Inhibition of stromal cell-derived factor-1α further impairs diabetic wound healing

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Objective: **Impaired diabetic wound healing is associated with abnormal stromal cell-derived factor (SDF)-1α production, decreased angiogenesis, and chronic inflammation. Lentiviral-mediated overexpression of SDF-1α can correct the impairments in angiogenesis and healing in diabetic wounds. We hypothesized that SDF-1α is a critical component of the normal wound-healing response and that inhibition of SDF-1α would further delay the wound-healing process.**

Methods: **dB/Db diabetic mice and Db/+ nondiabetic mice were wounded with an 8-mm punch biopsy and the wounds treated with a lentiviral vector containing either the green fluorescent protein (GFP) or SDF-1α inhibitor transgene. The inhibitor transgene is a mutant form of SDF-1α that binds, but does not activate, the CXCR4 receptor. Computerized planimetry was used to measure wound size daily. Wounds were analyzed at 3 and 7 days by histology and for production of inflammatory markers using real-time polymerase chain reaction. The effect of the SDF-1α inhibitor on cellular migration was also assessed.**

Results: **Inhibition of SDF-1α resulted in a significant decrease in the rate of diabetic wound healing, (3.8 vs 6.5 cm^2/day in GFP-treated wounds; $P = .04$), and also impaired the early phase of nondiabetic wound healing. SDF-1α inhibition resulted in fewer small-caliber vessels, less granulation tissue formation, and increased proinflammatory gene expression of interleukin-6 and macrophage inflammatory protein-2 in the diabetic wounds.**

Conclusions: **The relative level of SDF-1α in the wound plays a key role in the wound-healing response. Alterations in the wound level of SDF-1α, as seen in diabetes or by SDF-1α inhibition, impair healing by decreasing cellular migration and angiogenesis, leading to increased production of inflammatory cytokines and inflammation. Inhibition of SDF-1α further impairs diabetic wound healing. (J Vasc Surg 2011;53:774-84.)**

Clinical Relevance: **Diabetes results in a significant impairment in wound healing, leading to significant morbidity and health care expenditures. The pathophysiology that underlies this process is multifactorial, including abnormal growth factor production, cellular migration, and cellular function. Stromal cell-derived factor (SDF)-1α is a key chemokine involved in the wound-healing process and is involved in cellular recruitment and angiogenesis. SDF-1α is decreased in diabetic individuals. This study showed that inhibition of SDF-1α results in an even more dramatic delay in the diabetic wound-healing process and even results in a delay in the early phases of wound healing in normal mice, further supporting its role in wound healing. Inhibition of this chemokine promotes greater inflammatory cytokine production, inflammatory cell migration, and less vasculogenesis after dermal wounding. This study identifies SDF-1α as an essential component of normal wound healing and provides a potential therapeutic target to improve the diabetic wound-healing impairment.**

Diabetes has reached pandemic proportions in the United States and across the globe. Expenditures on diabetes care in the United States was >$174 billion in 2007.[1] An ulcer of the lower extremity precedes 84% of all diabetic lower extremity amputations and is the primary cause for hospitalization among diabetic individuals.[2] Despite the increasing prevalence of diabetes, current therapies have not decreased the incidence of amputation or lower extremity ulcer formation.

Hyperbaric oxygen, recombinant platelet-derived growth factor (PDGF), and biosynthetic grafts all have been extensively studied as alternative methods to promote the wound-healing process.[3-10] Although promising, their use has not been widespread. Hyperbaric oxygen has been demonstrated to increase numbers of endothelial progenitor cells (EPCs) in animal studies as well as in vitro studies, but not in humans.[11] Recombinant PDGF application to diabetic wounds demonstrates some reproducible improvement of the wound-healing process.[7] Apligraf (Novartis, East Hanover, NJ) or Dermagraft (Dermagraft, Westport, Conn) are both biosynthetic grafts used to promote wound closure in diabetic patients.[9,10] Although there is evidence to support the benefit of these therapies when used clinically, widespread use is tempered by their prohibitive costs.[12]

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The molecular physiology that underlies the diabetic wound-healing defect remains unclear. The wounds of diabetic individuals present with fewer EPCs, greater inflammation, and fewer growth factors.[13-19] Specifically, deficiencies in growth factors such as PDGF, keratinocyte growth factor, transforming growth factor-β (TGF-β), hepatocyte growth factor, and vascular endothelial growth factor, have all been implicated in the delayed healing rates observed in chronic diabetic wounds.[13-19] Chronic diabetic wounds have been shown to have deficiencies in the cellular response to growth factors, including decreased cellular recruitment and migration, decreased angiogenesis and granulation tissue production, impaired re-epithelialization, decreased extracellular matrix production, and impaired wound contraction.[13-19]

Stromal-derived factor-1α (SDF-1α), a CXC chemokine implicated in the wound-healing process[16-19] in the $Lepr^{db}$ mouse model of type II diabetes can correct this wound-healing defect when overexpressed.[20] Greater granulation tissue, smaller epithelial gap, and smaller wound size were all found on histologic analysis. To examine the role of SDF-1α in impaired and nonimpaired wound healing, we injected a lentiviral vector that expresses a mutant form of SDF-1α that binds, but does not activate, CXC chemokine receptor type 4 (CXCR4) and measured its effect on granulation tissue formation, angiogenesis, inflammation, cell migration, and wound healing.

METHODS

Lentiviral vector construction and fibroblast transduction. The *SDF* mutant we generated binds the CXCR4 receptor but does not activate it, based on studies by Choi et al,[21] using site-directed mutagenesis to evaluate the effect of specific mutations in the SDF-1α gene on CXCR4-mediated signal transduction. Replacement of the C-terminal proline amino acid with glycine generates a mutated form of SDF-1α that binds to the CXCR4 receptor but does not activate it.

A complementary DNA library was prepared from mouse tissues using Trizol and Superscript (Invitrogen, Carlsbad, Calif) according to the manufacturer's instructions. Sequence analysis was used to confirm the murine SDF-1α cDNA as well as the SDF-1α inhibitor.

The CS-CG HIV-1 transfer plasmid, modified as previously described,[22,23] was used to generate a self-inactivating lentiviral vector. This lentiviral vector allows expression of the green fluorescent protein (GFP) reporter gene (Clontech Laboratories, Mountain View, Calif) or the mutant SDF-1α construct with the GFP reporter gene as a single transcript under the control of a cytomegalovirus promoter. Vesicular stomatitis virus-G protein pseudotyped viral particles were generated by transfection into a 293T cell line and titered, as previously described.[24]

To test the ability of our viral construct to efficiently infect cells and produce transgene protein, we incubated passage five dermal fibroblasts with our lentiviral construct at a multiplicity of infection of 100 for 24 hours. Transduced fibroblasts were then plated in 12-well tissue culture plates at a seeding density of 5×10^5 cells/well. Tissue culture supernatants were aspirated from the plates 24 hours after transfection then frozen at −80°C. SDF-1α protein content was determined from thawed supernatants using a Quantikine enzyme-linked immunosorbent assay kit for murine CXCL12/SDF-1α (R&D Systems, Minneapolis, Minn) according to the manufacturer's instructions. All transduced cells produced >100 ng/mL of SDF-1α inhibitor.

