Decreased Circulating Progenitor Cell Number and Failed Mechanisms of Stromal Cell-Derived Factor-1α Mediated Bone Marrow Mobilization Impair Diabetic Tissue Repair

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OBJECTIVE—Progenitor cells (PCs) contribute to postnatal neovascularization and tissue repair. Here, we explore the mechanism contributing to decreased diabetic circulating PC number and propose a novel treatment to restore circulating PC number, peripheral neovascularization, and tissue healing.

RESEARCH DESIGN AND METHODS—Cutaneous wounds were created on wild-type (C57BL/J6) and diabetic (*Lepr*$^{db/db}$) mice. Blood and bone marrow PCs were collected at multiple time points.

RESULTS—Significantly delayed wound closure in diabetic animals was associated with diminished circulating PC number (1.9-fold increase vs. 7.6-fold increase in lin$^-$/sca-1$^+$/ckit$^+$ in wild-type mice; $P < 0.01$), despite adequate numbers of PCs in the bone marrow at baseline (14.4 ± 3.2% lin$^-$/ckit$^+$/sca1$^+$ vs. 13.5 ± 2.8% in wild-type). Normal bone marrow PC mobilization in response to peripheral wounding occurred after a necessary switch in bone marrow stromal cell-derived factor-1α (SDF-1α) expression (40% reduction, $P < 0.01$). In contrast, a failed switch mechanism in diabetic bone marrow SDF-1α expression (2.8% reduction) resulted in impaired PC mobilization. Restoring the bone marrow SDF-1α switch (54% reduction, $P < 0.01$) with plerixafor (Mozobil, formerly known as AMD3100) increased circulating diabetic PC numbers (6.8 ± 2.0-fold increase in lin$^-$/ckit$^+$, $P < 0.05$) and significantly improved diabetic wound closure compared with sham-treated controls (32.9 ± 5.0% vs. 11.9 ± 3% at day 7, $P > 0.05$; 73.0 ± 6.4% vs. 36.5 ± 7% at day 14, $P < 0.05$; and 88.0 ± 5.7% vs. 66.7 ± 5% at day 21, $P > 0.05$, respectively).

CONCLUSIONS—Successful ischemia-induced bone marrow PC mobilization is mediated by a switch in bone marrow SDF-1α levels. In diabetes, this switch fails to occur. Plerixafor represents a potential therapeutic agent for improving ischemia-mediated pathology associated with diabetes by reducing bone marrow SDF-1α, restoring normal PC mobilization and tissue healing. ***Diabetes*** **59:1974–1983, 2010**

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Adult bone marrow-derived progenitor cells (PCs) contribute to peripheral tissue repair and regeneration. Vasculogenic PCs mobilize from the bone marrow and participate in new blood vessel formation in a variety of physiologic and pathologic processes, such as wound healing, fracture repair, and myocardial revascularization (1–3). Since vasculogenic PCs contribute to nearly a third of new blood vessels in healing tissues, impaired PC response to injury may contribute to microangiopathic conditions (1,4).

We and others have previously demonstrated that diabetic vasculogenic PC dysfunction (i.e., impaired adhesion, proliferation, migration, tubule formation) impairs new blood vessel formation (5,6). Functional limitations alone, however, are unlikely to account for the magnitude of diabetic vasculopathy and wound-healing complications. Recent reports suggest that diabetic animals and humans exhibit fewer numbers of circulating vasculogenic PCs (5,7,8). For example, we have shown that type II diabetic animals contain significantly fewer circulating PCs at homeostasis and after cutaneous ischemia or excisional wounding (4,9). Others have noted diminished numbers of circulating PCs in type I diabetic animals after ischemic injury (7).

Whether circulating PC depletion is involved in the pathogenesis of delayed diabetic wound healing remains unknown. Peripheral injury normally stimulates bone marrow PC proliferation through cytokine-mediated signals (e.g., stem cell factor [SCF]) (10), whereas PC mobilization is mediated by other chemokinetic proteins such as matrix-metalloproteinase-9 (MMP-9) and stromal cell-derived factor-1-α (SDF-1α) (11–15). Recently, it has been demonstrated that modulation of bone marrow–SDF-1α levels with mobilizing agents 5-fluorouracil and granulocyte colony-stimulating factor induces PC mobilization (13–15), but whether this mobilization mechanism occurs physiologically remains unknown. Herein, we investigate the relationship between circulating vasculogenic PCs and diabetic wound closure; moreover, we explore the cause of diminished vasculogenic PCs in diabetic patients as well as the physiologic mechanism regulating PC release from the bone marrow. We hypothesize that impaired peripheral diabetic wound healing stems from a dysfunction in the central SDF-1α switch mechanism responsible for PC mobilization in the bone marrow.

RESEARCH DESIGN AND METHODS

Mice and wounding model. C57BL/6J (#664) wild-type and type II diabetic mice homozygous for *Lepr^{db}* (#642) age 8–12 weeks were purchased from Jackson Laboratories (Bar Harbor, ME). As previously described, these diabetic mice have persistently elevated weights and significantly elevated blood glucose levels compared with their wild-type controls (16,17). To confirm that mice used in this model were diabetic, fasting glucose levels of both wild-type and diabetic mice were measured using an AccuCheck Advantage glucometer and AccuCheck comfort strips (Roche; Branchburg, NJ). The body weights of the mice were also measured.

Cutaneous wounding was achieved by creating dorsal skin flaps or excisional wounds as previously described (4,18). Briefly, two 6-mm full-thickness wounds extending through the panniculus carnosus layer were excised from the dorsum of mice using a sterile circular punch biopsy (Fig. 1*A*) (Sklar Instruments; West Chester, PA). A silicone stent was secured around the perimeter of the wound to limit wound contracture (Fig. 1*B*). Time to closure, defined as the number of days for complete re-epithelialization, and percent wound closure, calculated as (1-[wound area]/[original wound area]), of excisional wounds were measured using photogrammetry. Digital photographs were taken on the day of wounding and every 7 days thereafter. Photographs were acquired with a 7-megapixel digital camera (Canon USA, Lake Success, NY) from a distance of 6.5 cm, with the lens oriented parallel to the wound. The wound area was measured using a digital selection (Photoshop CS3, Adobe Systems; San Jose, CA) and calibrated against the internal diameter of the silicon splint to correct for any subtle magnification, perspective, or parallax effect on the image. Ten animals were wounded in each experimental group studied. All experiments were performed in accordance with the New York University Medical Center Institutional Animal Care and Use Committee (IACUC#041104).

Isolation of peripheral and bone marrow mononuclear cells. Peripheral blood was obtained by intracardiac puncture at baseline, 2, 7, and 10 days after ischemia ($n = 8$ for each time point). Bone marrow was obtained by flushing each femur, tibia, humerus, and pelvis with PBS/10% FBS/5% EDTA (6,19). Peripheral blood and bone marrow mononuclear cells (MNCs) were separated by density gradient centrifugation with Histopaque 1,083 (Sigma, St. Louis, MO).

