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Fibroblast-Specific Deletion of Hypoxia Inducible Factor-1 Critically Impairs Murine Cutaneous Neovascularization and Wound Healing

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Abstract

Background—Diabetes and aging are known risk factors for impaired neovascularization in response to ischemic insult, resulting in chronic wounds, and poor outcomes following myocardial infarction and cerebrovascular injury. Hypoxia-inducible factor (HIF)-1 α , has been identified as a critical regulator of the response to ischemic injury and is dysfunctional in diabetic and elderly patients. To better understand the role of this master hypoxia regulator within cutaneous tissue, the authors generated and evaluated a fibroblast-specific HIF-1 α knockout mouse model.

Methods—The authors generated floxed HIF-1 mice (HIF-1^{loxP/loxP}) by introducing loxP sites around exon 1 of the HIF-1 allele in C57BL/6J mice. Fibroblast-restricted HIF-1 α knockout (FbKO) mice were generated by breeding our HIF-1^{loxP/loxP} with tamoxifen-inducible Col1a2-Cre mice (*Col1a2-CreER*). HIF-1 α knockout was evaluated on a DNA, RNA, and protein level. Knockout and wild-type mice were subjected to ischemic flap and wound healing models, and CD31 immunohistochemistry was performed to assess vascularity of healed wounds.

Results—Quantitative real-time polymerase chain reaction of FbKO skin demonstrated significantly reduced *Hif1* and *Vegfa* expression compared with wild-type. This finding was confirmed at the protein level ($p < 0.05$). HIF-1 α knockout mice showed significantly impaired revascularization of ischemic tissue and wound closure and vascularity ($p < 0.05$).

Conclusions—Loss of HIF-1 α from fibroblasts results in delayed wound healing, reduced wound vascularity, and significant impairment in the ischemic neovascular response. These findings provide new insight into the importance of cell-specific responses to hypoxia during cutaneous neovascularization.

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Diabetes and aging are known risk factors for impaired tissue recovery following ischemic insult, resulting in chronic wounds, and poor outcomes following myocardial infarction and cerebrovascular injury.¹⁻⁶ With a rapid expansion of the elderly segment of our population and a concurrent increase in the number of diabetic patients, these complications represent a significant and growing health care burden.^{7,8} New blood vessel formation is essential for the delivery of nutrients and maintenance of oxygen homeostasis in cutaneous tissue repair. Inadequate neovascularization has been implicated as a significant causal factor for the development of chronic wounds in the setting of diabetes and aging.^{1,9}

Impaired stability and function of the transcription factor hypoxia inducible factor (HIF)-1 has been identified as one mechanism underlying the compromised neovascularization associated with diabetes and aging.^{1,2,10} HIF-1 is the chief regulator of cellular responses to ischemia and functions as a master regulator of oxygen homeostasis^{11,12} (Fig. 1, *above*). It consists of a posttranslationally regulated α -subunit and a constitutively expressed β -subunit.¹³ Under hypoxic conditions, the hydroxylation of prolyl residues on the HIF-1 α subunit by the prolyl hydroxylases is diminished. This prevents the degradation of HIF-1 α ¹⁴ and allows it to initiate expression of multiple gene pathways that enhance oxygen delivery and increase metabolism, most notably vascular endothelial growth factor (VEGF).^{11,12} Conversely, in diabetes and aging, HIF-1 α is destabilized,^{2,10,15} resulting in impaired release of growth factors, poor neovascularization, and inadequate healing.

We and others have previously shown that up-regulation of HIF-1 α improves wound healing.^{2,13,16} To better understand the role of this master hypoxia regulator within the wound environment, we generated a fibroblast-specific HIF-1 α knockout mouse model using mice expressing Cre recombinase under the control of the collagen, type I, alpha 2, promoter¹⁷⁻¹⁹ and mice genetically engineered to introduce loxP sites around the *Hif-1* gene.²⁰ We found that loss of HIF-1 α from fibroblasts results in a significantly impaired neovascular response to ischemia, delayed wound healing, and reduced wound vascularity. These findings provide insight into the importance of the fibroblast hypoxia response in a number of cutaneous abnormalities.

MATERIALS AND METHODS

Ethics

All in vivo experiments were performed under the guidance of the Stanford University Veterinary Department and were approved by the Institutional Animal Care and Use Committee of Stanford University (protocol no. 12080).

