Sustained expression of *Hif-1α* in the diabetic environment promotes angiogenesis and cutaneous wound repair

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ABSTRACT

Impaired wound healing in diabetic patients is associated with deficiencies in the production of factors involved in cell proliferation and migration, such as vascular endothelial growth factor. However, it remains unclear how the transcriptional regulation of the genes encoding these factors is affected by the diabetic environment. Hypoxia-inducible factor-1α (Hif-1α), the regulatory subunit of the Hif-1 transcription factor, plays an important role in activating many of these genes. Therefore, we tested whether Hif-1α function is impaired in the diabetic wound environment and whether restoring Hif-1 function improves wound healing. Here, we show that Hif-1α protein levels are dramatically reduced in wounds of leptin receptor-deficient diabetic mice compared with nondiabetic littermates. Reduction in Hif-1α levels results in decreased DNA-binding activity and in decreased expression of several Hif-1 target genes, including vascular endothelial growth factor, heme oxygenase-1, and inducible nitric oxide synthase. Furthermore, we demonstrate that sustained expression of Hif-1α in leptin receptor-deficient diabetic wounds restores expression of these factors, enhances angiogenesis, and significantly accelerates wound healing. Taken together, these results suggest that Hif-1α function plays a significant role in wound healing and reduced levels of Hif-1α may contribute to impaired healing.

One consequence of diabetes mellitus is the disruption of the normal process of wound healing.[1] In diabetic patients and animal models, wound closure is profoundly delayed and the tensile strength of healed wounds is significantly decreased, often leading to wound reopening and chronic wounds. At the cellular level, wounds in the diabetic environment exhibit less proliferation of fibroblasts, endothelial cells, and keratinocytes, reduced collagen synthesis, reduced angiogenesis, and delayed reepithelialization. Unfortunately, in diabetic patients these deficiencies frequently result in chronic diabetic foot ulcers that require amputation. Patients with diabetes account for approximately 82,000 (more than 60%) of all nontraumatic amputations performed in the United States each year.[2]

The impaired healing observed in diabetic wounds has been associated with reduced levels of many growth factors also known to play a critical role during development and tissue remodeling, including keratinocyte growth factor (KGF), platelet-derived growth factor (PDGF), fibroblast growth factor (FGF), transforming growth factor-β (TGF-β), and vascular endothelial growth factor (VEGF).[3–6] Because VEGF has a potent role in angiogenesis, a process critical to efficient wound repair, animal studies and human trials have focused on it as a potential therapeutic agent. In murine models of diabetes, various methods have been used to increase VEGF protein concentration in the wound. The addition of recombinant protein,[7] introduction of adenovirus expressing VEGF,[8] and the manipulation of the molecular chaperone of VEGF, hypoxia up-regulated 1/oxygen-regulated protein 150 (Hyou1/Orp150), which restores VEGF secretion,[9] have all been moderately successful. However, clinical trials with recombinant protein therapy in humans have yielded only a modest improvement in wound healing and to date, only PDGF and KGF have received approval by the Federal Drug Administration for treatment of patients,[10–12] leading investigators to examine other relevant targets.

More recent studies have sought to identify additional proteins with altered expression levels in diabetic cells that may play an important role in tissue repair and regeneration. One such protein, inducible nitric oxide synthase (Nos2), has been implicated in wound healing. Rats with induced diabetes were found to have reduced levels of nitric oxide synthesis compared with controls,[13] and *Nos2*-deficient mice showed a significant impairment in wound healing. Restoring *Nos2* expression in these mice using adenoviral-mediated gene transfer accelerated healing.[14] Another protein, Heme oxygenase-1 (Hmox1), may also contribute to efficient wound repair, as it has been shown to provide a cytoprotective effect, both in stressed tissues[15] and during wound healing.[16] Thus, the list of proteins critical to wound healing that are reduced or lacking in

Hif-1	Hypoxia-inducible factor-1
Hmox1	Heme oxygenase 1
Lepr^{db}	Leptin receptor deficient [diabetic]
Nos2	Inducible nitric oxide synthase
VEGF	Vascular endothelial growth factor

diabetic wounds, and that improve wound healing when restored, continues to grow.

Hypoxia-inducible factor-1 (Hif-1), a transcription factor that functions upstream of *Vegf*, *Nos2*, and *Hmox1* as well as many other genes in response to a decrease in cellular oxygen concentration (hypoxia) and in response to injury,[17,18] is a heterodimer composed of Hif-1α and Hif-1β/Arnt. Hif-1α functions as the regulatory subunit and contains an oxygen-dependent degradation domain that affects its stability.[19] Hif-1 has been shown to be expressed in multiple cell types in response to injury, including keratinocytes, fibroblasts, and infiltrating immune cells.[18,20,21] Hif-1 controls the expression of numerous genes such as *Vegf*, *Nos2*, and *Hmox1*, which have been implicated in efficient tissue repair, angiogenesis, cell proliferation, and cell survival (reviewed in Semenza[22]). We therefore wanted to determine whether Hif-1 function is impaired in the diabetic wound environment.

In order to answer this question, we examined the expression of *Hif-1α* mRNA and protein in the wounds of leptin receptor-deficient diabetic mice ($Lepr^{db-/-}$), and assayed Hif-1α functional activation of downstream target genes in this model. Our results show that Hif-1α protein expression and function are impaired in $Lepr^{db-/-}$ diabetic wounds. In addition, we demonstrate that sustained expression of Hif-1α in the wounds of $Lepr^{db-/-}$ diabetic mice can restore Hif-1α function, reactivate Hif-1α target genes, promote angiogenesis, and significantly accelerate cutaneous healing.

MATERIALS AND METHODS

Mouse strains

Genetically diabetic mice and their nondiabetic littermates were obtained from Jackson Laboratories (strain B6.Cg-*m*+/+ $Lepr^{db}$/*J*, Bar Harbor, ME). Animals were housed in the University of California, San Francisco animal care facility. The Committee on Animal Research approved all procedures. The mice were between 8 and 14 weeks of age at the time of wounding and collection of tissue for cell, RNA, or protein isolation.

Statistical analyses

Statistical significance was determined using a Student's *t* test for experiments where control and experimental groups were being compared, as noted in figures or text. Statistical significance was determined using ANOVA, followed by a post hoc Tukey's test when more than two groups were being compared. A *p*-value of < 0.05 was considered to be significant.

