Activation of the ACE2/Angiotensin-(1–7)/Mas Receptor Axis Enhances the Reparative Function of Dysfunctional Diabetic Endothelial Progenitors

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We tested the hypothesis that activation of the protective arm of the renin angiotensin system, the angiotensin-converting enzyme 2 (ACE2)/angiotensin-(1-7) [Ang-(1-7)]/Mas receptor axis, corrects the vasoreparative dysfunction typically seen in the $CD34^+$ cells isolated from diabetic individuals. Peripheral blood $CD34^+$ cells from patients with diabetes were compared with those of nondiabetic controls. Ang-(1-7) restored impaired migration and nitric oxide bioavailability/cGMP in response to stromal cell–derived factor and resulted in a decrease in NADPH oxidase activity. The survival and proliferation of $CD34^+$ cells from diabetic individuals were enhanced by Ang-(1-7) in a Mas/phosphatidylinositol 3-kinase (PI3K)/Akt-dependent manner. ACE2 expression was lower, and ACE2 activators xanthenone and diminazine aceturate were less effective in inducing the migration in cells from patients with diabetes compared with controls. Ang-(1-7) overexpression by lentiviral gene modification restored both the in vitro vasoreparative functions of diabetic cells and the in vivo homing efficiency to areas of ischemia. A cohort of patients who remained free of microvascular complications despite having a history of longstanding inadequate glycemic control had higher expression of ACE2/Mas mRNA than patients with diabetes with microvascular complications matched for age, sex, and glycemic control. Thus, ACE2/Ang-(1-7)\Mas pathway activation corrects existing diabetes-induced $CD34^+$ cell dysfunction and also confers protection from development of this dysfunction. ***Diabetes*** **62:1258–1269, 2013**

Autologous endothelial progenitor cell (EPC) populations represent a novel treatment option for complications requiring therapeutic revascularization. However, the broadly dysfunctional cells of diabetic patients limit the feasibility of a cellular approach. The circulating progenitor cell numbers are reduced in patients with diabetes, and these cells show impaired reparative function in both in vitro and in vivo assays of angiogenesis (1–3). In patients with diabetes, the proliferation and migration in response to hypoxia-regulated cytokines or growth factors are highly impaired (1,4).

The renin angiotensin system (RAS) plays a vital role in regulating many physiological processes of the vascular system. Angiotensin II (Ang II), a product of angiotensin-converting enzyme (ACE), mediates its vasodeleterious effects such as vasoconstriction, proliferation, fibrosis, and inflammation through the activation of the AT_1 receptor. The vasoprotective arm of RAS involves ACE2, angiotensin-(1-7) [Ang-(1-7)], and Mas receptor. The vasoprotective effects of Ang-(1-7) involve stimulation of nitric oxide (NO) production and decreased production of reactive oxygen species (ROS); Ang-(1-7) activates endothelial nitric oxide synthase (eNOS) through an Akt-dependent mechanism and attenuates NADPH oxidase via the Mas receptor (5,6).

NO-dependent signaling events play a major role in the mobilization of progenitor cells from bone marrow (BM), homing to areas of vascular injury, and re-endothelialization (7,8). However, diabetes is typically associated with decreased NO bioavailability, due to either decreased eNOS activity/expression or increased ROS production via upregulated NADPH oxidase enzyme (9). The impaired reparative function of progenitor cells in patients with diabetes or ischemic cardiomyopathy can partially be reversed by restoring balance to NO and ROS levels, i.e., by increasing eNOS expression, by NO donors, or by decreasing NADPH oxidase-dependent ROS production (10–12).

Earlier studies have implicated progenitor cells in the vasoprotective effects of Ang-(1-7) (13). Chronic Ang-(1-7) treatment preserved endothelial function in rat models of myocardial ischemia and in-stent restenosis (14,15). Treatment with ACE2 or Ang-(1-7) corrected diabetic defects in therapeutic angiogenesis (16,17). However, no evidence has been shown for the direct effects of Ang-(1-7) on the vasoreparative function of progenitor cells.

This evidence coupled with the observation that some diabetic patients do not develop vascular complications lead us to hypothesize that EPCs from patients protected from the development of diabetes complications retain their vasoreparative potential despite the diabetic environment and that activation of the ACE2/Ang-(1-7)/Mas axis of the RAS in $CD34^+$ cells confers this protection.

RESEARCH DESIGN AND METHODS

Characteristics of patients and subjects. The characteristics of subjects and patients in cohorts 1 and 2 are listed in Table 1. The following exclusion criteria were used: evidence of ongoing acute or chronic infection (HIV, hepatitis B or C, or tuberculosis), ongoing malignancy, cerebral vascular accident or cerebral vascular procedure, current pregnancy, history of organ transplantation, presence of a graft, uremic symptoms, an estimated glomerular filtration rate of <20 cc/min (by modification of diet in renal disease equation), an albumin level of

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TABLE 1
Patient characteristics

Cohort 1	Healthy controls	Diabetic patients
Number (*n*)	31	45
Sex, male/female	14/18	19/25
Age, years	49 ± 5	55 ± 2
Duration of diabetes, years	—	12 ± 2
Type 1 diabetes, *n*	—	17
Type 2 diabetes, *n*	—	27
HbA_{1c}	4.9 ± 0.2	7.7 ± 0.2*
Retinopathy, *n*	—	10
Neuropathy, *n*	—	11
Nephropathy, *n*	—	4
CVD, *n*	—	5
Hypertension, *n*	—	24
Hypercholesterolemia, *n*	—	2
Smokers, *n*	—	4

Cohort 2	Healthy controls	Diabetic patients with complications	Diabetic patients without complications
Number, *n*	5	5	5
Duration of diabetes (years)	—	42 ± 6	42 ± 5
HbA_{1c}	5.4 ± 0.2	7.9 ± 0.4γ	7.4 ± 0.2*

Data presented as mean ± SEM, and *n* represents the number of donors used. CVD, cardiovascular disease. γ$P < 0.0005$ compared with healthy control group. *$P < 0.0001$ compared with healthy control group.

<3.6 (to avoid malnutrition as a confounding variable), or an inability or unwillingness to hold vasoactive medications for 24 h.

Cohort 1. Healthy subjects or diabetic patients with complications visiting clinics at the University of Florida, in accordance with the approved protocol by the institutional review board, were enrolled in this study. Participants were 28–60 years of age and included both males and females with type 1 or type 2 diabetes. Diabetic patients were evaluated for the presence of microvascular complications, degree of hyperglycemia, hypertension, and hyperlipidemia, and then peripheral blood (PB) was obtained.

Cohort 2. PB was drawn, and $CD34^+$ cells were isolated from diabetic individuals free of microvascular complications with a 40-year history of diabetes and consistently documented higher levels of HbA_{1c} (>7). $CD34^+$ cells were also isolated from age- and sex-matched control individuals and diabetic subjects with equal duration of diabetes but with advanced complications including proliferative diabetic retinopathy.

Enrichment of $CD34^+$ cells. PB was obtained in CPT tubes (Becton Dickinson) and centrifuged (1,000*g*, 30 min). Buffy coats containing total mononuclear cells were collected and washed three times with PBS (Mediatech, Manassas, VA) containing 2% FBS by centrifugation at 120*g* for 10 min. PB mononuclear cells were enriched for $lineage^-$ (Lin^-) cells using a negative selection kit (StemCell Technologies, Vancouver, BC, Canada) and then sorted for $CD45^{dim}CD34^+$ cells by fluorescence-activated cell sorting (FACS Aria, BD Biosciences). Lin^- cells were clearly resolved into $CD34^+$ and $CD45^+$ populations using conjugated mouse anti-human antibodies, pacific blue–CD34 (BioLegend) and PE-Cy7-CD45 (BD Pharmingen), as previously described (18) (Supplementary Fig. 1). In some experiments, Lin^- cells were enriched for $CD34^+$ cells by immunomagnetic selection (Stem Cell Technologies) using a positive selection kit (StemCell Technologies). Freshly isolated cells were used for most experiments. When needed, cells were plated in StemSpan containing cytokine cocktail (StemCell Technologies) in round-bottom 96-well plates (Nunc) for no more than 72 h.