HIF-1 α Knockout Mouse Model

We generated floxed HIF-1 mice (HIF-1^{loxP/loxP}) by introducing loxP sites around exon 1 of the HIF-1 allele in C57BL/6J mice purchased from The Jackson Laboratory (Bar Harbor, Me.). Fibroblast-restricted HIF-1 α knockout mice were generated by breeding our HIF-1^{loxP/loxP} with tamoxifen-inducible Col1a2-Cre mice (*Col1a2-CreER^{+/+}*) and then backcrossing progeny to HIF-1^{loxP/loxP} mice to obtain dermal specific HIF-1 knockout

progeny [*Col1a2-Cre^{+/+}*;HIF-1^{*loxP/loxP*} (FbKO)] for excisional wound and ischemic flap experiments (Fig. 1, *above* and *center*). FbKO mice were viable and fertile.

Tamoxifen Induction

Tamoxifen (Sigma-Aldrich, St. Louis, Mo.) was dissolved into sunflower seed oil (Spectrum, Lake Success, N.Y.) at a concentration of 10 mg/ml by shaking overnight at 37°C. FbKO and HIF-1^{*loxP/loxP*} mice were administered tamoxifen (75 mg/g body weight) by intraperitoneal injection once every 24 hours for 5 consecutive days. Mice were monitored closely for any adverse reactions to treatment. Tissue harvest for in vitro assays and all in vivo experiments were carried out 7 days after the final injection.

Primary Fibroblast Harvest

Primary fibroblasts were harvested from the skin of 10- to 12-week-old FbKO and HIF-1^{*loxP/loxP*} mice, as described previously,²¹ after tamoxifen induction, as described above. Briefly, mice were killed, shaved, and depilated. Next, the skin was cleansed with alcohol pads. The tissue was harvested and washed in a series of povidone-iodine dilutions in phosphate-buffered saline (100% povidone-iodine, 10% povidone-iodine, and 100% phosphate-buffered saline). The tissue was then minced, and incubated in Liberase (Hoffmann-La Roche, Indianapolis, Ind.) for 1 hour at 37°C with constant motion. The primary fibroblasts were cultured in Dulbecco's Modified Eagle Medium with sodium pyruvate (110 mg/liter) (Gibco/Invitrogen, Carlsbad, Calif.) supplemented with 10% fetal bovine serum (Axenia Biologix, Dixon, Calif.), 100 U/ml penicillin, 100 µg/ml streptomycin, 50 µg/ml Fungizone, and 40 µg/ml gentamicin (Gibco). After incubating for 48 hours at 37°C in 5% carbon dioxide, the media and nonadherent cells were aspirated and fresh medium was added. Medium was changed every 2 days until the cells were ready for experiments. All experiments were performed on cells at passage 1.

Quantitative Reverse-Transcription Polymerase Chain Reaction

Total RNA was isolated from cultured fibroblasts using an RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Total RNA was eluted in RNase-free water and quantified using a spectrophotometer at 260 and 280 nm. RNA was converted to cDNA by reverse transcription using the SuperScript III First-Strand Synthesis System (Invitrogen, Carlsbad, Calif.). For polymerase chain reactions, we used TaqMan Assays-on-Demand Gene Expression Products from Applied Biosystems (Foster City, Calif.): HIF-1, assay identification no. Mm00468869_m1; and VEGF α , assay identification no. Mm01281447_m1. Gene expression levels were normalized to B2M expression (assay identification no. Mm00437762_m1 and presented as relative values. Polymerase chain reaction was repeated three times for each assay.

Western Blotting

Primary fibroblasts were harvested in phosphate-buffered saline (pH 7.4) and centrifuged at 1000 rpm for 5 minutes at 4°C. The cell pellets were then lysed in 4°C radioimmunoprecipitation assay buffer supplemented with protease inhibitor cocktail (Sigma-Aldrich). Cells were sonicated briefly (10 seconds), placed on ice for 5 minutes, and

then sonicated again. After homogenization, samples were centrifuged at 10,000 *g* for 10 minutes at 4°C. Protein was quantified using the Quick Start Bradford Protein Assay Kit (Bio-Rad, Hercules, Calif.). Thirty-five micrograms of protein from each group was resolved on a sodium dodecyl sulfate–polyacrylamide gel (Invitrogen) and then transferred onto polyvinyl difluoride membranes (Invitrogen). Primary antibodies against mouse HIF-1 α (1:500; Novus Biological, Littleton, Colo.) and β -actin (1:1000; Santa Cruz Biotechnology, Santa Cruz, Calif.) were used for detecting the corresponding proteins. Western blots were developed using enhanced chemiluminescence (ECL Plus; GE Healthcare, Chalfont St. Giles, United Kingdom) and then analyzed using ImageJ software (National Institutes of Health, Bethesda, Md.). All blots were run in triplicate.

