

Transient Inhibition of Transforming Growth Factor- β 1 in Human Diabetic CD34⁺ Cells Enhances Vascular Reparative Functions

Ashay D. Bhatwadekar,¹ E.P. Guerin,^{1,4} Yagna P.R. Jarajapu,¹ Sergio Caballero,¹ Carl Sheridan,⁵ David Kent,⁴ Laurence Kennedy,² M. Cecilia Lansang,² Frank W. Ruscetti,⁶ Carl J. Pepine,³ Paul J. Higgins,⁷ Stephen H. Bartelmez,⁸ and Maria B. Grant¹

OBJECTIVE—Peripheral blood CD34⁺ cells from diabetic patients demonstrate reduced vascular reparative function due to decreased proliferation and diminished migratory prowess, largely resulting from decreased nitric oxide (NO) bioavailability. The level of TGF- β , a key factor that modulates stem cell quiescence, is increased in the serum of type 2 diabetic patients. We asked whether transient TGF- β 1 inhibition in CD34⁺ cells would improve their reparative ability.

RESEARCH DESIGN AND METHODS—To inhibit TGF- β 1 protein expression, CD34⁺ cells were treated ex vivo with antisense phosphorodiamidate morpholino oligomers (TGF- β 1-PMOs) and analyzed for cell surface CXCR4 expression, cell survival in the absence of added growth factors, SDF-1-induced migration, NO release, and in vivo retinal vascular reparative ability.

RESULTS—TGF- β 1-PMO treatment of diabetic CD34⁺ cells resulted in increased expression of CXCR4, enhanced survival in the absence of growth factors, and increased migration and NO release as compared with cells treated with control PMO. Using a retinal ischemia reperfusion injury model in mice, we observed that recruitment of diabetic CD34⁺ cells to injured acellular retinal capillaries was greater after TGF- β 1-PMO treatment compared with control PMO-treated cells.

CONCLUSIONS—Transient inhibition of TGF- β 1 may represent a promising therapeutic strategy for restoring the reparative capacity of dysfunctional diabetic CD34⁺ cells. *Diabetes* 59:2010–2019, 2010

From ¹Pharmacology and Therapeutics, University of Florida, Gainesville, Florida; the ²Division of Endocrinology, Diabetes, and Metabolism, University of Florida, Gainesville, Florida; the ³Division of Cardiology, University of Florida, Gainesville, Florida; ⁴The Vision Clinic, Circular Road, Kilkenny, Ireland; the ⁵School of Clinical Sciences University of Liverpool, Liverpool, U.K.; the ⁶Laboratory of Experimental Immunology, Center for Cancer Research, National Cancer Institute–Frederick, Frederick, Maryland; the ⁷Center for Cell Biology & Cancer Research Albany Medical College, Albany, New York; and ⁸BetaStem Therapeutics Inc., San Francisco, California.

Corresponding authors: Maria B. Grant, grantma@ufl.edu, and Stephen Bartelmez, bartelmezsh@yahoo.com.

Received 28 February 2010 and accepted 27 April 2010. Published ahead of print at <http://diabetes.diabetesjournals.org> on 11 May 2010. DOI: 10.2337/db10-0287.

A.D.B. and E.P.G. contributed equally to this study.

© 2010 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered. See <http://creativecommons.org/licenses/by-nc-nd/3.0/> for details.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Bone marrow derived progenitor cells (BMPCs) support vascular repair postnatally by direct integration into blood vessels and by the release of paracrine factors such as vascular endothelial cell growth factor, matrix metalloproteases, and angiopoietins to the neovessels (1,2). BMPCs possess dramatic ability to revascularize areas within 6–12 h after the injury (3), accounting for total 1–12% of the endothelial cells present in blood vessels (4). Lineage negative (lin⁻) cells from mice that express the cell surface antigens Sca-1 (Ly-6A/E) and c-kit can develop into endothelium, as can human lin⁻ cells expressing surface CD34 (1,5). Treatment with CD34⁺ cells presents an important therapeutic option for revascularization of ischemic vascular areas (6) and has been successful in numerous clinical trials (7,8).

However, diabetes significantly impairs the vasoreparative ability of CD34⁺ cells. Diabetic patients with peripheral vascular disease have decreased levels of CD34⁺ cells and suffer poor vessel growth in response to ischemia (9); this defect is linked to reduced precursor cell function (10). The widespread vasodegeneration seen in diabetic retinopathy may be attributed to the inability of BMPCs to compensate for the increased endothelial injury associated with diabetes. In particular, the diabetic BMPCs are unable to repair retinal vasculature (11); thus, the total rate of retinal cell loss greatly exceeds the reparative function of these cells. We showed that diabetic CD34⁺ cells fail to revascularize areas of retinal vascular injury (11) likely due to reduced migration. Diabetic peripheral neuropathy further hampers repair due to defects of circadian release of BMPCs from the bone marrow, creating an imbalance between the demand and supply of BMPCs during the vasodegenerative stage of diabetic retinopathy (12). Pharmacological manipulation of diabetic CD34⁺ cells (13) can serve as an important therapeutic strategy for their use as autologous cell therapy to facilitate vascular repair.

Transforming growth factor- β 1 (TGF- β 1) is a pleiotropic factor that regulates the balance between proliferation, differentiation, and quiescence of hematopoietic stem cells (HSCs), both as an extracellular and intracellular ligand (14,15). TGF- β 1 is elevated in the serum of diabetic patients and possibly intracellularly in CD34⁺ cells (16). Enhanced levels of endogenous TGF- β 1 have been reported in peripheral blood mononuclear cells of patients with diabetic nephropathy (17), and its increase provides a novel mechanism of cellular injury related to elevated glucose levels (18). Increased levels of TGF- β 1 induce

cellular senescence and growth arrest (19). Using blocking antibodies, we showed that transiently inhibiting TGF- β 1 in murine HSCs promoted survival of these cells in the absence of growth factors (20).

In this study, we investigated the effect of transient inhibition of endogenous TGF- β 1 in peripheral blood diabetic CD34⁺ cells using ex vivo treatment with phosphorodiamidate morpholino oligomers (PMOs). PMOs act by steric inhibition of protein synthesis by high affinity binding to 14–15 contiguous bases. PMOs are highly stable both intra- and extracellularly but are degraded after binding with a half-life of ~2–4 days in cells (21). We report here that transient inhibition of TGF- β 1 using TGF- β 1-PMO may represent a promising therapeutic strategy for restoring vascular reparative function in dysfunctional diabetic CD34⁺ cells.

RESEARCH DESIGN AND METHODS

All animal studies were approved by the institutional animal care and use committee, and studies were conducted in accordance with The Guiding Principles in the Care and Use of Animals (National Institute of Health) as well as the Association of Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research.

Isolation of murine HSCs. HSCs were harvested from the bone marrow obtained from femurs and tibiae of C57BL/6-Tg (UBC-GFP) 30Scha/J mice homozygous for green fluorescent protein (GFP) antibodies. Fluorescently labeled c-kit (CD117) and Sca-1 (BD PharMingen, San Diego, CA) were used to enrich HSCs from mononuclear cell fractions using a BD cell sorter (FACS Calibur, BD Bioscience, San Jose, CA). We have previously shown that this technique yields a 95% pure hemangioblast cell/HSC population (5).

Isolation of long-term repopulating HSCs. The detailed protocol for the isolation of long-term repopulating HSCs (LTR-HSCs) was previously described (22). Briefly, a dose response of LTR-HSCs (30–1,000 cells) pretreated for 1 h with anti-TGF- β 1 neutralizing antibody (clone 1D11.16) or IgG1K isotype control antibody, both at 20 μ g/ml (R & D Systems, Minneapolis, MN), were transplanted intravenously into lethally irradiated mice, and survival was examined over time.

Acute vascular injury: ischemia reperfusion injury model. Acute vascular injury was induced in mice using the ischemia reperfusion model, as detailed in an online appendix at <http://diabetes.diabetesjournals.org/cgi/content/full/db10-0287/DC1>. Briefly, the anterior chamber of the eye was then subjected to 2 h of increased hydrostatic pressure, resulting in retinal ischemia. This model results in the generation of acellular capillaries similar to what is observed in longstanding diabetic retinopathy but occurs after 7 days of injury (23). At this time, the animals received injections of isolated human CD34⁺ cells pretreated with either TGF- β 1-PMO or control PMO into their vitreous.

TGF- β 1 inhibition using PMO. TGF- β 1-PMOs or control PMOs (AVI-Biopharma, Portland, OR) were synthesized, lyophilized, dissolved in sterile distilled water, and serially diluted. CD34⁺ cells were incubated for 16 h at 37°C in Iscove's modified Dulbecco's medium containing 10% FBS, 10% selected horse serum, and 40 μ g/ml PMO. The detailed protocol for PMO treatment is described in an online appendix.