Isolation of wild type (WT) and diabetic fibroblasts and culture conditions

Fibroblasts were isolated as described previously.[23] Briefly, tissue explants were cultured in 10 cm dishes on plastic in Dulbecco's modified Eagle's medium (DMEM)+10% fetal bovine serum (FBS); cells migrating out of the explant were identified, grown to confluency, and passaged as needed. For cobalt chloride ($CoCl_2$) treatments, fibroblasts were incubated in media containing 200 μM $CoCl_2$ for 6 hours. After treatment, cells were harvested for RNA or protein as described below.

Animal wounding model

$Lepr^{db-/-}$ diabetic and $Lepr^{db+/-}$ nondiabetic littermate controls were anesthetized and the dorsum of the mouse was shaved and sterilized with betadine. A 2.0-cm-diameter open wound was excised including the *panniculus carnosus* layer. In $CoCl_2$ experiments, 0.5 mL of 200 μM $CoCl_2$ or phosphate-buffered saline (PBS) (control) was applied once at the time of wounding directly to open wounds. In gene transfer experiments, three control (empty vector) or $CMV\text{-}Hif\text{-}1\alpha^{\Delta ODD}$ expression plasmid pellets (each pellet consisted of 25 μg DNA incorporated into a 0.5% methylcellulose carrier, as described in Hansen and colleagues[24,25]) were placed directly onto open wounds. No dressing was applied, as dried DNA-methylcellulose disks adhere to and remain in the wound bed. Wound areas were measured at the time of wounding and thereafter every 5 days by planimetry (tracing of wound area). Traces were subsequently scanned and image analyses were performed using Adobe Photoshop CS 8.0 software. Measurements were made on at least six animals in each group. Additionally, at least three representative wounds from each group were harvested at the described time points by euthanizing the animal and removing the entire wound area, including a 2 mm region outside of the wound, as well as the granulation tissue within the wound. Tissue samples were snap-frozen in liquid nitrogen until processed for RNA or protein isolation.

RNA isolation, semi-quantitative, and quantitative real-time polymerase chain reaction (PCR)

Total RNA was harvested from cells using the RNeasy kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Total RNA was isolated from whole harvested wounds (at least three animals were used in each group) and homogenized in Trizol reagent (Life Technologies, Rockville, MD). One microgram of total RNA was used as a template for reverse transcription with random primers. Semi-quantitative PCR was performed at least three times each for *Vegf*, *Nos2*, and *Hmox1* using 1 μg of cDNA on an MJ Research PTC-200 thermal cycler (GMI, Ramsey, MN) for 30 cycles with a 55° annealing temperature, using an *18S* competitor/primer mix (Ambion, Austin, TX) as an internal control reference gene. *Vegf* and *Nos2* were amplified using primer sets purchased from R&D Systems (Minneapolis, MN). The *Vegf* primer set produces three bands: 613 bp, representing the $Vegf_{164}$ isoform; 541 bp, representing the $Vegf_{188}$ isoform; and 410 bp, representing the $Vegf_{120}$ isoform. The *Nos2* primer set produces a 513 bp band. For *Hmox1,* the following primers were used, which produce a 613 bp band: forward, 5′-agg-tgt-cca-gag-aag-gct-t-3′, reverse, 5′-tgc-acc-agg-cta-gca-3′. Quantitative real-time PCR was carried out in triplicate with a 10–20-fold dilution of first-strand cDNA using murine taqman probes and primers purchased as Assays on Demand (Applied Biosystems, Foster City, CA) for the following genes: β-glucuronidase (*Gus*) or TATA-binding protein (*Tbp*) (reference gene controls), *Hif-1α*, *Vegf*,

Nos2, and *Hmox1*. An ABI Prism SDS 7000 (Applied Biosystems, Foster City, CA) was used according to the manufacturer's instructions with the following cycling protocol: one cycle of 50 °C, 2 minutes; 95 °C, 10 minutes; 40 cycles of 95 °C, 15 seconds; 50 °C, 1 minute. Data were analyzed with ABI Prism SDS 7000 companion software and Microsoft Excel.

Protein isolation and Western blot analyses

Total protein was harvested from cultured cells in lysis buffer (10 mM HEPES, 10 mM KCl, 0.1 mM EDTA, and 0.4% NP40). Nuclear extracts were prepared from the cell lysate using an extraction buffer (20 mM HEPES, 400 mM NaCl, and 1 mM EDTA). Protein concentration was determined by bicinchonic acid (BCA) protein assay (Pierce, Rockford, IL). Twenty micrograms samples of protein from nuclear extracts were electrophoresed on a 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gel and transferred onto nitrocellulose; relative protein loading was confirmed by Ponceau staining. Blots were blocked with 5% milk in Tris-buffered saline with 0.1% Tween 20 (T-TBS). Hif-1α was detected with mouse monoclonal antibody NB 100-105 (Novus Biologicals, Littleton, CO) at a dilution of 1 : 500, followed by sheep anti-mouse immunoglobulin-G (IgG) linked to HRP (Amersham Biosciences, Piscataway, NJ) at 1 : 1,500 and chemiluminescent detection (Super Signal, Pierce). Total protein was isolated from harvested wound tissue treated with vector control or *CMV-Hif-1α*$^{\Delta ODD}$ plasmids by homogenization of wounds, including a 2 mm region of skin around the wound bed, in urea lysis buffer (8 M urea, 100 mM $Na_2H_2PO_4$, and 10 mM Tris). Protein concentration was determined by BCA protein assay (Pierce). Forty micrograms of total cell lysate was separated on a 10% SDS-PAGE gel and transferred to nitrocellulose. Relative protein loading was confirmed by Ponceau staining. Blots were blocked in 5% dry milk in T-TBS. 6XHis-tagged Hif-1α$^{\Delta ODD}$ was detected from cells or wound tissue using rabbit anti-His-tag (Novus Biologicals) at 1 : 1,000, followed by donkey anti-rabbit IgG linked to HRP (Amersham Biosciences) at 1 : 2,000 and chemiluminescent detection (Super Signal).

Electrophoretic mobility shift assays

Protein lysates were isolated from NIH-3T3 cells, WT primary dermal fibroblasts, or diabetic primary dermal fibroblasts after treatment with 200 μM $CoCl_2$ or control (PBS) in 10 mM HEPES, 10 mM KCl, 0.1 M EDTA, and 0.4% NP40. Nuclear extracts were then obtained after incubation in nuclear extraction buffer (10 mM HEPES, 400 mM NaCl, and 1 mM EDTA). 3.5 pmol of double-stranded oligonucleotides containing Hif-1α-binding sites (5′-TCT-GTA-CGT-GAC-CACACT-CAC-CTC-3′) were end-labeled with [γ^{32}P]-ATP. Ten micrograms of total nuclear extract was incubated for 20 minutes at room temperature in a binding reaction containing 70 fmol of labeled probe, 10 mM Tris (pH 7.5), 50 mM NaCl, 1 mM DTT, 1 mM EDTA, 5% glycerol, and 1 μg of poly (dI-dC). DNA–protein complexes were resolved on a 1× TBE 5% polyacrylamide gel, dried, and autoradiographed.