Experimental animals and surgical procedure. All experimental protocols used in this study were approved by the Institutional Animal Care and Use Committee at the Children's Hospital of Philadelphia. Genetically diabetic, 10- to 12-week-old, female C57BKS.Cg-m/$Lepr^{db}$ mice and age-matched nondiabetic heterozygous mice (Jackson Laboratory, Bar Harbor, Me) were used in this study. At the time of wounding, dB/Db mice weighed >45 g with blood glucose levels >400 mg/dL and Db/+ heterozygote mice weighed <25 g with blood glucose levels <250 mg/dL. Animals were given standard rodent chow and water ad libitum.

Each mouse was shaved and depilated before wounding. The dorsal skin was swabbed with alcohol and Betadine (Purdue Pharma, Stamford, Conn). Each mouse underwent a single dorsal full-thickness wound (including panniculus carnosum) with an 8-mm punch biopsy (Miltex Inc, York, Pa). After wounding, a Hamilton syringe was used to deliver 50 μL of the lentiviral SDF-1α inhibitor vector or a lentiviral GFP vector as a control. Ten microliters were injected intradermally at the 12, 3, 6, and 9 o'clock positions and at the wound base. Approximately 1×10^8 plaque forming units (PFUs) of the lentiviral constructs were used on each mouse. Next, a sterile Tegaderm (3M, St. Paul, Minn) was placed over the wound and left in place for 48 hours, after which it was removed.

Planimetry and wound size calculation. Photographs were obtained with a Nikon camera using a ruler for each image. ImageJ software (National Institutes of Health, Bethesda, Md; http://rsbweb.nih.gov/ij/) was used to calculate the wound area of each mouse daily. The same observer measured the size of each wound. Wound area was plotted daily as a function of time.

Immunohistochemistry and histologic analysis. Wounds were harvested at days 3 and 7 and immediately fixed in 10% neutral buffered formalin (Sigma, St. Louis, Mo). The tissue was processed using a histoprocessor (Leica TP1050, GMI Inc, Ramsey, Minn). Paraffin sections were cut 4 μm thick, mounted on slides, and incubated (Fisher Scientific, Pittsburgh, Pa) overnight. Slides were deparaffinized and washed in ethanol. Endogenous peroxidase was quenched with 0.3% hydrogen peroxide for 30 minutes at room temperature. Slides were pretreated with proteinase K (Dako, Carpinteria, Calif) for 10 minutes and rinsed with distilled water. Primary murine α-smooth muscle actin (α-SMA; 1:200), CD31 (1:50), and CD45 (1:50) antibodies (BD Biosciences, San Jose, Calif) were applied. They were developed with a Vectastain Elite ABC kit with secondary antibody (Vector Laboratories, Burlingame,

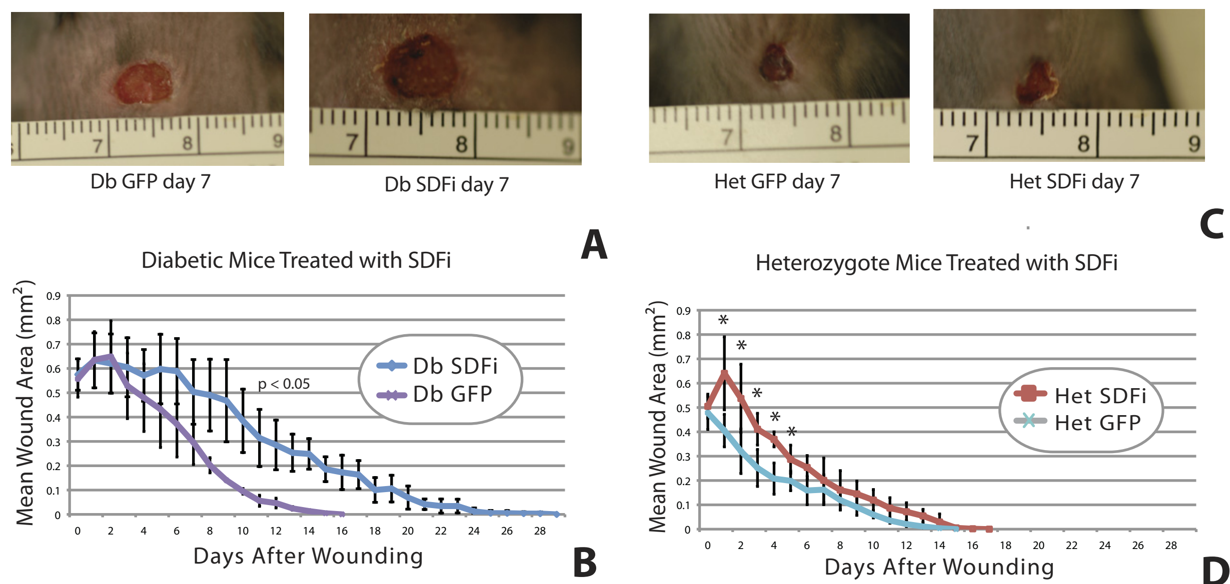


Fig 1. A, Diabetic wounds are shown at day 7 in the green fluorescent protein (*GFP*)-treated and stromal cell-derived factor inhibitor (*SDFi*)-treated mice. **B,** Mean wound area is shown for diabetic mice treated with SDFi or control GFP vector at each day after wounding (each data point, n = 5). **C,** Results for heterozygote (*Het*) mice are shown in both experimental groups at day 7. **D,** Daily mean wound area is shown for heterozygous mice in both groups until closure. The days after wounding at which there was a significant difference in wound size between the two groups is shown by the *asterisk* above the graph.

Calif). A blinded observer analyzed granulation tissue area, α-SMA+ vessel density, and numbers of CD31+ and CD45+ cells per high-power field (HPF). Three HPFs per slide were examined.

Real-time quantitative polymerase chain reaction. Wounds were harvested at days 3 and 7 after wounding and frozen in liquid nitrogen. Samples were homogenized in TRIzol (Invitrogen), and total RNA was extracted and purified following the manufacturer's instructions. RNA was converted into complementary DNA using the SuperScript First-Strand Synthesis System (Invitrogen). Real-time polymerase chain reaction (PCR) was performed with the ABI 7900 real-time PCR thermal cycler (Applied Biosystems, Foster City, Calif) to amplify samples in triplicate. All samples were amplified using TaqMan prepared primer assays. The average of the triplicate gene product for each gene was compared with 18S ribosomal RNA expression to generate a relative fold-expression. Results were reported as means ± SEM. A total of 19 mice (with 4 groups of 5 mice each) were sacrificed to perform PCR analysis, with a total of 57 PCR reactions performed.