Magnetic sorting and flow cytometry. Bone marrow PCs were magnetically separated from total bone marrow MNCs (Miltenyi Biotech, Auburn, CA). Cells were labeled with a lineage depletion cocktail (biotinylated anti CD5, CD45R (B220), CD11b, Gr-1 (Ly-6G/C), 7–4, and Ter-119 antibodies) in a 1:60 dilution, and secondary antibiotin microbeads (1:10). For characterization by flow cytometry, peripheral blood and bone marrow MNCs were stained with rat anti-mouse antibodies (fluorescein isothiocyanate-conjugated Ly-6A/E [1:100], phycoerythrin-conjugated VEFG-R2 [1:50], phycoerythrin-conjugated CXCR4 [1:100], allophycocyanin-conjugated CD117 [1:100], biotin-conjugated lineage depletion cocktail [1:20], and strepavidin-phycoerythrin-conjugated-Cy7 [1:200]) (BD Bioscience, San Jose, CA, and Miltenyi Biotech). All antibodies were titrated and optimized for appropriate detection. Cells were costained with 7-aminoactinomycin D (Invitrogen, Carlsbad, CA) to detect nonviable cells. Samples were collected using a BD FACSCaliber flow cytometer (Becton Dickinson, Franklin Lakes, NJ), and analyses were performed with FlowJo 8.0 software (TreeStar Inc., Ashland, OR) to delineate lin$^-$/sca-1$^+$(L^-S^+), lin$^-$/ckit$^+$ (L^-C^+), and lin$^-$/ckit$^+$/sca1$^+$ ($L^-C^+S^+$) populations of peripheral blood and bone marrow MNCs.

Chemotaxis assay. Recombinant mouse SDF-1α (250 ng/ml) (Peprotech, Rocky Hill, NJ) or saline was added to the bottom of 24-well plates (600 μl). Cells (5×10^4/100 μl) were suspended in EBM-2/10% FBS and seeded onto the membranes of Transwell inserts (5 μm pore) (Costar, Corning, NY). Assays were run in quadruplicate for 5 h at 37°C. The surface of the membranes was scraped with cotton swabs, fixed with 1% PFA, and mounted onto slides with DAPI-stained Vectashield (Vector, Burlingame, CA). Photographs were taken at ×4 magnification and the number of cells was quantified with a Kodak ID 3.5 Image Processor (Rochester, NY).

Proliferation assay. PCs (2×10^4/100 μl) suspended in EBM-2/10%FBS were seeded onto 96-well plates in quadruplicate. An additional 100 μl of SCF (100 ng/ml) or control media was added to each well. Plates were incubated at 37°C for 18 h. Bromodeoxyuridine (BrdU) reagent (BrdU Cell Proliferation Assay, Chemicon, Billerica, MA) was then added for 4 h, and proliferation was quantified with a spectrometer as a relative proliferation index.

Supplemented mobilization model. Ten days after wounding, wild-type and diabetic animals received the CXCR4 partial agonist Plerixafor (5 mg/kg; Sigma; $n = 15$) or PBS ($n = 15$) by subcutaneous injection 60 min before kill. Additional wild-type and diabetic animals received daily Plerixafor ($n = 15$) or PBS ($n = 15$) intraperitoneal injections beginning 3 days after wounding and continuing until time of wound closure.

ELISA protein quantification. SDF-1α protein levels were assessed by enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis, MN). Serum samples (from density gradient centrifugation) and bone marrow samples (from 500 μl of PBS flush) were run in triplicate and standardized with a BCA Protein Assay Kit (Pierce, Rockford, IL).

Statistical analysis. All data represent mean ± SEM. Pairwise comparisons between groups were assessed by Student t test. A one-way ANOVA and Tukey-Kramer post hoc analysis were performed when comparisons involved more than two groups. Significance was considered to be $P < 0.05$. The number of animals needed to demonstrate a significant difference between experimental groups with a power greater than 0.80 was calculated using G*Power (G*Power, Melbourne, Australia).

RESULTS

Diabetes significantly impairs cutaneous wound closure. The diabetic mice used in all experiments had significantly elevated serum glucose levels (485.7 ± 2.0 mg/dl vs. 140.3 ± 0.3 mg/dl, $P < 0.01$) and body weight (57.9 ± 0.1g vs. 25.9 ± 0.1g, $P < 0.01$) compared with age-matched wild-type mice.

Using an established excisional wound healing model, we demonstrated that a single punch biopsy produces a defect size of 6.1 ± 0.2 mm (Fig. 1*A–C*). Diabetic mice demonstrated significantly delayed rate of wound closure compared with wild-type animals (11.9 ± 3.0% vs. 47.5 ± 3.9% at day 7, $P < 0.01$; 36.5 ± 7.0% vs. 97.5 ± 1.8% at day 14, $P < 0.001$; and 66.7 ± 5.0% vs. 100 ± 0% at day 21, $P < 0.01$) (Fig. 1*D*). The average time to wound closure in wild-type animals was 15.0 ± 0.3 days. In contrast, the average time to wound closure in diabetic animals was significantly longer (26.4 ± 1.9 days; $P < 0.01$).

Impaired diabetic wound closure is associated with fewer circulating vasculogenic PCs. Having demonstrated that diabetic animals exhibit impaired cutaneous wound closure, we measured the number of circulating vasculogenic PCs in wild-type and diabetic animals during active wound healing. Two days after cutaneous wounding, wild-type animals had a moderate increase in circulating vasculogenic PCs compared with homeostatic levels (23.8 ± 2.0% vs. 13.5 ± 0.6% L^-S^+, $P < 0.05$; 3.1 ± 0.1% vs. 2.1 ± 0.3% L^-C^+, $P < 0.05$; 1.3 ± 0.1 vs. 0.5 ± 0.1% $L^-S^+C^+$, $P < 0.01$) (Fig. 2*A* and *B*). Seven days after wounding, circulating vasculogenic PCs in wild-type animals remained elevated (27.6 ± 1.8% L^-S^+, $P < 0.01$; 2.1 ± 0.1% L^-C^+; 1.3 ± 0.2 $l^-S^+C^+$, $P < 0.01$). The increase in wild-type circulating vasculogenic PCs was most pronounced at day 10 (32.2 ± 1.1% L^-S^+, $P < 0.01$; 7.4 ± 0.9% L^-C^+, $P < 0.01$; 4.0 ± 0.7% $L^-S^+C^+$, $P < 0.01$). In marked contrast, diabetic wounding led to a less substantive response in circulating diabetic vasculogenic PCs at identical time points: baseline (7.9 ± 1.2% L^-S^+, 1.7 ± 0.3% L^-C^+, 0.5 ± 0.2% $L^-S^+C^+$), day 2 (13.1 ± 2.6% L^-S^+, $P < 0.05$; 2.4 ± 0.2% L^-C^+, $P < 0.05$; 1.6 ± 0.4% $L^-S^+C^+$, $P < 0.05$), day 7 (15.6 ± 1.6% L^-S^+, $P < 0.05$; 3.7 ± 0.9% L^-C^+, $P < 0.05$; 0.8 ± 0.5% $L^-S^+C^+$), and day 10 (12.3 ± 1.2% L^-S^+, $P < 0.05$; 2.3 ± 0.4% L^-C^+, 1.0 ± 0.2% $L^-S^+C^+$, $P < 0.05$). The greatest difference between wild-type and diabetic circulating vasculogenic PC was observed 10 days after cutaneous wounding (32.2 ± 1.1% vs. 12.3 ± 1.2% L^-S^+, $P < 0.01$; 7.4 ± 0.9% vs. 2.3 ± 0.4% L^-C^+, $P < 0.05$; 4.0 ± 0.7% vs. 1.0 ± 0.2% $L^-S^+C^+$, $P < 0.01$, respectively).