Endogenous TGF- β 1 expression using flow cytometry. Lin⁻Sca-1⁺ cells (described above) were treated with anti-TGF- β 1 antibodies or TGF- β 1-PMO. Endogenous expression of TGF- β 1 was then studied after fixation and permeabilization using phycoerythrin-labeled TGF- β 1 antibodies (IQ Products, Groningen, Holland) and analyzed by flow cytometry (FACS Vantage DiVa 1 flow cytometer, San Jose, CA). Anti-actin antibodies (BD PharMingen) served as control.

Patient selection. Peripheral blood samples (50 ml) were collected from type 2 diabetic patients and healthy age-matched volunteers. The study was approved by the University of Florida Institutional Review Board. All participants gave written informed consent to participate. Criteria for inclusion and exclusion of participants are provided in an online appendix, and detailed patient characteristic are described in supplementary Tables 1 and 2 (also available in an online appendix).

Isolation of human CD34⁺ cells. Mononuclear cells were isolated from peripheral blood using Lymphoprep and then subjected to magnetic selection of lin⁺ cells yielding lin⁻ cells (Stem Cell Technologies, Vancouver, BC, Canada), which were then incubated with anti-CD34-phycoerythrin and anti-CD45-phycoerythrin-Cy7 (BD PharMingen) and other select antibodies. Lin⁻CD34⁺CD45^{mid} cells were clearly resolved from lymphocytes and other myeloid cells using a FACS Vantage DiVa flow cytometer. CD34⁺ cells

enriched by Miltenyi positive selection from human cord blood and bone marrow obtained from AllCells (Emeryville, CA) and were >95% pure as assessed by fluorescence-activated cell sorter (FACS) analysis. Viability was determined by 7AAD exclusion and was routinely >95% after washings (detailed protocol in an online appendix).

CD34⁺ survival in the absence of added growth factors. Lin⁻CD34⁺CD45^{mid} cells from cord blood or peripheral blood were directly sorted into 96-well plates (10 \pm 3 cells per well) containing plain medium, control PMO, or TGF- β 1-PMO. Cell viability and proliferation were assessed daily using direct light microscopy (\times 200), both before growth factors and after their addition. Recombinant hematopoietic growth factor cocktail was added on day 5 to each well that contained human TPO (20 ng/ml), stem cell factor (50 ng/ml), IL-3 (50 ng/ml), and IL-6 (20 ng/ml) (PeproTech, NJ). Cells were scored that survived after the initial 5-day period in the absence of growth factors.

Migration assay. Cell migration after overnight inhibition with TGF- β 1-PMOs or control PMOs was analyzed using a modified Boyden chamber assay as described previously (24).

CXCR4 expression on CD34⁺ cells. At $T = 0$ and after treatment with TGF- β 1-PMO for 16 h, healthy or diabetic CD34⁺ cells were incubated with CD34, CD45, and CXCR4 antibodies (BD Biosciences) to analyze CXCR4 expression using FACS (BD Bioscience).

Determination of NO production by 4-amino-5-methylamino-2-difluorofluorescein fluorescence imaging. NO production was quantified in peripheral blood CD34⁺ cells using NO-sensitive 4-amino-5-methylamino-2-difluorofluorescein (DAF-FM) fluorescence, as described previously (25).

Statistical analyses. Data are presented as mean values \pm SEM. Statistical analyses were performed using GraphPad InStat 3.0 (GraphPad Software, San Diego, CA). Statistical differences in the mean were assessed using one-way ANOVA followed by Tukey Carmer post hoc test or unpaired t test.

RESULTS

TGF- β 1 inhibition in vitro promotes engraftment of murine LTR-HSCs. To study the effect of TGF- β 1 inhibition on survival and engraftment of LTR-HSCs, mice were lethally irradiated with 950 rads and anti-TGF- β 1 antibody-treated LTR-HSCs were transplanted. As shown in Fig. 1A, isotype antibody-treated LTR-HSC did not mediate survival of lethally irradiated mice over a range of 30–500 cells per mouse, and even at 1,000 LTR-HSC per mouse, the six-month survival was only 10%. In striking contrast, anti-TGF- β 1 treatment for 1 h before transplant resulted in survival of ~70% of mice at 60 cells per mouse. However, if 250 LTR-HSC were treated with anti-TGF- β 1 antibody before transplant, essentially 100% of mice survived for >6 months post transplant.

Neutralizing antibody targets only cell surface TGF- β 1. We also wanted to evaluate the pharmacological effect of both neutralization of cell surface TGF- β 1 and intracellular TGF- β 1 and used PMOs. FACS analysis on murine lin⁻Sca-1⁺c-kit⁺ cells treated with either TGF- β 1-PMO or TGF- β 1 neutralizing antibody resulted in specific inhibition of endogenous TGF- β 1 (Fig. 1B). A functional assay that we used is survival of enriched progenitor cells in the absence of growth factors. Both murine LTR-HSC and human CD34⁺ cells exhibit a rapid death curve in the absence of stem cell growth factors. We observed that inhibition of endogenous TGF- β 1 in LTR-HSC resulted in prolonged survival of the cells even in single cell per well cultures (data not shown). Human CD34⁺ cells also survive in the absence of growth factors if TGF- β 1-PMO was present (supplementary Fig. 1, available in an online appendix). Unexpectedly, human CD34⁺ cells did not survive in the absence of growth factors when TGF- β 1 neutralizing antibody was present (in contrast to murine LTR-HSC) (supplementary Fig. 1) but did so when cultured with TGF- β 1-PMO. Human CD34⁺ cells not only proliferated in response to the added growth factors after 5 days but generated large numbers of progeny during the subsequent 7-day growth period with growth factors