Cellular migration. A standard 24-well colorimetric assay was used to assess cellular migration. Boyden chambers (Chemicon, Santa Cruz, Calif) equipped with 8-μm pore diameter polycarbonate filters were used. Cells were suspended in migration medium (1 μg/mL heparin/0.1% bovine serum albumin in Dulbecco's Modified Eagle Medium) and 2 × 105 cells were loaded into the upper chamber. The lower chambers were plated 24 hours before the migration assay with one of five conditions: (1) fibroblasts alone, (2) fibroblasts transfected with lenti-SDF-1α, (3) fibroblasts transfected with lenti-SDF-1α and fibroblasts transfected with the lenti-SDF-1α inhibitor at a ratio of 1:1 and (4) at a ratio of 1:2, and (5) no cells. The cells plated before migration were done so with 2 × 105 cells. This lower chamber was washed with phosphate-buffered saline and filled with migration medium. After 16 hours, cells in the migration medium were removed and quantified under a light microscope (original magnification ×20) after staining with 0.5% crystal violet. All migration experiments were done in triplicate.

Statistical methods. Data are presented as mean ± SEM to two significant figures. Statistical analysis was performed with one-way analysis of variance using SPSS 16.0 software (SPSS Inc, Chicago, Ill). Values were transformed (ln) when required to achieve normality of sampling. When significant differences ($P < .05$) were found, a least significant difference post hoc test, Mann-Whitney *U*, was used to identify differences between individual means.

RESULTS

Wound healing. Treatment of diabetic wounds with the lenti-SDF-1α inhibitor resulted in a significant and further impairment in wound healing. Diabetic wounds treated with lentiviral-SDF inhibitor were significantly larger at day 7 (0.25 ± 0.006 cm^2) compared with diabetic wounds treated with lentiviral GFP (0.16 ± 0.05 cm^2; $P = .009$; Fig 1, *A*). Treatment of diabetic wounds with the

lenti-SDF-1α inhibitor also resulted in a dramatic delay in the mean number of days to wound closure when compared with the lenti-GFP group (25 ± 2.5 vs 15.1 ± 1.3 days to closure; $P < .0021$). In addition, treatment of diabetic wounds with the lenti-SDF-1α inhibitor resulted in a dramatic decrease in the rate of wound closure of 3.8 ± 0.94 mm^2/d in the lenti-SDF-1α inhibitor group (n = 5) vs 6.5 ± 2.2 mm^2/d in the lenti-GFP group (n = 5; $P = .04$; Fig 1, *B*).

Treatment of nondiabetic wounds with the lenti-SDF-1α inhibitor demonstrated a significant impairment in the initial phase of wound healing, with increased wound area in days 1 to 5 vs the group treated with lenti-GFP ($P \leq .03$; Fig 1, *C*). However, the mean wound area after 5 days and the overall rate of wound closure for the nondiabetic wounds treated with SDF-1α inhibitor were not significantly different from GFP-treated wounds. The mean wound area over time is shown in Fig 1, *D*.

Granulation tissue. The effect of the lenti-SDF-1α inhibitor on granulation tissue was assessed at 7 days using trichrome staining and measurement of the granulation tissue volume. Representative sections of granulation tissue from diabetic mice treated with lenti-GFP and those treated with lenti-SDF inhibitor are displayed in Fig 2, *A* and *B*, respectively. Treatment with the lenti-SDF-1α inhibitor resulted in a significant decrease in the granulation tissue volume (1.8 ± 0.4/mm^2; $P = .031$; Fig 2, *E*) compared with the lenti-GFP treated diabetic wounds (1.1 ± 0.46/mm^2). Diabetic wounds also demonstrated significantly less granulation tissue than nondiabetic wounds (Fig 2, *C* and *D*).

Representative sections of granulation tissue from nondiabetic mice treated with lenti-GFP are shown in Fig 2, *C*, and from those treated with lenti-SDF-1α inhibitor in Fig 2, *D*. The mean granulation tissue volume was not significantly different (2.4 ± 0.32 vs 2.5 ± 0.94/mm^2; $P = .31$; Fig 2, *E*).

Angiogenesis. The effect of the lenti-SDF-1α inhibitor on angiogenesis was assessed using immunohistochemistry for CD31, an endothelial marker. Representative images of immunoperoxidase staining for CD31 at 7 days in diabetic wounds treated with lenti-GFP or lenti-SDF-1α inhibitor are demonstrated in Fig 3, *A* and *B*. Treatment with the SDF-1α inhibitor resulted in a significant decrease in the number of CD31+ cells/HPF (5.1 ± 1.83 [n = 5]; $P = .005$; Fig 3, *E*) vs lenti-GFP–treated wounds (7.1 ± 1.75).

Representative images of CD31 staining in nondiabetic wounds treated with lenti-GFP and lenti-SDF-1α inhibitor are displayed in Fig 3, *C* and *D*, respectively. Treatment of nondiabetic wounds with the lenti-SDF-1α inhibitor resulted in a significant decrease in the number of CD31+ cells/HPF (10.1 ± 4.45 [n = 10]; $P = .04$; Fig 3, *E*) compared with nondiabetic wounds treated with lenti-GFP (13.3 ± 3.75). In addition, the number of CD31+ cells/HPF was also significantly higher in nondiabetic wounds compared with diabetic wounds ($P < .05$).

Vasculogenesis. The effect of the lenti-SDF-1α inhibitor on vasculogenesis was assessed using immunohistochemistry for α-SMA. Representative images of immunoperoxidase staining for α-SMA at 7 days in diabetic wounds treated with lenti-GFP or lenti-SDF-1α inhibitor are demonstrated in Fig 3, *A* and *B*, respectively. Vessel density per HPF was then quantified. Treatment of diabetic wounds with the lenti-SDF-1α inhibitor resulted in a significant decrease in vessel density (15.1 ± 4.1 vessels/HPF [n = 5]; $P = .018$; Fig 4, *E*) compared with wounds treated with lenti-GFP (19.1 ± 4.5 vessels/HPF [n = 5]).