Characterization of ACE2 and Mas receptor expression in $CD34^+$ cells. Surface expression of ACE2 and Mas receptor was evaluated in PB Lin^- cells by flow cytometry (LSRII; BD Biosciences) using mouse anti-human ACE2 (Alexis Biochemicals) and rabbit anti-human Mas1 antibodies (Novus Biologicals). Antibodies were conjugated using Zenon Alexa-Fluor488 mouse IgG1 or Zenon Alexa-Fluor647 rabbit IgG labeling kits (Invitrogen). Nonimmune mouse IgG1 and rabbit IgG antibodies were used as isotype controls.

Microarray analysis is described in detail in Supplementary Fig. 3. The Ang-(1-7) fusion transgene construct used in our study was described by Santos et al. (19), and details are provided in Supplementary Fig. 6.

Functional studies using $CD34^+$ cells. Migration of $CD34^+$ cells was evaluated by the Chemicon QCMTM 96-well 5-μm Migration Assay (Chemicon International, Inc., Temecula, CA) and expressed as described previously (12). Intracellular levels of NO were evaluated by fluorescence microscopy using DAF-FM (Invitrogen) as described previously (12). Discoverx HitHunter cGMP assay kit (GE) was used for evaluating the production of cGMP in response to stromal cell–derived factor (SDF). NADPH oxidase activity was determined in whole-cell lysates using dihydroethidium (DHE) in a plate reader, and the activation of the enzyme was triggered by the addition of NADPH (20). Ang-(1-7) in the $CD34^+$ cell supernatants was analyzed by immunocompetition assay (Assaygate, Inc.).

The survival of cells was evaluated using the ViaLightPlus Kit (Lonza, Rockland, ME), as per the manufacturer's instructions. Cells were plated in basal medium with or without agonists/blockers, and the survival was evaluated after 48 h. Proliferation was by bromodeoxyuridine (BrdU) incorporation assay (Cell Proliferation ELISA; Roche Applied Science).

Real-time PCR studies. Total mRNA of human $CD34^+$ cells was isolated using the Aurum Total RNA Mini Kit (Bio-Rad), and the purity of RNA was determined by NanoDrop (ND-1000 UV-Vis spectrophotometer; NanoDrop Technologies). RNA was transcribed by using an iScript cDNA Synthesis Kit (Bio-Rad). Real-time PCR was performed by using Taqman Gene Expression Assay systems: ACE2, Hs00222343_m1; Mas receptor, Hs00267157_s1; β-actin, Hs99999903_m1. Results were analyzed by the $2^{-\Delta\Delta Ct}$ method and normalized to β-actin expression.

Evaluation of vascular engraftment potential of $CD34^+$ EPCs in an acute vascular injury model: mouse retinal ischemia/reperfusion. Mice were subjected to retinal ischemia/reperfusion (I/R) injury under isoflurane anesthesia by applying hydrostatic pressure in the anterior chamber of the eye for 2 h followed by reperfusion by withdrawing the pressure as described previously (3). The intravitreal injection of $CD34^+$ cells was performed 7 days after the insult, at which time retinal capillary damage was appreciable (21). After 48 h, the animals were killed, the eyes were enucleated, and the neural retinas were processed for immunohistochemistry to stain the vasculature (rhodamine-conjugated *Ricinus communis* agglutinin I) and the injected human cells (anti-human nuclear antigen) as described previously (3,21). Digital fluorescent images were acquired on a spinning disk confocal microscope (Olympus), and the vascular incorporation of $CD34^+$ EPCs was quantitatively evaluated by intensity correlation analysis using ImageJ software (ImageJ 1.37n; NIH) (3).

Data analysis and statistics. Results are expressed as mean ± SEM, and *n* represents the number of donors used. Results were analyzed for statistical significance by Student *t* test or one-way ANOVA with Newman-Keuls posttest, using GraphPad Prism software (GraphPad Software, La Jolla, CA). The nonparametric Kruskal-Wallis test was used where appropriate.

RESULTS

Ang-(1-7) stimulates migration in $CD34^+$ cells and restores migration of dysfunctional diabetic $CD34^+$ cells. Migration of $CD34^+$ cells in response to hypoxia-regulated cytokines is a signature of their in vivo

vasoreparative function, as they must be mobilized from the BM, enter the circulation, and then extravasate at sites of injury. We asked whether Ang-(1-7) can induce CD34$^+$ cell migration and found that in nondiabetic cells, Ang-(1-7) induced a robust migratory response. This migratory effect was observed in the concentration range of 10 to 100 nmol/L Ang-(1-7) and was comparable to that produced by 100 nmol/L SDF or 25 ng/mL vascular endothelial growth factor (VEGF) (Fig. 1*A*). The migration of diabetic cells in response to Ang-(1-7) at all three concentrations tested was similar to that observed in nondiabetic cells (Fig. 1*A*). Previously, we showed that migration to the physiologically relevant factors produced by ischemic tissue, SDF or VEGF, was significantly impaired in diabetic cells (11), and this impaired SDF migration was confirmed in this study ($P < 0.02$) (Fig. 1*A*). These data suggest that diabetic EPCs that do not respond to hypoxia-regulated factors are responsive to Ang-(1-7). Furthermore, expression of the Mas receptor was not altered in diabetic EPCs in comparison with nondiabetic cells, as measured by mRNA or as Mas receptor expressing Lin$^-$CD45^{dim}CD34$^+$ cells (Supplementary Fig. 2).

In nondiabetic CD34$^+$ cells, the Ang-(1-7)–mediated migratory response was sensitive to pretreatment with a nonspecific blocker of NOS, L-N^G-nitro-L-arginine methyl ester (L-NAME) (300 μmol/L; $P < 0.05$), or the Mas receptor antagonist, A-779 (1 μmol/L; $P < 0.05$) (Fig. 1*B*). Bradykinin (BK)-B2 receptors have been implicated in the vasodilatory and proangiogenic functions of Ang-(1-7) (22–24), and BK-B2 receptor and the kallikrein-kinin system are now known to play an important role in EPC function and in therapeutic neovascularization (25). Furthermore, interactions of Mas receptor with Ang-II receptors, AT1 and AT2, have been suggested (26). Therefore, we asked whether BK and Ang-II receptors are involved in the Ang-(1-7)–mediated migratory response. Nondiabetic CD34$^+$ cells were pretreated with selective inhibitors of the BK-B1 receptor R715 (300 nmol/L), the BK-B2 receptor Hoe 140 (100 nmol/L), the AT1 receptor

