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Reversal of Bone Marrow Mobilopathy and Enhanced Vascular Repair by Angiotensin-(1-7) in Diabetes

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The angiotensin (ANG)-(1-7)/Mas receptor (MasR) pathway activates vascular repair–relevant functions of bone marrow progenitor cells. We tested the effects of ANG-(1-7) on mobilization and vasoreparative functions of progenitor cells that are impaired in diabetes. The study was performed in streptozotocin-induced diabetic (*db/db*) mice. Diabetes resulted in a decreased number of Lineage$^-$Sca-1$^+$c-Kit$^+$ (LSK) cells in the circulation, which was normalized by ANG-(1-7). Diabetes-induced depletion of LSK cells in the bone marrow was reversed by ANG-(1-7). ρ-Kinase (ROCK) activity was increased specifically in bone marrow LSK cells by ANG-(1-7) in diabetes, and the beneficial effects of ANG-(1-7) were prevented by fasudil. ANG-(1-7) increased Slit3 levels in the bone marrow supernatants, which activated ROCK in LSK cells and sensitized them for stromal-derived factor-1α (SDF)–induced migration. Diabetes prevented the mobilization of LSK cells in response to ischemia and impaired the recovery of blood flow, both of which were reversed by ANG-(1-7) in both models of diabetes. Genetic ablation of MasR prevented ischemia-induced mobilization of LSK cells and impaired blood flow recovery, which was associated with decreased proliferation and migration of LSK cells in response to SDF or vascular endothelial growth factor. These results suggest that MasR is a promising target for the treatment of diabetic bone marrow mobilopathy and vascular disease.

Diabetes is a strong risk factor for microvascular and macrovascular diseases. Ischemic vascular diseases are by far the leading cause of increased mortality among individuals with diabetes. Endothelial dysfunction and impaired vascularization induced by chronic diabetes accelerate the progression of vascular disease and impair recovery from ischemic injury (1–3). Decreased nitric oxide (NO) availability and increased oxidative stress are widely accepted as major molecular mechanisms that accelerate diabetic vascular disease (4).

Bone marrow stem/progenitor cells (BMPCs) stimulate endothelialization, vascular regeneration, and tissue repair (2,5). BMPCs are mobilized from bone marrow in response to endothelial damage or ischemic injury, home to the areas of damage, and accomplish vascular repair by endothelial transdifferentiation or paracrine functions (6). Hypoxia-regulated factors, stromal-derived factor-1 (SDF), and vascular endothelial growth factor (VEGF) generated by ischemic injury induce mobilization and homing of BMPCs to ischemic regions (7,8).

Currently, cell-based therapies are being tested extensively for the treatment of cardiovascular diseases in several clinical trials (9–11). Individuals with diabetes who have cardiovascular complications are best treated with cell-based therapies; however, this approach is currently not feasible because diabetes causes severe impairment in BMPC mobilization, also known as bone marrow mobilopathy (2,3). The vasoreparative functions of BMPCs derived from individuals with diabetes are impaired, therefore the innate endothelial regeneration and vasculogenesis are diminished (1,12–14). It is now apparent that pharmacological strategies that stimulate mobilization and preserve the vasoreparative functions of BMPCs are ideal for the treatment of diabetic vascular disease or to accelerate the recovery of blood flow and tissue repair after ischemic insult (15,16).

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Angiotensin (ANG)-(1-7) is the cardiovascular protective member of the renin-ANG system, largely generated by ACE2 from the substrate ANG II. Increasing the availability of NO and decreasing oxidative stress via Mas receptor (MasR) largely contribute to its vasoprotective functions (17). Activation of the ACE2/ANG-(1-7)/MasR pathway stimulates vascular repair–relevant functions of BMPCs (18–21). Importantly, ANG-(1-7) reversed bone marrow oxidative stress and increased NO bioavailability in BMPCs in experimental diabetes (22) or $CD34^+$ cells from individuals with diabetes (20). This study tested the hypothesis that ANG-(1-7) reverses bone marrow mobilopathy and accelerates ischemic vascular repair in diabetes in mouse models of type 1 and type 2 diabetes. $Lineage^-Sca\text{-}1^+c\text{-}Kit^+$ (LSK) cells, which are known to be vasculogenic in mice (23,24), were enumerated in basal or ischemic conditions. This cell population has the highest potential of endothelial cell lineage development with vasoreparative functions, whereas LS or LK cells have comparable vasculogenic functions and are mobilized in response to ischemia (21,22). Molecular mechanisms involved in the reversal of mobilopathy by ANG-(1-7) were investigated. Last, the physiological significance of MasR in BMPC mobilization was determined by using MasR knockout (MasR-KO) mice.

RESEARCH DESIGN AND METHODS

All animal studies were approved by the Institutional Animal Care and Use Committee at North Dakota State University. Male C57BL/6N (Harlan Laboratories) and obese-diabetic (*db/db*) mice and their nondiabetic lean-control mice (The Jackson Laboratory) were used in this study. Type 1 diabetes was induced by streptozotocin (STZ) in male C57BL/6N mice (25). Mice were treated with STZ (40 mg/kg body wt i.p.) for 5 consecutive days. Blood glucose and HbA_{1c} levels were determined by test strips (Clarity Advanced) and A1C Now (Bayer), respectively. Mice with diabetes for 20 weeks were used (26). MasR-KO mice were generated by the *trans*-NIH Knock-Out Mouse Project (KOMP) and obtained from the KOMP repository.

Pharmacological Protocols

ANG-(1-7) or the vehicle, saline, were administered by subcutaneous osmotic pumps at the rate of 0.5 or 1 μg/kg/min, as indicated, for 4 weeks. Normal saline-treated mice served as control groups. Where applicable, A779, a MasR antagonist, was administered at the rate of 0.5 μg/kg/min s.c. (osmotic pumps for 4 weeks) (27), or fasudil, a ρ-kinase (ROCK) inhibitor, at a dose of 30 mg/kg i.p. for 14 days (28).

Flow Cytometry

Peripheral blood samples were collected, and white blood cells were obtained after red blood cell (RBC) lysis. Cells were suspended in staining buffer (BioLegend); treated with 0.5 μL of Trustain (BioLegend), followed by fluorescent-conjugated antibodies, Lineage cocktail-FITC, Sca-1-APC, and c-Kit-PE (BioLegend); and were incubated for 45 min at 4°C. Cells were resuspended in PBS, and 7-AAD (BD Pharmingen) was added to detect dead cells. No-stain control samples were prepared in the same way with isotype controls of respective antibodies. Flow cytometry was performed by using the Accuri-C6 System, and data were analyzed according to the gating strategy shown in Supplementary Fig. 1 to enumerate LSK cells (26).

Isolation of LSK Cells

RBC-lysed bone marrow suspension was Lineage depleted by using a negative selection kit, followed by enrichment for $c\text{-}Kit^+$ cells by using positive selection kit (StemCell Technologies) (26). LSK cells were used for proliferation or migration assays.

Colony-forming unit (cfu) assay was performed by using a single-cell suspension of RBC-lysed blood or bone marrow in MethoCult (STEMCELL Technologies) according to the protocol of the manufacturer. Colonies were enumerated as per the manufacturer guidelines.

Hindlimb ischemia (HLI) was performed by ligation and excision of the femoral artery (29). Blood flow was determined by imaging the RBC flux using a laser Doppler imaging system (Moor Instruments, Inc.).

Real-Time PCR was performed by isolating total RNA by using the RNeasy Plus Mini Kit and cDNA was synthesized from 50 ng of RNA using iScript cDNA Synthesis kit (Bio-Rad, Hercules, CA). Real-time PCR was performed by using SYBR Green gene expression assays for the expression of Slit1, Slit2, Slit3, Robo1, Robo2, Robo3, and Robo4 (GeneCopoeia, Rockville, MD) in the Quantitative PCR System (model 7500; Applied Biosystems). Primers are listed in Supplementary Table 1.

Genotyping of MasR-KO mice was performed by using ear-punch samples. PCRs were performed as described above, and the amplification products of MasR, LacZ, and β-actin genes were resolved by gel electrophoresis on 1% agarose.

Data Analysis

Results are expressed as the mean ± SEM. Regarding the number of experiments, *n* indicates the number of mice used in the experimental group. Treatment groups were compared using the Student *t* test or one-way or two-way ANOVA with Bonferroni post-test, as applicable (GraphPad Prism), and were considered significantly different at $P < 0.05$.