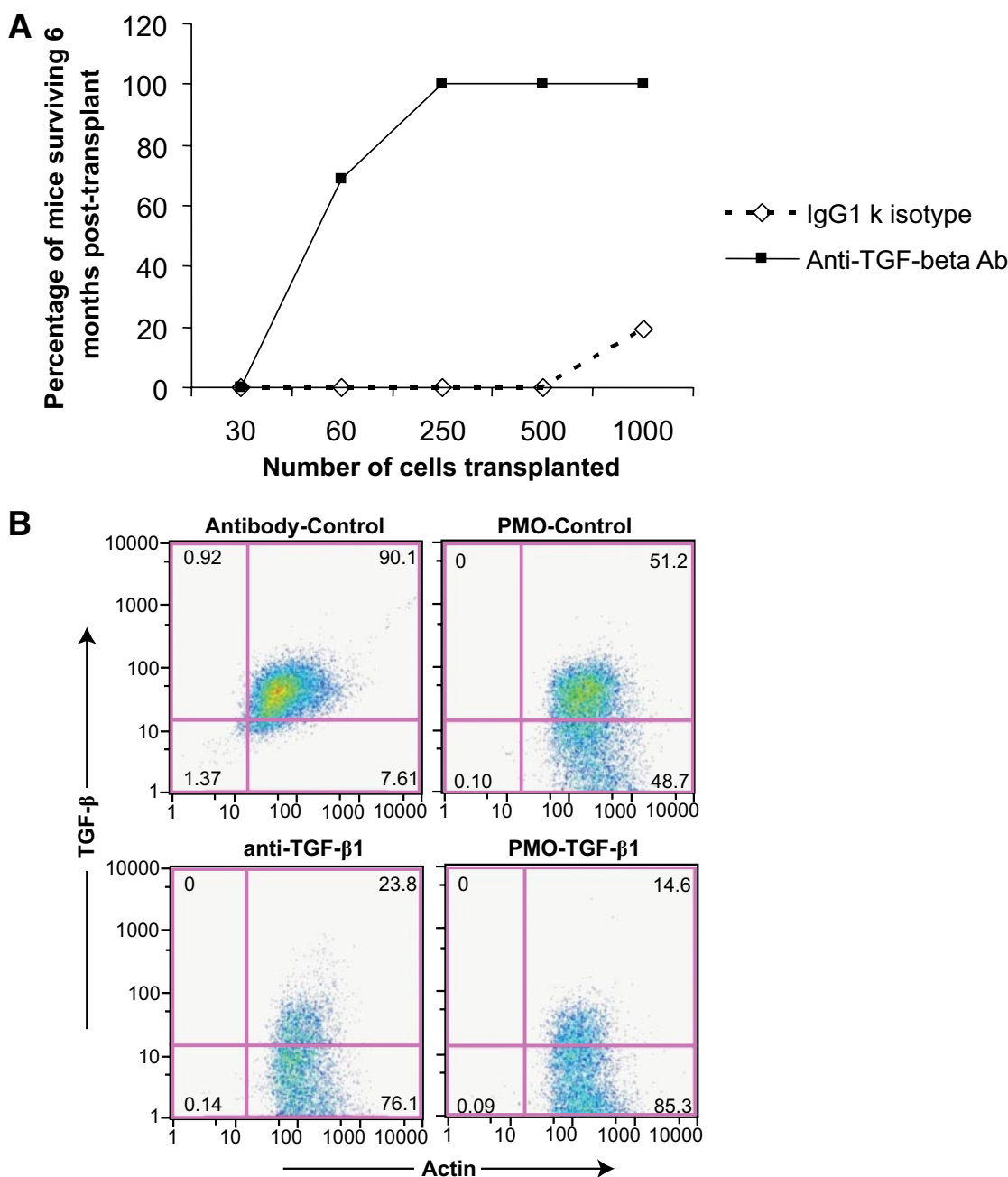


FIG. 1. Inhibition of endogenous TGF- β 1 in murine LTR-HSC accelerates engraftment and enhances repopulating efficiency. **A:** When low numbers of highly enriched bone marrow LTR-HSCs (see Methods) were treated with anti-TGF- β 1 neutralizing antibody just before transplant into lethally irradiated mice (950 rads) without the support of helper bone marrow, a large proportion of mice survived irradiation death. The proportion of mice that survived for six months after transplantation is shown. The datum is expressed as mean survival of 10–20 mice/group. **B:** Both anti-TGF- β 1 PMO and TGF- β 1 neutralizing cell surface antibodies inhibit endogenous expression of TGF- β 1 in primitive hematopoietic cells ($\text{lin}^- \text{Sca-1}^+$ -cells). Enriched bone marrow cells were treated with anti-TGF- β 1 PMOs or antibodies overnight, cells were fixed and permeabilized, and phycoerythrin-labeled TGF- β 1 antibodies were used to detect endogenous TGF- β 1. Anti-actin antibodies served as the permeabilization control. Flow cytometry dot plots were divided into four quadrants showing TGF- β 1 expression on the y-axis and actin expression on the x-axis. Values in the upper right quadrant of each individual plot indicate the percent of TGF- β 1 expressing cells. The bottom two plots show that use of either TGF- β 1 blocking antibody or TGF- β 1-PMO reduced the percentage of cells expressing TGF- β 1. For the TGF- β 1 blocking antibody studies, the percentage of cells expressing TGF- β 1 fell from 90.1 to 23.8%, while for the TGF- β 1-PMO studies, the percentage fell from 51.2 to 14.6% ($n = 10$ –20 mice/group). (A high-quality digital representation of this figure is available in the online issue.)

present. In contrast, control antibody, control PMO, and TGF- β 1 neutralizing antibody did not promote survival of CD34⁺ cells in the absence of growth factors, nor did they respond to added growth factors at day 5. At this point, we chose to exclusively use TGF- β 1-PMO and not TGF- β 1 neutralizing antibody for subsequent studies with human cells.

The PMOs used in these studies were 18–21 oligonucle-

otides in length, which showed excellent cellular uptake without the aid of any transfection reagent or peptide conjugation. To further establish kinetics PMO uptake by CD34⁺ cells, fluorescein isothiocyanate (FITC)-conjugated PMO were developed (FITC-PMO kindly provided by Pat Iversen, AVI-Biopharma). FITC-PMOs were rapidly taken up by peripheral blood CD34⁺ cells in an exponential manner; at 40 $\mu\text{g/ml}$ FITC-PMO, a plateau was observed

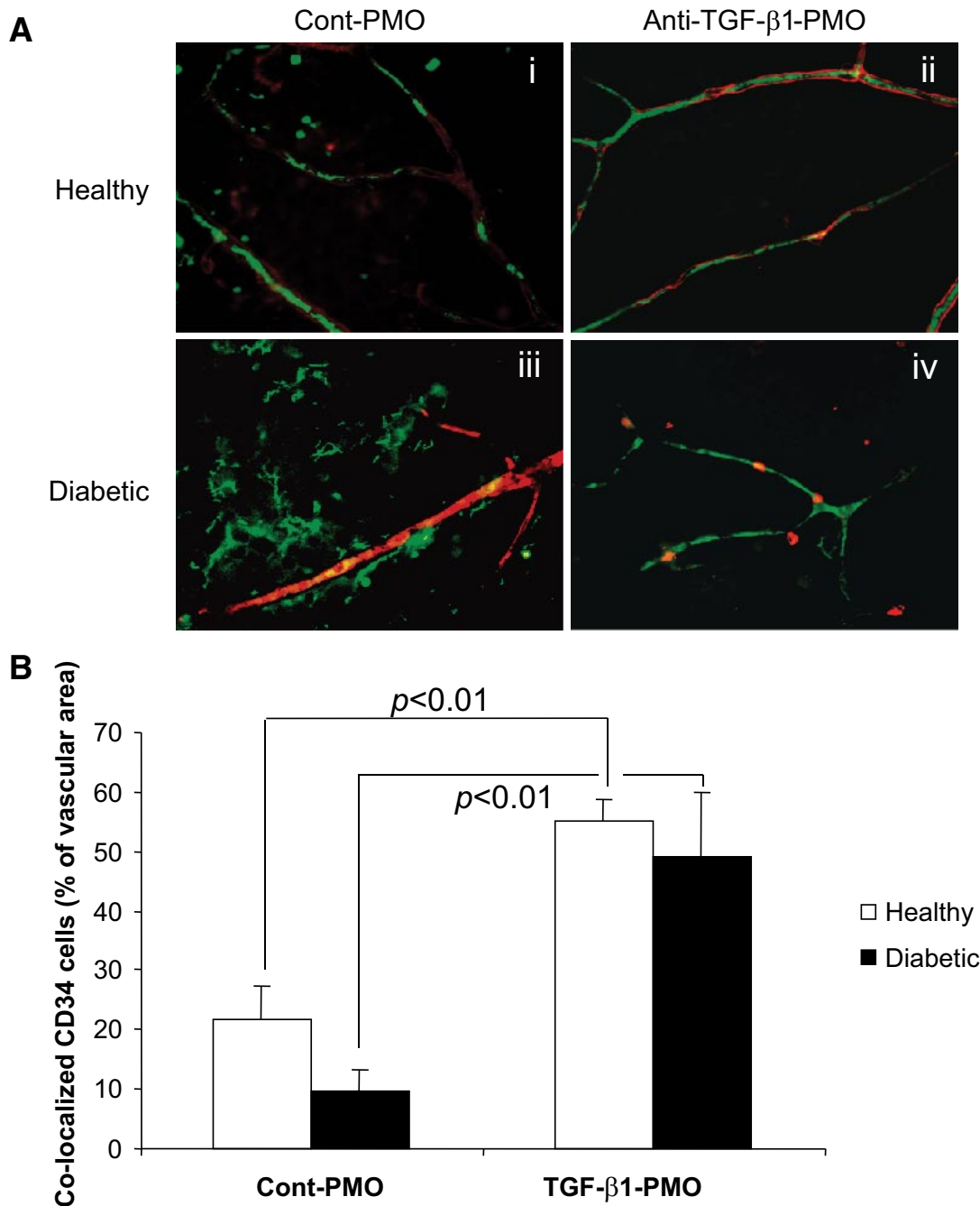


FIG. 2. TGF- β 1-PMO-treated HSCs integrate into degenerating capillaries in the ischemia reperfusion-damaged retina. **A:** Representative immunofluorescence images showing incorporation of intravitreally injected CD34⁺ cells (labeled green) into the retinal ischemic vasculature (red). Note that TGF- β 1-PMO-treated CD34⁺ cells show a substantial increase in incorporation into the vasculature. **B:** Morphometric quantification of blood vessel-colocalized CD34⁺ cells expressed as the percentage of the vascular area ($n = 5$ mice each group). Cont = control. (A high-quality digital representation of this figure is available in the online issue.)

after overnight ex vivo incubation, while at a concentration of 160 μ g/ml, saturation occurred within 1–3 h (supplementary Fig. 2, available in an online appendix).

TGF- β 1 inhibition in murine HSCs increases homing to areas of ocular injury. We pretreated murine HSCs ($lin^{-}c\text{-kit}^{+}Sca1^{+}$) with TGF- β 1 PMO or control PMO and tested cell homing to areas of laser injury. As shown in supplementary Fig. 3 (available in an online appendix), pretreatment with TGF- β 1-PMO substantially increased the number of GFP⁺ HSCs that were recruited to the sites of laser rupture of Bruch's membrane compared with control PMO-treated cells ($P < 0.05$).

Inhibition of endogenous TGF- β 1 in CD34⁺ cells increases vascular repair in the retinal ischemia reperfusion injury model. We next examined the reparative function of diabetic CD34⁺ cells after pretreatment with TGF- β 1-PMO or control PMO in the ischemia reperfusion model, which recapitulates key aspects of the vasodegenerate phase (acellular capillaries) of diabetic retinopathy. Analysis of retinal flat mounts showed that pretreatment of both diabetic and healthy CD34⁺ cells with TGF- β 1-PMO increased homing to areas of retinal injury to a greater degree than control PMO (Fig. 2A). Diabetic CD34⁺ cells showed a 2.2-fold lower colocaliza-

tion to injured retinal areas than healthy CD34⁺ cells (Fig. 2B). Pretreatment of healthy CD34⁺ cells with TGF- β 1-PMO resulted in a 2.51-fold ($P < 0.01$) increase in the numbers of CD34⁺ cells homing to injured vessels. On the other hand, treatment of diabetic CD34⁺ cells with TGF- β 1-PMO produced a robust reparative response ($P < 0.01$), as seen by a 5.4-fold ($P < 0.01$) increase in numbers of CD34⁺ cells colocalized with vessels supporting the restored ability of these cells to home to acellular capillaries (Fig. 2B).