Hif-1α siRNA, *Hif-1α* expression plasmid construction, and cell transfections

Annealed *Hif-1α* siRNAs (Ambion) with the following sequences: siRNA#1 5′-GGC-UCA-CCA-UCA-GUU-AUU-Utt-3′ (sense), 5′-AAA-UAA-CUG-AUG-GUG-AGC-Ctc-3′ (antisense), siRNA#2 5′-GGC-ACA-GAU-GGA-CUG-UUU-Utt-3′ (sense), 5′-AAAACA-GUC-CAU-CUG-UGC-Ctt-3′ (antisense), siRNA#3 5′-GGU-AUG-UGG-CAU-UUAUUU-Gtt-3′ (sense), and 5′-CAA-AUA-AAU-GCC-ACA-UAC-Ctt-3′ (antisense), were pooled and transfected into NIH-3T3 cells using a siPORT transfection reagent (Ambion) according to the manufacturer's instructions. NIH-3T3 cells stably transfected with a *Vegf-luciferase* reporter plasmid were incubated with the *Hif-1α* siRNA (or scrambled control siRNA) for 48 hours. The cells were lysed and luciferase assays were performed (n=3 for each group).

Hif-1α expression plasmids were constructed using RT-PCR to amplify murine *Hif-1α* cDNA from RNA isolated from 4-day-old wounds using the following primers: 5′-AGC-TGA ATT CCA CCG ATT GCG CAT GGA G-3′ (forward) and 5′-AGC-TCTCGA-GGT-TAA-CTT-GAT-CCA-AAG-CTC-3′ (reverse). The *Hif-1α* full-length PCR product was digested with *Eco*RI and *Xho*I and directionally cloned in frame into the pcDNA3.1 myc/his vector (Invitrogen, Carlsbad, CA). The constitutively active Hif-1α (Hif-1α$^{\Delta ODD}$) was amplified from the WT construct using two rounds of PCR to remove amino acids 401–613 (encoding the oxygen degradation domain, corresponding to amino acids 401–603 of human HIF-1α). All constructs were sequence-verified for accuracy (Biomolecular Resource Center, UCSF).

WT and diabetic fibroblasts were transiently transfected using FuGENE 6 transfection reagent (Roche, Indianapolis, IN) according to the manufacturer's instructions, with the *Vegf-luciferase* reporter plasmid. Co-transfection of *CMV-βgal* (Clontech, Palo Alto, CA) was performed to measure transfection efficiency. Cells were harvested after 24–36 hours for protein. For the constitutively active Hif-1α experiments, diabetic fibroblasts were transfected with either pcDNA3.1 (vector control, Invitrogen) or *CMV-Hif-1α*$^{\Delta ODD}$ plasmids, along with *CMV-βgal*. Cells were harvested after 48 hours for RNA or protein as described above, and β-gal assays were performed on each sample to normalize for transfection efficiency.

Analysis of angiogenesis, cell proliferation, and histological scoring

Wound tissue was isolated from *CMV-Hif-1α*$^{\Delta ODD}$-treated, vector control-treated, $CoCl_2$-treated, and PBS control-treated mice 4 and 7 days after wounding ($n \geq 4$ for each condition), and either frozen in O.C.T. compound (TissueTek, Torrance, CA) and stored at −80 °C until sectioning, or incubated in 10% formalin overnight and embedded in paraffin. Frozen wound tissue was cut into 10 μm sections, dried for 30 minutes at RT, fixed in acetone for 5 minutes, rehydrated, blocked, and incubated with rat anti-PECAM-1 (Pharmingen, San Diego, CA) at 1 : 100 in PBS+1% BSA, followed by anti-rat-FITC (Santa Cruz Biotechnology, Santa Cruz, CA) at 1 : 200, and 4',6-diamidino-2-phenylindole (DAPI) at 5 μg/mL in PBS.

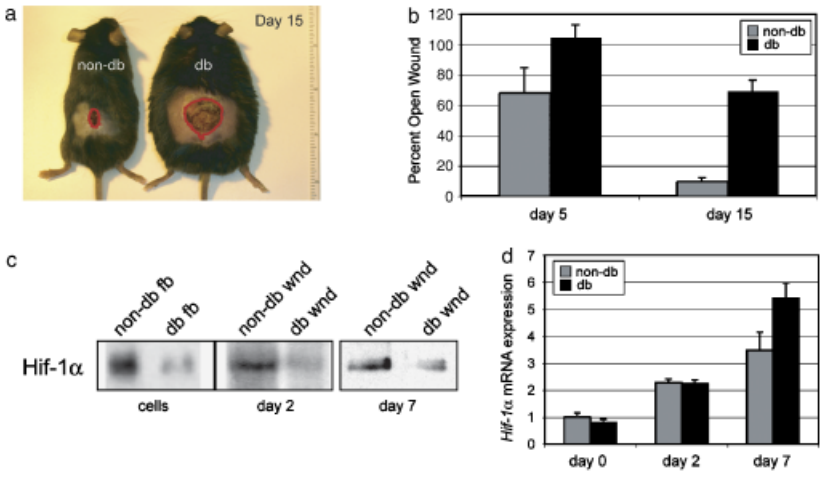

Figure 1. Impaired wound closure is associated with reduced levels of hypoxia inducible factor-1α (Hif-1α) protein. (A) Representative appearance of wounds (outlined) 15 days following wounding in $Lepr^{db+/-}$ nondiabetic (non-db) and $Lepr^{db-/-}$ diabetic (db) mice. (B) Quantification of wound area 5 and 15 days following wounding in nondiabetic (n=6) and diabetic (n=6) mice, error bars=SD. (C) Western blot analysis of Hif-1α from $Lepr^{db+/-}$ nondiabetic and $Lepr^{db-/-}$ diabetic mice in Days 2 and 7 wounds (n=3 in each group. (D) Quantitative real-time PCR of *Hif-1α* mRNA in $Lepr^{db+/-}$ nondiabetic (non-db) and $Lepr^{db-/-}$ diabetic (db) wounds in Days 2 and 7 wounds (n=3 for each group at each time point.