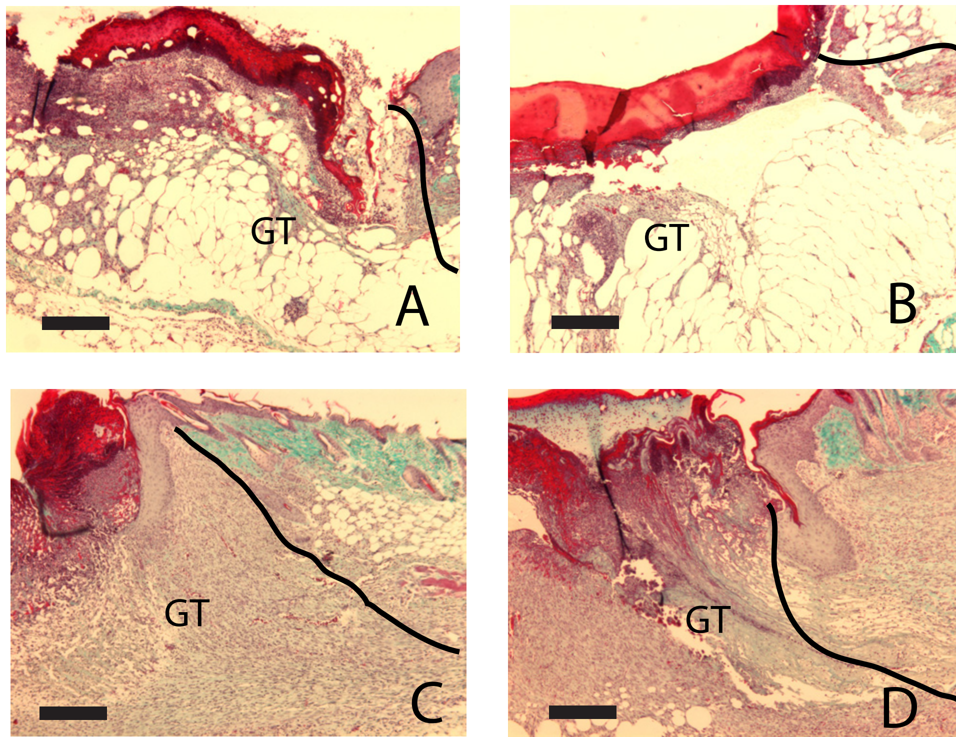
Representative images of immunoperoxidase staining for α-SMA at 7 days in nondiabetic wounds treated with lenti-GFP or lenti-SDF-1α are demonstrated in Fig 3, *C* and *D*, respectively. There was no significant difference in vasculogenesis between nondiabetic wounds treated with lenti-GFP or lenti-SDF-1α (27.9 ± 4.41 vs 25 ± 8.52 vessels/HPF [both n = 5]; Fig 3, *E*). However, diabetic wounds had a significant decrease in vessel numbers/HPF compared with nondiabetic wounds ($P < .0094$).

Inflammation. The effect of SDF-1α inhibition on inflammation was assessed using immunohistochemistry for CD45, the common leukocyte antigen. Representative images of immunoperoxidase staining for CD45 at 7 days in diabetic wounds treated with lenti-GFP or lenti-SDF-1α inhibitor are demonstrated in Fig 5, *A* and *B*, respectively. Diabetic wounds treated with the lenti-SDF-1α inhibitor demonstrated a significant increase in CD45+ cells/HPF of 22 ± 12.9 (n = 5; $P < .005$; Fig 5, *E*) compared with 18 ± 8.0 in lenti-GFP–treated diabetic wounds (n = 5).

Representative images of immunoperoxidase for CD45 at 7 days in nondiabetic wounds treated with lenti-GFP or lenti-SDF-1α inhibitor are demonstrated in Fig 5, *C* and *D*. There was no significant difference in inflammatory cell infiltration in the nondiabetic wounds treated with lenti-GFP or lenti-SDF-1α inhibitor; however, there were significantly fewer CD45+ cells in the nondiabetic wounds than in the diabetic wounds ($P < .002$; Fig 5, *E*).

Inflammatory cytokine gene expression. Real-time PCR was used to assess inflammatory cytokine gene expression in diabetic and nondiabetic wounds treated with lenti-GFP or lenti-SDF-1α inhibitor. Diabetic wounds treated with the lenti-SDF-1α inhibitor demonstrated increased interleukin (IL)-6 gene expression at day 3 after wounding vs lenti-GFP treated wounds (7.5 ± 2.1-fold vs 0.53 ± 0.14-fold; $P = .016$; Fig 6, *A*). By 7 days, there was no difference in IL-6 gene expression between groups. Diabetic wounds treated with the lenti-SDF-1α inhibitor also demonstrated increased gene expression of macrophage inflammatory protein-2 (MIP-2) at day 7 (1.98 ± 0.49-fold vs 1.36 ± 0.17-fold in lenti-GFP control; $P = .026$; Fig 6, *A*).

At 3 days, nondiabetic wounds treated with lenti-GFP demonstrated decreased IL-6 expression (1 ± 0.36-fold) compared with diabetic wounds treated with lenti-GFP (3.52 ± 1.2-fold; $P = .017$). No difference in IL-6 gene expression was seen between groups treated with lenti-GFP



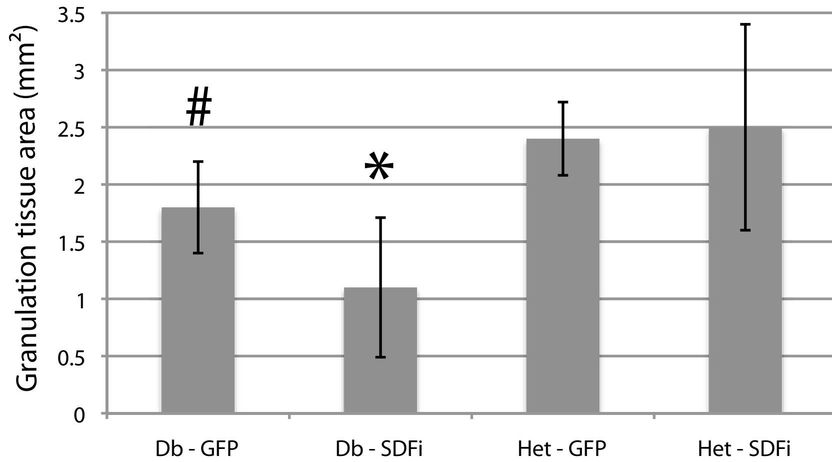
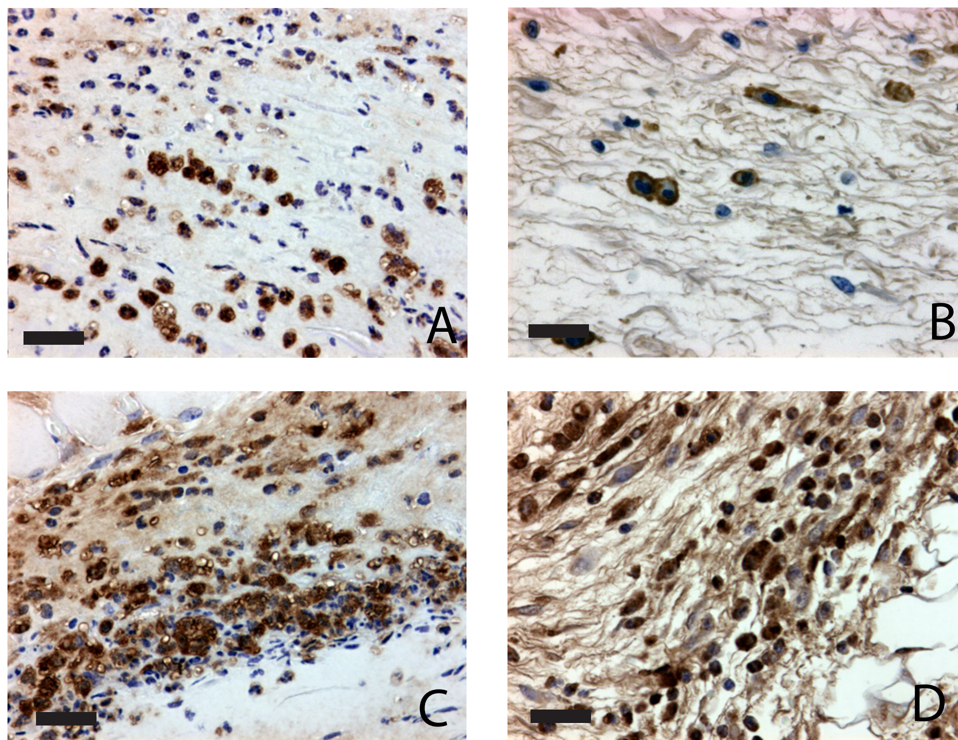