Inhibition of TGF- β 1 using PMO increases survival of diabetic CD34⁺ cells. Our animal studies showed that inhibition of TGF- β 1 improved homing to areas of injury, the critical first step in repair. To evaluate the mechanism responsible for the improvement in CD34⁺ cell homing after transient inhibition of endogenous TGF- β 1, we first tested the impact of PMO treatment on survival of human CD34⁺ cells. For these studies, we used CD34⁺ cells from sources that would be typically used for human revascularization trials, such as cord blood, bone marrow, and peripheral blood. Cord blood and bone marrow CD34⁺ cells were cultured in medium with or without PMOs for 5 days. In the absence of growth factors, 95% of human lin⁻ CD34⁺ cells died after 2–3 days, even though they were cultured in a rich medium (Iscove's modified Dulbecco's medium with 10% FBS and 10% horse serum). In contrast, TGF- β 1-PMO-treated normal cord blood and bone marrow cells survived ($P < 0.05$) in the absence of growth factors and continued their growth over the course of an additional 7 days (Fig. 3A). Next, peripheral blood CD34⁺ cells from diabetic subjects and age- and sex-matched control subjects were tested. As observed for cord blood and bone marrow CD34⁺ cells, the addition of growth factors elicited greater proliferation in TGF- β 1-PMO-treated peripheral blood cells than in control PMO-treated cells ($P < 0.05$) (Fig. 3B).

TGF- β 1-PMO improved migration of diabetic CD34⁺ cells to SDF-1 and increased CXCR4 expression. Inhibition of endogenous TGF- β 1 expression enhanced migration of both diabetic ($P < 0.05$ vs. control PMO) and healthy cells ($P < 0.01$ vs. control PMO) (Fig. 4). To understand the mechanisms involved in this enhanced migratory function of CD34⁺ cells, we analyzed expression of the SDF-1 receptor, CXCR4. Enriched CD34⁺ cells were gated for the CD34⁺ CD45^{mid} population (Fig. 5A), and CXCR4 expression was analyzed (Fig. 5B). We found that CXCR4 surface expression was greater in TGF- β 1-PMO-treated diabetic cells than in control PMO-treated cells ($P = 0.004$). A similar trend was observed in healthy cells; however, it did not achieve statistical significance.

Inhibition of endogenous TGF- β 1 increases SDF-1-induced NO generation in diabetic CD34⁺ cells. Bioavailable NO is reduced in diabetic progenitors, and this contributes to the migratory dysfunction observed in these cells (13,26). Because inhibition of TGF- β 1 in healthy and diabetic CD34⁺ cells increased migration and homing in both animal models, we asked whether this effect was coupled to enhanced NO generation. In healthy CD34⁺ cells, SDF-1 (100 nmol/l) increased NO generation by $45 \pm 4\%$ (Fig. 6). The signal transduction pathway involved in SDF-1-induced NO release in CD34⁺ cells has not been elucidated. To delineate this pathway, we tested different pharmacological inhibitors. SDF-1-induced NO release was significantly decreased when cells were pretreated with one of the following: 10 μ mol/l AMD3100, a specific nonpeptide CXCR4 antagonist ($3 \pm 2\%$, $P < 0.001$); 100

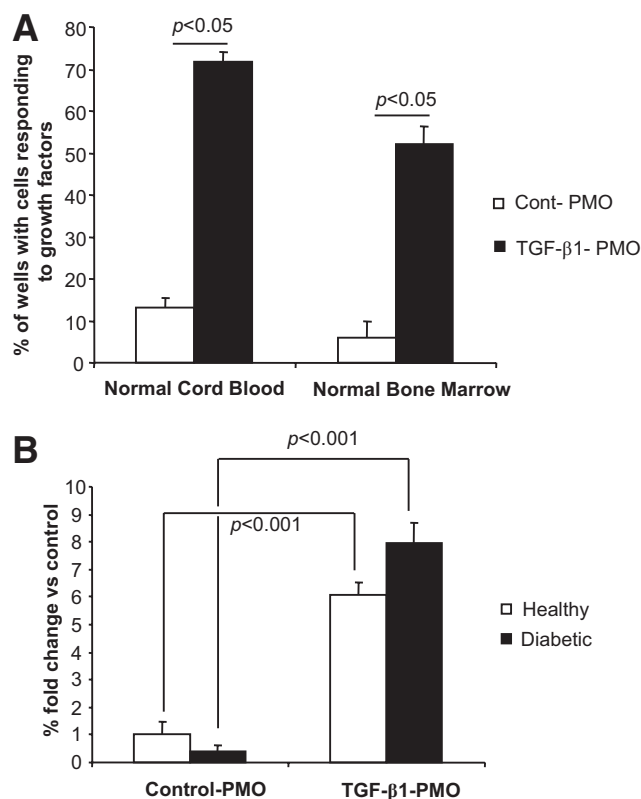


FIG. 3. TGF- β 1 inhibition improves survival of CD34⁺ cells from healthy and diabetic subjects. (A) Effect of TGF- β 1-PMO on the survival of CD34⁺ cells sorted from healthy human bone marrow or umbilical cord blood or (B) the survival of CD34⁺ cells sorted from healthy and diabetic human peripheral blood. Cells (10 cells per well, eight replicate wells) were treated with either TGF- β 1-PMO or control PMO. All cells were cultured in the absence of added growth factors for 5 days, after which growth factors were added to detect surviving (live) cells (final concentrations 20 ng/ml TPO, 50 ng/ml stem cell factor, 50 ng/ml IL-3, and 20 ng/ml IL-6). Results are expressed as percentage of wells containing growth-factor-responsive cells that attained >5,000 cells per well at day 12.

ng/ml pertussis toxin, a G_i protein inhibitor ($20 \pm 9\%$, $P < 0.01$); 20 μ mol/l LY294002, a phosphoinositide 3-kinase (PI3K) inhibitor ($12 \pm 3\%$, $P < 0.001$); or 30 μ mol/l

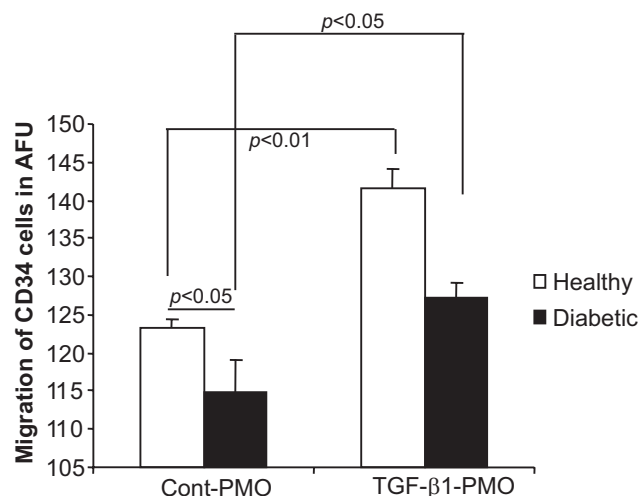


FIG. 4. Endogenous TGF- β 1 inhibition in CD34⁺ cells from peripheral blood of healthy and diabetic subjects improves their migration. Boyden chamber assay showing migration of cells to 100 nmol/l SDF-1 (representative of five separate experiments).