RESULTS

ANG-(1-7) Increases Circulating BMPCs That Were Decreased in Diabetes

Consistent with our recent study (26), diabetes resulted in a decreased number of progenitor cells in the circulation in both STZ-diabetic and *db/db* mice compared with their respective controls, as determined by flow cytometric enumeration of LSK cells or by cfu assay (Fig. 1), indicating dysfunctional mobilization of LSK cells from bone marrow (STZ-diabetic vs. control LSK cells $P < 0.01$, and cfus $P < 0.01$; *db/db* vs. lean LSK cells $P < 0.05$, and cfus $P < 0.05$) (Fig. 1*B*, *C*, *E*, and *F*). Treatment with ANG-(1-7)

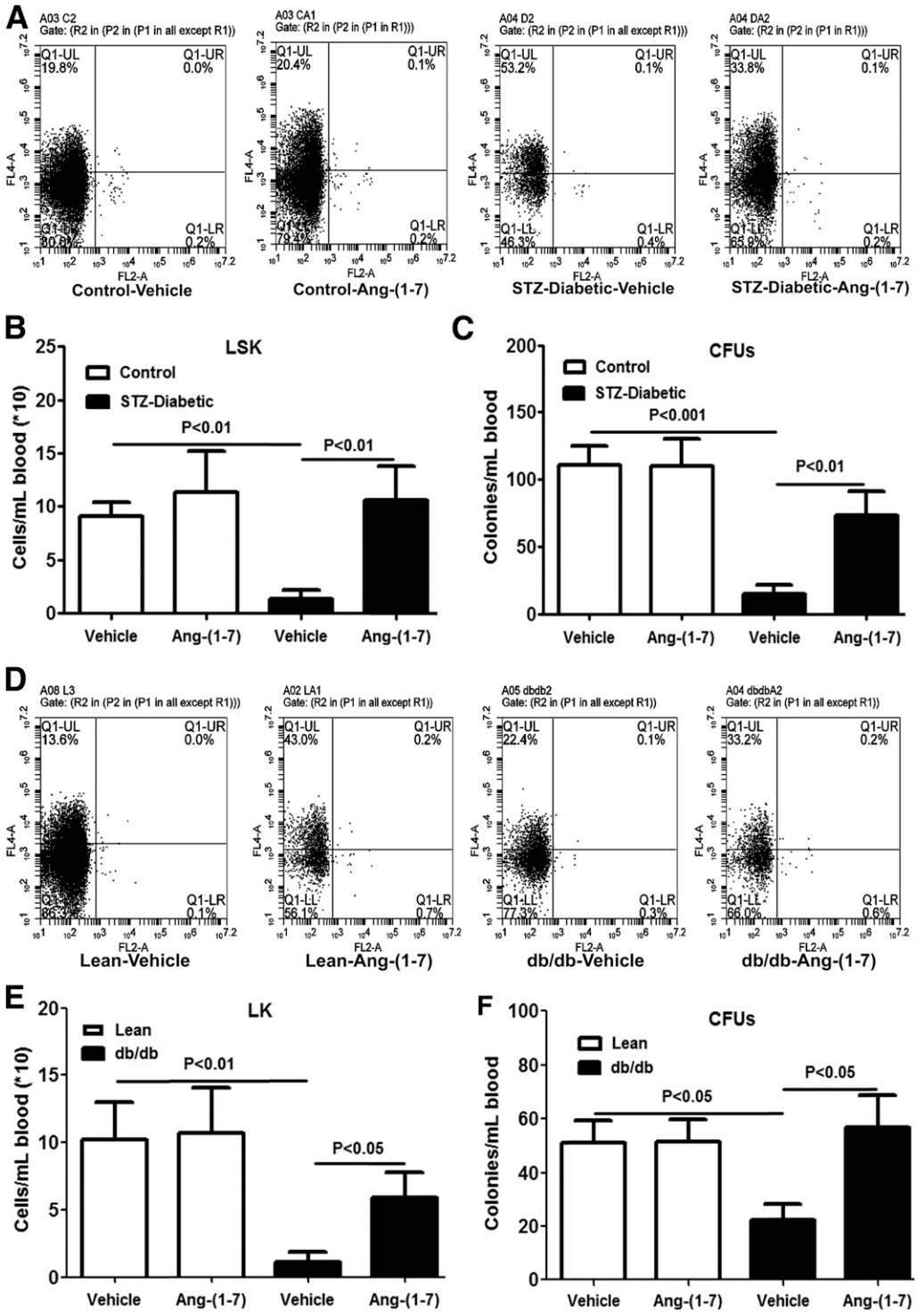

Figure 1—ANG-(1-7) increases the number of LSK cells and cfus in circulation in diabetes. *A* and *D*: Representative dot plots of flow cytometric enumeration of LSK cells in different treatment groups. *B* and *C*: The numbers of circulating LSK cells and cfus were decreased in chronic STZ-diabetic mice (compared with the control group), which were reversed by ANG-(1-7) treatment (n = 6). *E* and *F*: The numbers of circulating LK cells and cfus were decreased in *db/db* mice (compared with lean controls), which were restored to normal levels by ANG-(1-7) treatment (n = 8).

(1 μg/kg/min s.c.) restored the circulating progenitor cells to normal in STZ-diabetes cells ($P < 0.05$ compared with the untreated, n = 6) (Fig. 1*B* and *C*). In *db/db* mice, the number of LSK cells was not increased by ANG-(1-7); however, LK cells and cfus were restored to normal ($P < 0.05$ compared with the untreated, n = 8) (Fig. 1*E* and *F*).

Reversal was also observed in the diabetic decrease in total WBCs or Lineage$^-$ cells (Supplementary Fig. 2) in

both models. ANG-(1-7) at a lower dose (0.5 μg/kg/min) partially reversed diabetic mobilopathy (Supplementary Fig. 3); therefore, the dose of 1 μg/kg/min was used for the rest of the studies. Concurrent administration of A779 prevented the effect of ANG-(1-7) on circulating BMPCs in STZ-diabetic mice (Supplementary Fig. 4). It is important to note that treatment with ANG-(1-7) had no effect on hyperglycemia or HbA_{1c} levels and did not improve glucose tolerance (Supplementary Fig. 5) in both models.

Reversal of Diabetes-Induced Depletion of Bone Marrow–Resident Progenitor Cells by ANG-(1-7)

Then we tested whether diabetes results in the depletion of bone marrow–resident progenitor cells, which represent the BMPC reserve, and determined the effect of ANG-(1-7). The total number of LSK cells was decreased in both models of diabetes, which was reversed by ANG-(1-7) (Fig. 2*A* and *B*). In agreement with these observations, the proliferation of LSK cells derived from diabetic bone marrow was impaired in basal conditions or in response to SDF or VEGF (Fig. 2*C* and *D*). The proliferation of LSK cells derived from ANG-(1-7)–treated diabetic mice was similar to that of the respective nondiabetic controls in basal conditions or in response to SDF or VEGF (Fig. 2*C* and *D*) ($P < 0.05$, STZ-diabetic or *db/db* mice vs. respective controls), suggesting that ANG-(1-7) reverses the depletion of BMPCs induced by diabetes.

ANG-(1-7) Shifts SDF Gradient in Favor of BMPC Mobilization and Restores Migratory Function

SDF gradient is a strong stimulus for the mobilization of BMPCs (7,30). Therefore, we analyzed SDF levels in the circulation and in the respective bone marrow supernatants. STZ-diabetic mice have decreased plasma SDF levels ($P < 0.05$) when compared with the age-matched controls (Fig. 3*A*). Contrary to this, SDF levels were unchanged in bone marrow supernatants from STZ-diabetic mice (Fig. 3*B*). ANG-(1-7) treatment normalized plasma SDF levels in STZ-diabetic mice ($P < 0.05$, vs. STZ-diabetic mice) (Fig. 3*A*). In contrast, no differences were observed in plasma SDF levels (Supplementary Fig. 6) in *db/db* mice compared with lean mice, although the decrease in the number of circulating LSK cells was significant, as shown above. However, in both models of diabetes, the migration of LSK cells in response to SDF was impaired ($P < 0.05$, STZ-diabetic or *db/db* mice vs. respective controls), indicating the reduced sensitivity of LSK cells for mobilization in diabetes. This impairment was reversed by ANG-(1-7) ($P < 0.05$, ANG-(1-7)–treated STZ-diabetic or *db/db* mice compared with the respective untreated group) (Fig. 3*C* and *D*). VEGF was used as a reference stimulus for comparison, and similar impairment was observed in VEGF-induced migration in diabetic cells that was restored by ANG-(1-7) (Fig. 3*C* and *D*). These studies suggest that ANG-(1-7) restores the sensitivity of BMPCs for mobilization by hypoxia-regulated factors (e.g., SDF and VEGF).