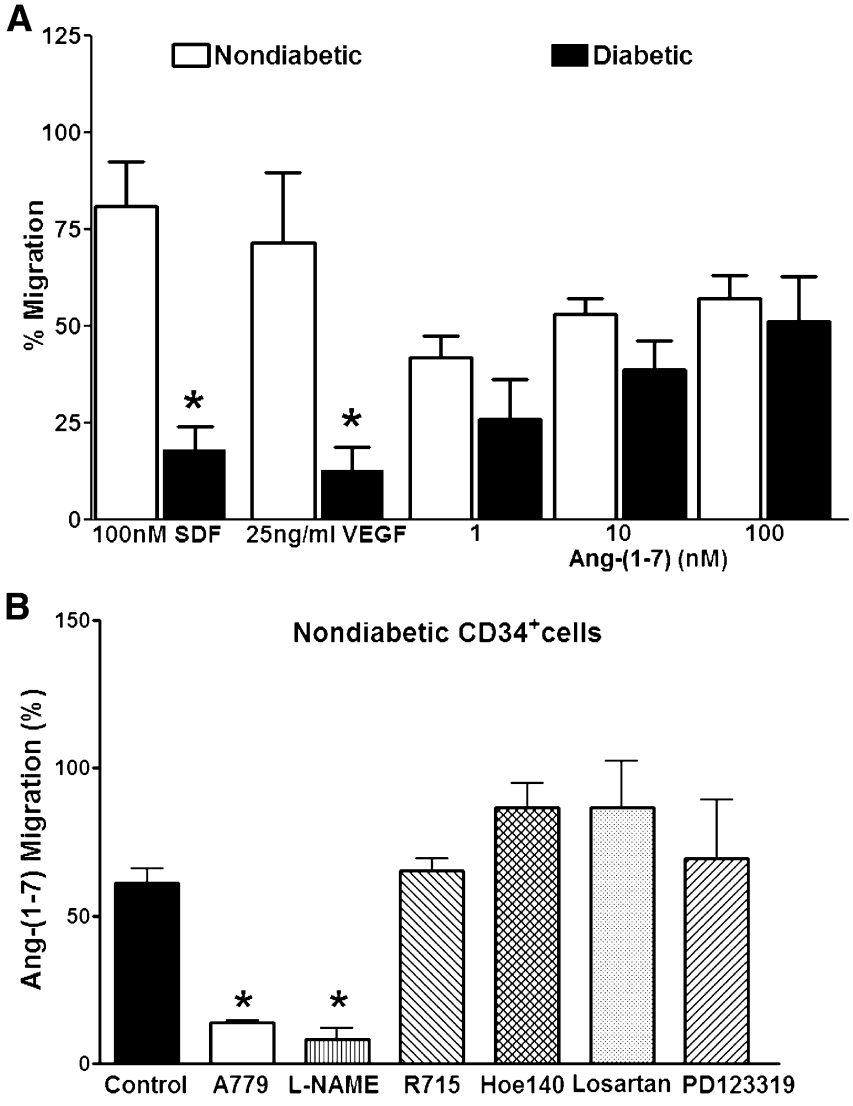

FIG. 1. Ang-(1-7) stimulates migration of healthy nondiabetic CD34$^+$ cells and restores migration of dysfunctional diabetic CD34$^+$ cells. *A*: Migration in response to SDF or VEGF was impaired in cells from diabetic patients with microvascular complications (*$P < 0.02$). Ang-(1-7) produced similar migratory responses in nondiabetic and diabetic cells at 1, 10, and 100 nmol/L. Responses observed at 100 nmol/L were comparable to those produced by SDF in nondiabetic cells. *B*: In nondiabetic CD34$^+$ cells, Ang-(1-7) (100 nmol/L)–mediated migration was inhibited by 1 μmol/L A779 (*$P < 0.05$; $n = 6$) or 300 μmol/L L-NAME (*$P < 0.05$, $n = 6$), and was resistant to blockade by R715 (300 nmol/L), Hoe 140 (100 nmol/L), losartan (100 nmol/L), or PD123319 (1 μmol/L).

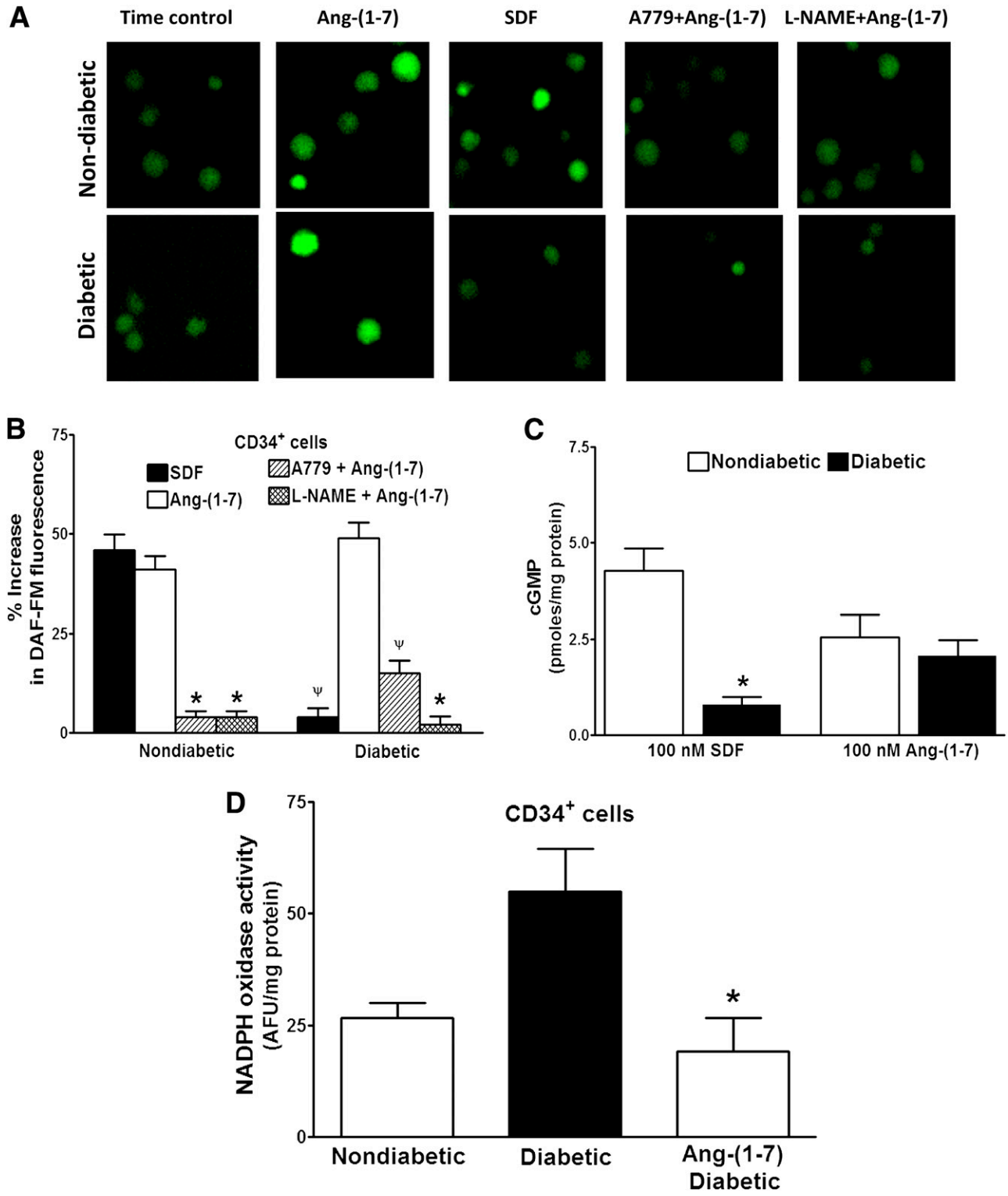

FIG. 2. Ang-(1-7) increases NO bioavailability and decreases NADPH oxidase activity. *A*: DAF-FM fluorescence images of intracellular NO levels in nondiabetic and diabetic cells with and without various treatments. *B*: Quantification of the effects of each treatment expressed as percent control; effect of SDF was decreased in diabetic cells. Ang-(1-7) stimulated NO generation to a similar extent in nondiabetic and diabetic cells. Ang-(1-7)–mediated NO release was blocked by 1 μmol/L A779 or 300 μmol/L L-NAME (nondiabetic: *$P < 0.01$; diabetic: Ψ$P < 0.05$ and *$P < 0.01$). *C*: Production of cGMP, a direct indicator of NO bioavailability, in response to SDF was lower in diabetic cells than nondiabetic cells (*$P < 0.05$). In contrast, Ang-(1-7) (100 nmol/L)–mediated cGMP production was similar in both nondiabetic and diabetic cells. *D*: NADPH oxidase activity was higher in the diabetic CD34$^+$ cells than nondiabetic cells, and treatment with 100 nmol/L Ang-(1-7) decreased the activity to normal (*$P < 0.05$).

losartan (100 nmol/L), or the AT2 receptor PD123319 (1 μmol/L) before evaluation of Ang-(1-7)–mediated migration. None of these inhibitors affected the Ang-(1-7)–mediated migratory response in CD34$^+$ cells (Fig. 1*B*), suggesting that BK and Ang-II receptors are not involved and that this response is mediated exclusively by the Mas receptor.