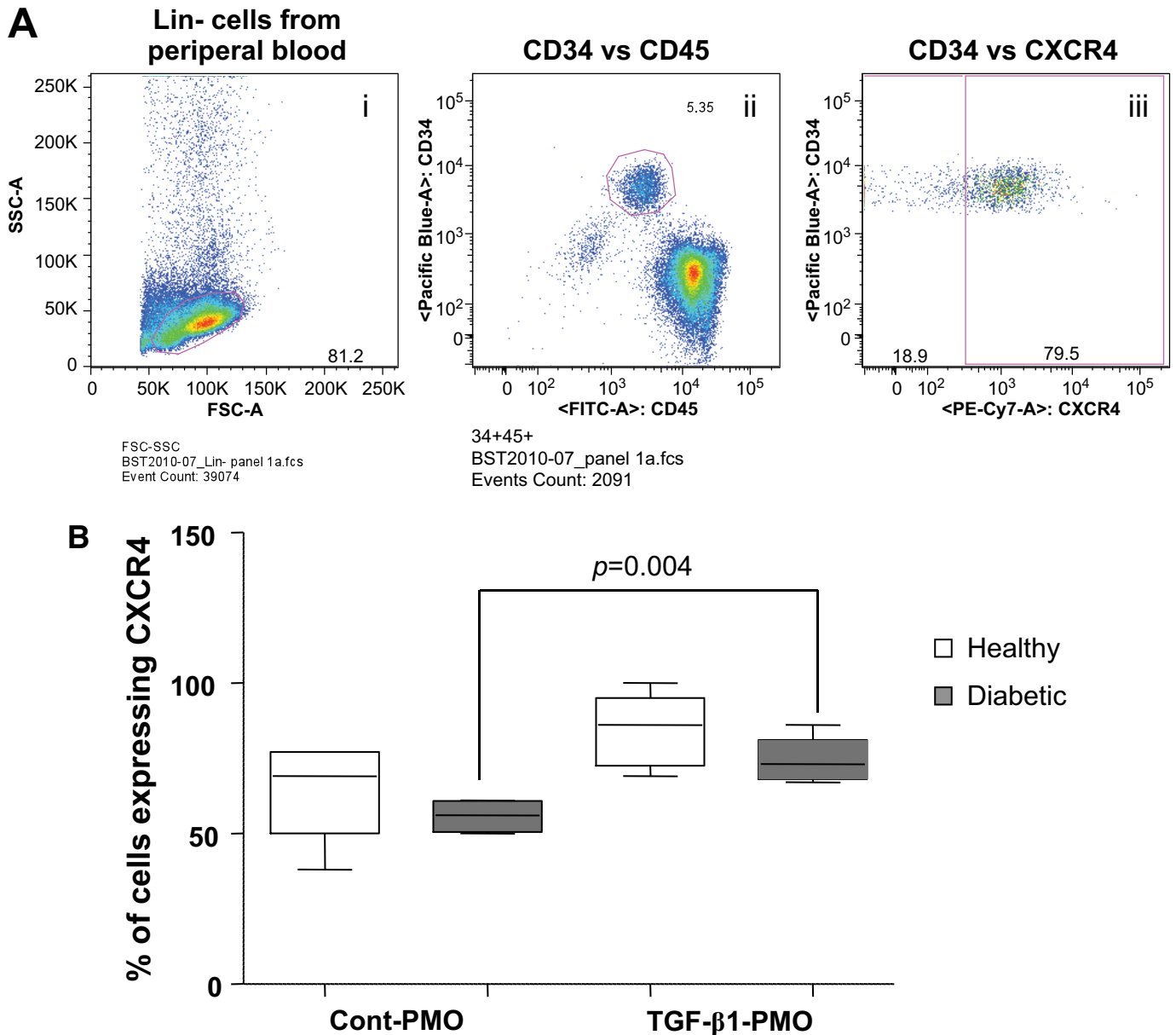


FIG. 5. Anti-TGF-β1-PMO upregulates CXCR4 expression in healthy and diabetic CD34⁺ cells. **A:** Flow cytometry dot plots of lin⁻ peripheral blood cells showing selection of (i) low forward and low side scatter cells and then (ii) selective gating for the CD34⁺ and CD45⁺ population. CXCR4 expression (iii) of the CD34⁺CD45⁺ population was studied using multicolored antibody labeling. **B:** Box plot showing increased CXCR4 expression by anti-TGF-β1-PMO in both healthy and diabetic CD34⁺ cells ($n = 5$ for each group). (A high-quality digital representation of this figure is available in the online issue.)

tricitin, an Akt inhibitor ($12 \pm 3\%$, $P < 0.01$) (Fig. 6). These results suggest that, in CD34⁺ cells, SDF-1-induced NO release involves the CXCR4/G_i-protein/PI3K/Akt pathway.

Next, we determined whether pretreatment with control or TGF-β1-PMO influenced NO release in these cells. We found that SDF-1 elicited a comparable degree of NO release in healthy, peripheral blood CD34⁺ cells that had been pretreated with either control PMO ($60 \pm 5\%$) or TGF-β1-PMO ($54 \pm 4\%$) (Fig. 7A, B). In contrast, SDF-1-induced marked NO generation in diabetic CD34⁺ cells treated with TGF-β1-PMO ($69 \pm 7\%$, $P < 0.0001$) but not in cells treated with control PMO (9.2%) (Fig. 7A, B). This ability of TGF-β1-PMO to restore NO release in diabetic CD34⁺ cells was diminished by the selective CXCR4 inhibitor, AMD3100 ($28 \pm 2\%$, $P < 0.01$), supporting that this effect was mediated by CXCR4 activation.

DISCUSSION

Therapeutic revascularization with endothelial progenitor cells holds promise as a treatment modality to prevent tissue damage and restore blood flow in individuals such as diabetic subjects who are not ideal candidates for standard revascularization procedures because of their small vessel disease. However, while cell therapy is needed in diabetic patients, this approach has limited utility because of endothelial progenitor cell dysfunction (27,28). Specifically, endothelial progenitors isolated from diabetic individuals demonstrate reduced proliferation, migration, and differentiation into endothelial cells. Exposure to high concentrations of glucose reduces endothelial nitric oxide synthase (eNOS) expression in these cells. HIF-1α modification by methylglyoxal, a consequence of increased reactive oxygen species formed during high

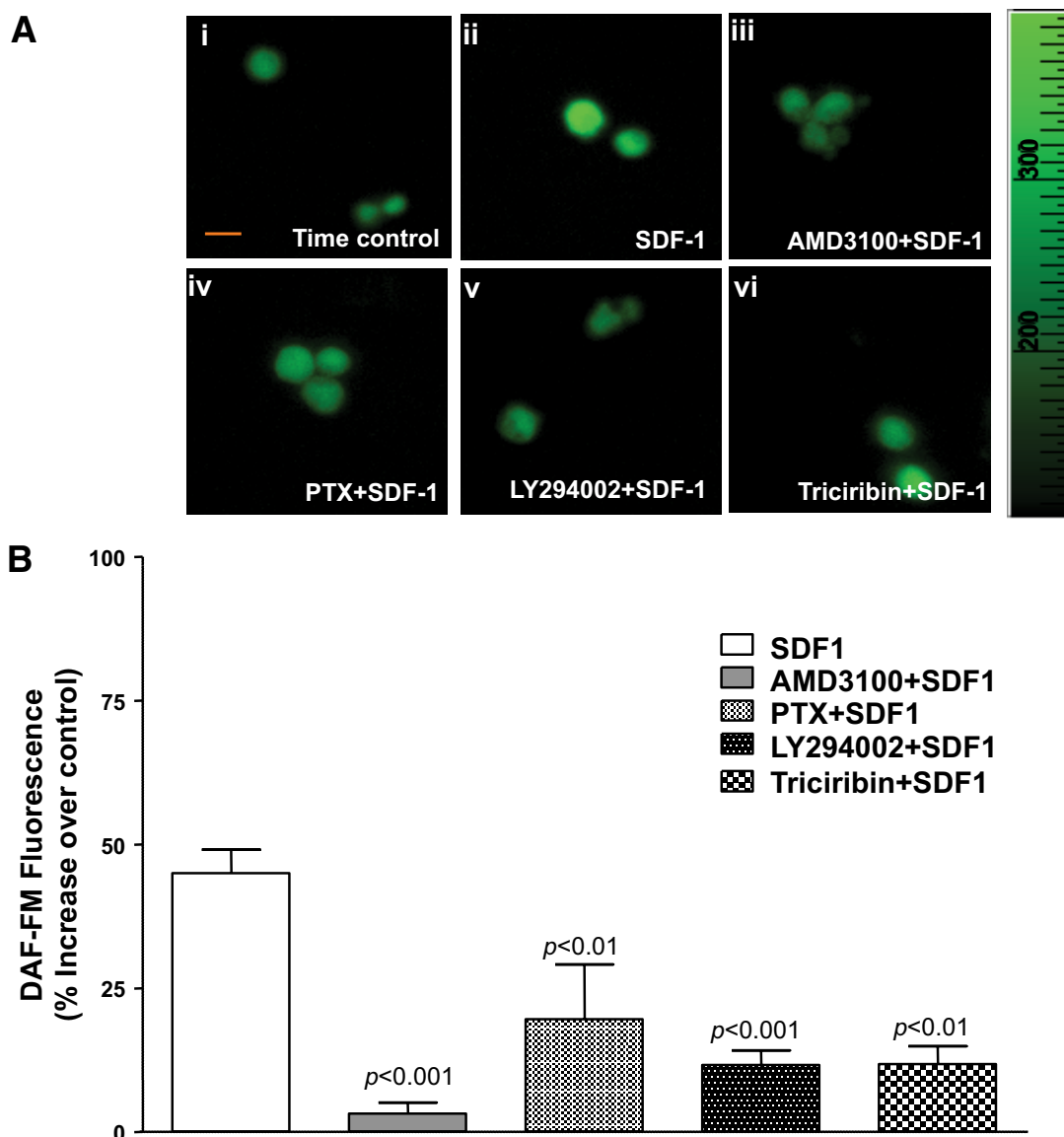


FIG. 6. SDF-1 induces NO release via activation of CXCR4/Gi-protein/PI3K/Akt pathway. (A) Representative images of DAF-FM fluorescence in the absence of any treatment (time control) (i) or after treatment with 100 nmol/l SDF-1 in the absence (ii) or presence of 10 μ mol/l AMD3100 (iii), 100 ng/ml pertussis toxin (PTX) (iv), 20 μ mol/l LY294002 (v), or 20 μ mol/l triciribin (vi). The color scale for DAF-FM fluorescence is shown to the right. **B:** Effects of different pharmacological treatments on SDF-1-mediated NO release expressed as a percent increase over the time control ($n = 4$ patients and up to 75 cells imaged per donor). (A high-quality digital representation of this figure is available in the online issue.)

glucose exposure, is likely responsible (27). BMPCs from *db/db* mice show reduced expression of eNOS and phospho-eNOS (29). Consistent with this finding, we have shown that human diabetic CD34⁺ cells have reduced NO bioavailability, which is associated with decreased migration that can be restored through exposure to NO donors (13).