Diabetic bone marrow contains adequate numbers of vasculogenic PCs at baseline and in response to cutaneous wounding. Since diabetic mice showed impaired cutaneous wound closure and fewer circulating vasculogenic PCs associated with impaired healing, we also investigated the number of PCs in wild-type and

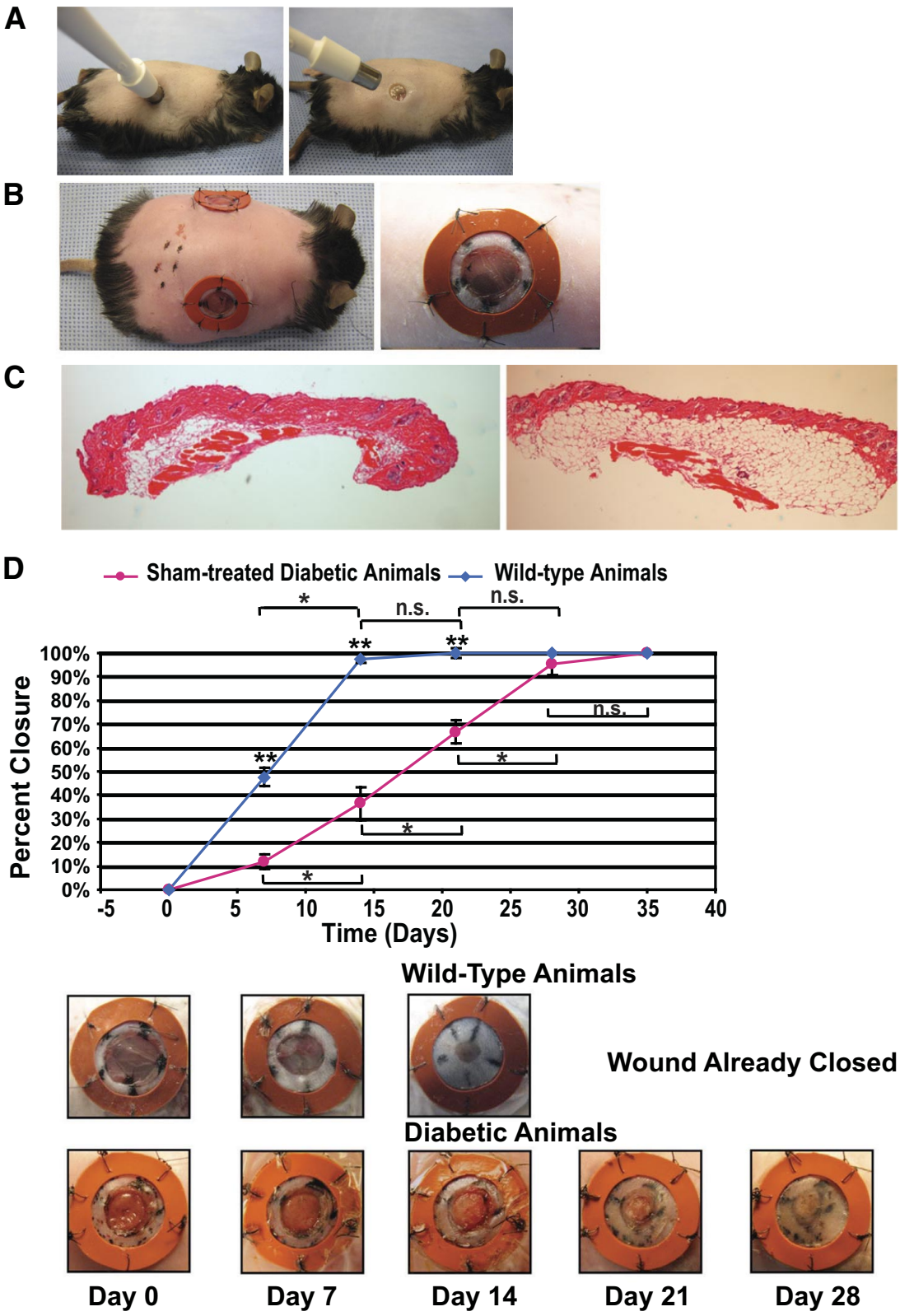

FIG. 1. Diabetic mice experience delayed wound closure compared with wild-type mice. *A*: A 6-mm punch biopsy was performed on the dorsal surfaces of mice. *B*: Silicone stents were then secured to the perimeter of the wound to prevent contraction. *C*: Histology confirmed the full thickness nature of wild-type (*left*) and diabetic (*right*) wounds. *D*: Representative images of wounds at days 0, 7, 14, 21, and 28 are shown (from *left* to *right*, respectively). Diabetic mice (blue) have significantly decreased wound closure rates compared with wild-type animals (pink) in a stented excisional wound model. n.s., not significant; *$P < 0.05$; **$P < 0.01$. (A high-quality color digital representation of this figure is available in the online issue.)

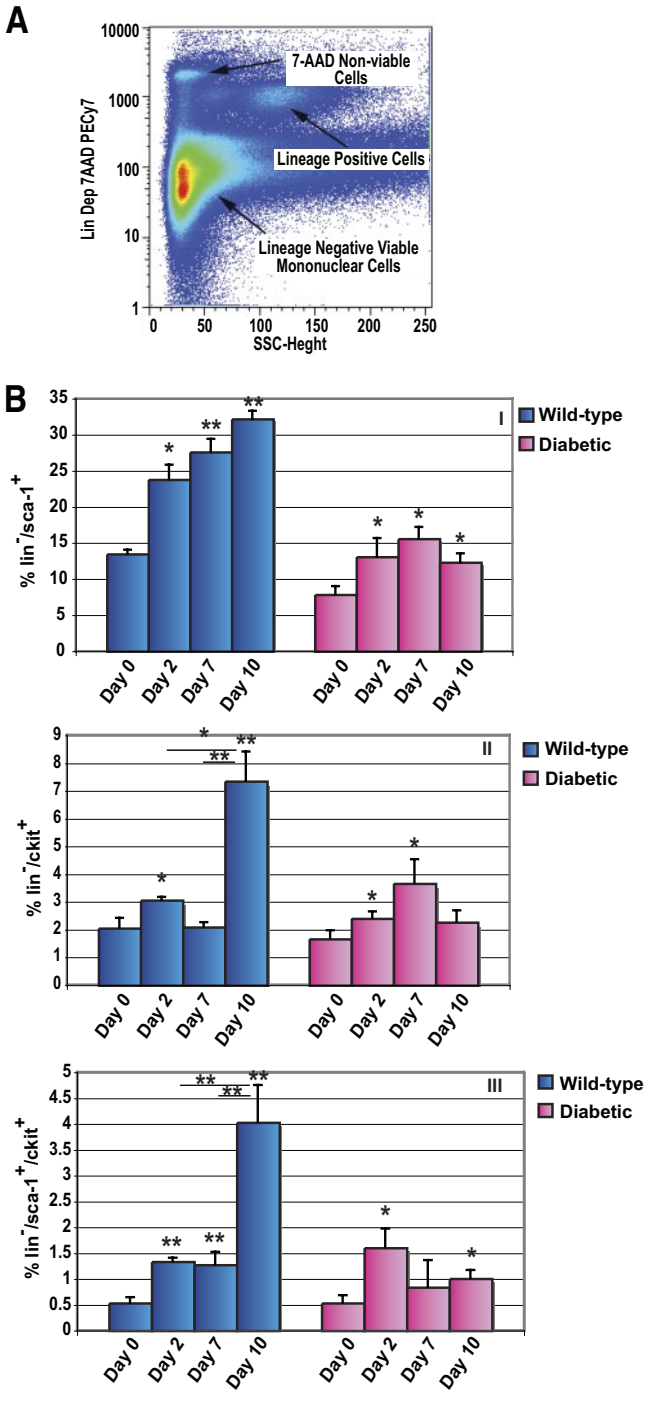

FIG. 2. Peripheral ischemia and circulating PC number. *A*: Peripheral blood mononuclear cells were analyzed by fluorescent activated cell sorting; a representative scatter of circulating cells is shown. *B*: Three distinct PC populations were analyzed in experimental groups: lin$^-$/sca-1$^+$ (*top*), lin$^-$/ckit$^+$ (*middle*), and lin$^-$/sca-1$^+$/ckit$^+$ (*bottom*). Peripheral ischemia led to an increase in circulating PCs of wild-type animals, peaking at day 10. In diabetic animals, an increase in circulating PC number was either stunted or absent. n.s., not significant. *$P < 0.05$; **$P < 0.01$. (A high-quality color digital representation of this figure is available in the online issue.)