Matrix metalloprotease-9 plays an important role in the bone marrow egress of stem/progenitor cells via releasing kit-ligand (31). In the current study, no changes were observed in bone marrow matrix metalloprotease-9 levels in STZ-diabetes mice, and those levels were not affected by ANG-(1-7) (Supplementary Fig. 7).

ROCK Mediates ANG-(1-7)–Induced Sensitization of BMPCs for Mobilization in Diabetes

ROCK is an important mediator of several cellular functions in stem/progenitor cells including proliferation and migration (32,33) while producing detrimental effects in cardiovascular tissues (34,35). ANG-(1-7) treatment increased the ROCK activity in LSK cells derived from STZ-diabetic or *db/db* mice ($P < 0.05$, vs. untreated STZ-diabetic or *db/db* mice, respectively) (Fig. 4*A* and *B*). Interestingly, we observed no change in the ROCK activity in either heart or aorta (Supplementary Fig. 8), which indicates that the effect of ANG-(1-7) on ROCK is specific for BMPCs. Then, we tested whether pharmacological inhibition of ROCK prevents the beneficial effect of ANG-(1-7) on the circulating LSK cells in STZ-diabetes mice. Fasudil was administered for 2 weeks, starting on day 14 of the ANG-(1-7) treatment. ANG-(1-7) reversed the diabetes-induced decrease in the number of circulating LSK cells, as observed above, and this reversal was abolished in mice that were concurrently treated with fasudil (Fig. 4*C* and *D*). No changes were observed in mice treated only with fasudil (Fig. 4*C*). In agreement with this and our previous studies in human $CD34^+$ cells (19), SDF- or VEGF-induced migration of LSK cells in vitro was attenuated by Y-27632, a ROCK inhibitor ($P < 0.05$, vs. SDF) (Fig. 4*E*).

ANG-(1-7) Stimulates Slit3 Secretion From Bone Marrow Mesenchymal Cells, Which in Turn Activates ROCK in BMPCs

Slits belong to a family of secreted glycoproteins that consists of highly homologous Slit1, Slit2, and Slit3. They mediate diverse cellular functions by acting on the roundabout (Robo) family of receptors in different cell types, including stem/progenitor cells (36,37). Slit3 was shown to be secreted by bone marrow mesenchymal cells (BM-MSCs), which activate angiogenic signaling (36,38) partly by modulating ROCK activity (36). We have identified mRNA transcripts for Slit1, Slit2, and Slit3 in BM-MSCs and Robos (Robo1, Robo3, and Robo4) in mouse LSK cells (Supplementary Fig. 9). Among the three Slit homologs, Slit3 was shown to stimulate migration in endothelial cells by activating ROCK (38); therefore, we hypothesized that Slit3 is involved in ANG-(1-7)–mediated ROCK activation in mouse LSK cells. ANG-(1-7) treatment resulted in increased Slit3 levels in the bone marrow supernatants derived from control mice, and to a larger extent in STZ-diabetic mice, compared with their respective untreated groups (Fig. 5*A*). Diabetes did not alter the bone marrow Slit3 levels.

We further tested whether ANG-(1-7)–induced Slit3 is derived from BM-MSCs. Monolayers of BM-MSCs were cultured from the bone marrow and treated with ANG-(1-7) (100 nmol/L). Cell supernatants were analyzed for Slit3 levels. BM-MSCs constitutively secreted Slit3, which

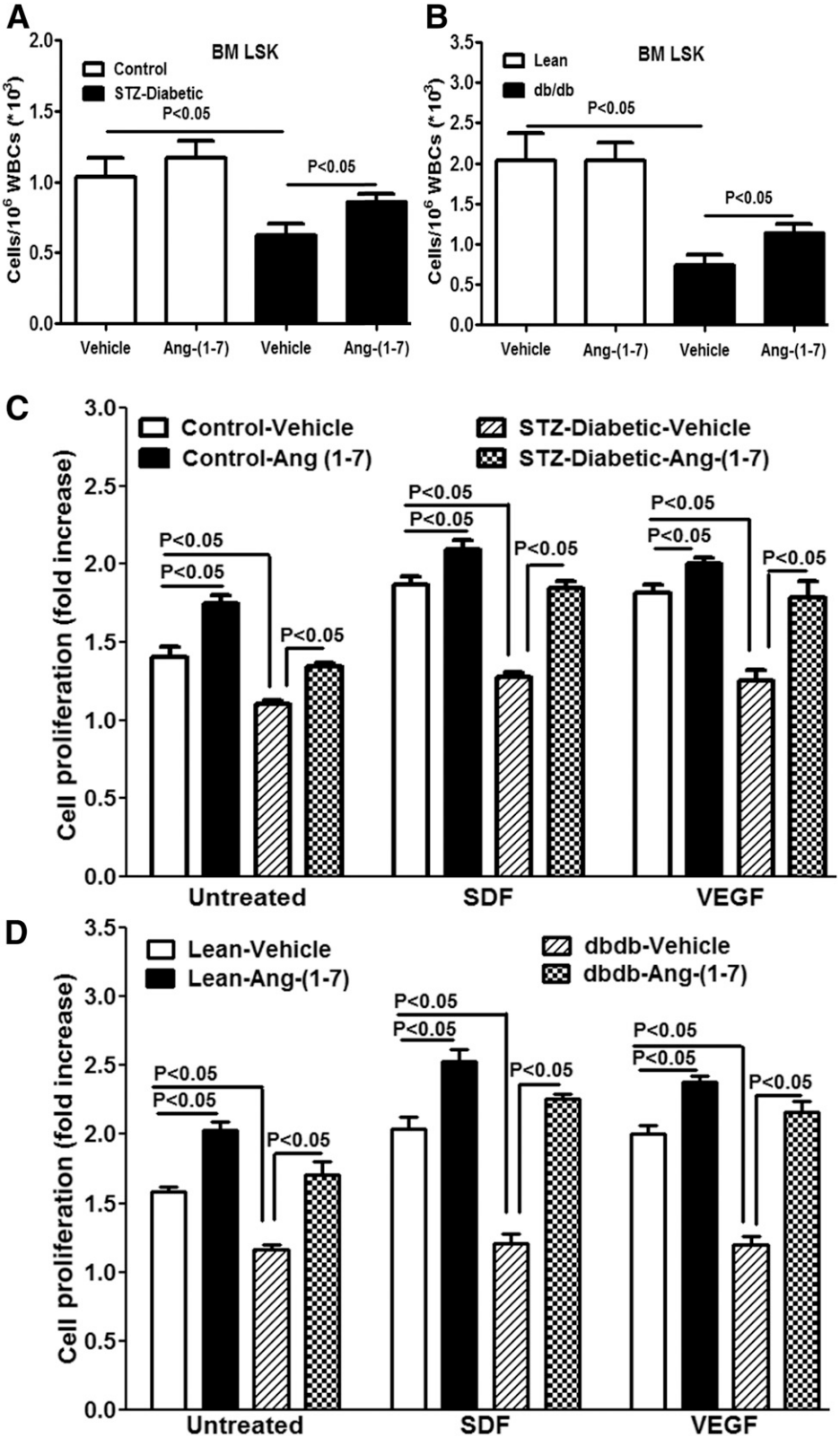

Figure 2—Reversal of diabetes-induced depletion of bone marrow (BM)–resident LSK cells and impairment of proliferation by ANG-(1-7) treatment. *A* and *B*: The number of BM LSK cells was decreased in STZ-diabetic or *db/db* mice, which was reversed by ANG-(1-7) treatment (n = 6–8). *C* and *D*: The proliferation of cells was determined using a BrdU proliferation assay kit (Roche Bioscience) by using 10,000 cells/well in RPMI 1640 medium for 48 h with or without drug treatments. Proliferation was expressed as a fold increase relative to the effect of mitomycin (1 μmol/L), an inhibitor of proliferation. LSK cells derived from STZ or *db/db* mice showed decreased proliferation in basal conditions or in response to SDF or VEGF. Proliferation was restored to normal in cells derived from ANG-(1-7)–treated diabetic mice (n = 6). WBCs, white blood cells.