In this study, we tested whether the reparative function of diabetic progenitors could be enhanced through inhibition of TGF- β 1, a novel approach that we hypothesized could correct multiple functional defects in these cells. We found that pretreating CD34⁺ progenitors with TGF- β 1-PMO enhanced proliferation and migration in vitro as well as homing in two preclinical in vivo models of injury. Endogenous TGF- β 1 has been shown to be a primary factor maintaining HSCs in G0/G1. However, exogenous TGF- β 1 (in plasma and tissue), SDF-1/CXCR4 signaling, and circulating growth factors could also regulate HSC cell cycle status. Although the majority of TGF- β 1 is expressed

as a latent, inactive form, HSCs and BMPCs express transglutaminase and other proteolytic enzymes that can activate endogenous TGF- β 1 (S.H.B., unpublished data). Accordingly, active intracellular TGF- β 1 has been identified in HSCs and endothelial progenitors (30). HSC quiescence, division, and daughter cell fate decisions have been shown to be mediated by the TGF- β 1/hematopoietic growth factor axis (22,31).

Previously, it has been reported that inhibition of TGF- β 1 induces G0-to-G1 transition (32). Here we find that it promotes cell migration and proliferation and improves homing of CD34⁺ cells to the vasculature. If intracellular or extracellular TGF- β 1 is withdrawn from CD34⁺ cells in the presence of optimal hematopoietic growth factors, then G0 cells rapidly shift to G1 and the G2/M/S interface. TGF- β 1 inhibition drives CD34⁺ cells into G1 (33) and has been shown to increase the engraftment potential of HSCs (34). In contrast to TGF- β 1 inhibition, withdrawal of

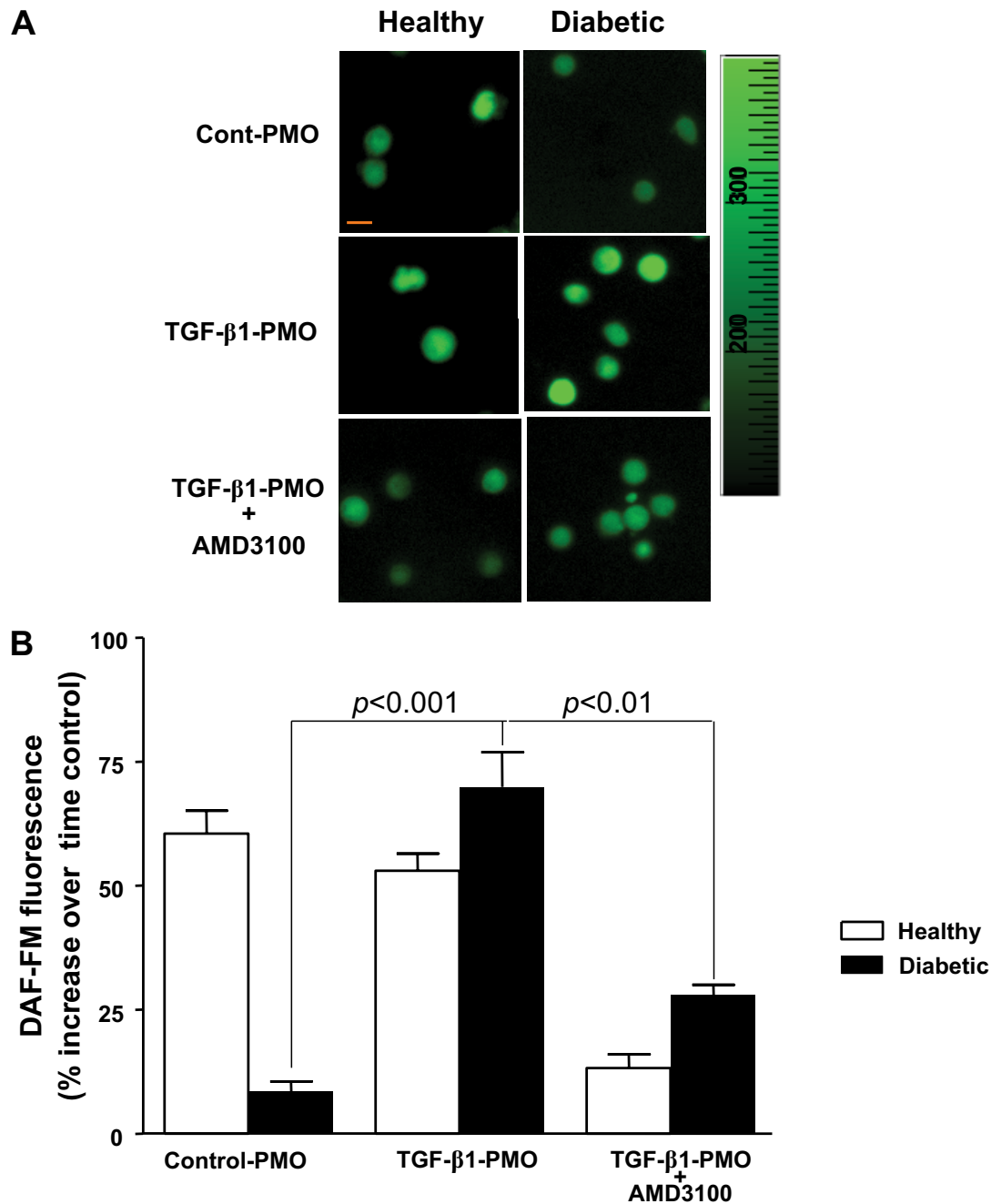


FIG. 7. SDF-1 induced NO release via activation of CXCR4 expression. **A:** Representative DAF-FM fluorescence images of healthy and diabetic CD34 cells treated with control or TGF-β1-PMO with or without CXCR4 inhibitor (10 μmol/l AMD3100) and stimulated with 100 nmol/l SDF-1; the DAF-FM fluorescence color scale is shown to the right. **B:** Quantification of the effects of PMOs on NO release in CD34⁺ cells expressed as a percent of NO release upon stimulation by SDF-1 (100 nmol/l) as compared to respective time control for the individual group ($n = 4$ patients and up to 75 cells imaged per donor). (A high-quality digital representation of this figure is available in the online issue.)

hematopoietic growth factors results in CD34⁺ cell death, some of which is mediated by TGF-β1 (35).

Here we show that if TGF-β1 is inhibited intracellularly in the absence of hematopoietic growth factors, human CD34⁺ cells can survive for >1 week ex vivo. In vivo we observe an enhanced reparative response that is likely due not only to increased migration of the TGF-β1-PMO-treated human CD34⁺ cells but also to increased proliferation at the site of vascular injury. Similar to engraftment into the bone marrow of LTR-HSC after TGF-β1-PMO treatment, we observed correction of defective homing in the diabetic CD34⁺ cells treated with TGF-β1-PMO. To

better understand the mechanisms of this beneficial effect, we first focused on the SDF-1/CXCR4 pathway that has been shown to be central to mediating tissue repair after injury. SDF-1 is highly expressed by ischemic tissue and can also increase progenitor cell homing back to the bone marrow (36,37). SDF-1 plays an important role in vascular development (38), and ablation of the SDF-1/CXCR4 gene in mice produces defects in blood vessel formation. As shown in Fig. 1A, anti-TGF-β-treated LTR-HSC demonstrates increased homing to the bone marrow, and as shown in Fig. 2 and supplementary Fig. 3, anti-TGF-β-treated cells migrate better to injured tissue. This can be due to increased CXCR4

expression or increased proliferative and migratory potential. Our results revealed that the migration of human CD34⁺ cells to SDF-1 was reduced in diabetic cells and that this migratory defect was corrected by endogenous TGF- β 1 inhibition, suggesting that efficient signal transduction of the SDF-1/CXCR4 pathway was restored.

To further understand the influence of TGF- β 1 inhibition on this pathway, we evaluated NO release in response to SDF-1 and used different pharmacological inhibitors to delineate the involvement of CXCR-4 receptor signaling pathway in SDF-1-induced NO release. Importantly, our studies also indicate that SDF-1-induced NO release occurred via activation of CXCR-4, and this involved G_i protein, PI3K and subsequent Akt activation. Akt activates eNOS by phosphorylation at Ser¹¹⁷⁷, resulting in NO production (39,40). Our findings are consistent with earlier studies showing that SDF-1-induced chemotaxis of T lymphocytes involves G_i protein-coupled PI3K activation and Akt phosphorylation (41,42).