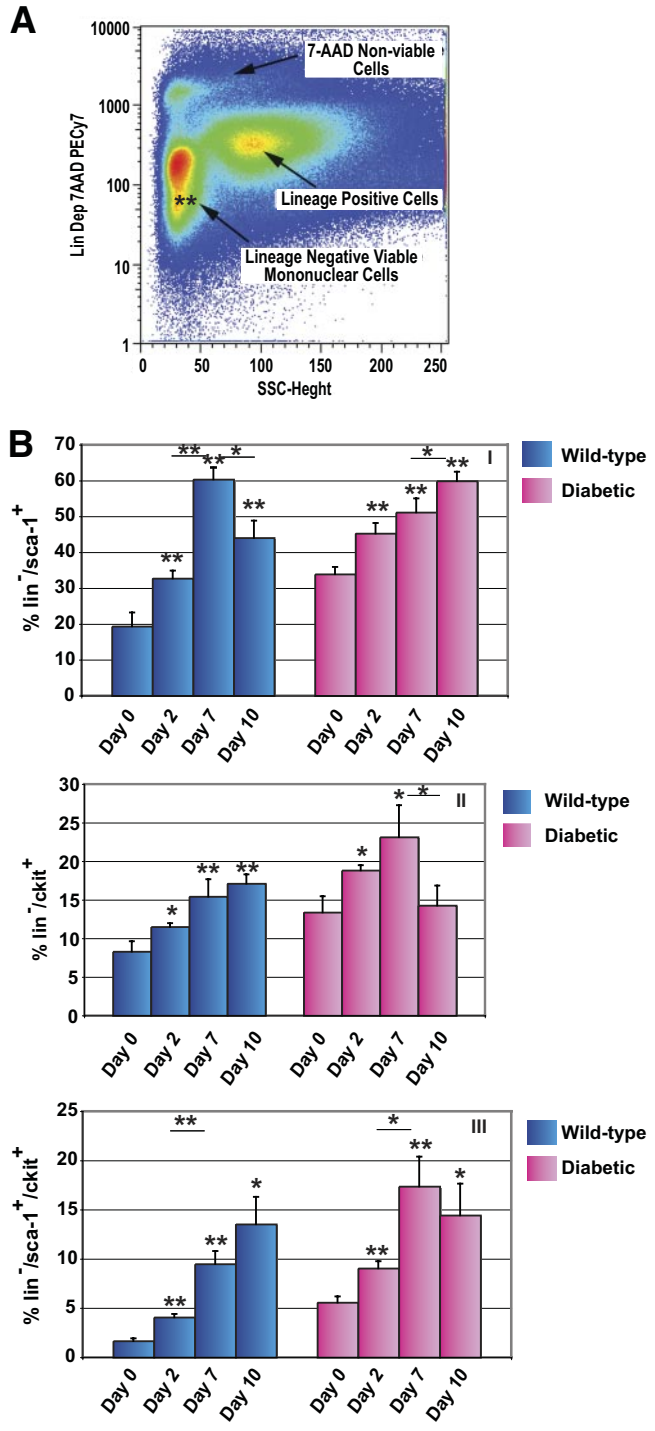

FIG. 3. Peripheral ischemia and bone marrow PC number. *A*: Representative scatter plot of bone marrow cells and the discrete populations of lineage negative, lineage positive, and nonviable cells. *B*: PC populations were also quantified in the bone marrow: lin$^-$/sca-1$^+$ (*top*), lin$^-$/ckit$^+$ (*middle*), and lin$^-$/sca-1$^+$/ckit$^+$ (*bottom*). Both wild-type and diabetic animals exhibited a steady increase in the number of bone marrow PCs at days 2, 7, and 10 after ischemia. *$P < 0.05$; **$P < 0.01$. (A high-quality color digital representation of this figure is available in the online issue.)

diabetic bone marrow. Surprisingly, bone marrow fluorescence-activated cell sorter analysis demonstrated fewer numbers of certain PC populations at baseline in wild-type compared with diabetic mice (19.4 ± 3.9% vs. 33.9 ± 2.0% L^-S^+, $P < 0.05$; 8.3 ± 1.3% vs. 13.4 ± 2.1% L^-C^+, $P = 0.08$; and 1.7 ± 0.3% vs. 5.6 ± 0.6% $L^-S^+C^+$, $P < 0.01$) (Fig. 3*A* and *B*). After cutaneous wounding, wild-type bone marrow PC number increased markedly: day 2 (32.7 ± 2.2% L^-S^+, $P < 0.01$; 11.5 ± 0.5% L^-C^+, $P < 0.05$; 4.1 ± 0.3% $L^-S^+C^+$, $P < 0.01$), day 7 (60.4 ± 3.2% L^-S^+, $P < 0.01$; 15.4 ± 2.2% L^-C^+, $P < 0.01$; 9.5 ± 1.3% $L^-S^+C^+$, $P < 0.01$), and day 10 (44.1 ± 4.8% L^-S^+, $P < 0.01$; 17.1 ± 1.2% L^-C^+, $P < 0.01$;

13.5 ± 2.8% $L^-S^+C^+$, $P < 0.05$). In a similar fashion, diabetic bone marrow PCs proliferated in response to cutaneous wounding: day 2 (45.3 ± 2.9% L^-S^+, $P < 0.01$; 18.8 ± 0.7% L^-C^+, $P < 0.05$; 9.5 ± 0.7% $L^-S^+C^+$, $P < 0.01$) day 7 (51.1 ± 3.9% L^-S^+, $P < 0.01$; 23.1 ± 4.1% L^-C^+, $P < 0.05$; 17.4 ± 3.0 and $L^-S^+C^+$, $P < 0.01$) and day 10 (59.9 ± 2.6% L^-S^+, $P < 0.01$; 14.3 ± 2.6% L^-C^+; 14.4 ± 3.2% $L^-S^+C^+$, $P < 0.05$).

Diabetic and wild type bone marrow vasculogenic PCs have similar functional capabilities. Since wild-type and diabetic animals had a similar number of bone marrow PCs at baseline and in response to cutaneous wounding, we examined the functional capacity of wild-type and diabetic bone marrow PCs. We first studied the effects of cutaneous injury on bone marrow PC proliferation; wild-type bone marrow PC proliferation remained at baseline levels for 2 days (1.0 ± 0.3-fold increase from baseline), but increased significantly by day 7 (2.3 ± 0.1-fold increase, $P < 0.01$). Diabetic bone marrow PC proliferation increased substantially at 2 days (3.3 ± 0.2-fold increase from baseline, $P < 0.01$) after cutaneous wounding and continued to remain elevated through day 7 (3.2 ± 0.1-fold increase, $P < 0.01$) (Fig. 4*A*).

We next investigated the effects of SCF, a key PC growth factor, (10,20) on wild-type and diabetic bone marrow PC proliferation. SCF treatment of wild-type and diabetic bone marrow PCs before cutaneous wounding induced a 3.1 ± 0.5-fold and 2.8 ± 0.4-fold increase in proliferation in both wild-type and diabetic bone marrow PCs, respectively ($P < 0.01$). Similarly, SCF treatment induced a 2.5 ± 0.2-fold and 2.7 ± 0.6-fold increase in wild-type and diabetic bone marrow PCs 7 days after cutaneous wounding ($P < 0.01$) (Fig. 4*B*).