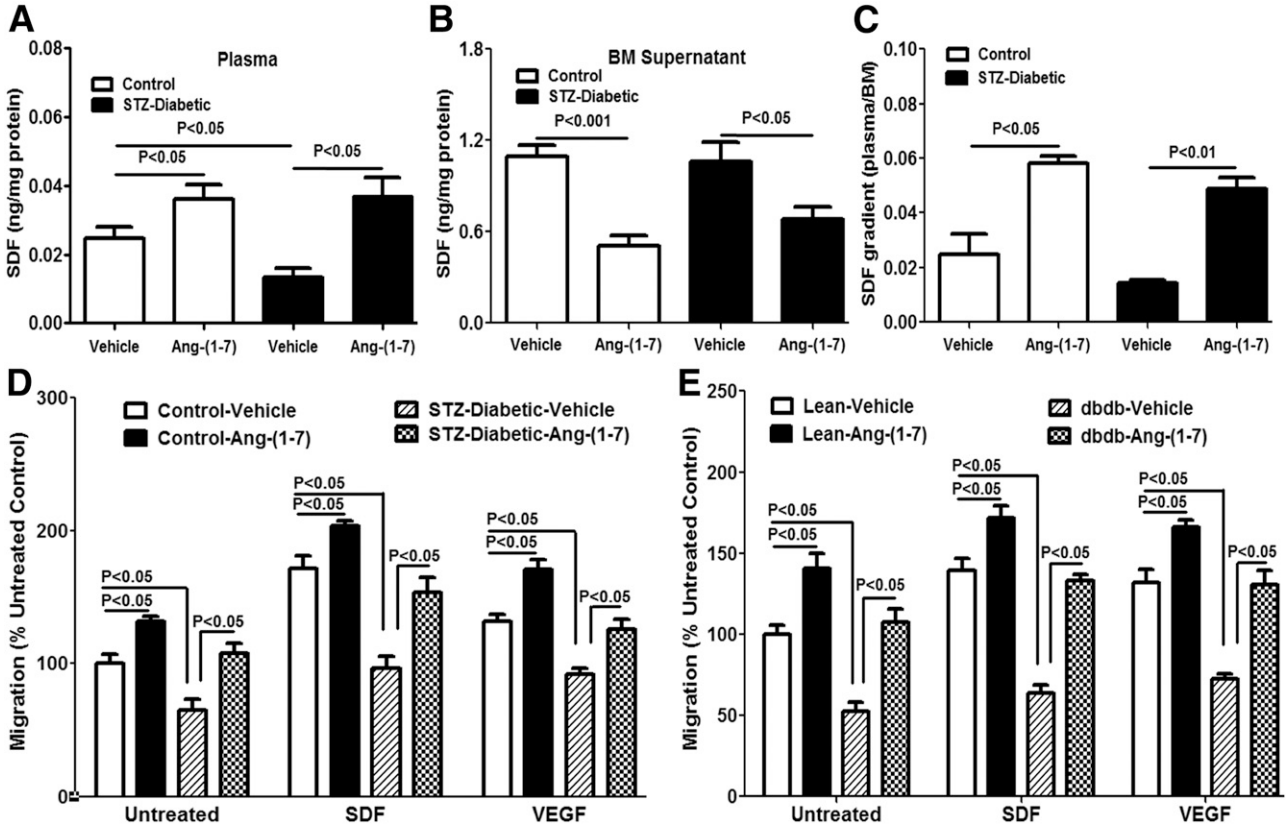

Figure 3—ANG-(1-7) shifts the SDF gradient across the blood-bone marrow (BM) interface in favor of BMPC mobilization. SDF was quantified by ELISA (R&D Systems). *A*: Plasma SDF levels decreased in STZ-diabetic mice and ANG-(1-7) treatment restored these levels (n = 5–7). *B*: BM supernatants were obtained by flushing the BM with sterile PBS and centrifugation at 1,200 rpm for 10 min at 4°C. SDF levels were decreased in the BM supernatants of STZ-diabetic mice after ANG-(1-7) treatment (n = 5–7). *C*: Plasma-to-BM ratio of SDF levels was decreased in diabetes, which was restored by ANG-(1-7) treatment. *D* and *E*: The migration of cells was evaluated using the QCM Chemotaxis Assay Kit (EMD Millipore) by using 20,000 cells/well in Hanks' balanced salt solution. Each sample was tested in duplicate with and without treatments, and the migration of cells was allowed for 5 h before evaluation. Responses were determined in arbitrary fluorescence units and expressed as the percentage increase over untreated controls of the respective group. Basal, SDF-induced, or VEGF-induced migration of LSK cells was decreased in STZ-diabetic or *db/db* mice, and this dysfunction was reversed by ANG-(1-7) treatment (n = 6).

was augmented by ANG-(1-7) ($P < 0.05$) (Fig. 5*B*). Then, we tested whether Slit3 treatment would sensitize LSK mobilization to SDF or VEGF, which was tested by the migration assay in vitro. Slit3 itself did not induce migration in LSK cells; however, it potentiated SDF- or VEGF-induced migration in a concentration-dependent fashion (Figs. 5*C*). Importantly, Slit3 stimulated ROCK activity in LSK cells in vitro (Fig. 5*D*). At a concentration of 400 ng/mL, Slit3-mediated ROCK activity is indeed higher than that induced by a known activator of ROCK, lysophosphatidic acid (3 nmol/L) (39). These results suggest that ANG-(1-7) causes sensitization of LSK cells for mobilization to SDF or VEGF gradients by stimulating Slit3 secretion by MSCs, which in turn activates ROCK in LSK cells and thus restores mobilization in diabetes.

Impaired Mobilization of BMPCs by Ischemic Injury in Diabetes Is Reversed by ANG-(1-7)

Ischemic injury is a strong stimulus for the mobilization of LSK cells, which migrate to the areas of ischemia, accelerate vascularization, and restore blood flow (40,41). This innate protective reparative function is severely impaired in diabetes (1,42). Therefore, we sought to determine whether ANG-(1-7) treatment causes the reversal of bone marrow mobilopathy in response to ischemia and accelerates vascular repair in diabetes. This was tested in diabetic mice undergoing HLI. In response to ischemia, control mice showed the highest mobilization of LSK cells on day 2 after HLI and remained higher until day 7; on day 10, the number of cells returned to the preischemic levels (Fig. 6*B*). ANG-(1-7) treatment increased the peak mobilization by twofold on day 2 after HLI ($P < 0.001$, n = 6) in control mice compared with the untreated mice. In STZ-diabetic mice, the mobilization of LSK cells in response to ischemia is especially blunted on days 2, 3, 5, and 7 after HLI, which is reflected in their respective areas under the curve ($P < 0.05$ compared with the control) (Fig. 6*B* and *C*). ANG-(1-7) treatment reversed this dysfunction and restored the peak mobilization that appeared on day 3 postischemia ($P < 0.001$) (Fig. 6*B*).