Fig 2. Representative sections of wound tissue are shown 7 days after initial wounding (Masson's trichrome stain; *black bar* = 200 μm): in diabetic wounds treated with **(A)** lenti-green fluorescent protein (*GFP*) or **(B)** with lenti-stromal cell-derived factor-1α inhibitor (*SDFi*), and heterozygote (*Het*) wounds treated with **(C)** GFP or **(D)** lenti-SDFi. **E,** The graph shows mean volume of granulation tissue (*GT*) at day 7 in Db and Het mice treated with GFP and SDFi (*error bars* show the standard error of the mean).

at day 7. Nondiabetic wounds treated with lenti-GFP demonstrated decreased MIP-2 gene expression at day 3 (4.36 ± 0.98-fold v 1 ± 0.20-fold at day 3; P = .004) and postwounding day 7 (0.73 ± 0.17-fold vs 0.08 ± 0.009-fold at day 7; P = .002).

Nondiabetic wounds treated with the lenti-SDF-1α inhibitor did not demonstrate any significant difference in IL-6 gene expression at 3 or 7 days. There was, however, significant increase in MIP-2 gene expression in nondiabetic wounds treated with the lenti-SDF-1α inhibitor at 7



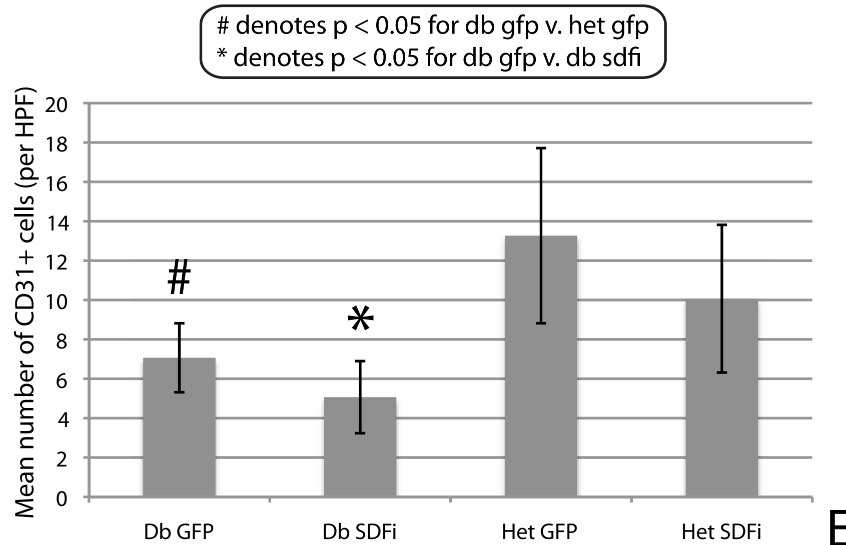


Fig 3. CD31+ cells are shown at day 7 in a diabetic mice treated with **(A)** lenti-green fluorescent protein (*GFP*) or **(B)** lenti-stromal cell-derived factor-1α inhibitor (*SDFi*), and in heterozygote (*Het*) mice treated with **(C)** GFP or **(D)** lenti-SDFi (the *black bars* represent 25 μm). **E,** The graph shows the mean number of CD31+ cells counted for both groups (the *error bars* show the standard error of the mean).

days (1.37 ± 0.20-fold) vs nondiabetic wounds treated with lenti-GFP (0.08 ± 0.009-fold; $P = .003$). Fig 6, *B* displays the relative-fold expression for IL-6 and MIP-2, respectively, in both lenti-SDF-1α inhibitor and lenti-GFP-treated nondiabetic wounds.

Cellular migration. Fibroblasts transduced with lenti-SDF-1α resulted in a significant increase in the migration of splenic leukocytes (253 ± 45 cells) compared with non-transduced fibroblasts (160 ± 39 cells; $P = .004$; Fig 7) or no cells (144 ± 40 cells; $P = .017$). The addition of fibroblasts transduced with the lenti-SDF-1α inhibitor, without changing the number of total fibroblasts, resulted in a dose-dependent decrease in the migration of splenic leukocytes.