PECAM-1-positive/DAPI-positive cell staining adjacent to the wound bed was analyzed by planimetry, using Adobe Photoshop to capture the stained cell area (or DAPI-stained area), which was then analyzed by histogram analysis to obtain a PECAM/DAPI value. This ratio was used to assess angiogenesis. To measure cell proliferation around the wound site, paraffin-embedded wound tissue was cut into 5 μm sections, deparaffinized, rehydrated, subjected to high-temperature antigen unmasking, blocked, and incubated with mouse monoclonal antibody PC10 (Novocastra Laboratories, Newcastle-upon-Tyne, UK) against proliferating cell nuclear antigen (PCNA) at 1 : 100 in block, followed by incubation with biotinylated goat anti-mouse at 1 : 1,000 in TBS+1% BSA. Detection was carried out using the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA), and PCNA-positive cells were counted blind. Histological scoring was carried out blind on paraffin sections of wound tissue isolated 4 days after wounding according to the method of Greenhalgh et al.[4] Briefly, each specimen was given a score of 1–12: 1–3, none to minimal cell accumulation and granulation tissue or epithelial migration; 4–6, thin immature granulation tissue dominated by inflammatory cells; 7–9, moderately thick granulation tissue, ranging from being dominated by inflammatory cells to more fibroblasts and collagen deposition; and 10–12, thick, vascular granulation tissue dominated by fibroblasts and extensive collagen deposition.

RESULTS

Delayed wound healing correlates with a reduction in Hif-1α protein levels in genetically diabetic mice

Mice homozygous for a mutation in the leptin receptor ($Lepr^{db}$) exhibit a phenotype similar to adult-onset diabetes mellitus (type II), including a significant wound-healing impairment when compared with their nondiabetic littermates.[26–28] In this study, a 2.0-cm-diameter full-thickness excisional wound model was used, providing a larger wound bed area than in previous studies. However, similar to the results described in previous studies, we found that within 5 days after wounding, wound areas were smaller at all time points in age-matched nondiabetic, heterozygous ($Lepr^{db+/-}$) mice when compared with diabetic, homozygous ($Lepr^{db-/-}$) mice. By 15 days after wounding, the difference between these two groups was dramatic, with nondiabetic mice displaying on average only 10% open wound area remaining relative to the initial wound area, while their diabetic littermates displayed 70% open wound area still remaining (Figure 1A and B). By Day 45, wounds were completely closed in both groups (not shown).

We analyzed Hif-1α protein levels in primary fibroblasts isolated from $Lepr^{db-/-}$ and $Lepr^{db+/-}$ skin, as well as Days 2 and 7 $Lepr^{db-/-}$ and $Lepr^{db+/-}$ wounds, to determine whether there was an effect of the diabetic wound environment on this important transcriptional regulator. Hif-1α protein levels were significantly reduced in cultured primary fibroblasts as well as wounds harvested from $Lepr^{db-/-}$ at both time points (Figure 1C), although the mRNA levels were similar and slightly increased by Day 7 (Figure 1D). This finding shows that although *Hif-1α* gene expression is induced in diabetic wounds, Hif-1α protein expression and/or stabilization is impaired in the $Lepr^{db-/-}$ diabetic environment during wound repair.

In situ hybridization analysis of *Hif-1α* mRNA in Day 7 wound tissue from both nondiabetic and diabetic mice revealed high expression levels of *Hif-1α* mRNA in keratinocytes and fibroblasts of wounded skin in both $Lepr^{db+/-}$ and $Lepr^{db-/-}$ mice at Day 7, with no apparent difference in spatial organization (data not shown). No signal was obtained using the *Hif-1α* sense control probe (not shown).

Reduction of Hif-1α protein levels is associated with the reduction of *Vegf, Hmox1,* and *Nos2* expression in $Lepr^{db-/-}$ diabetic wounds

As reported previously, expression of Hif-1 target genes *Vegf*, *Hmox1*, and *Nos2* mRNA was low to undetectable in unwounded skin (data not shown and Hanselmann and colleagues[16,29]). However, 2 days after wounding, mRNA levels increased for all three genes in wounds from $Lepr^{db+/-}$ nondiabetic mice, but remained low in $Lepr^{db-/-}$