Next, we examined the migratory ability of wild-type and diabetic bone marrow PCs. Bone marrow PCs isolated from wild-type and diabetic animals migrated equally effectively to SDF-1α (wild-type 246 ± 25 cells/HPF, $P < 0.05$; diabetic 250 ± 46, $P < 0.05$) (Fig. 4*C*). Similarly, no change was noted in the migratory ability of bone marrow PCs isolated from diabetic and wild-type mice after ischemia (data not shown).

The bone marrow SDF-1α switch-mechanism is dysfunctional in diabetic mice. Since diabetic bone marrow contains an adequate number of functional PCs, we next investigated potential mechanisms responsible for the impaired mobilization of vasculogenic PCs into the circulation. SDF-1α is a key bone marrow PC mobilizing factor (12–15); therefore, we assayed serum and bone marrow SDF-1α levels in wild-type and diabetic mice. Serum SDF-1α levels were not significantly different in wild-type compared with diabetic mice before (340 ± 50 vs. 430 ± 40 pg/ml) or after cutaneous wounding (1,008 ± 240 vs. 420 ± 80 pg/ml at day 2; 520 ± 150 vs. 870 ± 190 pg/ml at day 7; and 360 ± 50 vs. 390 ± 60 pg/ml at day 10) (Fig. 5*A*). However, based on recent evidence that bone marrow, rather than serum, SDF-1α expression may be involved in granulocyte colony-stimulating factor (G-CSF) and 5-fluorouracil (5-FU) mediated PC mobilization, (15,21) we hypothesized that bone marrow SDF-1α may act as a switch to regulate PC release after cutaneous wounding. Seven days after cutaneous wounding, wild-type bone marrow SDF-1α expression decreased precipitously (640 ± 110 pg/ml vs. 1,007 ± 210 pg/ml at baseline, $P < 0.01$). This change in bone marrow SDF-1α expression immediately preceded the most pronounced increase (day 10) in wild-type circulating vasculogenic PCs (Fig. 2*A*–*C*).

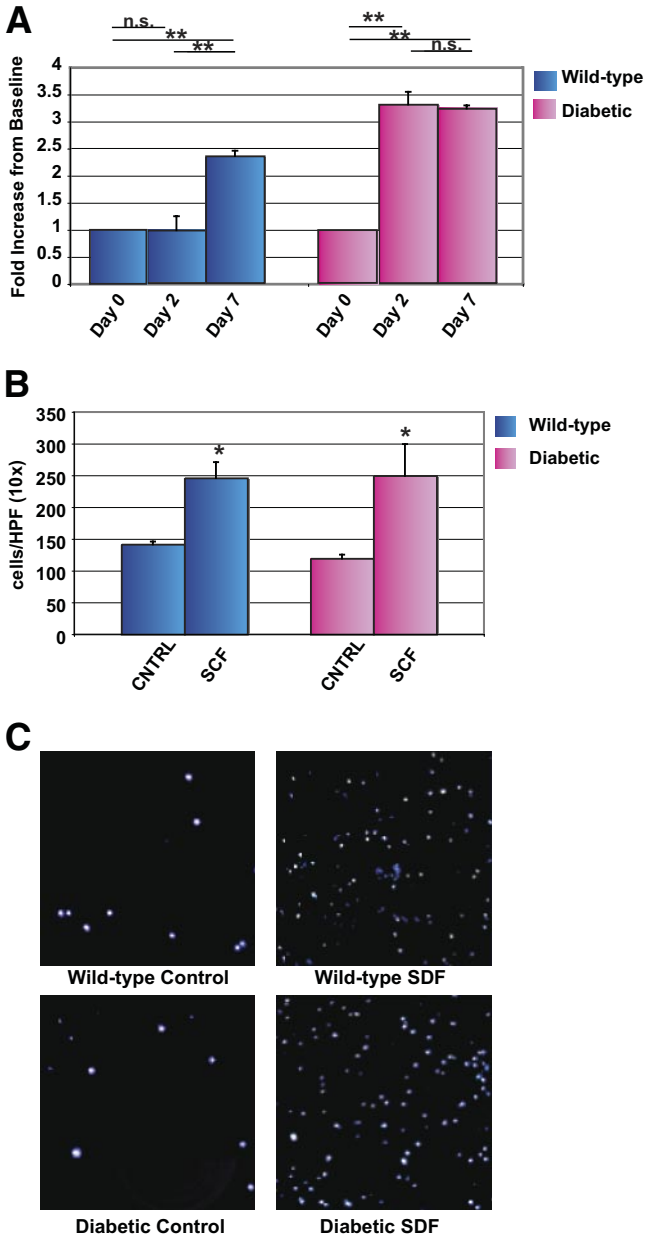

FIG. 4. Peripheral ischemia and bone marrow PC function. *A*: BrdU assay demonstrated that wild-type bone marrow PC proliferation remained at baseline for 2 days after cutaneous injury, but increased significantly by day 7. Diabetic bone marrow PC proliferation, however, significantly increased by day 2 after wounding and remained elevated through day 7. *B*: After SCF treatment, BrdU assay demonstrated that wild-type and diabetic bone marrow PC number increased appropriately both before cutaneous wounding (day 0) and 7 days after cutaneous wounding. (Data for Fig. 4*A* and *B* are presented as fold change from baseline, where baseline values are equal to 1.) *C*: The migratory response of PCs to SDF-1α in vitro was not significantly different in wild-type and diabetic animals. Representative photomicrographs of transwell membranes with DAPI-stained PCs are shown. CNTRL, control; n.s., not significant; *$P < 0.05$; **$P < 0.01$. (A high-quality color digital representation of this figure is available in the online issue.)

In marked contrast, diabetic bone marrow SDF-1α expression remained constant (1,000 ± 170 pg/ml at baseline, 1,400 ± 120 at day 2, 1,390 ± 210 at day 7, and 1,140 ± 130 at day 10) (Fig. 5*B*).

Plerixafor corrects the diabetic bone marrow SDF-1α switch and restores PC mobilization. Having found that a switch in wild-type bone marrow SDF-1α expression

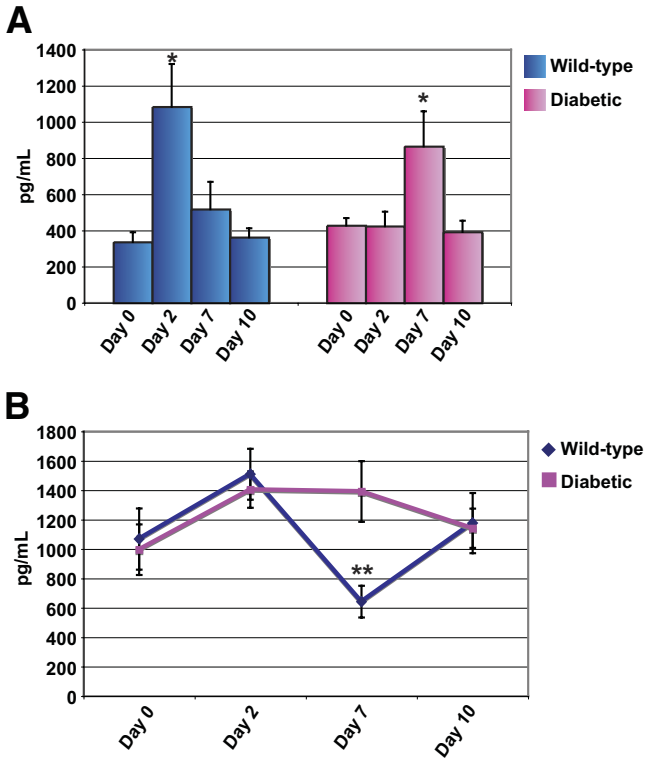

FIG. 5. Impaired mobilization of diabetic bone marrow PC. *A*: Quantification of serum SDF-1α demonstrated an increase in SDF-1α levels in both wild-type and diabetic animals after ischemia. *B*: SDF-1α levels in the bone marrow were also quantified, revealing a sharp decline in wild-type bone marrow SDF-1α at postischemia day 7. No corresponding drop in bone marrow SDF-1α was noted in diabetic animals. n.s., not significant; *$P < 0.05$; **$P < 0.01$. (A high-quality color digital representation of this figure is available in the online issue.)