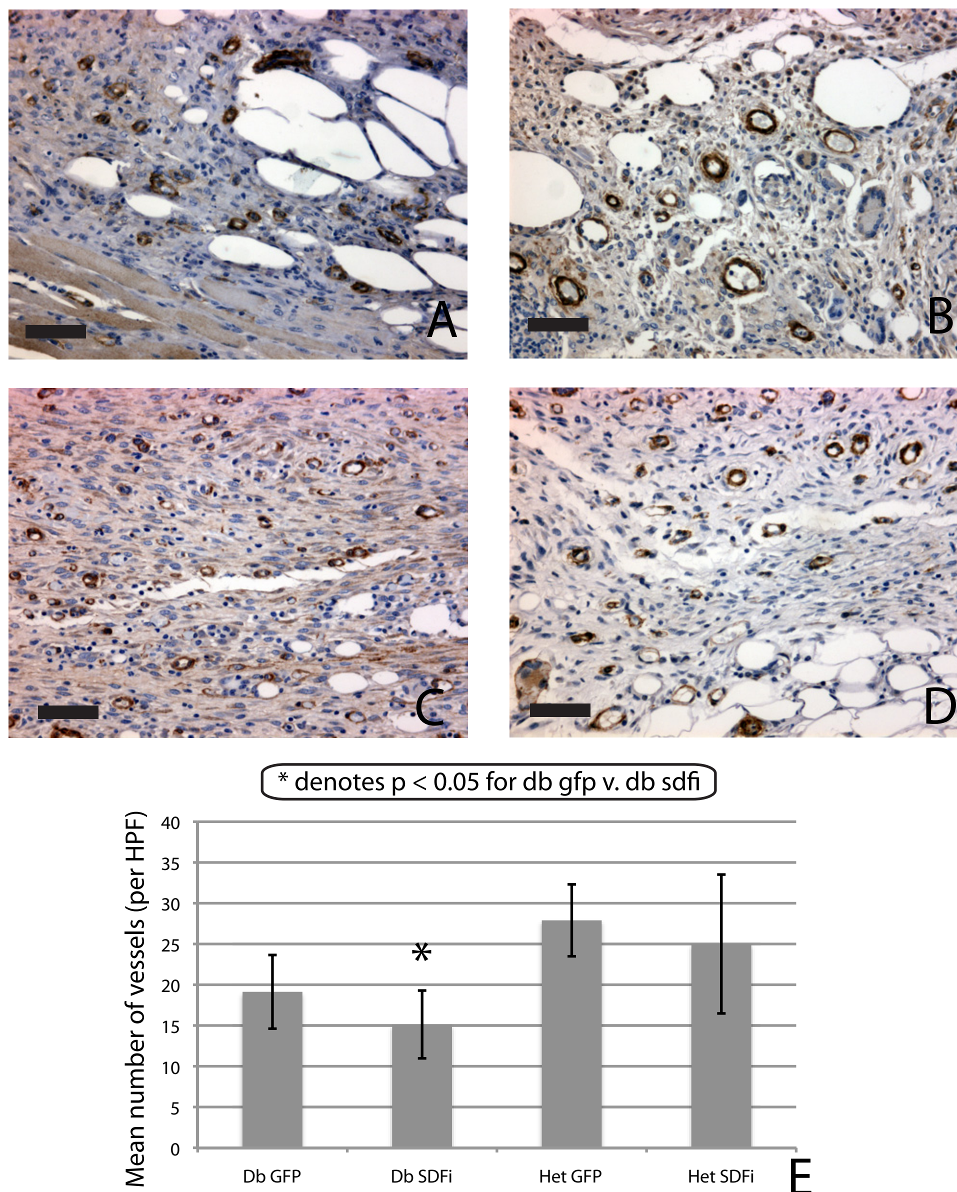


Fig 4. α-Smooth muscle actin staining at 7 days after wounding is shown in diabetic mice treated with **(A)** lenti-green fluorescent protein (*GFP*) or **(B)** lenti-stromal cell-derived factor-1α inhibitor (*SDFi*), and in heterozygote (*Het*) mice treated with **(C)** lenti-GFP or with **(D)** lenti-SDFi (*black bar* = 25 μm). **E,** Graph shows the mean number of vessels at day 7 (*error bars* show the standard error of the mean).

DISCUSSION

We demonstrate that SDF-1α is a key component in the wound healing process and that competitive inhibition of the activation of the CXCR4 receptor can alter the rate of wound healing—especially in the diabetic mouse. Competitive inhibition of SDF-1α significantly impairs the rate of wound healing, decreases angiogenesis, and increases inflammation in the diabetic mouse. These are all features of a chronic wound phenotype. In nondiabetic mice, the inhibitor also decreases rate of wound healing early on and is associated with increased inflammation. These findings suggest a delay in wound healing, with the increased duration of an open wound, which may result in increased inflammatory response to the persistent wound. Nondiabetic wounds may be able to overcome this increased inflammatory response, but diabetic wounds, which already have a disordered inflammatory response, may be unable to compensate for the increased inflammation.

Diabetic wounds are deficient in SDF-1α,[11] and correction of this deficiency improves healing.[20] Because there was