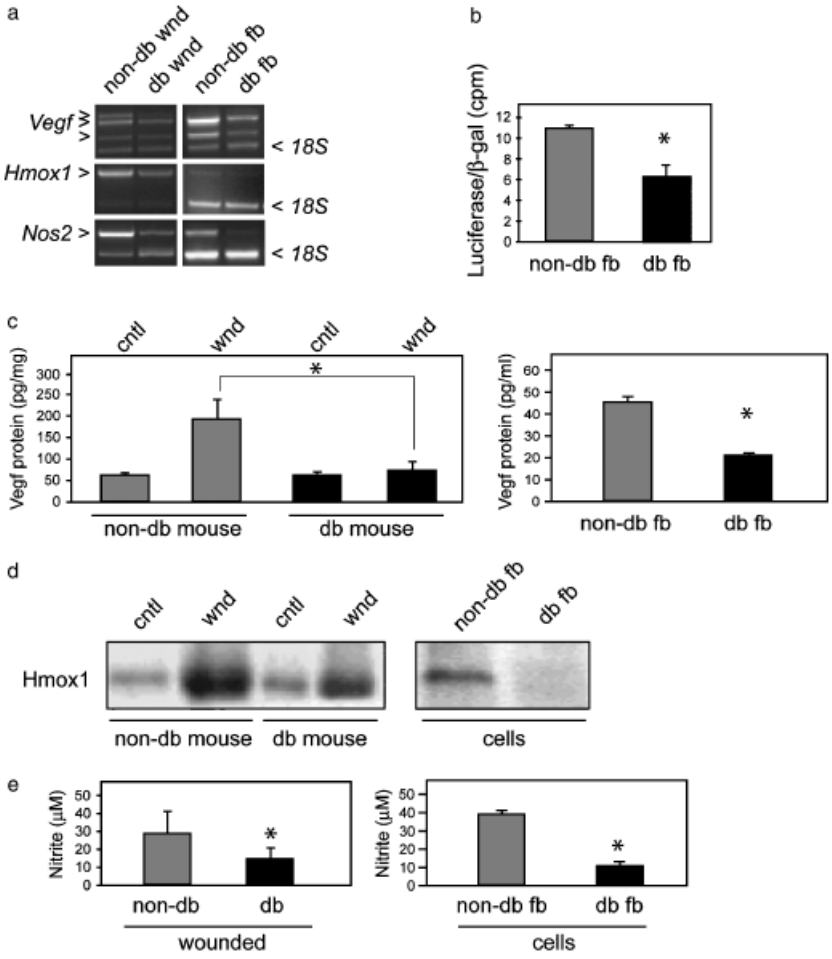

Figure 2. Attenuation of *Vegf*, *Hmox1*, and *Nos2* in the $Lepr^{db-/-}$ diabetic environment occurs at the transcriptional level and is associated with a reduction of Hif-1α. (A) Representative semi-quantitative RT-PCR of *Vegf*, *Hmox1*, and *Nos2* in $Lepr^{db+/-}$ nondiabetic and $Lepr^{db-/-}$ diabetic wound tissue at Day 2, and primary fibroblasts. The *Vegf* primer set amplifies three bands representing murine isoforms $Vegf_{188}$ (613 bp), $Vegf_{164}$ (541 bp), and $Vegf_{120}$ (410 bp). (B) Hif-1-responsive luciferase reporter assay in $Lepr^{db+/-}$ nondiabetic and $Lepr^{db-/-}$ diabetic primary fibroblasts. (C) Vegf protein levels assayed using ELISA from $Lepr^{db+/-}$ nondiabetic and $Lepr^{db-/-}$ diabetic unwounded (cntl) and wounded (wnd) skin at Day 2, respectively, and primary fibroblasts ($n=3$ for each group, error bars=SD, $*p < 0.05$ between non-db wnd and db wnd groups). (C) Representative Western blot of Hmox1 protein levels from $Lepr^{db+/-}$ nondiabetic and $Lepr^{db-/-}$ diabetic unwounded and wounded skin at Day 2, respectively, and primary fibroblasts. (D) Nitrite assay measuring Nos2 activity from Day 2 wounds of nondiabetic and diabetic mice and primary fibroblasts ($n=3$ for each group, error bars=SD, $*p < 0.05$).

diabetic mice (Figure 2A). Reduced levels of *Vegf*, *Hmox1*, and *Nos2* mRNA were also observed in primary fibroblasts isolated from $Lepr^{db-/-}$ mice compared with nondiabetic cells (Figure 2A). Moreover, transfection of an Hif-1-responsive luciferase reporter into primary fibroblasts isolated from nondiabetic and diabetic mice showed significantly reduced activation in diabetic fibroblasts (Figure 2B). Not surprisingly, Vegf, Hmox1, and Nos2 protein levels were also reduced in the diabetic wounds and cultured primary fibroblasts compared with their nondiabetic counterparts (Figure 2C–E).

Sustained expression of Hif-1α results in restored expression of Hif-1 target genes in vivo in $Lepr^{db-/-}$ diabetic mice

$CoCl_2$ blocks the degradation of Hif-1α in a variety of cultured cells, including dermal fibroblasts explanted from $Lepr^{db-/-}$ mice, and thus increases the total cellular concentration of Hif-1α.[30] To test the response of sustained expression of Hif-1α in vivo, 200 μM $CoCl_2$ or PBS (vehicle) was applied to full-thickness excisional wounds of $Lepr^{db-/-}$ and $Lepr^{db+/-}$ mice. $CoCl_2$ treatment of wounds in $Lepr^{db-/-}$ mice resulted in a marked increase in Hif-1α protein concentration in vivo by 2 days after wounding compared with control-treated mice (Figure 3A), and restored Hif-1 DNA-binding activity in diabetic wounds (Figure 3B). Furthermore, enhanced Hif-1 DNA-binding activity in wounds of $Lepr^{db-/-}$ diabetic mice resulted in an increase in activation of *Vegf*, *Nos2*, and *Hmox1* mRNA expression, as measured by quantitative RT-PCR. As shown in Figure 3C–E, all three genes were up-regulated in vivo by 2 days after wounding in $Lepr^{db-/-}$ diabetic mice treated with $CoCl_2$ compared with $Lepr^{db-/-}$ mice treated with PBS.

Additionally, the $CoCl_2$-mediated increase in Hif-1 DNA-binding activity increased the activation of the *Vegf* promoter in cell culture in an Hif-1α-dependent manner, as knockdown of Hif-1α expression using small interfering RNAs (siRNA) resulted in an attenuated response of the *Vegf*-luciferase reporter to $CoCl_2$ (approximately 50% knockdown) (Figure 3F).

Constitutively active Hif-1α is sufficient to activate Hif-1 target genes in vivo in $Lepr^{db-/-}$ diabetic mice

To test the specificity of sustained Hif-1α expression in the diabetic wound environment, we constructed a