Ang-(1-7) restores NO availability in diabetic CD34$^+$ cells by reducing ROS production. NO-mediated signaling pathways are major determinants of SDF migration in CD34$^+$ cells (7,11); therefore, we used DAF-FM fluorescence microscopy to assess NO production in response to Ang-(1-7) in these cells (Fig. 2*A*). Ang-(1-7)–stimulated NO production was comparable in nondiabetic (42 ± 4%) and diabetic (49 ± 6%) CD34$^+$ cells, whereas NO production in response to SDF was greatly decreased in diabetic cells (10 ± 2%; $P < 0.01$) compared with nondiabetic cells (46 ± 4%) (Fig. 2*B*). NO production induced by Ang-(1-7) was blocked by pretreatment with L-NAME (300 μmol/L) or A779 (1 μmol/L) (Fig. 2*B*). These results suggest that the increased NO bioavailability associated with Ang-(1-7) is responsible for restoring the migratory response of diabetic CD34$^+$ cells. These findings were further confirmed by determining cGMP generation, a direct indicator or NO bioavailability. Levels of cGMP generated by SDF were significantly lower in diabetic cells. In contrast, Ang-(1-7) increased cGMP production similarly in both nondiabetic and diabetic cells (Fig. 2*C*).

Increased ROS generation in diabetic cells has been shown to be largely responsible for the decreased bioavailability of NO and reduced EPC migration (9). Previously, we showed that NOX2 is the major isoform of NADPH oxidase in human CD34$^+$ cells, and that in diabetic CD34$^+$ cells, the production of superoxide, expression of NOX2, and activity of NADPH oxidase are significantly higher. Thus, we asked whether restoration of NO availability in diabetic CD34$^+$ cells was due to the Ang-(1-7)–mediated decrease in the activation of NADPH oxidase. To test this, NADPH oxidase activity was determined in nondiabetic and diabetic EPCs. Cells were treated with Ang-(1-7), and the cell lysates were assayed for NADPH oxidase (20). Diabetic cells showed

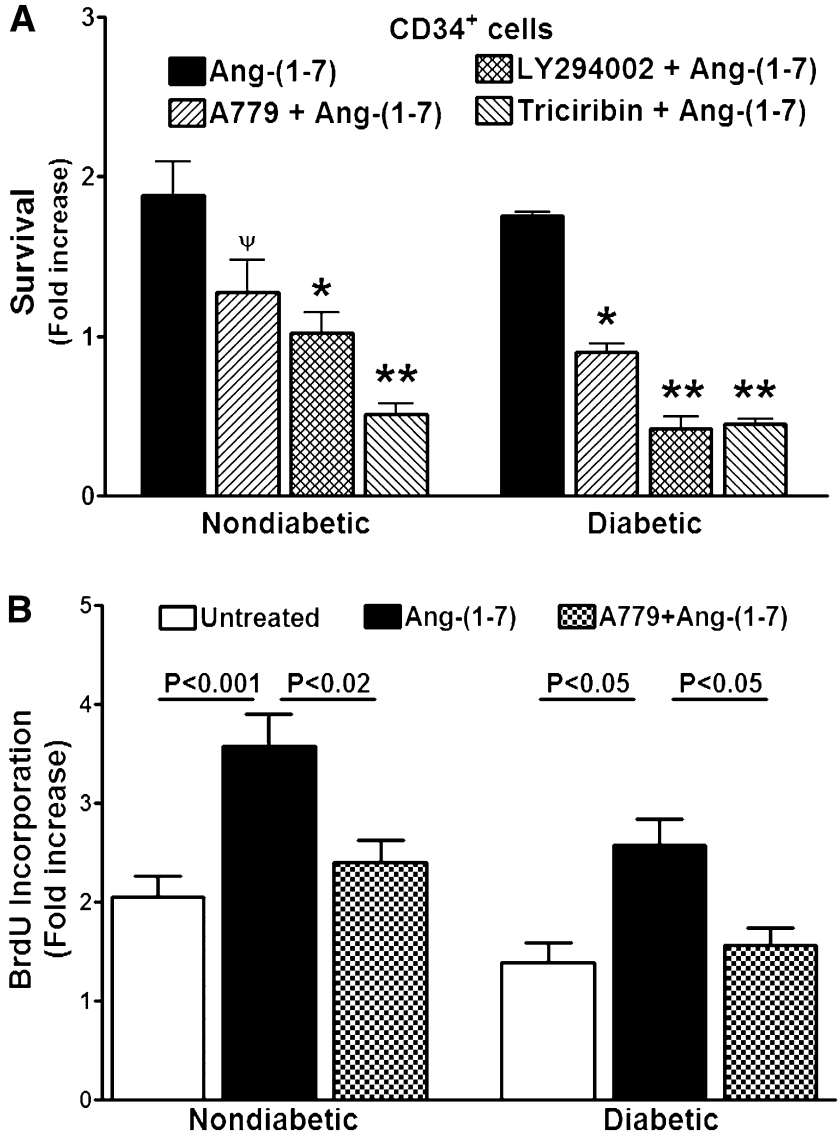

FIG. 3. Ang-(1-7) stimulates survival and proliferation in CD34$^+$ cells via the Mas/PI3K/Akt pathway. *A*: A stimulatory effect of Ang-(1-7) was observed in cells plated in basal culture medium and is expressed as fold-increase compared with untreated cells. Ang-(1-7) (100 nmol/L) enhanced the survival of CD34$^+$ cells of nondiabetic (1.75-fold; $P < 0.004$) and diabetic origin (1.84-fold; $P < 0.004$). The stimulatory effect was attenuated by treatment with A779, LY294002, or triciribin pretreatment ($\Psi P < 0.05$; $*P < 0.01$; $**P < 0.001$). *B*: Proliferation of cells was determined by BrdU incorporation after 48-h treatment with 100 nmol/L Ang-(1-7), and the fold increase in proliferation was expressed relative to the effect of mitomycin-C (10 μmol/L) in the respective group of cells. Ang-(1-7) increased proliferation of both nondiabetic and diabetic cells, which was inhibited by simultaneous treatment with A779 (1 μmol/L).