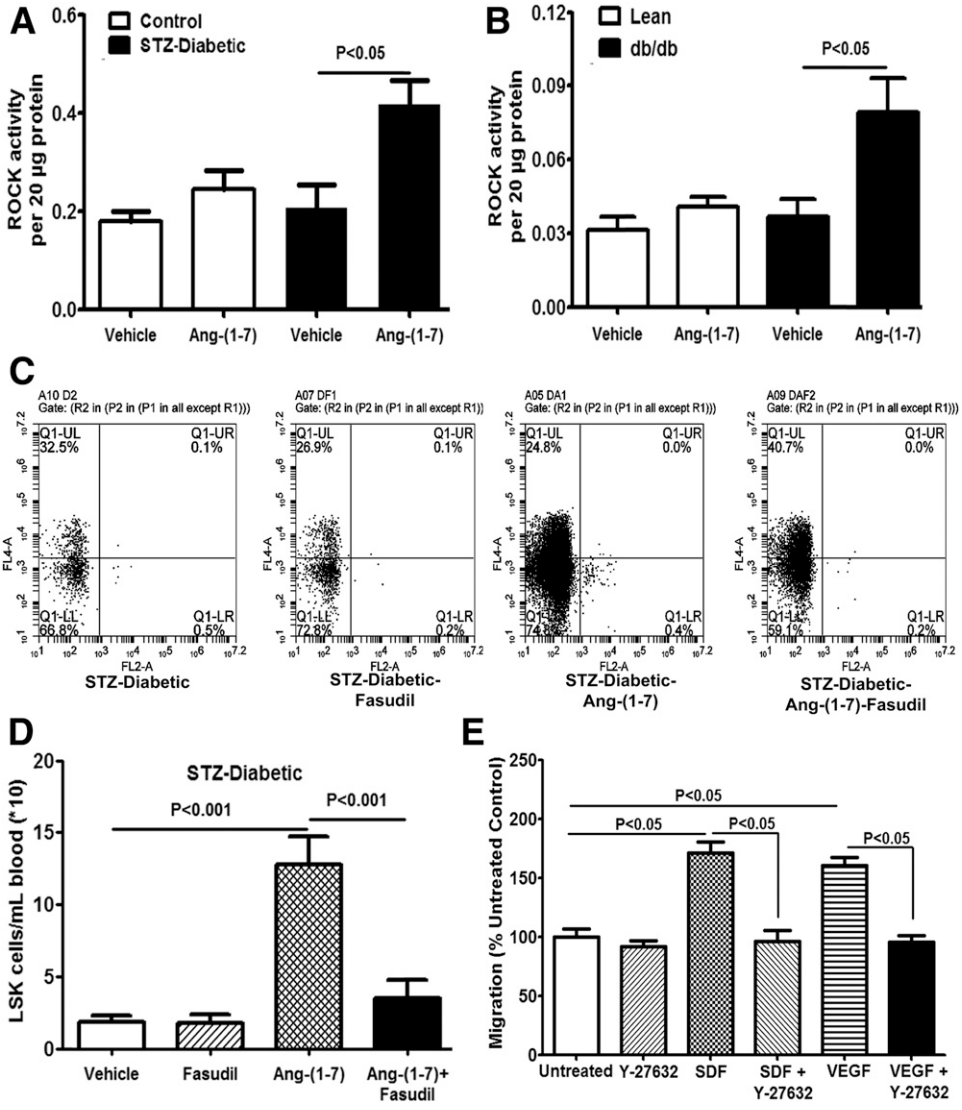

Figure 4—ROCK mediates ANG-(1-7)–induced mobilization of BMPCs in diabetes. ROCK activity was determined using an assay kit (Cyclex) and was expressed as Y-27632–sensitive activity units. *A* and *B*: ANG-(1-7) treatment increased ROCK activity in LSK cells derived from STZ-diabetic or *db*/*db* mice compared with their respective untreated group (n = 5–7). *C*: Representative flow cytometric dot plots of LSK cells in different treatment groups. *D*: The increase in the number of circulating LSK cells by ANG-(1-7) treatment in STZ-diabetic mice was blunted by fasudil, a ROCK inhibitor (n = 6). *E*: SDF- or VEGF-induced migration of LSK cells was blunted by Y-27632, a ROCK inhibitor. Y-27632 did not modify migratory response on its own (n = 5).

In lean control mice, the pattern of mobilization responses was different, and the maximum fold mobilization observed was lower compared with that of C57BL/6 control mice, as observed above. Lean mice responded to ischemic injury by mobilizing LSK cells on day 2, which was increased by ANG-(1-7) treatment (Fig. 6*E* and *F*). In contrast to C57BL/6 mice, the number of LSK cells returned to preischemic levels by day 3. However, in agreement with STZ-diabetic mice, ischemic injury in *db*/*db* mice did not mobilize LSK cells, and treatment with ANG-(1-7) restored mobilization in response to ischemia on day 2, which reached the maximum level on day 3 and returned to preischemic levels by day 7.

ANG-(1-7) Restores Vascularization and Blood Flow Recovery to Ischemic Areas in Diabetes

In control mice, the recovery of blood flow to the ischemic limb after HLI was 94 ± 2% by day 14, which continued to increase and stabilized at 104 ± 2% on day 21 (Fig. 6*A*). In STZ-diabetic mice, blood flow recovery was slower and lower than that of control mice (44 ± 4% on day 10, $P < 0.001$, n = 6; and 66 ± 10% on day 21, $P < 0.01$, n = 6) (Fig. 7*B*). The occurrence of partial or whole foot amputation was 20% in the diabetic group. Treatment with ANG-(1-7) accelerated blood flow recovery in STZ-diabetic mice, and the flux of blood observed at days 10 and 21 was 81 ± 5% and 102 ± 7% ($P < 0.001$ and $P < 0.05$ vs. untreated STZ-diabetic), respectively, and completely normalized at

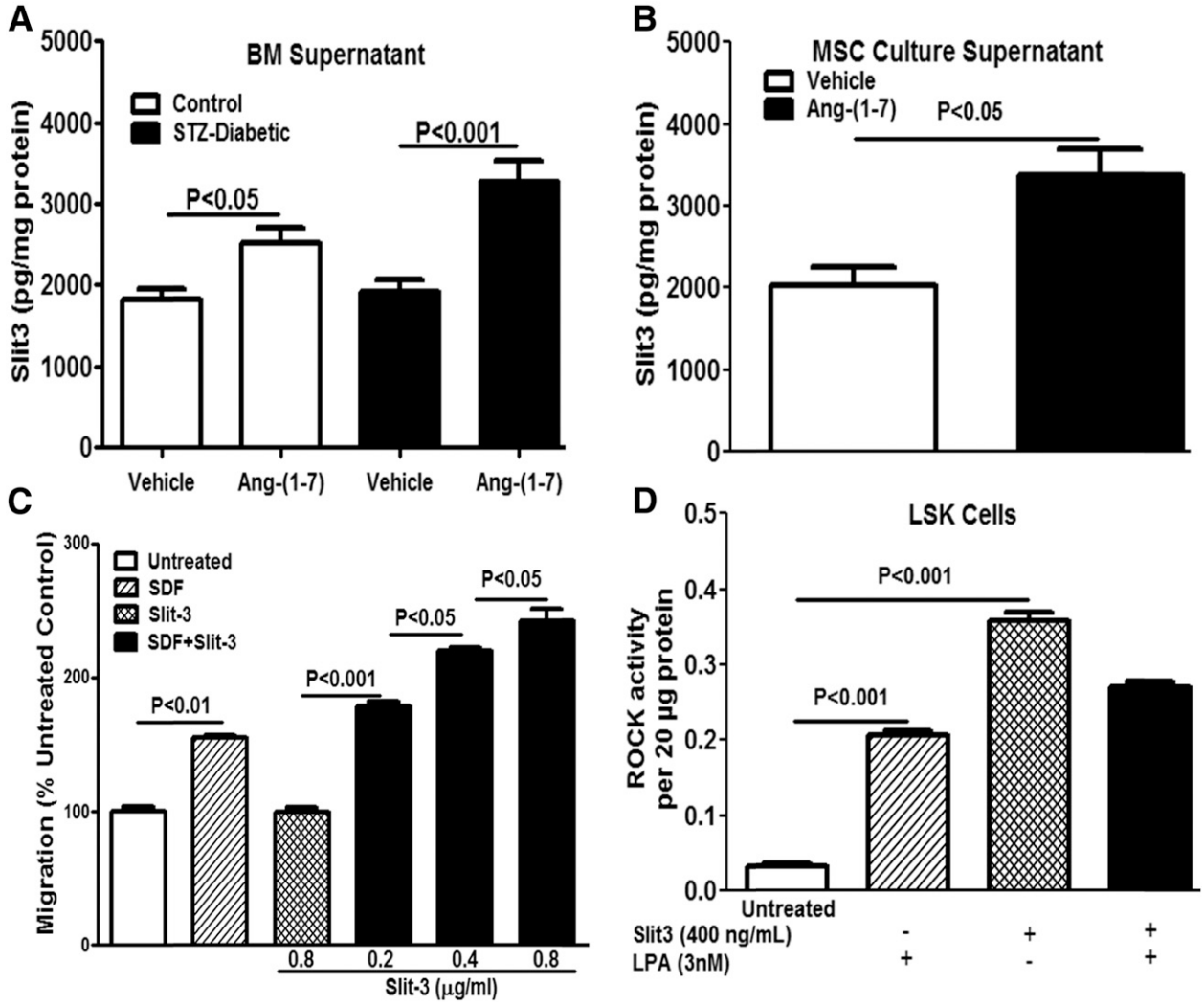

Figure 5—ANG-(1-7) stimulates Slit3 release from MSCs, which in turn activates ROCK in LSK cells. BM-MSCs were obtained by plating a single-cell suspension of bone marrow (BM), after RBC lysis, in RPMI 1640 media with 15% FBS and penicillin streptomycin (100 international units/mL penicillin and 100 μg/mL streptomycin) in 24-well culture plates at a density of 4×10^6 cells/cm^2. Plates were incubated at 37°C with 5% CO_2. Media containing nonadherent cells were discarded after 24 h and replaced with fresh medium and weekly thereafter. Adherent cells were cultured for at least 2 weeks before experimentation. Slit3 levels were determined by ELISA (Biomatik). *A*: In vivo treatment with ANG-(1-7) increased Slit3 levels in BM supernatants in control and STZ-diabetic mice (n = 5–7). *B*: In cultured BM-MSCs, ANG-(1-7) stimulated Slit3 release (n = 4). *C*: Slit3 did not induce the migration of LSK cells but potentiated SDF-induced migration in a concentration-dependent manner (n = 4). *D*: Slit3 stimulated ROCK activity in LSK cells (n = 5). Lysophosphatidic acid (LPA) was used as positive control (n = 3).

day 21. Importantly, no evidence for amputation was observed in ANG-(1-7)–treated diabetic mice. In agreement with these findings, tissue necrosis in the ischemic skeletal muscle was higher in diabetic mice compared with control mice, and the necrosis was remarkably lower in diabetic mice that had received ANG-(1-7) treatment (Fig. 7*C*). Furthermore, the vascularization of ischemic areas is impaired in diabetic mice, as determined by the staining of vasculature with isolectin-B4 and endothelial cells by CD31 ($P < 0.001$, n = 6, compared with control mice) (Fig. 7*C* and *F*). ANG-(1-7) treatment increased the capillary density in the ischemic limbs of diabetic mice (Fig. 7*C* and *F*).