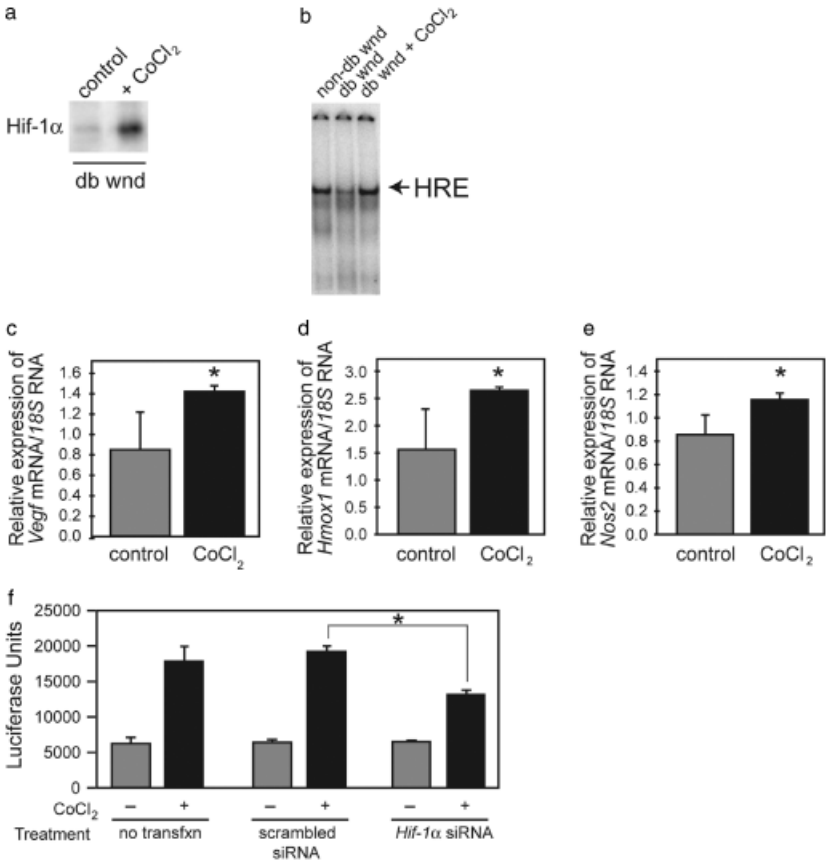

Figure 3. Cobalt chloride ($CoCl_2$) restores hypoxia inducible factor-1α (Hif-1α) protein levels and DNA-binding activity in $Lepr^{db-/-}$ diabetic wounds resulting in Hif-1 target gene up-regulation. (A) Representative Western blot of Hif-1α-isolated from Day 2 wounds of $Lepr^{db-/-}$ diabetic mice treated with phosphate-buffered saline (PBS) (control) or $CoCl_2$. (B) Representative EMSA of Hif-1α binding site (HRE) oligos with protein extracts from $Lepr^{db+/-}$ nondiabetic, $Lepr^{db-/-}$ diabetic, and $CoCl_2$-treated $Lepr^{db-/-}$ diabetic wounds at Day 2. (C–E) Quantitative RT-PCR analyses of (c) *Vegf*, (D) *Hmox1*, and (E) *Nos2* mRNA expression from Day 2 wounds of diabetic mice treated with PBS (control) or $CoCl_2$, respectively ($n=3$ for each group, error bars=SD, $*p < 0.05$). (F) *Vegf* promoter activation in NIH-3T3 fibroblasts transfected with a *Vegf-luciferase* reporter construct (containing HRE consensus-binding sites) and treated with scrambled control siRNA or *Hif-1α* siRNA. *CMV-βgal* was cotransfected (1 : 10) to normalize for transfection efficiency. siRNA knockdown of $CoCl_2$-induced Hif-1α protein expression resulted in an ~50% decrease in *Vegf-luciferase* activity ($n=9$, error bars=SD, $*p < 0.05$). PCR, polymerase chain reaction.

constitutively active form of murine Hif-1α referred to as Hif-1$\alpha^{\Delta ODD}$, which lacks the oxygen-dependent degradation domain (Figure 4A). Human HIF-1$\alpha^{\Delta ODD}$ has previously been shown to be stable in normoxia in cell culture.[19] Nuclear localization of the 6×-His-tagged murine Hif-1$\alpha^{\Delta ODD}$ isoform was verified after transfection of this expression plasmid into cultured fibroblasts (Figure 4B). Western blot analysis revealed that this isoform has an apparent molecular weight of ~68 kD (Figure 4C). To introduce the *CMV-Hif-1*$\alpha^{\Delta ODD}$ expression plasmid into mouse skin, a 2.0-cm-diameter full-thickness wound was excised and 75 μg of DNA in 0.5% methylcellulose (air dried) was immediately applied directly to wound tissue in $Lepr^{db-/-}$ diabetic and $Lepr^{db+/-}$ nondiabetic mice, as described previously (and in "Materials and methods").[24,25] Expression of the transgene was monitored by performing Western blot analysis on protein extracts from homogenized wound tissue, and was clearly detectable four days after application (Figure 4D). Transgene expression reached the limits of detection between 7 and 15 days after application (not shown), consistent with previous transgene expression profiles in this model.[24,25]

Expression of Hif-1 target genes *Vegf*, *Nos2*, and *Hmox1* in animals treated with *CMV-Hif-1*$\alpha^{\Delta ODD}$ or empty vector control was measured by quantitative RT-PCR. This analysis revealed that expression of the constitutive form of Hif-1α is sufficient to significantly upregulate at least three Hif-1 target genes, *Vegf*, *Nos2*, and *Hmox1*, in the $Lepr^{db-/-}$ diabetic wound environment (Figure 4E–G).

Sustained expression of Hif-1α accelerates wound healing and promotes multiple aspects of tissue repair

To test the biological significance of sustained expression of Hif-1α in the $Lepr^{db-/-}$ diabetic wound environment, we compared wound closure rates of $Lepr^{db-/-}$ diabetic and control $Lepr^{db+/-}$ nondiabetic mice treated with the *CMV-Hif1*$\alpha^{\Delta ODD}$ expression plasmid or vector control, or treated with $CoCl_2$ or PBS. $Lepr^{db-/-}$ diabetic mice expressing *Hif-1*$\alpha^{\Delta ODD}$ showed a statistically significant acceleration in healing compared with control wounds treated with vector alone (Figure 5A). This difference was readily apparent by Day 15 (third time point) and persisted throughout the study (Figure 5A). Similar results were obtained when a single application of $CoCl_2$ immediately after wounding was compared with PBS treatment of wounds (data not shown). Thus, both methods used to induce sustained Hif-1α expression resulted in a statistically significant improvement in the rate of wound healing.

We analyzed the overall morphology of *Hif-1*$\alpha^{\Delta ODD}$-treated, $CoCl_2$-treated, and control-treated wounds from $Lepr^{db-/-}$ mice by blind histological scoring according to the method of Greenhalgh and colleagues (described in detail in "Materials and methods").[4] Histological evaluation of wounds from untreated $Lepr^{db-/-}$ mice have