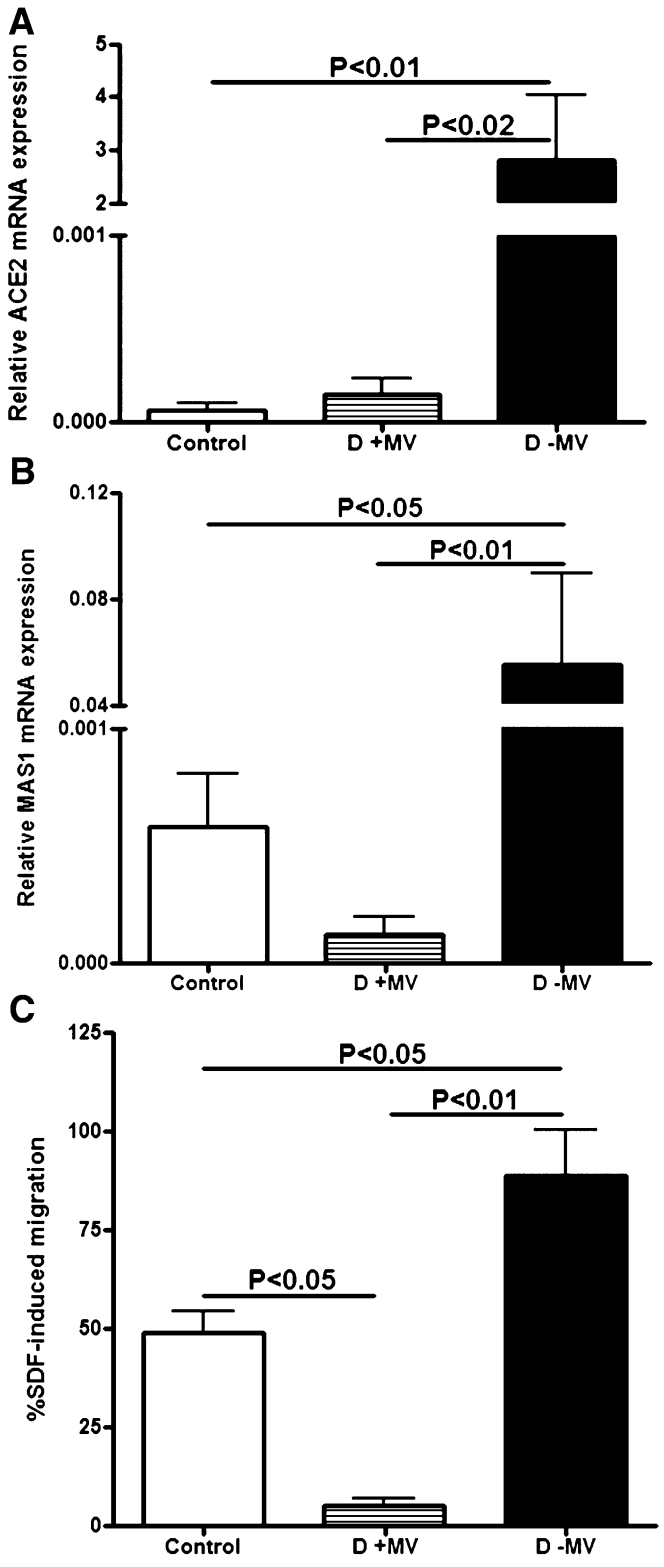

FIG. 4. Expression of genes in the protective arm of RAS is increased in the CD34$^+$ cells of diabetic individuals resistant to the development of microvascular complications, despite poor glycemic control. Quantitative RT-PCR revealed a significant increase in the mRNA levels of ACE2 (*A*) and Mas receptor (*B*) (Mann-Whitney test; $n = 5$) in the CD34$^+$ cells of patients with longstanding diabetes without microvascular complications (D −MV) compared with cells from either control or patients with diabetes with microvascular complications (D +MV). Datum was expressed as relative mRNA expression normalized to β-actin as a housekeeping gene. *C*: Migration in response to SDF was higher in CD34$^+$ cells from patients with diabetes without microvascular complications (D −MV) or control cells. Compared with controls, this response was decreased in cells from patients with diabetes with microvascular complications (D +MV) ($n = 5$, one-way ANOVA, Newman-Keuls posttest).

significantly higher activity of NADPH oxidase compared with the nondiabetic cells ($P < 0.05$), and this activity was reduced to normal levels by pretreatment with Ang-(1-7) ($P < 0.05$) (Fig. 2*D*).

Ang-(1-7) enhances the survival and proliferation of CD34$^+$ cells. We next asked whether Ang-(1-7) supports the survival and proliferation of CD34$^+$ cells. Survival of Ang-(1-7)–treated cells was determined by evaluating ATP levels (Supplementary Data), and proliferation was determined by evaluating BrdU incorporation over 48 h. Survival of both nondiabetic and diabetic cells was increased by Ang-(1-7) (100 nmol/L) in the basal medium, suggesting a prosurvival function of this peptide (Fig. 3*A*). This protective response was not observed when cells were pretreated with A779 or phosphatidylinositol 3-kinase (PI3K) inhibitor, LY294002, or Akt inhibitor, triciribin, suggesting that the Mas receptor/PI3K/Akt pathway is involved in this response. Ang-(1-7) increased proliferation of both nondiabetic and diabetic cells, which was inhibited by simultaneous treatment with A779 (10 μmol/L) (Fig. 3*B*).

Cluster analysis identifies ACE2 and the Mas receptor as increased transcripts in diabetic patients who are resistant to the development of microvascular complications. We reasoned that the CD34$^+$ cells of patients resistant to the development of microvascular complications might have a more robust reparative phenotype than those that develop complications. We also considered that diabetic individuals with longstanding diabetes and poor glucose control might represent a unique population in that they would likely be repairing any diabetes-initiated insults in a constant and effective manner. We performed microarray studies in patients who met these criteria (cohort 2) (Table 1) and found that, compared with patients with a comparable duration of diabetes and degree of glucose control, the protected patients had different expression levels of genes involved in RAS, NO, and NADPH oxidase, as well as IL-6, thrombin, cAMP, endothelin-1, and p53 (Supplementary Fig. 3). The protective arm of the RAS, ACE2/Ang-(1-7)/Mas receptor pathway is closely coupled to other key pathways, such as NO/cGMP, NADPH oxidase-ROS, and PI3K/Akt, that have been implicated in the pathogenesis of diabetes complications (Supplementary Fig. 4). We hypothesized that by modulating multiple protective pathways, activation of the Mas receptor by Ang-(1-7) may represent the key regulatory event of EPC function. We next confirmed this gene signature by RT-PCR of ACE2 and the Mas receptor. ACE2 expression was significantly higher in the protected patients compared with patients with complications ($P < 0.02$) or control ($P < 0.01$) (Fig. 4*A*). Mas receptor expression was also increased in the diabetic subjects protected from development of complications compared with those with complications ($P < 0.05$) or with control subjects ($P < 0.01$). Importantly, in vitro migration of CD34$^+$ cells to SDF was higher in diabetic individuals without microvascular complications compared with control subjects ($P < 0.05$) and patients with diabetes with complications ($P < 0.01$). Compared with control cells, this response was decreased in cells from patients with diabetes with microvascular complications ($P < 0.05$), consistent with that observed in patients with diabetes from cohort 1 (Fig. 4*C*). These data implicate a key role for the

protective axis of RAS (ACE2/Mas expression) in maintaining the reparative potential of CD34$^+$ cells in diabetes.

We next asked whether ACE2 activation can restore the vasoreparative functions of dysfunctional diabetic CD34$^+$ cells from subjects in cohort 1. We used two small-molecule ACE2 activators, xanthenone (XNT) and DIZE (27,28). Although XNT and DIZE stimulated migration of nondiabetic CD34$^+$ cells (Fig. 5*A*), their effects on diabetic cells was lower at all concentrations tested (Fig. 5*A*). Next, we measured the Ang-(1-7) secretion from CD34$^+$ cells in response to the ACE2 activators. Basal levels of Ang-(1-7) were significantly lower in supernatants of diabetic cells than of nondiabetic cells. In the presence of exogenous Ang-II (100 nmol/L), Ang-(1-7) levels were increased by 30-fold in nondiabetic cells, whereas only a threefold increase was observed in the diabetic cells. Treatment with DIZE further increased the levels of Ang-(1-7) up to 100-fold (13 ng/mL; 14.43 nM) in nondiabetic cells, whereas in diabetic cells, only 1.2 ng/mL (1.33 nM) was detected (Fig. 5*B*). We next evaluated ACE2 expression in both nondiabetic and diabetic CD34$^+$ cells. Real-time PCR showed that the ACE2 mRNA expression was significantly lower in diabetic CD34$^+$ cells (Fig. 5*C*). Consistent with this, ACE2 surface expression, as evaluated by flow cytometry, was significantly lower in diabetic cells (Fig. 5*C* and Supplementary Fig. 5). These results suggest that ACE2 expression and Ang-(1-7) generation are significantly decreased in dysfunctional CD34$^+$ cells from diabetic individuals with microvascular complications.