Along similar lines, blood flow recovery after HLI was lower in *db/db* mice compared with lean control mice after HLI (day 10: 55 ± 6% vs. 101 ± 5% in lean mice, $P < 0.001$; day 21: 68 ± 2% vs. 105 ± 6% in lean mice, $P < 0.001$ n = 6). After ANG-(1-7) treatment, blood flow recovery was higher compared with that in the untreated *db/db* mice (87 ± 5% $P < 0.05$, n = 6), whereas no difference was observed in lean mice (105 ± 5%, n = 6). In addition, the tissue necrosis and impaired vascular regeneration that were observed in *db/db* mice after HLI were reversed by ANG-(1-7) treatment (Supplementary Fig. 10).

Endogenous Expression of MasR Is Required for Mobilization of BMPCs

Last, we hypothesized that the endogenous expression of MasR is essential for the mobilization of progenitor cells

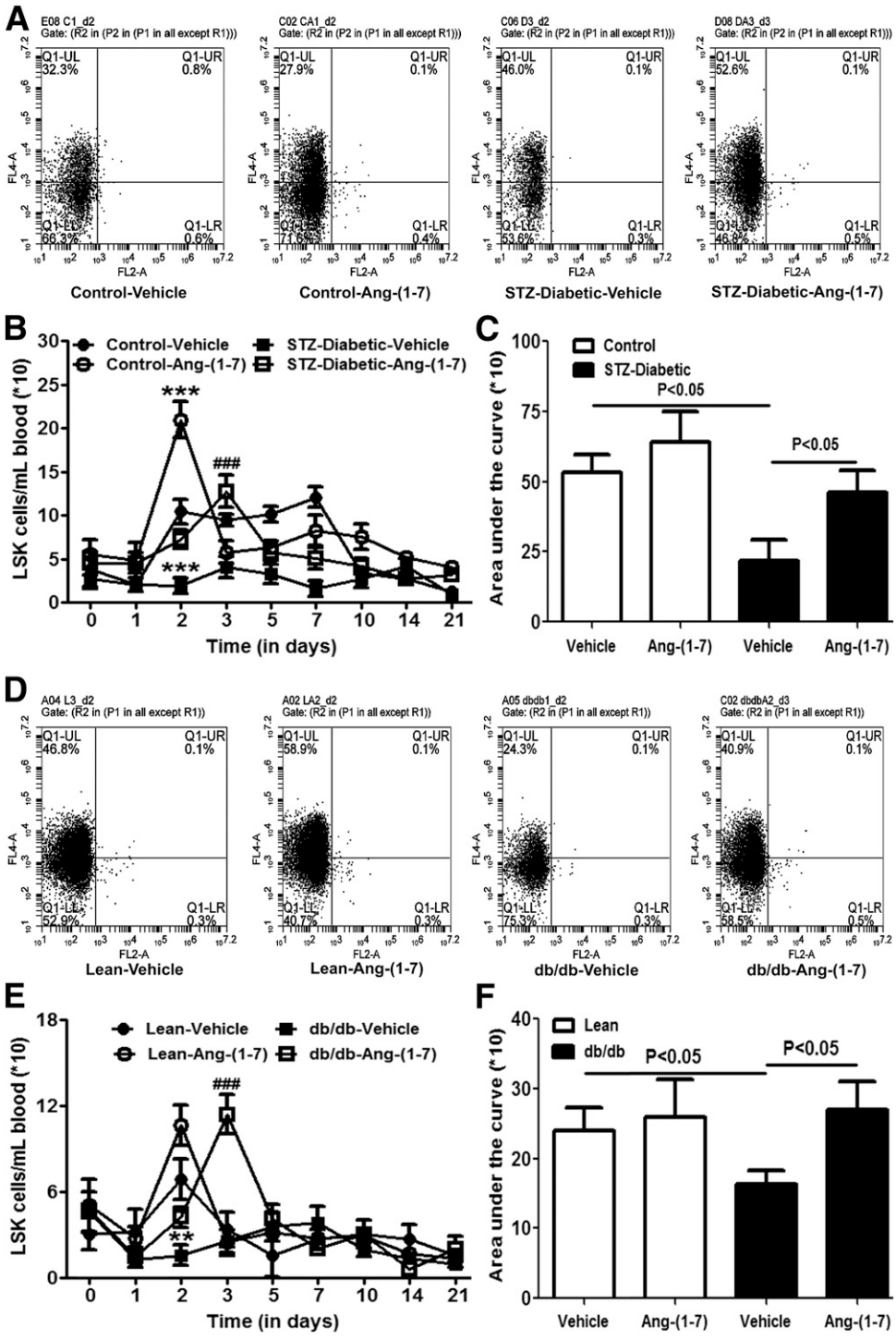

Figure 6—Reversal of diabetic dysfunction in the mobilization of BMPCs in response to ischemia by ANG-(1-7) treatment. *A*: Representative flow cytometric dot plots of progenitor cells at the time of peak mobilization after HLI in different treatment groups involving control and STZ-diabetic mice. *B*: LSK cell mobilization kinetics in different treatment groups involving control and STZ-diabetic mice over a period of 21 days after HLI (n = 6–8), where day 0 was prior to HLI. ***P < 0.001 vs. control-vehicle; ###P < 0.001 vs. STZ-diabetic-vehicle as analyzed by using two-way ANOVA. *C*: The area under the curve of mobilization kinetics after HLI decreased in STZ-diabetic mice, which was reversed by ANG-(1-7) treatment (n = 6–8). *D*: Representative flow cytometric dot plots of progenitor cells at the time of peak mobilization after HLI in treatment groups involving lean and *db*/*db* mice. *E*: LSK cell mobilization kinetics in different treatment groups involving lean and *db*/*db* mice over a period of 21 days after HLI (n = 5), where day 0 was prior to HLI. **P < 0.01 vs. lean-vehicle; ###P < 0.001 vs. *db*/*db*-vehicle as analyzed by two-way ANOVA. *F*: The area under the curve of mobilization kinetics after HLI was decreased in *db*/*db* mice, which was restored to normal by ANG-(1-7) treatment (n = 5).