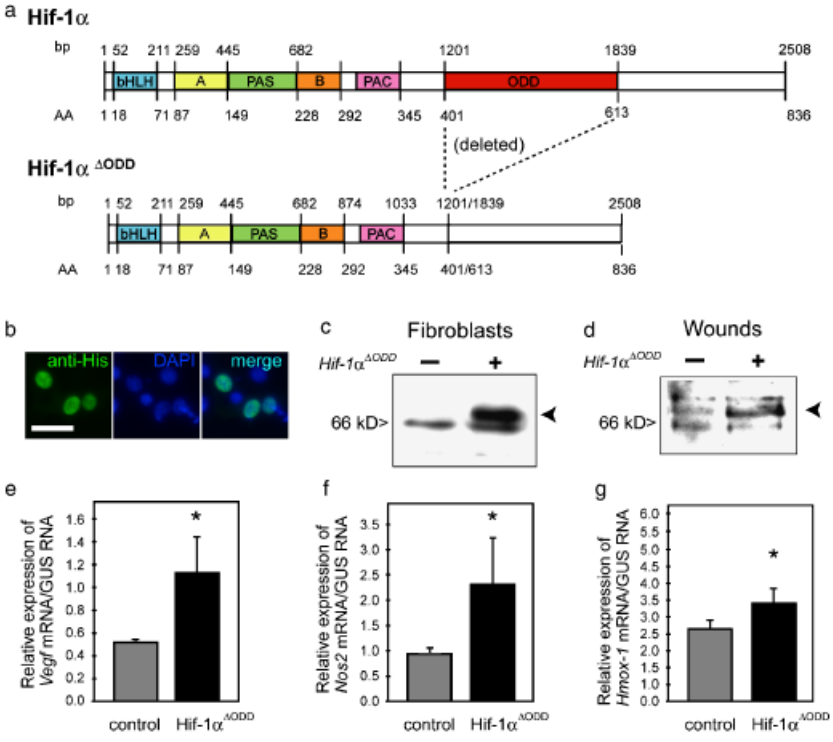

Figure 4. Constitutive expression of *Hif-1α*$^{\Delta ODD}$ in diabetic wounds results in Hif-1 target gene up-regulation in vivo. (A) Schematic representation of wild-type Hif-1α (upper panel), with known domains indicated, and Hif-1α$^{\Delta ODD}$ construct used in subsequent experiments (lower panel) indicating region deleted (the ODD domain). (B) Transfection of 6×-Histidine-tagged *CMV-Hif-1α*$^{\Delta ODD}$ in fibroblasts results in constitutive nuclear localization under normoxic conditions: (left panel) His-tag immunolocalization, (middle panel) DAPI staining, (right panel) merge (scale bar=40 μm). (C) Representative Western blot from fibroblasts expressing *CMV-Hif-1α*$^{\Delta ODD}$ (+) or vector control (−) using an antibody against the 6×-histidine tag produces a band of ∼68 kD. (D) Representative Western blot of *CMV-Hif-1α*$^{\Delta ODD}$ transgene expression in sample Day 4 wounds from vector control (−) or *CMV-Hif-1α*$^{\Delta ODD}$-treated mice (+). (E–G) Relative expression of *Vegf*, *Nos2*, and *Hmox1* mRNA, respectively, in vector control and *CMV-Hif-1α*$^{\Delta ODD}$-treated wounds of diabetic mice at Day 4 (n=6 for each group, error bars=SD, *$p < 0.05$). DAPI, 4′,6-diamidino-2-phenylindole; Hif-1α, hypoxia inducible factor-1α.

consistently displayed less granulation tissue, angiogenesis, extracellular matrix deposition, and slower epithelial regeneration compared with nondiabetic mice in our wound model (data not shown) as well as previously used models.[4,6,31] Sustained expression of Hif-1α in the diabetic wound environment, using either gene transfer of *Hif-1α*$^{\Delta ODD}$ expression plasmid or $CoCl_2$ treatment, resulted in dramatically higher overall wound histology scores, in *Lepr*$^{db-/-}$ diabetic mice compared with empty vector or PBS-treated controls (Figure 5B).

Sustained expression of Hif-1α also specifically promoted angiogenesis in skin proximal to the wound bed, as revealed by PECAM-1 staining (Figure 5C and D). PECAM-1-positive cells were quantified in skin 7 days after wounding, during peak angiogenesis, and were significantly increased in tissue treated with $CoCl_2$ compared with controls (Figure 5E). Similar results were obtained after treatment with *CMV-Hif-1α*$^{\Delta ODD}$ (data not shown). PCNA immunolocalization revealed no significant difference in cell proliferation at this time point (not shown). These results indicate that sustained expression of Hif-1α in the *Lepr*$^{db-/-}$ diabetic wound environment can rescue the impaired healing phenotype via up-regulation of Hif-1 target genes during the initial phase of wound healing, resulting in an increase in angiogenesis and subsequently more efficient wound repair during the secondary phase of healing.

DISCUSSION

The diabetic wound environment is characterized by a dramatic reduction in proteins that regulate repair and regeneration, including growth factors, growth factor receptors, components of signal transduction pathways, and transcription factors. In this study, we have investigated the expression and activity of the transcription factor Hif-1α and the expression of its downstream target genes *Vegf*, *Hmox1*, and *Nos2* in diabetic cells and diabetic wounds from *Lepr*$^{db-/-}$ mice, to determine whether the expression, stability, or function of this important transcriptional regulator is impaired in the diabetic environment. Our results indicate that Hif-1α protein levels are greatly reduced in this environment. Although this study does not distinguish between effects due to the diabetic environment per se and effects due to the leptin receptor deficiency, previous work has indicated that impaired hypoxic responses of diabetic fibroblasts from *Lepr*$^{db-/-}$ mice fail to induce *Vegf* expression.[32] Nonetheless, sustained expression of Hif-1α with $CoCl_2$ or by gene transfer of a constitutively active form of Hif-1α restores expression of Hif-1 target genes and dramatically accelerates wound repair in the *Lepr*$^{db-/-}$ mouse model of type II diabetes. Interestingly, although transgene expression peaks in vivo at approximately days 4–7, acceleration of wound closure was not measured until approximately Day 15. This apparent delay in phenotypic change following genotypic change is not surprising. We were able to detect enhanced Hif-1 DNA-binding activity and reactivation of Hif-1 target genes within the first few days following sustained Hif-1α expression. Subsequently, the increase in angiogenesis measured at Day 7 is a necessary prerequisite for improved granulation tissue formation and subsequent reepithelialization. Differences in gross closure rates cannot be expected to be significant until a later time point. Thus, sustained expression of Hif-1α initiates a series of early changes in gene expression that culminate in improved wound repair and closure.