Ang-(1-7) gene modification of the dysfunctional diabetic CD34$^+$ cells restores their function in vitro. We next determined whether gene modifications that cause CD34$^+$ cells to express Ang-(1-7) could restore the reparative function of diabetic cells. CD34$^+$ cells were transduced with lentiviral vector carrying the Ang-(1-7) fusion transgene [lenti-Ang-(1-7)] or a control vector (lenti-control). Transduction with lenti–enhanced green fluorescent protein (eGFP) resulted in ~40% of cells expressing eGFP within 48 h, as confirmed by fluorescence microscopy or flow cytometry (Supplementary Fig. 6), which is consistent with previous observations (29). Ang-(1-7) expression in CD34$^+$ cells was measured in medium conditioned with 100,000 cells for 10 h and found to be 3 ng/mL (3.33 nM) (Fig. 6*A*), which is similar to levels produced by healthy nondiabetic cells (see above). Ang-(1-7) was not detected in the preconditioned medium of untransduced or lenti-control–transduced diabetic cells (Fig. 6*A*). Ang-(1-7)–modified diabetic cells demonstrated robust cGMP production in response to SDF, which was sensitive to blockade by L-NAME (Fig. 6*B*). The migration of Ang-(1-7)–expressing cells to SDF was similar to that observed for nondiabetic cells (Fig. 6*C*). These observations confirm that lentiviral expression of Ang-(1-7) in the dysfunctional diabetic CD34$^+$ cells restores their function in vitro that likely reflects their in vivo vasoreparative potential.

To test the in vivo vasoreparative function of Ang-(1-7), CD34$^+$ cells were infected with either lenti-Ang-(1-7) or lenti-control. Modified cells were evaluated using a mouse model of I/R injury that recapitulates many features of diabetic retinopathy, including the presence of acellular capillaries. Cells were injected intravitreally into injured eyes, and the homing of cells to areas of injury, a direct indicator of the in vivo migration of cells, was expressed as percent of the total vascular area. As shown in Fig. 7*A*, *i* and *ii*, after I/R injury, mouse retinas showed evidence of injured vasculature with markedly attenuated vessels and poor staining with rhodamine-labeled lectin compared with the vessels of uninjured retinas. The I/R retinas that received nondiabetic CD34$^+$ cells exhibited 52 ± 8% incorporation of cells into the injured vascular structures (Fig. 7*A*, *iii*). Nondiabetic cells transduced with lenti-control or lenti-Ang-(1-7) showed a similar vascular

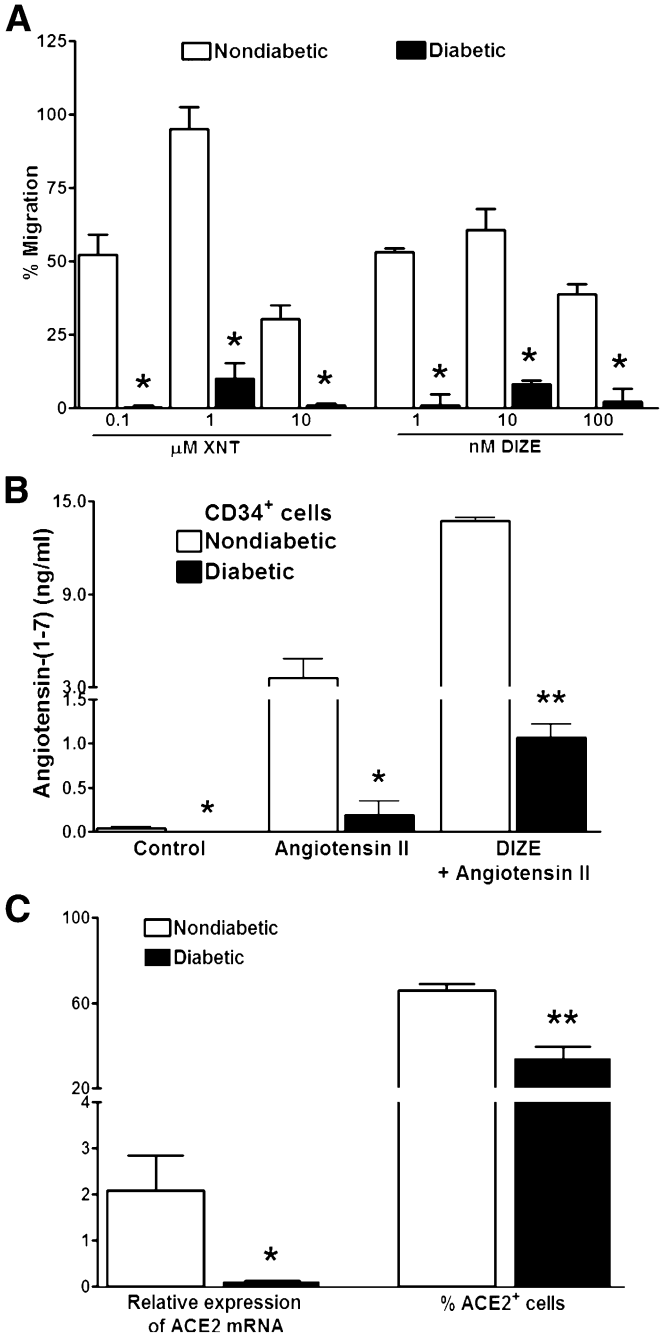

FIG. 5. Diabetic CD34$^+$ cells have impaired ACE2-dependent function. *A*: ACE2 activators, XNT and DIZE, at three different concentrations produced migration in CD34$^+$ cells of nondiabetic origin. This function is impaired in CD34$^+$ cells of diabetic origin (*P < 0.0001 at all three concentrations of XNT or DIZE) (control cells: 1 μmol/L vs. 0.1 μmol/L XNT, P < 0.05; 1 nmol/L vs. 10 nmol/L DIZE, not significant). *B*: The production of Ang-(1-7) was lower in diabetic cells. Both basal release and the release in the presence of angiotensin-II were lower in diabetic cells (*P < 0.001). In the presence of 10 nmol/L DIZE and angiotensin-II, the release of Ang-(1-7) was lower in diabetic cells (P < 0.0001). *C*: Expression of ACE2 mRNA and the percentage of Lin$^-$CD45^{dim}CD34$^+$ cells expressing ACE2 were decreased in diabetic compared with nondiabetic cells (*P < 0.02, n = 8; **P < 0.001, n = 12).**