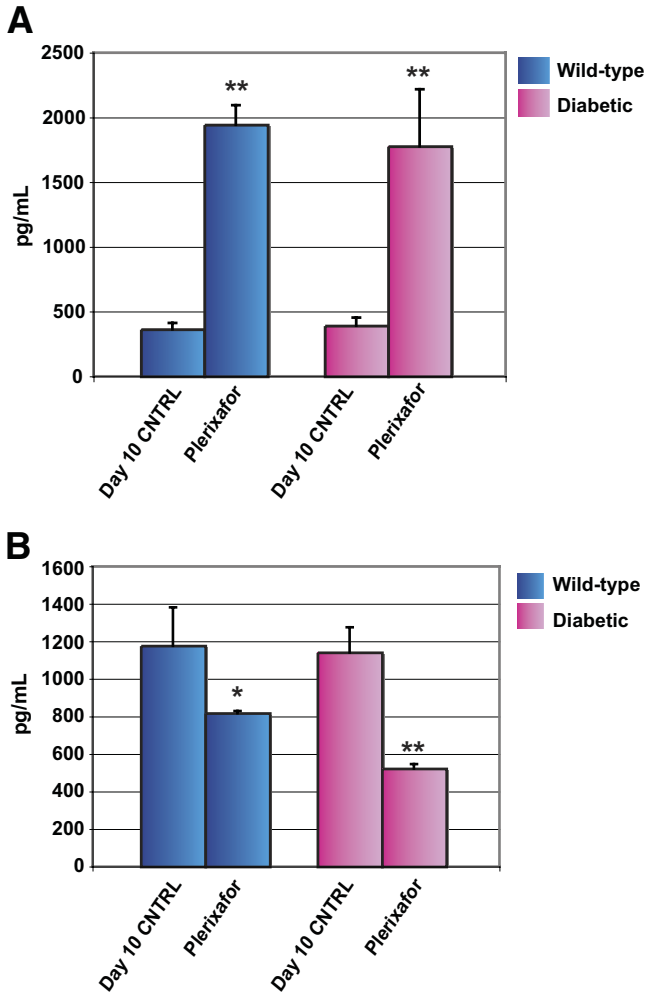

FIG. 6. Effects of plerixafor on postischemic SDF-1α levels and PC mobilization. *A*: ELISA of plerixafor-treated animals at day 10 after ischemia demonstrated significant increases in both wild-type and diabetic serum–SDF-1α compared with control day-10 animals. *B*: ELISA of plerixafor-treated animals revealed significant decreases in both wild-type and diabetic bone marrow–SDF-1α compared with controls. CNTRL, control; *$P < 0.05$; **$P < 0.01$. (A high-quality color digital representation of this figure is available in the online issue.)

immediately preceded PC mobilization, we examined the effects of the SDF-1α receptor (CXCR4) partial agonist, plerixafor, on serum and bone marrow SDF-1α levels. Treatment of nonwounded wild-type and diabetic mice with plerixafor resulted in a rise in serum SDF-1α (1,940 ± 150 vs. 367 ± 33 pg/ml, $P < 0.01$; 1,880 ± 300 vs. 380 ± 45 pg/ml, $P < 0.01$, respectively) similar to the rise in serum SDF-1α postcutaneous wounding (Fig. 6*A*). Likewise, bone marrow levels of SDF-1α significantly declined in both wild-type and diabetic animals treated with plerixafor 10 days after wounding (820 ± 10 vs. 1,180 ± 200 pg/ml, $P < 0.05$; 520 ± 20 vs. 1,140 ± 130 pg/ml, $P < 0.01$, respectively) (Fig. 6*B*). Finally, a single dose of plerixafor administered 10 days after cutaneous injury led to increased bone marrow PC mobilization in wild-type (1.9 ± 0.1-fold increase in L^-S^+, $P < 0.01$; 7.5 ± 0.5-fold increase in L^-C^+, $P < 0.01$; 3.7 ± 1.0-fold increase in $L^-S^+C^+$, $P < 0.05$) and diabetic mice (3.6 ± 0.1-fold increase in L^-S^+, $P < 0.01$; 6.0 ± 1.8-fold increase in L^-C^+, $P < 0.05$; 4.5 ± 1.7-fold increase in $L^-S^+C^+$, $P < 0.05$) (Fig. 7).

Restoration of diabetic bone marrow PC mobilization restores wound closure. Since decreased circulating PCs in diabetic mice is associated with impaired wound closure, and treatment with plerixafor restored the switch in bone marrow SDF-1α expression as well as circulating PC numbers in diabetic mice, we tested the effects of plerixafor on diabetic wound closure. Although a single plerixafor intraperitoneal injection did not significantly improve wound closure in either animal model (data not shown), daily plerixafor intraperitoneal injections improved diabetic percent wound closure compared with sham-treated diabetic mice at 7, 14, and 21 days (32.9 ± 5.0% vs. 11.9 ± 3%, $P > 0.05$; 73.0 ± 6.4% vs. 36.5 ± 7%, $P < 0.05$; and 88.0 ± 5.7% vs. 66.7 ± 5%, $P > 0.05$, respectively) (Fig. 8*A*). Additionally, the improvement in plerixafor-treated diabetic wound closure correlated with an increase in circulating PCs cells (3.7 ± 1.0-fold increase in L^-S^+ at 1 h, $P < 0.01$; 11.9 ± 1.7-fold increase in L^-S^+ at day 7, $P < 0.01$; and 19.6 ± 1.9-fold increase in L^-S^+ at day 14, $P < 0.01$) (Fig. 8*B*).

DISCUSSION

The effects of ischemic injury on circulating PC number have been well documented in both animal and human studies (3,12,13,19). In healthy subjects, peripheral ischemia leads to a significant rise in the number of circulating PCs, thereby increasing the quantity of PCs that can participate in tissue repair. With certain clinical conditions, such as diabetes, the quantity of circulating PCs is reduced (4,6–9). Although it has been shown that diabetic humans and animals exhibit fewer numbers of circulating PCs at rest and in response to ischemia, the mechanisms