Ischemic Flap Model

FbKO and HIF-1^{*loxP/loxP*} mice were subjected to an in vivo model of soft-tissue ischemia ($n = 3$).¹ This was achieved by creating a random-pattern skin flap (1.25 $\times$ 2.5 cm) on the dorsum of each mouse. The tissue was elevated from the underlying muscle bed and a 0.13-mm-thick silicone sheet (Invotec International, Jacksonville, Fla.) was inserted to separate the skin from the underlying tissue bed before the flap was sutured back in place. The silicone sheet blocks blood supply from the underlying wound bed and forces the flap to rely on the vascular supply from its base for perfusion. The flap was sutured using interrupted 6-0 nylon sutures along its three elevated sides and covered with Tegaderm (3M, St. Paul, Minn.). Digital images of the flaps were taken on days 0 and 9. The viable tissue area was evaluated using ImageJ software.

Immunohistochemistry

After fixation in 4% paraformaldehyde, tissue samples were embedded in paraffin and serially cut into sections of 8- μ m thickness. Vascular density was detected using a CD31 monoclonal antibody (1:500 dilution; Abcam, Cambridge, United Kingdom) with a secondary Alexa-Fluor 594-linked anti-rabbit immunoglobulin G antibody (1:1000 dilution; Invitrogen). All samples were counterstained with 4',6-diamidino-2-phenylindole. Slides were mounted with the Vectashield Mounting Medium (Vector Laboratories, Burlingame, Calif.) and cover-slipped. A Zeiss Axioplan 2 fluorescence microscope was used to image the slides (Carl Zeiss, Inc., Thornwood, N.Y.). CD31 staining intensity was quantified using ImageJ software.

Excisional Wound Model

FbKO and HIF-1^{*loxP/loxP*} mice were housed with a 12-hour light/dark cycle and provided ad libitum with standard food and water. Female mice, aged 10 to 12 weeks, were used for surgery ($n = 6$). After depilation, two 6-mm full-thickness wounds extending through the panniculus carnosus were made on the dorsa of mice as described previously.²² Each wound was stented open by a circular silicone ring fixed to the skin using an immediate-bonding adhesive (Krazy Glue; Elmer's, Inc., Columbus, Ohio) and 6-0 nylon sutures to prevent wound contraction. All wounds were covered with an occlusive dressing (Tegaderm). Digital photographs were taken every other day until closure. Wound area was measured relative to the area of the silicone ring, mitigating the effects of interphotograph variability, using

ImageJ software. The percentage change in wound size over time, relative to the initial margins, was used as a measure of wound healing kinetics.

Statistical Analysis

Data were analyzed by a two-tailed *t* test for bivariate analyses and analysis of variance for multivariate analyses and are presented as mean $\pm$ standard error. A value of $p < 0.05$ was considered statistically significant.

RESULTS

Validation of HIF-1 α Knockout

FbKO mice displayed no overt phenotype and appeared grossly similar to wild-type C57BL/6J mice (Fig. 2, *above, left*). We confirmed tamoxifen-dependent excisive recombination of HIF-1 α in FbKO mice using polymerase chain reaction (Fig. 2, *above, right*). To assess whether Cre-mediated recombination was targeted appropriately, quantitative reverse-transcription polymerase chain reaction of wild-type and FbKO skin was carried out, and demonstrated significantly reduced *Hif1* and *Vegfa* expression (Fig. 2, *below, left*). These findings were also confirmed at the protein level, with significantly reduced HIF-1 α protein in FbKO skin compared with wild-type, demonstrated by Western blot (Fig. 2, *below, right*).