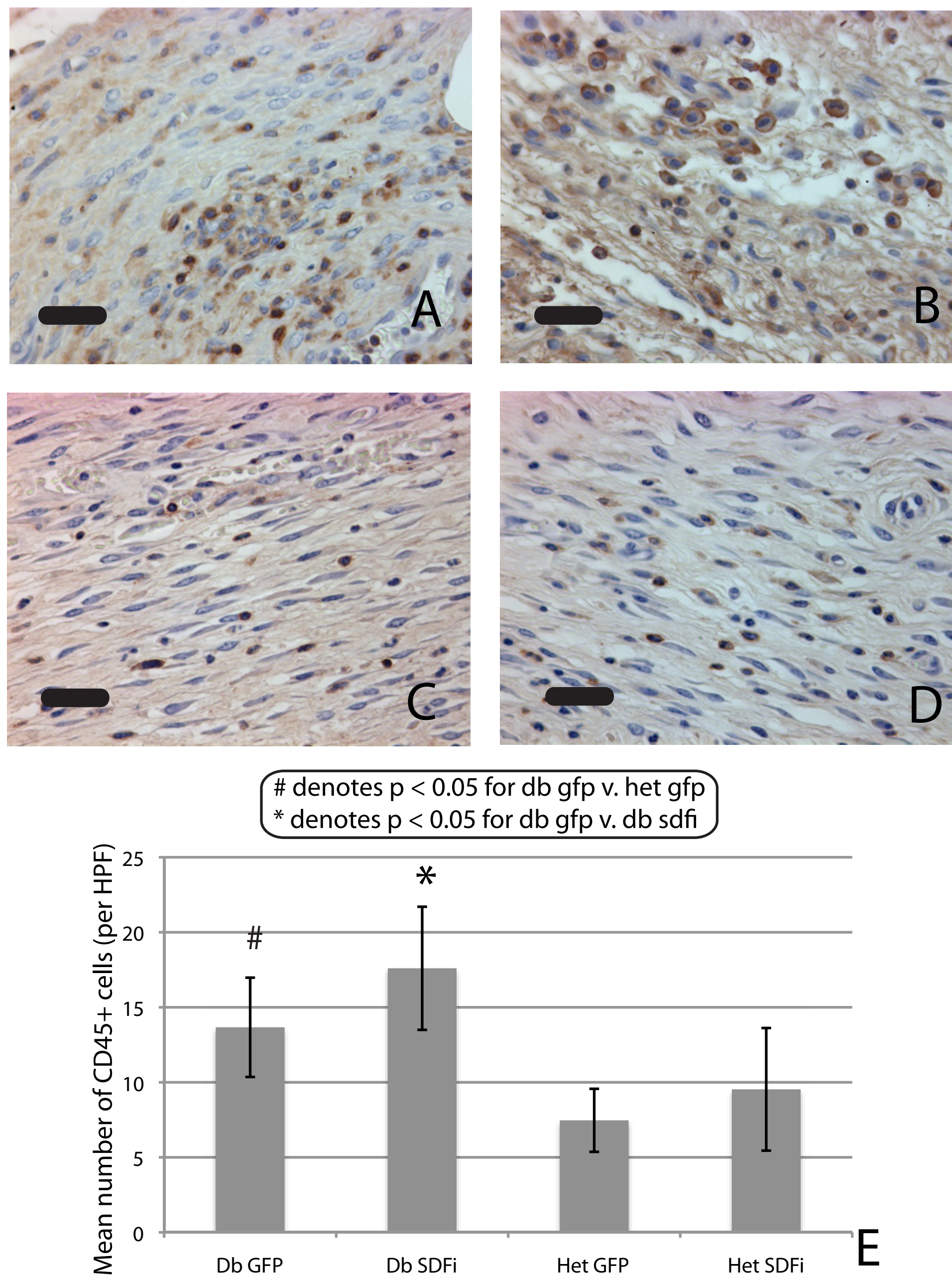


Fig 5. CD45 staining at day 7 is shown in diabetic mice treated with **(A)** lenti-green fluorescent protein (*GFP*) or **(B)** lenti-stromal cell-derived factor-1α inhibitor (*SDFi*), and in heterozygote (*Het*) mice treated with **(C)** lenti-GFP or **(D)** with lenti-SDFi (*black bar* = 25 μm). **E,** Graph shows the mean number of CD45+ cells per high-powered field (*HPF*; the *error bars* show the standard error of the mean).

no SDF-1α deficiency in nondiabetic mice, the level of SDF-1α production required to overcome the effects of the inhibitory vector are lower. These findings suggest a role for SDF-1α in normal wound healing, but SDF-1α may have an even more critical role in the pathogenesis of diabetic wound healing.

SDF-1α is a chemotactic factor regulating the migration of EPCs as well as leukocytes.[25,26] EPC migration is also regulated by activated endothelial nitric oxide synthase. In addition to SDF-1α deficiency, diabetic wounds have also been shown to be deficient in endothelial nitric oxide synthase.[11] Decreased EPC recruitment and function have been implicated in the impairment of diabetic wound healing.[27,28] A recent study demonstrated that db/db mice have fewer circulating EPCs and that wound healing was subsequently significantly improved when more EPCs were mobilized.[29] These findings, combined with our observa-

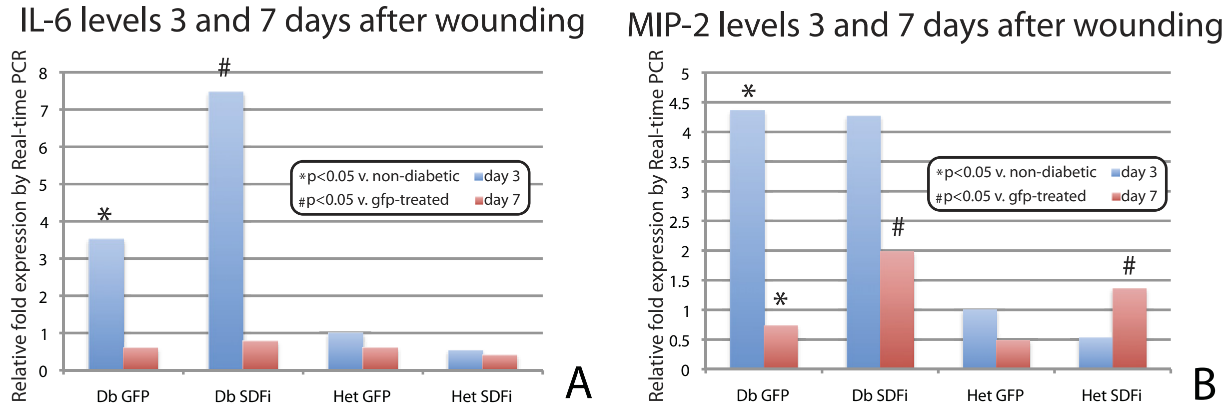


Fig 6. Real-time quantitative polymerase chain reaction analysis of whole wounds at days 3 and 7 after wounding shows **(A)** interleukin-6 (*IL-6*) levels in diabetic and nondiabetic mice and **(B)** macrophage inflammatory protein-2 (*MIP-2*) levels (n = 5 for each experimental group). *GFP,* Green fluorescent protein; *Het*, heterozygote; *SDFi,* stromal cell-derived factor-1α inhibitor.

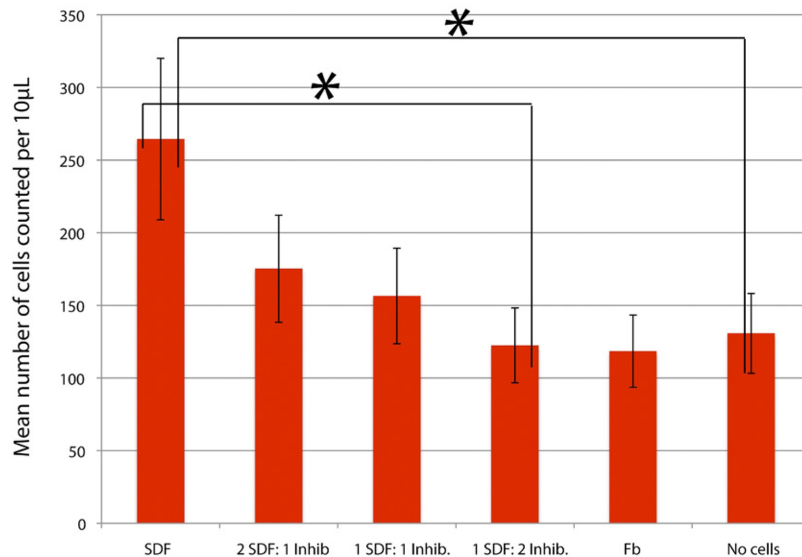


Fig 7. Splenic leukocyte migration assay shows there was a decrease in leukocyte migration with increasing concentrations of lenti-stromal cell-derived factor (*SDF*)-1α inhibitor transfected cells. On average, 256 ± 42 cells were counted in the group with lenti-SDF-1α cells. When the lower chamber had an increased ratio of lenti-SDF-1α inhibitor, fewer cells migrated; when 1.5×10^5 lenti-SDF-1α inhibitor cells were plated, 126 ± 51 cells were counted (*$P < .05$). The *error bars* show the standard error of the mean.

tions of further wound healing impairment with SDF-1α inhibition, further support a role for SDF-1α in wound healing and the diabetic impairment in healing.