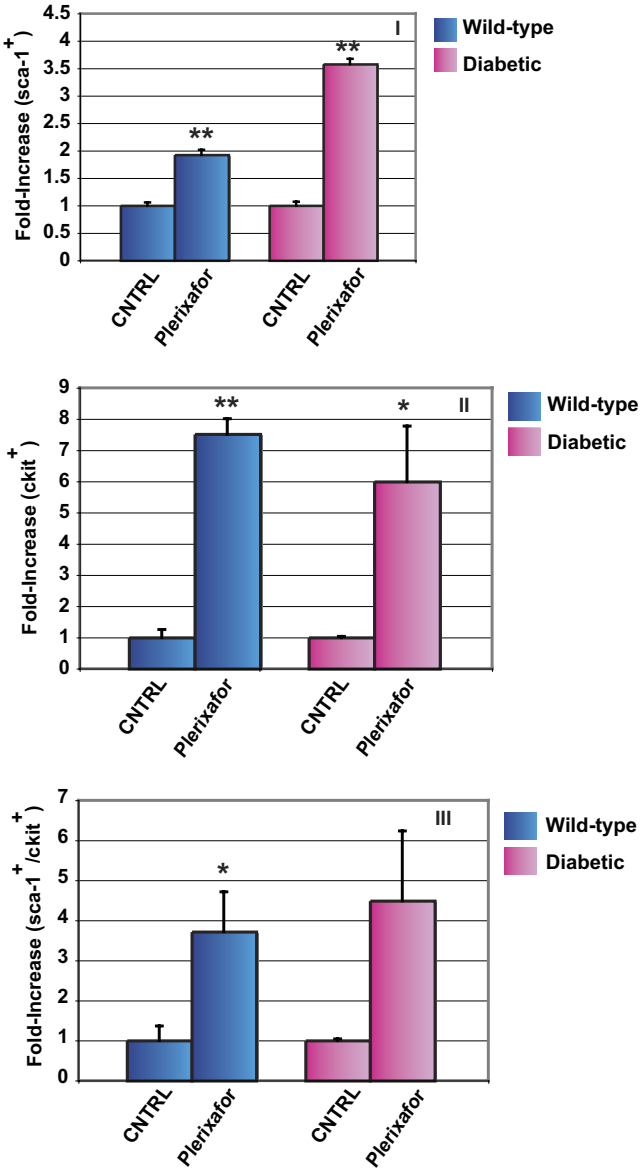

FIG. 7. Restoration of diabetic bone marrow PC mobilization with plerixafor treatment. Fluorescence activated cell sorting of peripheral blood indicated significant elevation of PC populations in both wild-type (blue) and diabetic (pink) animals at day 10 after treatment with plerixafor: $lin^-/sca\text{-}1^+$ (*top*), $lin^-/ckit^+$ (*middle*), and $lin^-/sca\text{-}1^+/ckit^+$ (*bottom*). CNTRL, control; *$P < 0.05$; **$P < 0.01$. (A high-quality color digital representation of this figure is available in the online issue.)

underlying this deficiency and the resulting impact on wound healing remain unknown. The present study is the first to report a decline in bone marrow SDF-1α levels as a key regulator of PC mobilization in response to peripheral ischemia and further identifies this SDF-1α–mediated pathway as a primary mechanism of insufficient PC mobilization in diabetes. We provide further support that decreased numbers of circulating PC results in poor diabetic wound closure, and also demonstrate that diabetic wound closure may be improved by restoring this pathway with the CXCR4 partial agonist, AMD3100 (9).

Our initial experiments demonstrated elevated circulating PCs in wild-type animals after ischemia (3.6-fold L^-C^+, 7.6-fold $L^-S^+C^+$ at day 10), with only minor changes in circulating PC numbers in diabetic animals (1.4-fold L^-C^+, 1.9-fold $L^-S^+C^+$ at day 10). Similar findings were noted by Fadini et al., who reported a peak in S^+ and S^+C^+ cells 2 to 3 days after hind-limb ischemia (approximately a four-fold increase), with no comparable increase in circulating PCs of type I diabetic animals (22). Although the kinetics of peak mobilization vary between our study and that of Fadini et al. (day 10 vs. day 2), we confirm, in a different model of peripheral ischemia, that type 2 diabetes is associated with diminished circulating PCs. Additionally, our findings offer further evidence that different subpopulations of bone marrow PCs may be mobilized at different times after peripheral wounding, (23,24) as the $lin^-/sca\text{-}1^+$ population in wild-type mice was mobilized earlier and to a greater extent than the other populations of PCs compared with diabetic mice (Fig. 2*B*). Conversely, the number of PCs found at baseline in the bone marrow and peripheral blood of wild-type mice correspond with those reported by Yamamoto et al. (25).

Subsequent analysis indicated that this phenomenon was not due to differences in the availability of cells from the bone marrow, as the number of PCs in the bone marrow did not differ among wild-type and diabetic animals at any time point studied. Similarly, no differences were noted in bone marrow PC proliferative capacity. These data supported our initial hypothesis that fewer circulating PCs in diabetes is the result of aberrant mechanisms of mobilization.

We then explored chemokinetic-signaling pathways that have been implicated in PC mobilization. Previous reports suggest that PC mobilization is regulated in part by serum SDF-1α levels (12,21). Though we observed significant increases in serum SDF-1α in both animal types, we believe that these changes were not the primary mechanism responsible for PC mobilization. First, maximum levels of serum SDF-1α in wild-type animals occurred at day 2 after ischemia, whereas peak PC mobilization occurred at a much later time point (day 10). Second, diabetic animals achieved levels of serum SDF-1α equal to wild-type, but did not experience substantial mobilization of PCs at any time point. Finally, diabetic PCs exhibited a normal migratory response to SDF-1α, as demonstrated by an in vitro chemotaxis assay.

In contrast to serum SDF-1α, bone marrow SDF-1α levels significantly affected PC mobilization. Our data revealed a temporal relationship between changes in bone marrow SDF-1α and PC mobilization. Wild-type bone marrow SDF-1α levels declined at day 7 after ischemia, immediately before mobilization, which subsequently peaked at day 10. Moreover, a failure of bone marrow SDF-1α to decrease after peripheral wounding correlated with the impaired mobilization observed in diabetic animals. Finally, pharmacologic reduction of bone marrow SDF-1α levels with plerixafor in both wild-type (32%) and diabetic (54%, relative to control) animals led to significant PC mobilization.

By investigating both serum and bone marrow SDF-1α levels in relation to PC mobilization, we also addressed the long-standing question of whether a SDF-1α bone marrow-to-peripheral blood gradient is necessary for successful PC mobilization. Previously, De Falco et al. (12) detected a positive SDF-1α gradient (bone marrow levels less than peripheral blood levels) in a hind-limb ischemia model. Others have argued against such a gradient, claiming that bone marrow levels remain higher than peripheral blood levels throughout the mobilization process (14). Although we identified a switch in bone marrow SDF-1α expression, a significant