HIF-1 α Knockout Results in Impaired Recovery from Tissue Ischemia

To clarify the role of fibroblast-specific HIF-1 α in cutaneous neovascularization, an ischemic flap model was used (Fig. 3, *above*).¹ After 10 days, there was significantly more tissue necrosis in FbKO mice compared with floxed control mice (Fig. 3, *below, left*). Specifically, we detected 57.8 ± 7.6 percent flap survival in the floxed control group versus only 10.9 ± 1.7 percent survival in the FbKO group ($p < 0.05$) (Fig. 3, *below, right*).

Fibroblast-Specific Knockout of HIF-1 α Reduces Flap Vascularity

Based on the reduced ischemic tissue survival rate in FbKO mice and the prominent role of HIF-1 α in new blood vessel formation,² we suspected an impairment of flap vascularization in the HIF-1 α knockout group. Concordant with this expectation, we observed significantly decreased vascular density in the flap tissue of FbKO mice compared with control using CD31 staining (Fig. 4) ($p < 0.05$). This suggests that fibroblast-specific HIF-1 α has a significant role in cutaneous neovascularization following injury, which may be related to fibroblast-specific expression of downstream angiogenic factors and may represent the mechanism by which it promotes cutaneous healing.

Fibroblast-Specific Knockout of HIF-1 α Impairs Wound Healing

Given the significance of the HIF-1 α pathway in the response to tissue injury,^{10,11} a murine excisional wound-healing model²² was used to assess the importance of fibroblast-specific expression of HIF-1 α during in vivo wound closure (Fig. 5, *above*). As early as day 5 after wounding, statistically significant differences in wound healing kinetics were observed, with slower healing in FbKO mice (Fig. 5, *center, and below, left*). This resulted in significantly

earlier wound closure in floxed control mice compared with FbKO (11.25 ± 0.3 days versus 14.75 ± 0.2 days after injury; $p < 0.05$) (Fig. 5, *below, right*). These data suggest that fibroblast-specific expression of HIF-1 α plays a critical role in the response to tissue injury during cutaneous wound healing. As expected, the FbKO phenotype was also associated with decreased vascular density in healed wounds, as shown by CD31 staining (Fig. 6).

In aggregate, our findings suggest that we have successfully constructed a conditional HIF-1 α knockout mouse model and that reducing the expression of this master transcription factor in fibroblasts leads to a dramatically reduced neovascular response, resulting in increased tissue necrosis and impaired wound healing.

DISCUSSION

Diabetes and aging are both known risk factors for the development of chronic wounds, myocardial infarction, and cerebrovascular injury.¹⁻⁶ A more detailed understanding of the cellular and signaling changes occurring in these setting is needed to inform future targeted therapies. The impaired neovascularization associated with diabetes and aging^{1,9} has been strongly linked to dysfunction of the HIF-1 α pathway.^{2,10,15}

In this study, we generated and validated a fibroblast-specific HIF-1 α knockout mouse to understand cell-specific hypoxic responses in the setting of cutaneous ischemia. In our random-pattern ischemic flap model, there are no axial vessels incorporated into the flap, resulting in a gradation of ischemia, which is proportional to the distance from the base. More distally, tissue survival is dependent on the ability of cells to cope with hypoxia and correct the vascular deficit through local vessel repair.^{1,23} Our study showed significantly reduced tissue survival in ischemic flaps raised on FbKO mice. In contrast to an excisional wound, the formation of granulation tissue is less important in the setting of pure tissue ischemia than the ability to support the growth of new microvasculature. Therefore, this assay provides excellent assessment of the impaired ability for neovascularization in this model and further illustrates that fibroblasts have a critical role in microvascular angiogenesis.²⁴ Considered together, this suggests that fibroblast expression of HIF-1 α may influence cell survival and critically mediate microvascular angiogenesis in ischemic tissue.

We further confirmed these findings by demonstrating that loss of HIF-1 α from fibroblasts critically impairs wound healing and that this was associated with decreased vascular density. This may be associated with decreased VEGF expression by fibroblasts. Our initial validation of the FbKO mouse demonstrated reduced VEGF expression in skin, and fibroblast expression of VEGF has been implicated as critically mediating vascularization and the formation of stroma, particularly in the setting of cancer.²⁵⁻²⁷ Therefore, the mechanism for impaired wound healing with the loss of HIF-1 α from fibroblasts may be related to a decreased VEGF signal influencing the formation of normal vascularized granulation tissue.

