus for cell-based therapies for ischemic vascular diseases [7,42]. CD34⁺ cell therapy in patients with refractory angina significantly improved exercise capacity, reduced adverse cardiac events, healthcare costs and mortality [43,44].

Clinical trials in diabetic setting are very few as chronic diabetic environment not only decreases the number of cells that can be collected for therapy but also negatively impacts regenerative outcomes [16]. A small randomized controlled trial on diabetic patients with critical limb ischemia (CLI), i.m administration of CD34⁺ cells that were mobilized by G-CSF, has improved perfusion indices and wound healing [45]. Clinical benefits of CD34⁺ cell therapy were reported to be preserved in individuals with insulin resistance but not in those with diabetes [46]. Therefore *ex vivo* modification of cells to enhance their potential to sustain the diabetic environment is needed to enhance regenerative and revascularization outcomes of cell therapies for diabetic vascular diseases.

Earlier studies have demonstrated *ex vivo* modification to enhance eNOS expression by molecular or pharmacological approaches would enhance reparative outcomes of cell therapies in animal models of cardiopulmonary diseases [47,48]. However, in diabetic condition, which is known to increase oxidative stress and increased generation of reactive oxygen species (ROS), increasing eNOS expression would likely aggravate oxidative stress by increasing the generation of peroxynitrite [33,49]. ACE2 and Ang-(1-7) pharmacology has been well documented in cardiopulmonary tissues, and largely involves both increasing NO bioavailability and decreasing oxidative stress. Studies in diabetic BM and vasculogenic progenitor cells in mouse models and human disease have demonstrated similar protective mechanisms [22,50,51]. Therefore increasing ACE2 expression or Ang-(1-7) generation would be a promising approach for enhancing regenerative outcomes of cell-based therapies.

Current study provided evidence for ACE2/ACE imbalance in diabetic CD34⁺ cells. Decreased ACE2 expression and ACE2/ACE ratio negatively correlated with HbA1C a commonly used clinical indicator of the severity of diabetes. Our previous studies have consistently showed impaired proliferation and migration to hypoxia-regulated factors that are *in vitro* functional signatures of vascular repair [22,33]. Diabetic individuals in the small cohort of patients in the current study have increased number of vascular complications with increased HbA1C or decreased ACE2/ACE ratio, thus supporting the hypothesis that increasing ACE2 expression would reverse vasoreparative dysfunction in diabetic cells. In the current study, *in vitro* experiments confirmed secretion of soluble ACE2 from CD34⁺ cells modified with LV-ACE2, which generates Ang-(1-7) in the circulation and promote vasoprotective and angiogenic functions in an autocrine and paracrine fashion. Increased local concentrations of Ang-(1-7) would reverse diabetic impairments in the migration and proliferation of cells and promote the recruitment of cells to the ischemic areas, and the angiocrine functions on peri-ischemic endothelium. Concurrently, increased ACE2 expression would decrease circulating levels of Ang II resulting in attenuated detrimental effects of via AT1R activation. Our results also infer that decreasing ACE expression or ACE-inhibitors will have beneficial effects on vasoreparative outcomes however this hypothesis is not tested in the current study. ACE2/ACE imbalance has been demonstrated in other organ systems in both diabetic and nondiabetic setting. Seminal discoveries in diabetic renal glomeruli provided compelling evidence for ACE2/ACE imbalance [52–54] and many other studies have demonstrated this phenomenon as an underlying pathological mechanism in cardiac, pulmonary and gastrointestinal complications [55–58]