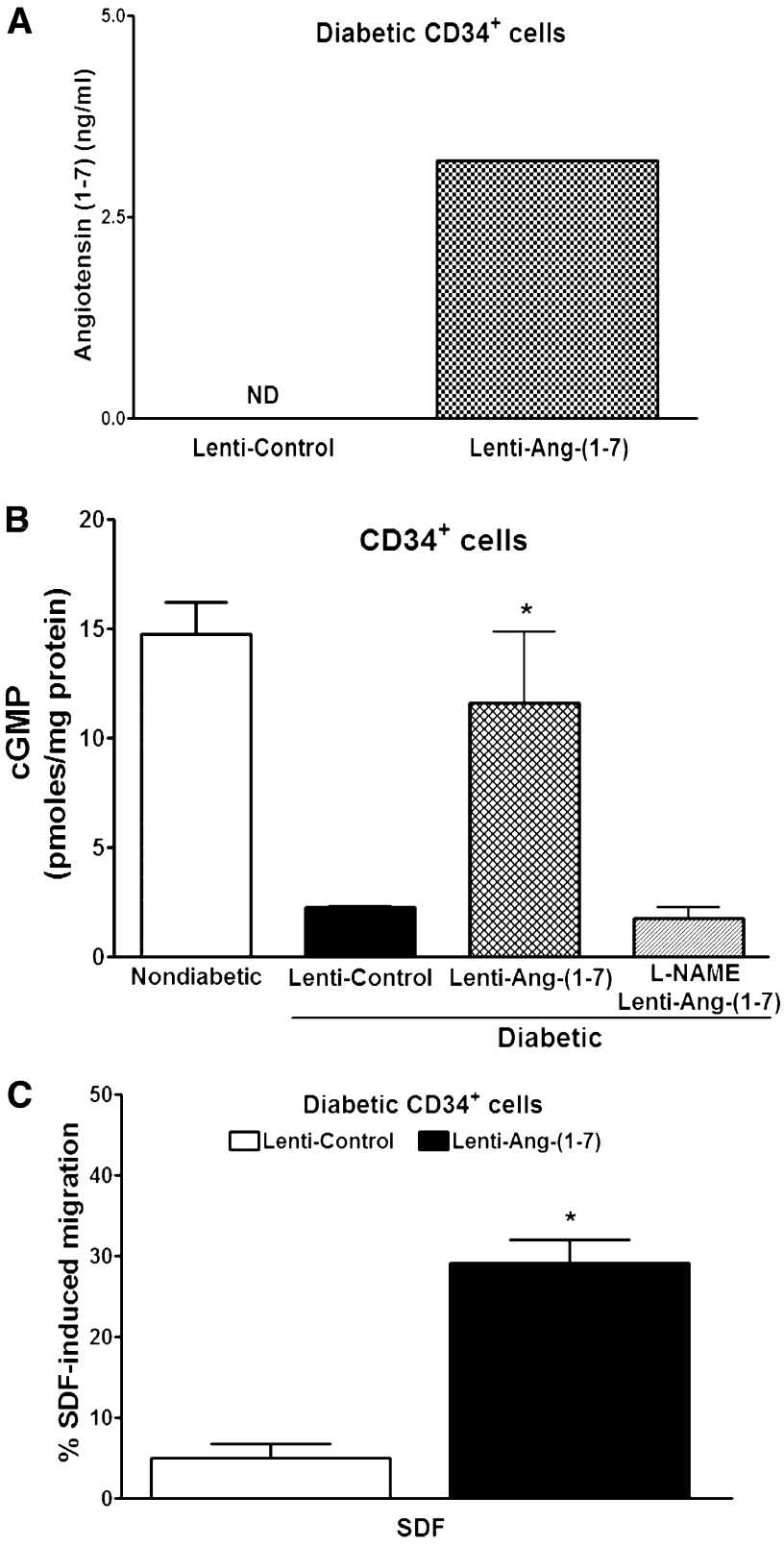

FIG. 6. Lentiviral Ang-(1-7) expression in the dysfunctional diabetic CD34$^+$ cells restores their function in vitro. *A*: Release of Ang-(1-7) by diabetic CD34$^+$ cells 72 h after lentiviral Ang-(1-7) gene modification. Ang-(1-7) release was not detectable (ND) in cells that were modified by control lentivirus. *B*: cGMP production in response to 100 nM SDF treatment was increased in diabetic cells modified with lenti-Ang-(1-7) compared with lenti-control cells (*$P < 0.05$, $n = 4$). Pretreatment with L-NAME decreased cGMP production (*$P < 0.05$, $n = 4$). *C*: Migration to 100 nM SDF is enhanced in diabetic CD34$^+$ cells by lentiviral Ang-(1-7) expression compared with the diabetic cells modified by control lentivirus ($P < 0.05$).

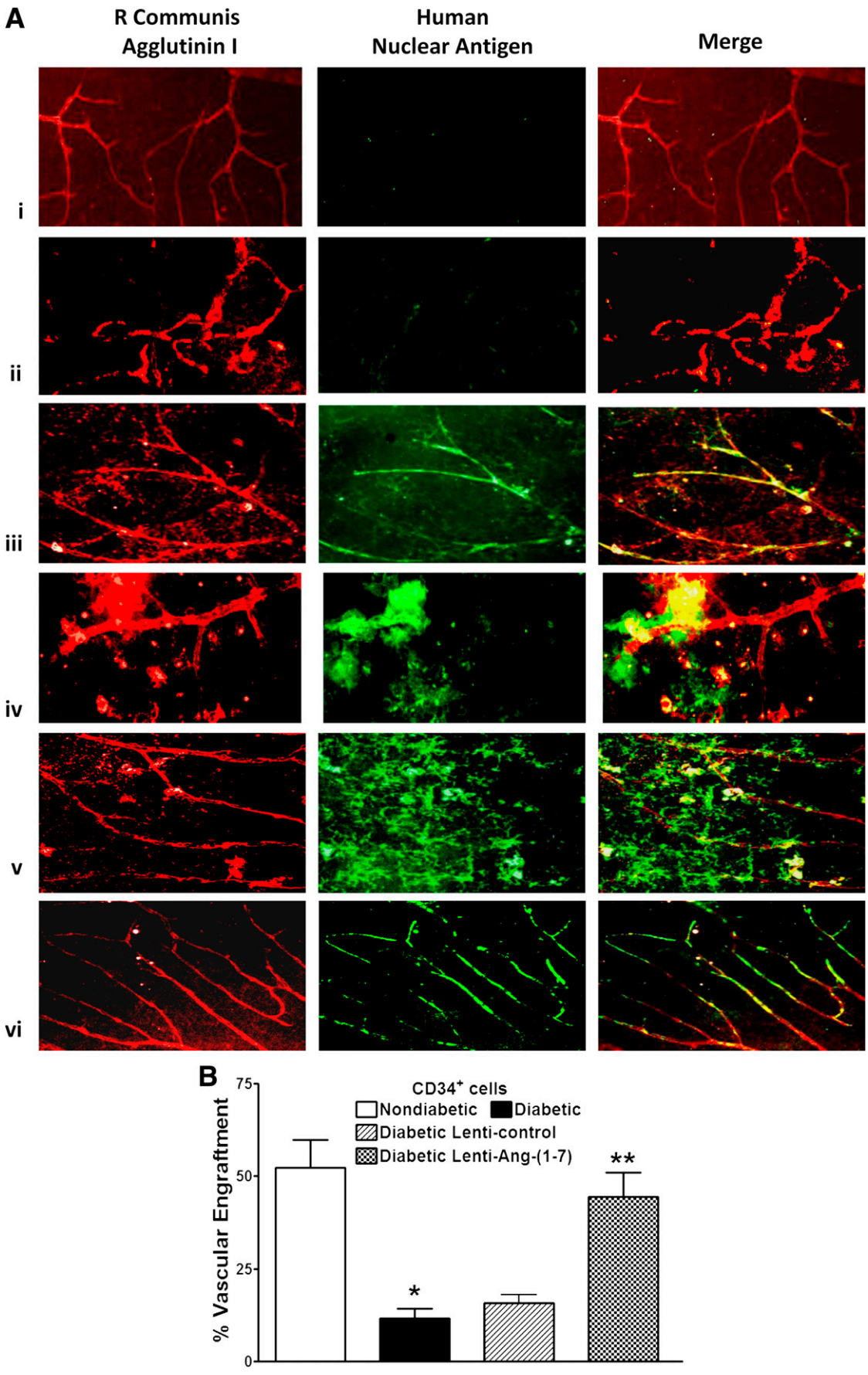

FIG. 7. Ang-(1-7) gene expression restores vasoreparative function in diabetic CD34+ cells in a mouse retinal I/R injury model. *A*: Representative images (original magnification ×10). *i*: Healthy vasculature (red) from retinas obtained from uninjured eyes that were not treated with cells. *ii*: Retinas obtained from eyes subjected to I/R injury but not injected with cells, showing injured/degenerate vessels. *iii*: Retinas obtained from eyes

engraftment potential compared with untreated cells (Supplementary Fig. 7). Previously, we showed that cells of diabetic origin display markedly reduced homing to areas of injury and markedly less incorporation. Diabetic cells form aggregates on the surface of the vitreous and do not associate with the retinal vasculature (3). In agreement with our previous findings, diabetic cells showed a marked impairment, with only 7 ± 3% vascular engraftment ($P < 0.001$, compared with nondiabetic cells) (Fig. 7*A*, *iv*). Lenti-control transduction of diabetic cells did not improve the homing efficiency of diabetic cells (15 ± 2%; $P < 0.001$ vs. nondiabetic cells) (Fig. 7*A*, *v*). In marked contrast, Ang-(1-7) gene modification by lenti-Ang-(1-7) transduction of diabetic cells resulted in significantly higher incorporation of cells into the injured vascular structures (44 ± 7%; $P < 0.0001$ vs. untreated diabetic cells) (Fig. 7*A*, *vi*), which was comparable to that observed for nondiabetic cells.