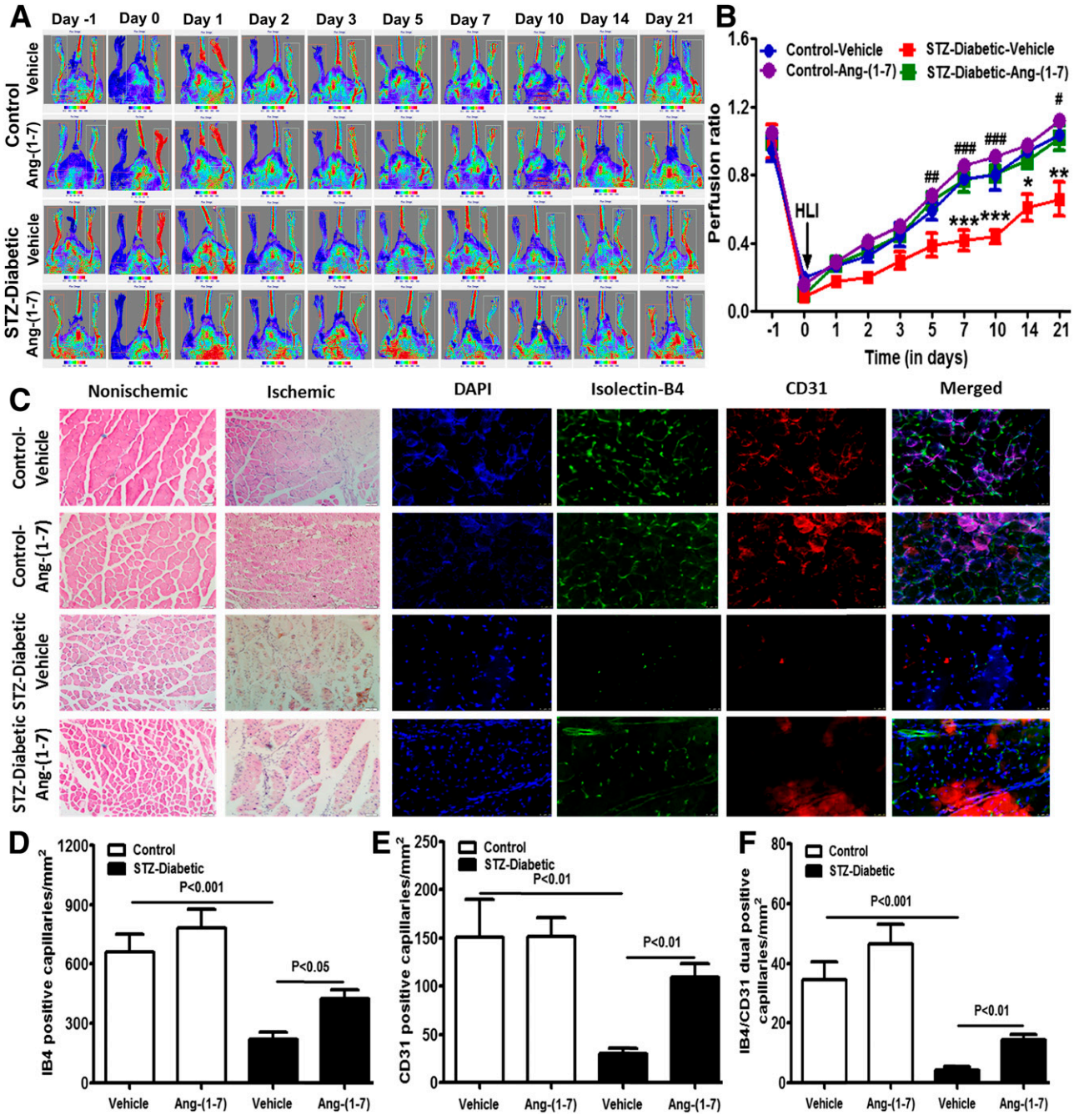

Figure 7—Reversal of diabetic impairments in the blood flow recovery and neovascularization of ischemic areas by ANG-(1-7): *A*: Representative laser Doppler images of blood flow in different treatment groups before and after HLI. *B*: Blood flow was quantified by RBC flux (blood × area^{-1} × time^{-1}) expressed as a percentage of the respective contralateral limb. Blood flow recovery was lower in STZ-diabetic mice, which was restored to normal by ANG-(1-7) treatment (n = 6). *P < 0.05, **P < 0.01, and ***P < 0.001 vs. control-vehicle; #P < 0.05, ##P < 0.01, and ###P < 0.001 vs. STZ-diabetic-vehicle using two-way ANOVA. *C*: Immunohistochemistry for tissue necrosis and capillary density. Shown were representative light microscopic images of hematoxylin-eosin (H-E) staining, and fluorescence images of isolectin-B4 (IB4) or CD31 staining of gastrocnemius muscle sections. Muscle samples were fixed in 3.8% paraformaldehyde and embedded in paraffin blocks to obtain 8 micron sections. For H-E staining, sections were deparaffinized, hydrated, and stained with Harris Hematoxylin Solution (Electron Microscopy Sciences), followed by counterstaining with Eosin Y Phloxine B Solution (Electron Microscopy Sciences). Then, the sections were dehydrated and mounted in Permount Mounting Medium (Electron Microscopy Sciences). For determining the capillary density, sections were deparaffinized in histoclear and hydrated. Antigen retrieval was performed in citrate buffer of pH 6.0. Nonspecific receptors were blocked by using normal horse serum (Vector Laboratories), then stained with CD31 primary antibody (Santa Cruz Biotechnology) followed by secondary antibody (Vector Laboratories) and IB4 (Enzo Life Sciences, Inc.). Imaging was performed in DAPI mounting media (Vector Laboratories) using a fluorescence microscope (Olympus). *D–F*: Capillary density was quantified by IB4, CD31, or dual positive capillaries in the muscle sections. Capillary density decreased in STZ-diabetic mice and was normalized by ANG-(1-7) treatment (n = 6). Scale bar, 25 μm.

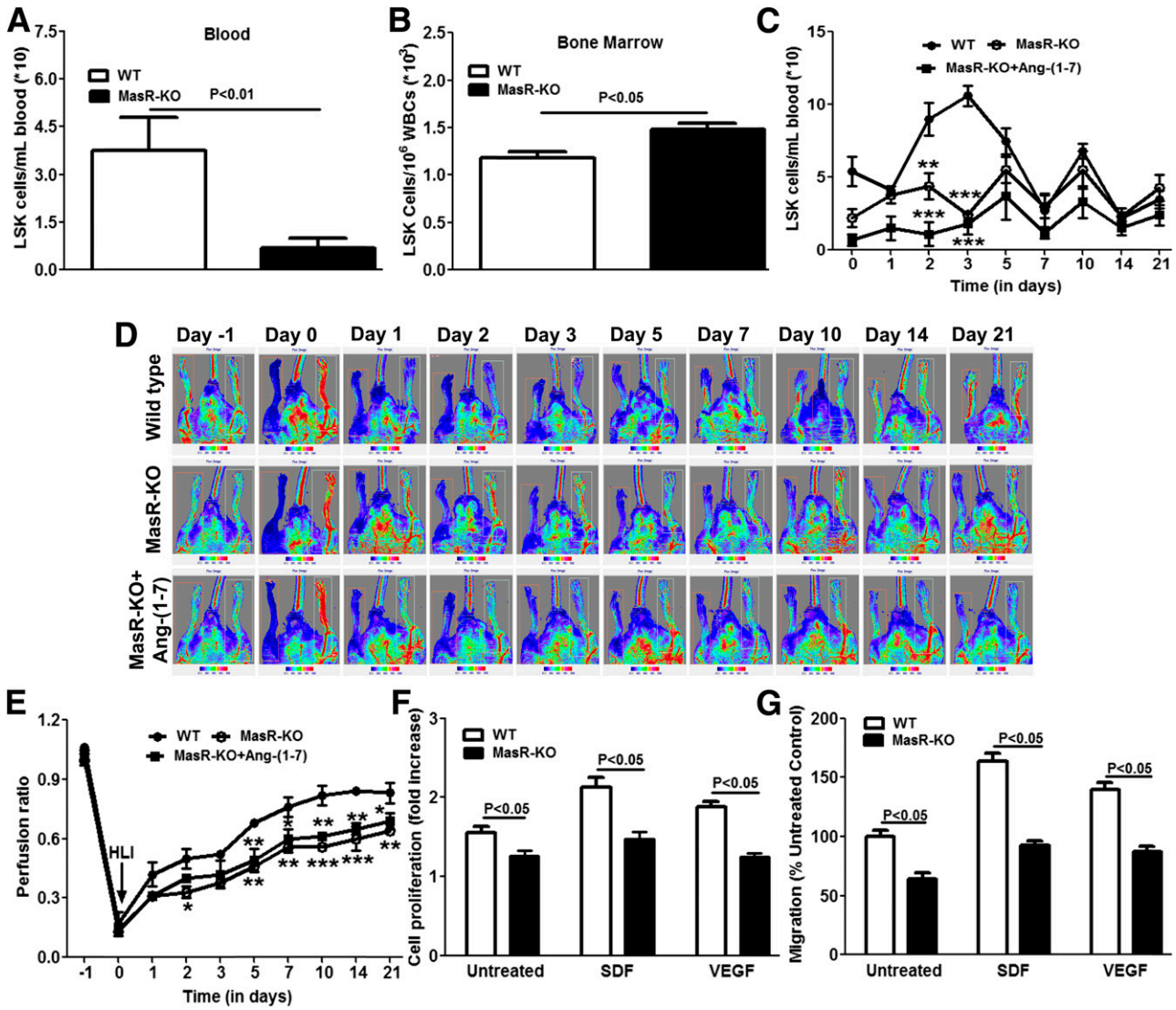

Figure 8—Endogenous MasR expression is essential for the mobilization of BMPCs. *A*: The number of circulating LSK cells was decreased in MasR-KO mice (n = 6) compared with WT mice. *B*: Bone marrow–resident LSK cells were unaltered in MasR-KO mice (n = 3). *C*: The mobilization of LSK cells in response to ischemia was monitored over a period of 21 days, which was impaired, and ANG-(1-7) treatment did not increase mobilization in MasR-KO mice (n = 4), where day 0 was prior to HLI. $^{**}P < 0.01$ and $^{***}P < 0.001$ vs. WT mice (two-way ANOVA). *D*: Representative laser Doppler images of blood flow in WT and MasR-KO mice before and after HLI. *E*: Blood flow recovery, quantified as the flux of residual blood flow and expressed relative to flux in the respective contralateral limb, was lower in MasR-KO mice compared with WT mice (n = 4). ANG-(1-7) did not increase blood flow recovery in MasR-KO mice. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ vs. WT mice (two-way ANOVA). *F*: Basal, SDF-induced, or VEGF-induced proliferation in LSK cells was decreased in MasR-KO mice (n = 4). *G*: Basal, SDF-induced, or VEGF-induced migration in LSK cells was decreased in MasR-KO mice (n = 4). WBCs, white blood cells.