Our *in vivo* studies with CD34⁺ cells from nondiabetic or diabetic older adults administered to nondiabetic or diabetic mice showed interesting findings. Nondiabetic human cells have no effect on the blood flow recovery to ischemic areas in nondiabetic mice as the innate vasculogenic functions are intact including mobilization of cells from BM into the bloodstream, migration to areas of ischemia and angiocrine functions. However, unexpectedly diabetic cells negatively impacted the normal vasoreparative process and reduced the blood flow recovery to the ischemic areas. A previous study in db/db model of diabetes by Stepanovic et al [32] reported similar observations in wound-revascularization that were attributed to the altered paracrine profile with predominant pro-inflammatory and antiangiogenic factors (see below). Diabetes induces dysregulated hematopoiesis with myelopoietic bias resulting in increased monocyte-derived macrophage generation, which creates pro-inflammatory microenvironment that likely opposes revascularization [70–72]. Additionally, recent studies implicated calprotectin (S100A8/A9) in mediating defective angiocrine signaling in CD34⁺ cells from sedentary individuals [73,74]. Diabetic cells with ACE2 gene transfer indeed restored blood flow recovery similar to that observed in untreated mice. In contrast, in diabetic mice CD34⁺ cells from neither nondiabetic nor diabetic older adults restored blood flow recovery following ischemic injury suggesting that apart from the angiocrine dysfunction in administered cells, diabetic environment of the recipient tissue has profound negative impact on the vasoreparative potential of cells from even a nondiabetic individual. These findings at least in part explain the lack of adequate therapeutic outcomes with cell-based therapies in diabetic individuals. ACE2 gene transfer restored blood flow recovery in both groups of cells and in both nondiabetic and diabetic mice. It is important to note that diabetic mice are severely deficient of vasculogenic functions that includes impaired mobilization from BM, reduced numbers in the circulation and impaired migration to areas of ischemia and angiocrine dysfunction [16–18,59] therefore the observed effects on blood flow recovery in diabetic mice are exclusively derived from the administered human cells. Beneficial effects of ACE2-gene transfer can be explained by the protective effects of Ang-(1-7) on the diabetic tissue or vascular environment. Previous studies have shown Ang-(1-7) via MasR activation normalizes diabetic inflammatory and oxidative environment resulting in increased NO availability and expression of angiogenic factors, and decreased pathologic response to receptor for advanced glycation-end products (RAGE) activation, which provides a strong mechanistic basis [23,60]. Based on a limited number of experiments using A779, we have confirmed that the observed beneficial effects of ACE2-gene transfer *in vivo* are mediated by MasR. This is in agreement with previous reports that showed compelling evidence for the role of MasR on the beneficial effects of Ang-(1-7) on BM-derived vasculogenic cells by using molecular or pharmacological approaches and MasR-knockout mice [24,61].

We have determined the effect of Ang-(1-7) on paracrine profile CD34⁺ HSPCs, by analyzing selected cytokines and growth factors that are known to have autocrine and angiocrine functions. Krenning et al [9] showed direct evidence for angiocrine functions of CD34⁺ cells and identified IL-8, monocyte chemoattractant protein-1 (MCP1), HGF, bFGF and VEGFa as candidate angiocrine mediators. SCF, HGF and TPO have multiple functions on vasculogenic cells such as pro-angiogenic and pro-proliferative, and regulate differentiation and migration [8,62,63]. Increased pro-inflammatory cytokines (IL-1 β , IL6, TGF- β 1) and decreased angiogenic factors (HGF, Angiopoietin-1, and TPO) were observed in the diabetic CM compared with nondiabetic while bFGF and VEGFa were not detected. Ang-(1-7) differentially modulated the secretion of factors with inter-individual variation. Key findings are increased HGF and TPO and decreased TGF- β 1 and IL6 by Ang-(1-7). Decorin, which is known to negatively modulate TGF- β 1 availability and intracellular signaling [64,65], is decreased in diabetic cells, which in turn promotes detrimental effects of TGF- β 1 in CD34⁺ cells and endothelium. Ang-(1-7) decreased TGF- β 1 expression but had no effect on decorin secretion. Decreasing TGF- β 1 alone was shown to restore NO generation, migration and proliferation in diabetic cells likely via decreasing thrombospondin-1 expression [66]. G-CSF and GM-CSF are pro-proliferative in HSPCs however increased secretion as observed in diabetic CM may exert pro-inflammatory effects on the environment [34,67]. Levels of these two factors were not affected by Ang-(1-7). MCP1 and tumor necrosis factor α (TNF α), which were shown to mediate angiocrine functions of murine Lin- cells [68], were unchanged in diabetic CM. Paracrine factors are not limited to cytokines and growth factors as evidence has been shown for a role of exosomal miRNAs in angiocrine communication [69]. This novel concept is yet to be studied in the setting of diabetes and for a possible modulation by ACEs and angiotensins. Collectively, this set of findings suggest that Ang-(1-7) stimulates angiocrine functions in diabetic CD34⁺ cells with decreased release of pro-inflammatory and increased angiogenic factors.