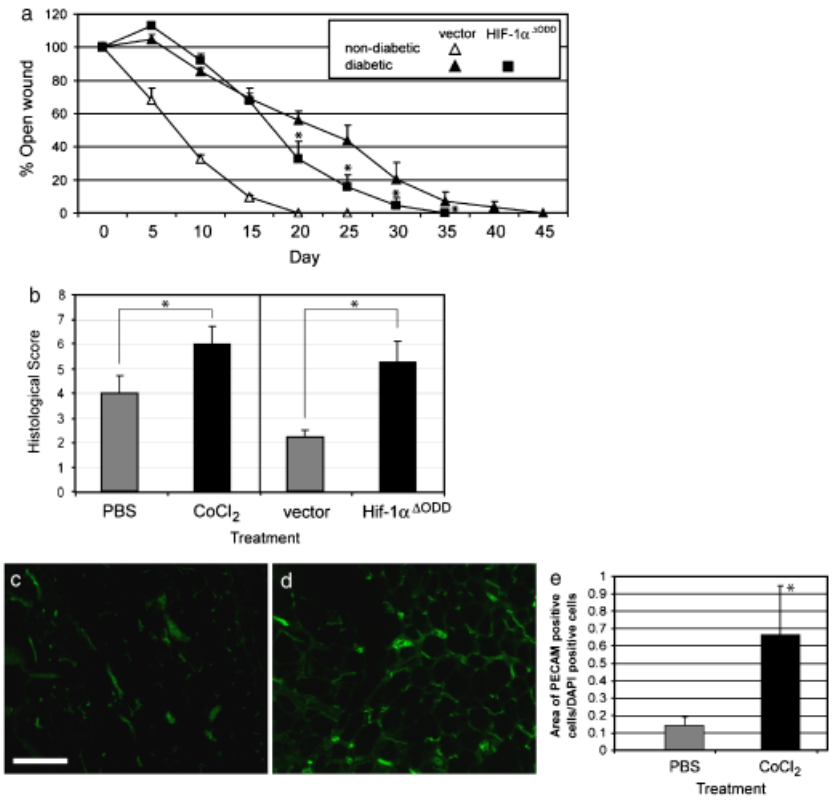

Figure 5. Sustained expression of Hif-1α accelerates wound healing, enhances angiogeneis, and promotes cutaneous wound repair in vivo. (A) Percent open wound at each time point of $Lepr^{db+/-}$ nondiabetic mice treated with vector control gene transfer (open triangle), and $Lepr^{db-/-}$ diabetic mice expressing *CMV-Hif-1α*$^{\Delta ODD}$ (closed square) or vector control gene transfer (closed triangle). Difference in average wound area was statistically significant between $Lepr^{db+/-}$ nondiabetic and $Lepr^{db-/-}$ diabetic mice at all time points, and between vector-treated and *CMV-Hif-1α*$^{\Delta ODD}$-treated diabetic mice after Day 15 through closure (original wound diameter=2.0 cm, n=6 in each group, error bars=SD, *$p < 0.05$). (B) Histological scoring of wound tissue samples 4 days after wounding from diabetic mice treated with PBS, $CoCl_2$, expression plasmid vector, or Hif-1α$^{\Delta ODD}$ expression plasmid as indicated (n=3 for each group, error bars=SD, $p < 0.05$ between PBS and $CoCl_2$ and between vector and *Hif-1α*$^{\Delta ODD}$). (C–E) Analysis of angiogenesis using PECAM-1 staining of wounds from $Lepr^{db-/-}$ diabetic mice harvested at Day 7, treated with (C) PBS, or (D) $CoCl_2$ (scale bar=50 μm). (E) Quantification of angiogenesis in Day 7 wounds from diabetic mice treated with PBS or $CoCl_2$ (n=4 for each group, error bars=SD, *$p < 0.05$). Hif-1α, hypoxia inducible factor-1α; PBS, phosphate-buffered saline.

Previous studies have yielded inconclusive findings regarding the correlative effects of wound healing and the application of growth factors, such as Vegf. Although Vegf is undoubtedly required for angiogenesis and wound healing,[5,33,34] the addition of Vegf to open wounds does not consistently result in improved wound healing.[35] This is likely due to the fact that multiple factors, in addition to Vegf, contribute to efficient wound repair. Excessive proteolytic activity in the diabetic wound environment can rapidly degrade recombinant growth factors applied to wound tissue. For example, several studies have shown that the combination of increased concentrations of matrix metalloproteinases (MMPs) and decreased concentrations of tissue inhibitors of metalloproteinases (TIMPs) contributes to an increased proteolytic environment in diabetic foot ulcers and diabetic mouse models.[36,37]

To circumvent this problem, we utilized two different methods to constitutively express the Hif-1α transcription factor, which not only activates *Vegf* expression, but multiple genes important to wound healing, thereby effectively enhancing expression of many Hif-1 downstream factors within the wound tissue. These downstream Hif-1α effector proteins act to coordinate multiple events during tissue repair, and ultimately would be more effective than the addition of a single gene. Indeed, recently, it has been shown that viral gene transfer of stabilized HIF-1α is superior to VEGF in inducing angiogenesis,[38] and that peptide-matrix-based gene delivery of *HIF-1α*$^{\Delta ODD}$ enhanced wound-induced angiogenesis better than VEGF.[39] Other recent studies have shown that gene transfer of NF-κB, SMADs, Egr-1, and Hox transcription factors to wounds can lead to the modulation of many downstream target genes that can significantly affect the repair process.[24,25,40–42]

Activation of several known Hif-1 target genes due to increased Hif-1α expression, stabilization, or both has previously been showed in response to wounding in nondiabetic animals.[18,21,43] However, to our knowledge, there have been no studies to date on the expression of Hif-1α in diabetic wounds. Our finding that Hif-1α protein levels are reduced in the wounds of $Lepr^{db-/-}$ diabetic mice compared with nondiabetic controls, and that sustained expression of this protein rescues many of the defects associated with impaired wound healing in the $Lepr^{db-/-}$ diabetic environment, most notably angiogenesis, underscores the importance of transcription factor function during the repair process.

The reduced level of Hif-1α protein in the diabetic environment may be due to either a failure in the translation of *Hif-1α* mRNA or in the stabilization of the protein in response to injury. Although the most extensively studied pathway known to stabilize Hif-1α protein has been hypoxia, the immediate increase in Hif-1α protein levels in response to injury is independent of hypoxic conditions.[21] Additionally, diabetic wound tissue has a lower oxygen tension than normal wound tissue.[44] Therefore, the

decreased level of Hif-1α protein in the diabetic environment is unlikely to be related to hypoxia.

Nonhypoxic mediators that may be involved in Hif-1α protein expression or stabilization in response to injury include tumor necrosis factor-α (TNF)-α. TNF-α has been shown to induce Hif-1α expression in primary inflammatory cells independent of hypoxia.[21] Interestingly, TNF-α expression is attenuated in diabetic mice. It remains to be determined what other cell types, if any, may lead to an increase in Hif-1α levels in response to TNF-α. Furthermore, in vascular smooth muscle cells, angiotensin II was shown to dramatically up-regulate *Hif-1α* mRNA expression and translation independent of hypoxia via the protein kinase C and phosphatidylinositol 3 kinase pathways, respectively.[45] In addition, the insulin pathway in particular is likely to be affected by the diabetic environment. Exposure of nondiabetic cells to insulin, IGF-1, or IGF-2 has been shown to result in the induction of Hif-1α protein expression,[46] and IGF-1 can induce Hif-1α-mediated *Vegf* gene expression by increasing *Hif-1α* mRNA translation.[47] Moreover, IGF-1 has been shown to stimulate diabetic wound healing in rats via the IGF-1 receptor (IGF-1R) pathway.[48]

Understanding how the diabetic environment impacts Hif-1 function will undoubtedly provide new insights into the processes underlying both normal and delayed wound healing. Regardless, we have shown that it is possible to overcome the deficiencies in endogenous signaling pathways leading to Hif-1α protein expression or stabilization in the $Lepr^{db-/-}$ diabetic environment by directly activating factors downstream of Hif-1 that are sufficient to enhance tissue repair. This study underscores a potentially critical role for transcriptional regulation of groups of genes in response to injury, and that future wound-healing therapies may benefit from utilizing transcription factors to bypass the complications of the chronic wound environment and directly activate tissue repair programs more effectively.

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