in response to ischemic injury, which was tested by using MasR-KO mice on a C57BL/6J background. Representative genotyping is shown in Supplementary Fig. 11. Blood glucose levels and glucose tolerance of MasR-KO mice were similar to those observed in wild-type (WT) mice (Supplementary Fig. 12). The number of circulating LSK cells is lower in MasR-KO mice compared with the WT mice (cells/mL blood: 37.4 ± 10.4 vs. 6.7 ± 3.2 in WT, n = 6) (Fig. 8*A*). This difference was not due to reduced progenitor reserve in the bone marrow as the number of bone marrow–resident LSK cells was similar in both groups (Fig. 8*B*). In MasR-KO mice undergoing HLI, the mobilization of LSK cells was not observed, whereas the WT mice responded to ischemia by mobilizing LSK cells (Fig. 8*C*) in a manner similar to that observed in C57BL/6 mice, as described above. The recovery of blood flow was significantly decreased in MasR-KO mice compared with WT mice (blood flow flux ratio 64 ± 2% vs. 83 ± 5% in WT mice, n = 4) (Fig. 8*D* and *E*). ANG-(1-7) did not increase the mobilization or blood recovery in MasR-KO mice (Fig. 8*D* and *E*). The proliferative potential of LSK cells derived from MasR-KO mice was lower in basal conditions or upon exposure to SDF or VEGF compared with

WT cells (Fig. 8*F*). Along similar lines, migration induced by SDF or VEGF was significantly impaired in LSK cells derived from MasR-KO mice compared with WT mice ($n = 4$) (Fig. 8*G*).

DISCUSSION

This study reports several novel findings. ANG-(1-7) treatment reversed diabetic mobilopathy in physiological conditions or in response to ischemic insult. The study provides evidence for a novel pathway involving MasR/Slit3/ROCK in the mobilization of progenitor cells by ANG-(1-7) treatment. Beneficial effects of ANG-(1-7) were observed without normalizing blood glucose levels, suggesting that MasR signaling is not affected by diabetes and that ANG-(1-7) would be effective even in the individuals with poor control of hyperglycemia. Importantly, endogenous expression of MasR appears to be indispensable for the mobilization of BMPCs and that genetic ablation of MasR recapitulates diabetic bone marrow mobilopathy and impaired vascular regeneration.

The dose of ANG-(1-7) that we used was either similar or lower than the doses used in previous studies in mice (82 μg/kg/h s.c. for 6 weeks) (43,44). ANG-(1-7) at doses as low as 300 μg/kg/day s.c. potentiated the effects of NEUPOGEN on the recovery of circulating blood cells in mice after chemotherapy; however, higher doses of 1,200 μg/kg/day s.c. were needed for stimulating the recovery of progenitors of all lineages in the absence of NEUPOGEN (45). According to the toxicokinetic studies (46), with doses up to 10 mg/kg/day s.c. for 28 days, no toxicity was observed; therefore, the dose used in the current study is safe and is in agreement with those used in the previous studies.

ANG peptides are known to stimulate the proliferation of human and murine BMPCs (47). In particular, ANG-(1-7) accelerated hematopoietic recovery after chemoradiation therapy, which was attributed to its ability to enhance proliferation (47). The proliferation of BMPCs is diminished in diabetes (1,20), which was apparent by the reduced number of LSK cells in the bone marrow and in circulation. ANG-(1-7) increased the number of cells in both models of diabetes and restored the proliferative potential of cells. Increased proliferation of resident bone marrow cells likely increases their basal mobilization into circulation; however, the SDF gradient across the blood-BM barrier is a stimulus for the mobilization of cells, particularly in response to ischemia (7). Our studies indicated that the circulating SDF levels were decreased in STZ-diabetes mice but not in *db*/*db* mice, although the number of circulating cells is decreased in both models. Although ANG-(1-7) treatment indeed restored SDF levels in STZ-diabetic mice, the sensitization of cells for mobilization to SDF or VEGF gradients appears to mediate the beneficial effects of ANG-(1-7) treatment in both models. Increased ROCK activity is a strong indicator of the migratory propensity of cells (32,33). ANG-(1-7) treatment increased ROCK activity specifically in BMPCs. MasR activation is not involved in this response because in vitro treatment of LSK cells with ANG-(1-7) did not stimulate ROCK. We focused on Slit matrix proteins that are known to regulate cell migration via the Robo/ROCK pathway. In endothelial cells, Slit3 increased ROCK and induced migration, resulting in angiogenesis (38), whereas Slit2 inhibited migration (48). In endothelial cells, stimulated migration results in an enhanced angiogenic response (36). In the current study, Slit3 levels were increased by ANG-(1-7) in the bone marrow in vivo, which was further confirmed in vitro in BM-MSCs. Slit3-stimulated ROCK itself did not induce migration but potentiated migration in the presence of SDF. Although mRNA transcripts of all four isoforms of Robo receptors were detected in LSK cells, only Robo4 protein was shown to be expressed in mouse progenitor cells (48). Further investigations are required to confirm that Robo4 mediates the Slit3-dependent effects of ANG-(1-7) in the current experimental setting. Collectively, these results demonstrated for the first time that ANG-(1-7) orchestrates progenitor mobilization by stimulating Slit3 from BM-MSCs, which in turn causes the sensitization of cells for mobilization by endogenous mobilizers.

Diabetes, in both models, impaired mobilization in response to ischemia, decreased blood flow recovery, and increased tissue necrosis. It is important to note that the magnitude and pattern of mobilization differed in both models of diabetes, suggesting either a strain-dependent difference or a role for leptin receptor in the mobilization of BMPCs, which needs further investigation. ANG-(1-7) reversed diabetic dysfunctions in response to the ischemic response in both models, which was associated with increased SDF release in response to ischemia. Importantly, the vascularization of ischemic areas was stimulated by ANG-(1-7), which in turn decreased tissue necrosis and accelerated tissue repair in both models of diabetes. The revascularization of ischemic areas is largely due to increased mobilization of BMPCs in response to ischemia and decreased oxidative stress in the ischemic environment (8). Thangarajah et al. (49) demonstrated that oxidative stress results in decreased hypoxia-inducible factor-1α activity but not stability in experimental diabetes, which eventually results in the reduced expression of SDF. In the current study, restoration of SDF levels by ANG-(1-7) is most likely due to the reduced oxidative stress, which in turn normalizes hypoxia-inducible factor-1α activity, that has been shown consistently in previous studies (17).

Genetic ablation of MasR recapitulated the diabetic phenotype with a decreased number of BMPCs in circulation and impaired mobilization and reduced blood flow recovery after ischemia, suggesting that endogenous MasR expression is indispensable for the mobilization of BMPCs that are responsible for tissue maintenance and repair. Decreased numbers of circulating cells in MasR-KO mice appear to be largely due to the decreased sensitivity for SDF- or VEGF-induced proliferation and migration of progenitor cells. It is important to note that MasR-KO mice in the C57BL/6 background did not show hyperglycemia

or glucose intolerance. However, the deletion of MasR resulted in the dysregulation of lipid and glucose metabolism similar to metabolic syndrome on the FVB/N background, which is evidently more sensitive to changes induced by gene deletion (50).

In conclusion, our study shows that MasR is a promising target for the reversal of bone marrow mobilopathy and to accelerate vascular repair in diabetes after ischemic insult. MasR plays an important role in the mobilization of progenitor cells that involves paracrine interaction of MSCs and progenitor cells via the MasR/Slit3/ROCK pathway. The activation of MasR is a novel mechanism-based approach for enhancing the neovascularization outcomes of cell-based therapies for the treatment of diabetic vascular disease.

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Author Contributions. G.V. researched the data and prepared the manuscript. S.J. researched the data. S.E.T. and L.A.C. provided materials and contributed to discussion. S.H.B. contributed to discussion. Y.P.R.J. conceived the idea for the study; contributed to discussion; and prepared, critically reviewed, and approved the manuscript. Y.P.R.J. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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