Limitations of the study

This study has a few limitations some of which may require further investigation while others cannot be overcome. 1. Number of cells that can be obtained from an individual diabetic individual is limited therefore cells that were transduced with LV-control and LV-ACE2 are not paired. Cells obtained from a patient were just sufficient for a single administration. 2. Majority of cells were derived from type 2 diabetic individuals and the mouse model used for determining the *in vivo* functions was a widely used model for type 1 diabetes. A few studies have reported a protocol for development of type 2 diabetes or metabolic syndrome in athymic nude mice, which yet requires better understanding of the disease profile in this model as they lack important populations of immune cells. 3. Individual variation in the paracrine profile of CD34⁺ cells regardless of the presence of diabetes, which requires further investigation involving larger cohort. 4. Cells from either male or female individuals were used in the study but male mice were exclusively used for *in vivo* experimentation.

Clinical perspectives

- Vascular regeneration potential of CD34⁺ HSPCs is attenuated in diabetes. This study tested the hypothesis that the vasoprotective enzyme of renin angiotensin system, ACE2 in diabetic CD34⁺ HSPCs is down-regulated and that reparative dysfunction is reversed by ACE2 gene transfer.
- Older diabetic individuals have reduced number of CD34⁺ HSPCs and decreased ACE2 activity compared with nondiabetic individuals. Intramuscular administration of cells derived from older diabetics attenuated blood flow recovery in nondiabetic mice undergoing hindlimb ischemic injury (HLI) but not the cells derived from nondiabetic individuals. Neither the control nor the older diabetic cells could restore blood flow in diabetic mice. LV ACE2 gene transfer reversed the reparative dysfunctions and enhanced restoration of blood flow to ischemic areas. *In vitro* Ang-(1-7) treatment modified paracrine profile of control or diabetic CD34⁺HSPCs.
- Success of autologous cell-based therapies for the treatment of ischemic vascular diseases in diabetic older adults is still questionable due to reparative dysfunction in HSPCs. *Ex vivo* molecular modification to increase ACE2 expression would enhance revascularization outcomes of cell-based therapies.

Data Availability

Data sharing is not applicable to the present paper as all supporting data related to study are included in the manuscript.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

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CRediT Author Contribution

Y.P. Jarajapu: Conceptualization, Funding acquisition, Project administration, Resources, Supervision, Writing—original draft, Writing—review and editing. **S. Joshi:** Investigation, Methodology, Validation. **I. Montes de Oca:** Methodology, Project administration. **A. Maghrabi:** Methodology, Project administration. **C. Lopez-Yang:** Methodology, Project administration. **J. Quiroz-Olvera:** Project administration. **C.A. Garcia:** Project administration, Resources.

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Abbreviations

ACE, angiotensin-converting enzyme; ACE2, angiotensin-converting enzyme 2; Ang II, angiotensin II; Ang-(1-7), angiotensin-(1-7); AT1R, angiotensin II type 1 receptor; bFGF, basic fibroblast growth factor; BM, bone marrow; CM, conditioned medium; eGFP, enhanced green fluorescent protein; eNOS, endothelial nitric oxide synthase; GM-CSF, granulocyte monocyte-colony stimulating factor; G-CSF, granulocyte-colony stimulating factor; HbA1C, hemoglobin A1C; HGF, hepatocyte growth factor; HLI, hindlimb ischemia; HRP, horseradish peroxidase; HSPC, hematopoietic stem/progenitor cell; IL, interleukin; i.m., intramuscular; Lin⁻, lineage-negative; LV, lentiviral; MasR, Mas receptor; MCP1, monocyte chemoattractant protein-1; MNC, mononuclear cell; MOI, multiplicity of infection; SCF, stem cell factor; SDF, stromal derived factor-1 α ; STZ, streptozotocin; TGF- β 1, transforming growth factor- β 1; TPO, thrombopoietin; VEGF, vascular endothelial growth factor; 7-AAD, 7-aminoactinomycin D.

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