We found that healthy CD34⁺ cells demonstrated robust NO release in response to SDF-1 and TGF- β 1 inhibition did not increase NO release further. This suggests that CXCR-4 activation required for NO release in response to SDF-1 was already maximal in the nondiabetic cells, before TGF- β 1 inhibition using PMOs. In contrast, TGF- β 1 inhibition in diabetic CD34⁺ cells corrected defective NO release in response to SDF-1, in part by significantly increasing the expression of CXCR-4 on the diabetic cells, which was not the case for the nondiabetic cells (Fig. 5B). This implies that, in healthy nondiabetic CD34⁺ cells, activation of CXCR-4 triggers NO-independent signaling pathways, such as tyrosine phosphorylation of Wiskott-Aldrich syndrome protein (WASp). WASp is a critical regulator of actin cytoskeleton remodeling after CXCR-4 activation. SDF-1 exposure results in Cdc42 activation and WASp tyrosine phosphorylation as well as in WASp association with Fyn and Pyk-2 tyrosine kinases (43). Here, we observed that cells were recruited to areas of vascular injury in two distinct but complementary injury models. Systemic administration of TGF- β 1-PMO-treated murine LTR-HSCs increased the homing of these cells to sites of neovascularization (supplementary Fig. 3) and intravitreal administration of TGF- β 1-PMO-treated CD34⁺ cells enhanced homing to injured retinal vessels (Fig. 2). Both the laser injury model and the ischemia reperfusion model result in release of angiogenic growth factors (44). We have previously shown that laser injury alone is sufficient to induce stem cell recruitment as early as 1 week after injury (45). In this model, SDF-1 antibodies reduce the recruitment of HSCs to the choroidal neovascularization lesion, suggesting that SDF-1/CXCR-4 participates in the recruitment of cells to sites of laser injury (46). Our current study suggests that treatment of the murine HSCs and human CD34⁺ cells with TGF- β 1-PMO increases their homing to areas of injury by enhancing their migratory potential. This is likely due to increased surface expression of CXCR-4 mediated by TGF- β 1-PMO exposure. Activation of CXCR-4 generates NO, which is critical to CD34⁺ cell migration (13,47).

Retinal and subretinal ischemia contributes to visual impairment and blindness in diseases as diverse as retinopathy of prematurity, diabetic retinopathy, and age-related macular degeneration. The ischemia reperfusion model mimics many aspects of the pathophysiology of retinal ischemia and leads to the development of acellular capillaries. We have previously shown that, in this model,

healthy CD34⁺/endothelial precursors reendothelialize ischemic capillaries; however, diabetic CD34⁺/endothelial precursor cells do not (11). In the present study, inhibition of TGF- β 1 enhanced the recruitment of diabetic as well as healthy CD34⁺ cells to sites of retinal injury and corrected this defect in diabetic CD34⁺ cells.

In conclusion, our studies show that transient inhibition of TGF- β 1 in CD34⁺ cells ex vivo enhances repair after vascular damage. This finding may have a profound impact on disease states associated with vascular dysfunction such as ischemic heart disease and diabetic vascular complications. While an attempt is being made to replace traditional approaches for alleviating tissue ischemia (e.g., stents, angioplasty, or vessel grafts) with cell therapy, autologous cellular therapy has not been feasible in diabetic patients because of dysfunctional cells. Transient inhibition of TGF- β 1 may represent a promising therapeutic strategy for restoring vascular reparative function in diabetic CD34⁺ cells and may increase the likelihood of successful cellular therapy in diabetic individuals.

ACKNOWLEDGMENTS

This work was supported by grants to M.B.G. from the National Institutes of Health (EY-007739 and EY-012601), U01-HL-087366, the Loftus family, and the Juvenile Diabetes Research Foundation and to A.D.B. from the American Heart Association. S.H.B. received a grant from BetaStem Therapeutics Inc. No other potential conflicts of interest relevant to this article were reported.

A.D.B., E.P.G., Y.P.R.J., S.H.B., and S.C. generated and analyzed data. C.S., D.K., F.W.R., C.J.P., and P.J.H. contributed to discussion. L.K. and M.C.L. provided access to the diabetic clinic at University of Florida. S.H.B. and M.B.G. analyzed data and wrote the manuscript.

The authors thank Dr. Martin Friedlander (The Scripps Research Institute, La Jolla, CA) and Dr. George King (Joslin Diabetes Center, Boston, MA) for their helpful discussions during the completion of these studies and the preparation of this manuscript. We also thank Dr. Patrick L. Iversen (AVI Biopharma Inc, Portland, OR) for many helpful discussions concerning the approaches used in these studies and for his ability to design PMOs that are effective.

REFERENCES

- Asahara T, Murohara T, Sullivan A, Silver M, van der Zee R, Li T, Witzenbichler B, Schatteman G, Isner JM. Isolation of putative progenitor endothelial cells for angiogenesis. *Science* 1997;275:964-967
- Hristov M, Weber C. Progenitor cell trafficking in the vascular wall. *J Thromb Haemost* 2009;7(Suppl. 1):31-34
- Gill M, Dias S, Hattori K, Rivera ML, Hicklin D, Witte L, Girardi L, Yurt R, Himel H, Rafii S. Vascular trauma induces rapid but transient mobilization of VEGFR2(+)/AC133(+) endothelial precursor cells. *Circ Res* 2001;88:167-174
- Schatteman GC. Adult bone marrow-derived hemangioblasts, endothelial cell progenitors, and EPCs. *Curr Top Dev Biol* 2004;64:141-180
- Grant MB, May WS, Caballero S, Brown GA, Guthrie SM, Mames RN, Byrne BJ, Vaughn T, Spoerri PE, Peck AB, Scott EW. Adult hematopoietic stem cells provide functional hemangioblast activity during retinal neovascularization. *Nat Med* 2002;8:607-612
- Schatteman GC, Hanlon HD, Jiao C, Dodds SG, Christy BA. Blood-derived angioblasts accelerate blood-flow restoration in diabetic mice. *J Clin Invest* 2000;106:571-578
- Kawamoto A, Katayama M, Handa N, Kinoshita M, Takano H, Horii M, Sadamoto K, Yokoyama A, Yamanaka T, Onodera R, Kuroda A, Baba R, Kaneko Y, Tsukie T, Kurimoto Y, Okada Y, Kihara Y, Morioka S, Fukushima M, Asahara T. Intramuscular transplantation of G-CSF-mobilized CD34(+) cells in patients with critical limb ischemia: a phase I/IIa, multicenter, single-blinded, dose-escalation clinical trial. *Stem Cells* 2009;27:2857-2864