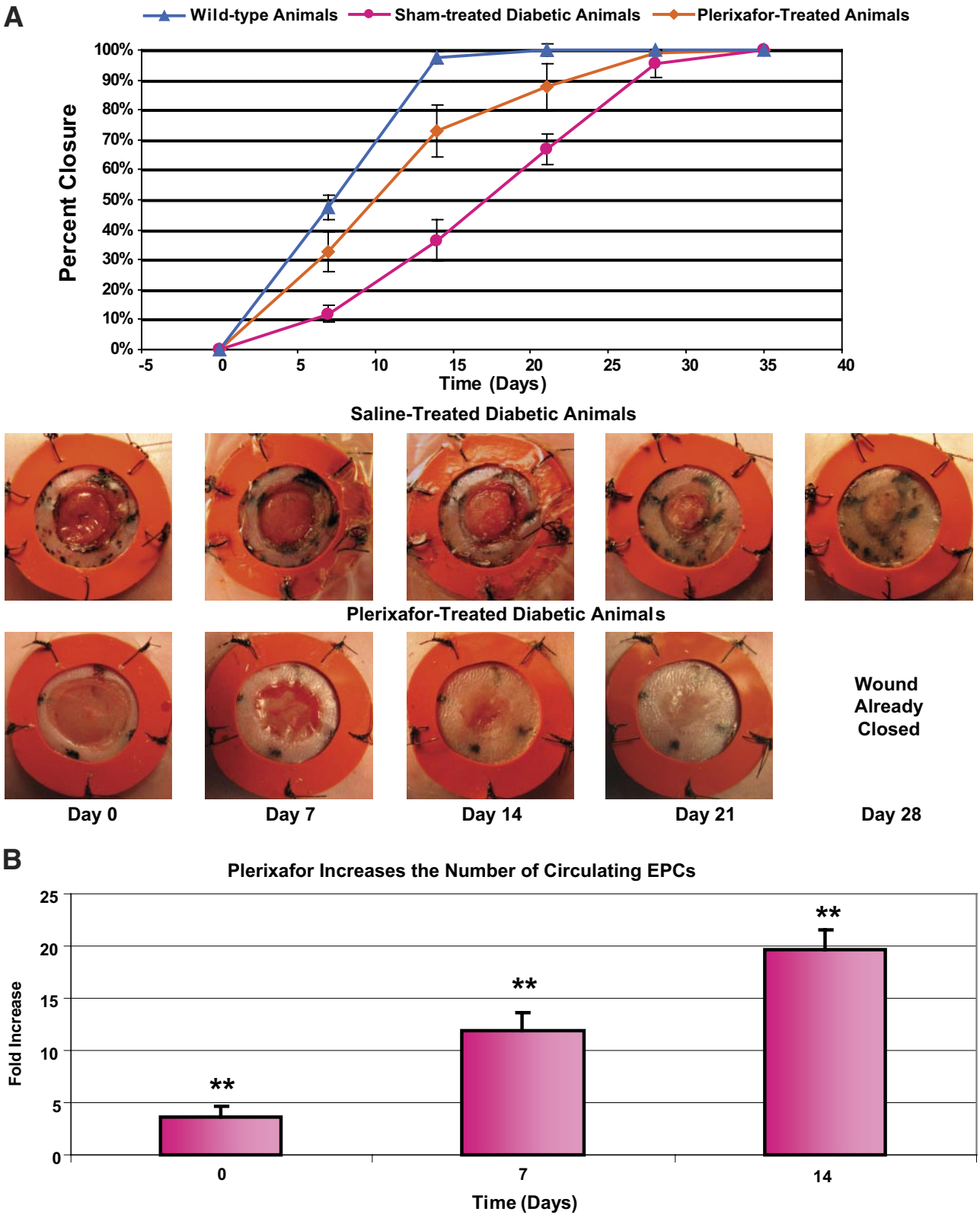

FIG. 8. Restoration of diabetic bone marrow PC mobilization and diabetic wound closure. *A*: Wounds of plerixafor-treated diabetic mice (orange) had decreased time to closure rates when compared with sham-treated diabetic mice (pink). Pictures of the wound closure progression from a sham-treated (*above*) and plerixafor-treated (*below*) diabetic mice are shown, demonstrating improved diabetic wound closure with plerixafor treatment. *B*: Peripheral blood harvested 1 h after plerixafor administration demonstrated a linear increase in the population of circulating vascular PC ($lin^-/sca\text{-}1^+$) through postwounding day 14. EPCs, endothelial progenitor cells; *$P < 0.05$; **$P < 0.01$. (A high-quality color digital representation of this figure is available in the online issue.)

gradient from bone marrow to peripheral blood was never established in response to peripheral ischemia alone. Conversely, treatment with plerixafor generated a 1:2.4 and 1:3.6 SDF-1α gradient (bone marrow:peripheral blood), resulting in greater PC mobilization in wild-type (1.9 ± 0.1-fold increase in L^-S^+, $P < 0.01$; 7.5 ± 0.5-fold increase in L^-C^+, $P < 0.01$; 3.7 ± 1.0-fold increase in $L^-S^+C^+$, $P < 0.05$) and diabetic mice (3.6 ± 0.1-fold increase in L^-S^+, $P < 0.01$; 6.0 ± 1.8-fold increase in L^-C^+, $P < 0.05$; 4.5 ± 1.7-fold increase in $L^-S^+C^+$, $P < 0.05$) 10 days after cutaneous injury, respectively. Based on these experiments, we hypothesize that a bone marrow:peripheral blood SDF-1α gradient enhances, but is not critical for, ischemia-induced PC mobilization.

Although this study demonstrates that a reduction in bone marrow SDF-1α serves as a critical mechanism for peripheral ischemia-induced PC mobilization, studies to investigate quotidian bone marrow SDF-1α regulation are needed. Moreover, the mechanisms mediating the observed switch in bone marrow SDF-1α remain unknown. Since much attention has been focused on the influence of MMP-9 and kit ligand on PC mobilization, subsequent studies from our laboratory investigated a potential mechanism involving these factors (13,26). To date, we have been unable to demonstrate a correlation between bone marrow or serum MMP-9 levels and the impaired SDF-1α switch in diabetic mice (data not shown). One potential factor may be neutrophil elastase (NE), which has been shown to break down SDF-1α within the bone marrow niche (27,28). Although it is possible that diabetic bone marrow contains less NE, this seems unlikely in light of previous reports that demonstrate elevated levels of circulating NE in diabetic patients (29,30). It is also plausible that diabetes affects the functional activity, rather than the amount, of NE in the bone marrow. Future studies will help identify the role of various proteinases in bone marrow SDF-1α stability, as well as their involvement in diabetic PC mobilization.

In addition to identifying a novel mechanism for normal and impaired PC mobilization, this study highlights the potential clinical utility of plerixafor as an agent for PC augmentation in human patients. Clinical trials are currently underway to evaluate the efficacy of augmented PC mobilization and direct PC delivery to sights of vascular occlusion (31). Ongoing trials with plerixafor are encouraging, offering strong evidence for robust mobilization of proangiogenic cells (32,33). Indeed, our group has found that plerixafor significantly improves time to re-epithelialization (closure) in a diabetic wound model via restoration of bone marrow PC mobilization. Although a single dose of plerixafor resulted in reduction of SDF-1α in the bone marrow and a subsequent release of bone marrow progenitor cells, this did not alter the rate of closure in diabetic animals. This finding is consistent with previous studies using our wound healing model in which repeated delivery of supraphysiologic levels of VEGF was necessary to document increased rates of closure (9). Moreover, bone marrow-derived progenitor cells are known to rapidly return to the bone marrow or extramedullary sites to replenish progenitor cell populations, (14), and thus it is not surprising that we did not see physiologic effects on wound closure after a single dose. Therefore, we choose to deliver repeated doses of plerixafor based on delivery protocols that have been previously reported to have a biologic effect (34). And although these findings do not infer that plerixafor results in normal wound healing, we feel that faster wound closure with this treatment is of clinical importance.

Although the putative mechanism of plerixafor-mediated PC mobilization is CXCR4 agonism, (35) we identify an additional physiologic effect of this pharmacologic agent: a significant decrease in bone marrow SDF-1α levels. This finding is consistent with previous data demonstrating increased progenitor cells mobilization with G-CSF delivery (15); however, the mechanism by which plerixafor achieves decreased levels of bone marrow SDF-1α remains unknown. At present, it is unclear whether SDF-1α levels decreased through altered mechanisms of translocation, degradation, or some yet unidentified mechanism (36). Current data suggest an apparent paradox in the SDF-1α–CXCR4 signaling pathway of progenitor cell trafficking. Petit et al. (37) recently proposed the notion of a "biphasic mobilization pattern" in which CXCR4 inhibition allows for recruitment of stem cells from the perivascular niche, whereas activation of CXCR4 promotes the mobilization of stem cells in a different niche that are tethered to the stroma. Further investigation is warranted, however, as the long-term effects of plerixafor treatment on bone marrow and peripheral blood SDF-1α levels remain unknown. Since diabetes impairs the bone marrow SDF-1α switch after peripheral ischemia and diabetic patients often require vascular therapeutic interventions, our findings offer clinical significance for diabetic wound healing. Future studies should help elucidate the therapeutic implications of targeting the bone marrow SDF-1α–vasculogenic PC mechanism to augment diabetic progenitor cell mobilization.

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O.M.T. and J.C. contributed equally to this study.

O.M.T., J.C., and R.J.A. researched data, contributed to discussion, wrote the manuscript, and reviewed/edited the manuscript. C.C.C., C.D.L., R.T., and S.M.G. researched data and contributed to discussion. J.P.L., P.B.S., and S.M.W. contributed to discussion and reviewed/edited the manuscript.

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