DISCUSSION

This study reports several novel observations, including the first demonstration of the protective role of the ACE2/Ang-(1-7)/Mas receptor pathway activation in diabetic $CD34^+$ cells. The maintained activation of this key signaling pathway in diabetic $CD34^+$ cells was associated with protection from the development of microvascular complications. Interestingly, by history and physical examination, the patients also appeared to be free of macrovascular complications. Expression of Ang-(1-7) in diabetic $CD34^+$ cells restored the vasoreparative function of these cells in a Mas receptor–dependent manner. This suggests that ex vivo Ang-(1-7) overexpression represents a promising approach for correcting vasoreparative dysfunction in $CD34^+$ cells from diabetic patients to facilitate the use of autologous cells as cell therapy in these patients.

Gene expression profiling of $CD34^+$ cells from diabetic patients with no complications despite poor glycemic control provided molecular insights into the novel signaling mechanisms contributing to the unique vasoprotective functions of these cells, and supporting that activation of the ACE2/Ang-(1-7)/Mas receptor pathway may have high clinical significance. Consistent with this, our study shows that either exogenous treatment or endogenous expression of Ang-(1-7) restored the vasoreparative functions of dysfunctional diabetic cells by activating Mas receptor and the NO, ROS, and PI3K/Akt signaling pathways.

Our data support the contention that restoration of vasoreparative function in dysfunctional diabetic cells by Ang-(1-7) is the result of both decreased ROS production by NADPH oxidase and increased bioavailability of NO. This leads to improvement in in vitro migration of diabetic $CD34^+$ cells and their enhanced homing to areas of vascular injury in vivo. Furthermore, we showed that ACE2 expression and Ang-(1-7) release were significantly reduced in $CD34^+$ cells from diabetic patients with microvascular complications.

$CD34^+$ cells of individuals with longstanding diabetes (>40 years duration and with proliferative diabetic retinopathy) (Fig. 4*A*, D +MV) showed higher ACE2 mRNA than $CD34^+$ cells from individuals with a shorter duration of diabetes (~12 years and less severe complications) (Fig. 5*C*). This may represent compensation in cells from longstanding patients with diabetes. Although direct comparisons between humans and rodents cannot be made, the increase in ACE2 mRNA in the $CD34^+$ cells of individuals with advanced complications is consistent with our previous study (30) that shows an increase in ACE2 expression in the hearts of Akita mice. In this rodent study, we suggest that the upregulation of ACE2 is supportive of compensatory mechanisms, activating the protective arm of RAS and increasing ACE2 expression activity, which could help minimize vascular complications in states of insulin deficiency. Our gene array study showed that the protective arm of RAS was robustly activated in diabetic individuals resistant to the development of vascular complications, despite poor glycemic control (Fig. 4*A*, D −MV).

NO is an important factor for the migratory function of progenitor cells to areas of ischemia, and increasing eNOS expression enhances the reparative function of healthy BM-derived progenitor cells (10). However, simply increasing eNOS expression without correcting the highly oxidative environment of diabetes further increases ROS production via formation of peroxynitrite and limits the feasibility of this approach (31). In parallel studies, we observed that the blockade of NADPH oxidase in diabetic $CD34^+$ cells restores their vasoreparative function by enhancing NO bioavailability (12). Therefore, decreasing oxidative stress by normalizing ROS production is essential for the success of cell-based therapies that depend on the restoration of adequate levels of bioavailable NO. The current study used two unique properties of Ang-(1-7), the ability to decrease ROS production and to stimulate NO release, and provides the proof-of-concept for the therapeutic use of ex vivo Ang-(1-7)–modified $CD34^+$ cells as a novel strategy for correcting diabetic vascular dysfunction.

This study also identifies defective ACE2 signaling in patients with vascular complications. However, interestingly, despite low levels of ACE2, Mas receptor expression was unaltered and its functional coupling to eNOS activation, NADPH inhibition, and PI3K/Akt activation were intact, thereby making the dysfunctional diabetic cells responsive to Ang-(1-7).

We observed decreased ACE2 expression, decreased production of Ang-(1-7), reduced migration, and reduced NO bioavailability by the ACE2 activators XNT or DIZE in the cohort of patients with complications. These results suggest that short-term use of ACE2 activators might be ineffective in correcting diabetic EPC dysfunction in patients; however, the therapeutic benefit of a long-term treatment with ACE2 activators cannot be ruled out. Importantly, patients protected from complications have maintained ACE2 expression in the presence of hyperglycemia, which would suggest that ACE2 overexpression

subjected to I/R injury and injected with nondiabetic $CD34^+$ cells showing a high degree of vascular incorporation ($n = 6$). *iv*: I/R-injured retinas injected with diabetic $CD34^+$ cells did not show clear vascular incorporation; instead clumps of green/red fluorescence were observed ($n = 6$). *v*: I/R-injured retinas injected with lenti-control–treated diabetic $CD34^+$ cells did not show clear vascular incorporation; instead clumps of green/red fluorescence were observed ($n = 4$). *vi*: I/R-injured retinas injected with lenti-Ang-(1-7) diabetic $CD34^+$ cells showed incorporation of cells into degenerate vasculature ($n = 6$). *B*: Quantification of the vascular engraftment of $CD34^+$ cells of nondiabetic and diabetic origin, with or without lentiviral gene modification. Vascular incorporation of diabetic cells was lower than nondiabetic cells (*$P < 0.001$). Vascular engraftment of diabetic cells was increased by Ang-(1-7) gene transduction ($P < 0.002$) compared with untreated diabetic cells.**

represents an alternative approach for sustained correction of diabetic EPC dysfunction in the presence of persistent hyperglycemia. This unusual feature of expressing either ACE2 or Ang-(1-7) is particularly relevant in that pharmacological or genetic manipulation of CD34$^+$ cells will correct the diabetic EPC dysfunction and enhance the reparative function of the injected cells.

Earlier reports implicated EPCs in the cardiovascular-protective functions of the ACE2/Ang-(1-7) pathway (14–17). Ang-(1-7) enhanced the in vitro proliferation of hematopoietic stem cells and accelerated hematopoietic recovery after myelosuppression in rodent model and clinical studies (32,33). Recently, an elegant study in rodents provided evidence for the enhanced recruitment of c-Kit$^+$ cells, a murine marker of stem/progenitor cells, to the areas of myocardial injury as an underlying mechanism for the Ang-(1-7)–mediated cardiac recovery after experimental cardiac ischemia (34). The current study complements these observations and provides direct evidence for the involvement of Ang-(1-7) in the vasoreparative potential of human EPCs. Our results suggest that Ang-(1-7) represents a viable therapeutic option even for diabetic patients with poor glucose control.

In conclusion, this study identifies the ACE2/Ang-(1-7)/Mas receptor axis as a novel functional signature for the vasoprotective potential of circulating progenitor cells, providing a strong impetus for a paradigm shift in our understanding of the molecular mechanisms of diabetic vascular complications and novel therapeutic strategies.

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Y.P.R.J. designed experiments, researched the data, contributed to discussion, and wrote the manuscript. A.D.B., S.C., S.H., and R.M. researched the data. V.S. provided reagents or technology. D.K. provided clinical samples. A.W.S. contributed to discussion. C.T. and E.M.F. provided reagents or technology and contributed to discussion. M.K.R. and M.B.G. conceived the study idea, contributed to discussion, and critically reviewed and approved the manuscript. M.K.R. and M.B.G. are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis.

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