8. Sekiguchi H, Ii M, Losordo DW. The relative potency and safety of endothelial progenitor cells and unselected mononuclear cells for recovery from myocardial infarction and ischemia. *J Cell Physiol* 2009;219:235–242
9. Fadini GP, Sartore S, Schiavon M, Albiero M, Baesso I, Cabrelle A, Agostini C, Avogaro A. Diabetes impairs progenitor cell mobilisation after hindlimb ischaemia-reperfusion injury in rats. *Diabetologia* 2006;49:3075–3084
10. Fadini GP, Miorin M, Facco M, Bonamico S, Baesso I, Grego F, Menegolo M, de Kreutzenberg SV, Tiengo A, Agostini C, Avogaro A. Circulating endothelial progenitor cells are reduced in peripheral vascular complications of type 2 diabetes mellitus. *J Am Coll Cardiol* 2005;45:1449–1457
11. Caballero S, Sengupta N, Afzal A, Chang KH, Li Calzi S, Guberski DL, Kern TS, Grant MB. Ischemic vascular damage can be repaired by healthy, but not diabetic, endothelial progenitor cells. *Diabetes* 2007;56:960–967
12. Busik JV, Tikhonenko M, Bhatwadekar A, Opreanu M, Yakubova N, Caballero S, Player D, Nakagawa T, Afzal A, Kielczewski J, Sochacki A, Hasty S, Li Calzi S, Kim S, Duclas SK, Segal MS, Guberski DL, Esselman WJ, Boulton ME, Grant MB. Diabetic retinopathy is associated with bone marrow neuropathy and a depressed peripheral clock. *J Exp Med* 2009;206:2897–2906
13. Segal MS, Shah R, Afzal A, Perrault CM, Chang K, Schuler A, Beem E, Shaw LC, Li Calzi S, Harrison JK, Tran-SON-Tay R, Grant MB. Nitric oxide cytoskeletal-induced alterations reverse the endothelial progenitor cell migratory defect associated with diabetes. *Diabetes* 2006;55:102–109
14. Ruscetti FW, Bartelmez SH. Transforming growth factor beta, pleiotropic regulator of hematopoietic stem cells: potential physiological and clinical relevance. *Int J Hematol* 2001;74:18–25
15. Keller JR, Mantel C, Sing GK, Ellingsworth LR, Ruscetti SK, Ruscetti FW. Transforming growth factor beta 1 selectively regulates early murine hematopoietic progenitors and inhibits the growth of IL-3-dependent myeloid leukemia cell lines. *J Exp Med* 1988;168:737–750
16. Herder C, Zierer A, Koenig W, Roden M, Meisinger C, Thorand B. Transforming growth factor-beta1 and incident type 2 diabetes: results from the MONICA/KORA case-cohort study, 1984–2002. *Diabetes Care* 2009;32:1921–1923
17. Nam JS, Cho MH, Lee GT, Park JS, Ahn CW, Cha BS, Lim SK, Kim KR, Ha HJ, Lee HC. The activation of NF-kappaB and AP-1 in peripheral blood mononuclear cells isolated from patients with diabetic nephropathy. *Diabetes Res Clin Pract* 2008;81:25–32
18. Anjaneyulu M, Berent-Spillion A, Inoue T, Choi J, Cherian K, Russell JW. Transforming growth factor-beta induces cellular injury in experimental diabetic neuropathy. *Exp Neurol* 2008;211:469–479
19. Untergasser G, Gander R, Rumpold H, Heinrich E, Plas E, Berger P. TGF-beta cytokines increase senescence-associated beta-galactosidase activity in human prostate basal cells by supporting differentiation processes, but not cellular senescence. *Exp Gerontol* 2003;38:1179–1188
20. Ruscetti FW, Akel S, Bartelmez SH. Autocrine transforming growth factor-beta regulation of hematopoiesis: many outcomes that depend on the context. *Oncogene* 2005;24:5751–5763
21. Summerton JE. Morpholino, siRNA, and S-DNA compared: impact of structure and mechanism of action on off-target effects and sequence specificity. *Curr Top Med Chem* 2007;7:651–660
22. Sitnicka E, Ruscetti FW, Priestley GV, Wolf NS, Bartelmez SH. Transforming growth factor beta 1 directly and reversibly inhibits the initial cell divisions of long-term repopulating hematopoietic stem cells. *Blood* 1996;88:82–88
23. Zheng L, Gong B, Hatala DA, Kern TS. Retinal ischemia and reperfusion causes capillary degeneration: similarities to diabetes. *Invest Ophthalmol Vis Sci* 2007;48:361–367
24. Chang KH, Chan-Ling T, McFarland EL, Afzal A, Pan H, Baxter LC, Shaw LC, Caballero S, Sengupta N, Li Calzi S, Sullivan SM, Grant MB. IGF binding protein-3 regulates hematopoietic stem cell and endothelial precursor cell function during vascular development. *Proc Natl Acad Sci U S A* 2007;104:10595–10600
25. Kielczewski JL, Jarajapu YP, McFarland EL, Cai J, Afzal A, Li Calzi S, Chang KH, Lydic T, Shaw LC, Busik J, Hughes J, Cardounel AJ, Wilson K, Lyons TJ, Boulton ME, Mames RN, Chan-Ling T, Grant MB. Insulin-like growth factor binding protein-3 mediates vascular repair by enhancing nitric oxide generation. *Circ Res* 2009;105:897–905
26. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K, Zeiher AM, Dimmeler S. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 2003;9:1370–1376
27. Ceradini DJ, Yao D, Grogan RH, Callaghan MJ, Edelstein D, Brownlee M, Gurtner GC. Decreasing intracellular superoxide corrects defective ischemia-induced new vessel formation in diabetic mice. *J Biol Chem* 2008;283:10930–10938
28. Egan CG, Lavery R, Caporali F, Fondelli C, Laghi-Pasini F, Dotta F, Sorrentino V. Generalised reduction of putative endothelial progenitors and CXCR4-positive peripheral blood cells in type 2 diabetes. *Diabetologia* 2008;51:1296–1305
29. Yan J, Tie G, Park B, Yan Y, Nowicki PT, Messina LM. Recovery from hind limb ischemia is less effective in type 2 than in type 1 diabetic mice: roles of endothelial nitric oxide synthase and endothelial progenitor cells. *J Vasc Surg* 2009;50:1412–1422
30. Fernandez T, Amoroso S, Sharpe S, Jones GM, Bliskovski V, Kovalchuk A, Wakefield LM, Kim SJ, Potter M, Letterio JJ. Disruption of transforming growth factor beta signaling by a novel ligand-dependent mechanism. *J Exp Med* 2002;195:1247–1255
31. Ploemacher RE, van Soest PL, Boudewijn A. Autocrine transforming growth factor beta 1 blocks colony formation and progenitor cell generation by hemopoietic stem cells stimulated with steel factor. *Stem Cells* 1993;11:336–347
32. Dao MA, Hwa J, Nolte JA. Molecular mechanism of transforming growth factor beta-mediated cell-cycle modulation in primary human CD34(+) progenitors. *Blood* 2002;99:499–506
33. Morita N, Yamamoto M, Tanizawa T. Correlation of c-kit expression and cell cycle regulation by transforming growth factor-beta in CD34+ CD38- human bone marrow cells. *Eur J Haematol* 2003;71:351–358
34. Dao MA, Taylor N, Nolte JA. Reduction in levels of the cyclin-dependent kinase inhibitor p27(kip-1) coupled with transforming growth factor beta neutralization induces cell-cycle entry and increases retroviral transduction of primitive human hematopoietic cells. *Proc Natl Acad Sci U S A* 1998;95:13006–13011
35. Xiao M, Oppenlander BK, Dooley DC. Transforming growth factor-beta(1) induces apoptosis in CD34(+)CD38(-/low) cells that express Bcl-2 at a low level. *Exp Hematol* 2001;29:1098–1108
36. Glimm H, Tang P, Clark-Lewis I, von Kalle C, Eaves C. Ex vivo treatment of proliferating human cord blood stem cells with stroma-derived factor-1 enhances their ability to engraft NOD/SCID mice. *Blood* 2002;99:3454–3457
37. Voermans C, Kooi ML, Rodenhuis S, van der Lelie H, van der Schoot CE, Gerritsen WR. In vitro migratory capacity of CD34+ cells is related to hematopoietic recovery after autologous stem cell transplantation. *Blood* 2001;97:799–804
38. Lima e Silva R, Shen J, Hackett SF, Kachi S, Akiyama H, Kiuchi K, Yokoi K, Hatara MC, Lauer T, Aslam S, Gong YY, Xiao WH, Khu NH, Thut C, Campochiaro PA. The SDF-1/CXCR4 ligand/receptor pair is an important contributor to several types of ocular neovascularization. *FASEB J* 2007;21:3219–3230
39. Dimmeler S, Fleming I, Fisslthaler B, Hermann C, Busse R, Zeiher AM. Activation of nitric oxide synthase in endothelial cells by Akt-dependent phosphorylation. *Nature* 1999;399:601–605
40. Fulton D, Gratton JP, McCabe TJ, Fontana J, Fujio Y, Walsh K, Franke TF, Papapetropoulos A, Sessa WC. Regulation of endothelium-derived nitric oxide production by the protein kinase Akt. *Nature* 1999;399:597–601
41. Cherala RP, Ganju RK. Stromal cell-derived factor 1 alpha-induced chemotaxis in T cells is mediated by nitric oxide signaling pathways. *J Immunol* 2001;166:3067–3074
42. Sotsios Y, Whittaker GC, Westwick J, Ward SG. The CXC chemokine stromal cell-derived factor activates a Gi-coupled phosphoinositide 3-kinase in T lymphocytes. *J Immunol* 1999;163:5954–5963
43. Stabile H, Carlino C, Mazza C, Giliani S, Morrone S, Notarangelo LD, Notarangelo LD, Santoni A, Gismondi A. Impaired NK-cell migration in WAS/XLT patients: role of Cdc42/WASp pathway in the control of chemokine-induced beta2 integrin high-affinity state. *Blood* 2010;115:2818–2826
44. Boyd SR, Zachary I, Chakravarthy U, Allen GJ, Wisdom GB, Cree IA, Martin JF, Hykin PG. Correlation of increased vascular endothelial growth factor with neovascularization and permeability in ischemic central vein occlusion. *Arch Ophthalmol* 2002;120:1644–1650
45. Sengupta N, Caballero S, Mames RN, Butler JM, Scott EW, Grant MB. The role of adult bone marrow-derived stem cells in choroidal neovascularization. *Invest Ophthalmol Vis Sci* 2003;44:4908–4913
46. Sengupta N, Caballero S, Mames RN, Timmers AM, Saban D, Grant MB. Preventing stem cell incorporation into choroidal neovascularization by targeting homing and attachment factors. *Invest Ophthalmol Vis Sci* 2005;46:343–348
47. Li Calzi S, Purich DL, Chang KH, Afzal A, Nakagawa T, Busik JV, Agarwal A, Segal MS, Grant MB. Carbon monoxide and nitric oxide mediate cytoskeletal reorganization in microvascular cells via vasodilator-stimulated phosphoprotein phosphorylation: evidence for blunted responsiveness in diabetes. *Diabetes* 2008;57:2488–2494