In this study, we demonstrate that the loss of HIF-1 α expression in fibroblasts significantly impairs tissue survival in the setting of cutaneous ischemia. Fibroblast expression of HIF-1 α is also critical for excisional wound healing. This wound healing deficit is associated with a

decrease in vascular density, likely influencing the formation of granulation tissue. Our findings reinforce the importance of HIF-1 α regulation of the fibroblast transcriptional profile during the vascular response to ischemia. Future studies assessing the influence of other cell types and signaling pathways on the fibroblast transcriptional profile would further elucidate the complex interplay regulating neovascularization.

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Fig. 1.

The HIF-1 α pathway and creation of an HIF-1 α knockout mouse model. (*Above*) HIF-1 α is a critical mediator in the cellular and tissue response to hypoxia. The function of this transcription factor is impaired by chronic hyperglycemia and aging, leading to impaired neovascularization and ischemic tissue recovery in diabetic and aged patients. (*Center*) Exon 1 of the *Hif1* gene, which is flanked by loxP sites, is excised in cells expressing Cre recombinase under the *Col1a2* promoter. (*Below*) Recombination after tamoxifen induction results in a fibroblast knockout of HIF-1 α , allowing the assessment of dermal-specific

deletion of HIF-1 α during cutaneous wound healing and ischemic flap survival. *PHD*, prolyl hydroxylase; *HRE*, hypoxia response element.

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Fig. 2.

Validation of the HIF-1α knockout mouse model. (*Above, left*) Photograph of age-matched wild-type and HIF-1α knockout mice. (*Above, right*) Polymerase chain reaction confirmation of tamoxifen-dependent recombination of HIF-1. *HIF^{loxP}*, floxed HIF-1 band; *loxP*, recombined band after HIF-1 excision; *HIF^{WT}*, wild-type band. (*Below, left*) mRNA expression of *Hif1* and *Vegfa* in unwounded skin. (*Below, right*) Quantification of HIF-1α Western blot densitometry from unwounded skin ($n = 3$) (* $p < 0.05$). All data are means $\pm$ 1 SEM.

Fig. 3.

HIF-1 α knockout results in impaired recovery from tissue ischemia. (*Above*) Creation of a full-thickness peninsular skin flap with a reproducible ischemic gradient. (*Below, left*) Tissue survival after day 10 showing dramatically pronounced necrosis in the HIF-1 α knockout group. (*Below, right*) Quantification of tissue survival ($n = 3$) (* $p < 0.05$). All data are means $\pm$ 1 SEM.

Fig. 4. HIF-1 α knockout reduces ischemic flap vascularity. (*Left*) CD31 staining confirmed a significant decrease in microvessels among the HIF-1 α knockout mouse group. The nuclear stain was 4',6-diamidino-2-phenylindole. *Scale bar* = 100 μ m. (*Right*) Quantification of CD31-stained pixels ($n = 3$). *Scale bar* = 50 μ m (* $p < 0.05$). All data are mean $\pm$ 1 SEM. *KO*, knockout; *CTRL*, control; *HPF*, high-power field.

Fig. 5.

Knockout of HIF-1 α impairs wound healing. (*Above*) Murine excisional wound model. Full-thickness excisional wounds are created on the dorsa of mice. Ring stents are applied to prevent wound contracture by means of the panniculus carnosus. *E*, epidermis; *D*, dermis; *H*, hypodermis; *asterisk*, panniculus carnosus. (*Center*) Representative images of HIF-1 α knockout and control wounds demonstrate a slower and delayed time to wound closure among HIF-1 α knockout mice. (*Below, left*) Wound healing kinetics. (*Below, right*) Wound closure time ($n = 6$) (* $p < 0.05$). All data are mean $\pm$ 1 SEM.

Fig. 6. HIF-1 α knockout reduces wound vascularity. (*Left*) CD31 staining confirmed a significant decrease in microvessels among the HIF-1 α knockout mouse group. The nuclear stain was 4',6-diamidino-2-phenylindole. *Scale bar* = 100 μ m. (*Right*) Quantification of CD31-stained pixels ($n = 6$) (* $p < 0.05$). *Scale bar* = 50 μ m. All data are means $\pm$ 1 SEM. *KO*, knockout; *CTRL*, control; *HPF*, high-power field.