SDF-1α is partly regulated through the transcriptional activator hypoxia inducible factor-1α.[25] After injury, wound-induced tissue hypoxia leads to upregulation of hypoxia inducible factor-1α.[30] Toksoy et al[31] has demonstrated that there is a clear increase in the expression of SDF-1α at the ischemic margin of a wound in a normal wound-healing environment. This coincides with the region where most vasculogenesis occurs. In vitro, SDF-1α has been shown to promote the proliferation and migration of endothelial cells,[32,33] and in vivo, it augments the growth of granulation tissue and endothelial and progenitor cells.[11,20,34]

Inhibition of SDF-1α activity resulted in decreased CD31+ cells and vessels in the wound and decreased granulation tissue formation, which correlated with impaired rates of wound healing and delayed wound closure.

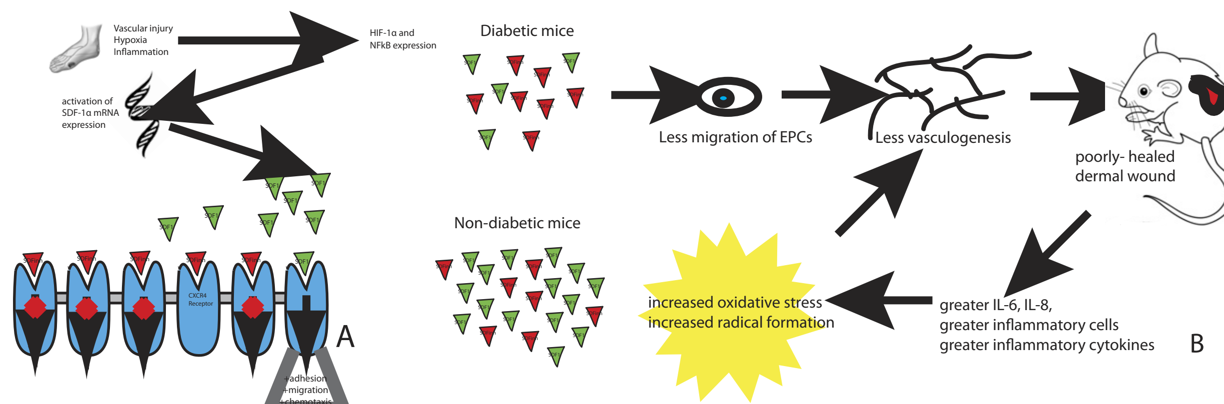


Fig 8. **A,** Schematic of the mechanism by which the stromal cell-derived factor (*SDF*)-1α inhibitor has an effect on the intracellular transduction at the CXCR4 receptor. Although the inhibitor competitively inhibits at CXCR4 through binding, it does not activate a series of important intracellular signaling cascades. **B,** Inhibition of SDF-1α in diabetic wounds may be easier due to intrinsically low levels. This prevents angiogenesis and subsequently promotes a chronic wound. *EPC,* Endothelial progenitor cells; *HIF-1α,* hypoxia inducible factor-1a; *IL,* interleukin; *NFκB,* nuclear factor κB.

These findings suggest that a lack of CXCR4 activation results in impaired wound healing through decreased recruitment of EPCs and subsequent angiogenesis. Overexpression of SDF-1α hastens wound healing in the diabetic mouse and augments angiogenesis.[20] Furthermore, mesenchymal stem cells have also been shown to correct the deficiency of SDF-1α and promote granulation tissue formation and improved wound healing.[34]

Diabetic wounds are known to be more abundant in proinflammatory cytokines, and chronic inflammation has been implicated in the pathogenesis of the diabetic wound-healing impairment.[14,19,35] We hypothesized that with inhibition of the CXCR4 receptor, the delay in wound healing may be due to chronic inflammation, supported by our finding that there was greater CD45 infiltrate not only in diabetic mice treated with control vector compared with heterozygote mice treated with a control vector but also in diabetic mice treated with the inhibitor. Impaired wound healing may in fact increase the proinflammatory cytokines circulating in the wound and ultimately result in a chronic inflammatory state. Although no clear relationship exists at the moment between inhibition of the CXCR4 pathway and upregulation of inflammatory cytokines, these proinflammatory cytokines have a possible role in the development and persistence of chronic wounds.[35]

We demonstrated increased inflammatory cells in diabetic wounds treated with the SDF-1α inhibitor at 7 days compared with those treated with GFP. This increased inflammation correlates with increased expression of IL-6 at 3 days and MIP-2 at 7 days. The impaired wound healing in nondiabetic wounds treated with the SDF-1α inhibitor was early in the course of wound healing and was corrected by day 6. The correlation of this gene expression data and wound closure is complicated because of the lag between gene expression and the end result of the gene product.

Previous studies have demonstrated that expression of SDF-1α will promote cellular and leukocyte migration.[32] The observation of a decline in leukocyte migration with greater expressed levels of mutant SDF-1α confirms this mechanism of impaired cellular migration. That our animal model is a leptin-resistant diabetic mouse may confound the picture. Leptin is known to promote angiogenesis, so the differences illustrated in our study at baseline may be exaggerated in a background of no leptin activity.

Novel approaches to treat diabetic wound healing are lacking. The application of growth factors, as in the case of recombinant PDGF, has been used to improve human tissue healing, but the results have not been dramatic. Biosynthetic grafts and hyperbaric oxygen therapy have been used as a novel therapy to improve the diabetic wound-healing process. These, too, have been met with marginal success and, at times, prohibitive costs.[36]

CONCLUSIONS

SDF-1α is a highly conserved chemokine that plays a critical and multifaceted role in the wound-healing process in normal and diabetic environments. We hypothesized that further decreases in SDF-1α functional activity would result in further impairments in diabetic healing and might affect nondiabetic healing. In addition, the diabetic wound-healing impairment has been associated with chronic inflammation. Chronic leg ulcers were significantly more infiltrated with inflammatory cells than one would expect in a well-healing wound.[37]

We hypothesized that delayed healing would result in increased production of proinflammatory cytokines and increased inflammation as seen in diabetic wounds. Fig 8 displays what we suspect is the mechanism by which our inhibitor has an effect on wound healing. Specifically, we believe that the impaired angiogenesis that occurs as a result of the inhibitor sets into play a wound that heals poorly and is stymied by chronic inflammation. Our results here appear to support these findings, with a marked increase in inflammatory cells and inflammatory cytokines with further inhibition

of SDF-1α activity. Although clearly more studies are needed to define this relationship, SDF-1α may prove to be a key factor in the wound-healing process that could be targeted to correct the diabetic wound-healing defect.

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AUTHOR CONTRIBUTIONS

Conception and design: DB, KL
Analysis and interpretation: DB, JX
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Statistical analysis: DB
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