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EXPERIMENTAL

Overexpressed HIF-2α in Endothelial Cells Promotes Vascularization and Improves Random Pattern Skin Flap Survival

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Background: The local skin flap procedure is very useful for reconstruction. However, flap necrosis caused by circulatory failure can occur at its distal portion. Hypoxia-inducible factors (HIFs) in endothelial cells (ECs) help to maintain ECs and promote vascularization, and HIF-2α is abundantly expressed in ECs. However, the mechanisms of action of HIF-2α in ECs are not yet fully understood. The aim of this study was to evaluate the in vivo effects of overexpression of HIF-2α in ECs on skin flap survival.
Methods: A random pattern skin flap (1.0×3.0 cm) was elevated on the dorsum of transgenic mice (Tg mice) with EC-specific HIF-2α conditional overexpression and wild-type littermate control mice ($n = 6$). Flap survival was evaluated on postoperative day 7. Tissue samples from the skin flaps were harvested and analyzed using Western blotting, quantitative reverse transcriptase-polymerase chain reaction, and immunohistochemistry.
Results: The *HIF-2α* mRNA and protein levels were significantly increased in the Tg mice when compared with control mice. Tg mice had significantly increased skin flap survival areas (72.0% ± 2.7%) when compared with wild-type mice (45.7% ± 1.1%). Moreover, histological examination revealed an increase in the subcutaneous blood vessel counts in the Tg mice.
Conclusions: Specific overexpression of HIF-2α in ECs promoted vascularization and enhanced skin flap survival in vivo in a mouse model. *(Plast Reconstr Surg Glob Open 2014;2:e132; doi: 10.1097/GOX.0000000000000083; Published online 4 April 2014.)*

Covering tissue defects using skin flaps is a basic surgical strategy for plastic and reconstructive surgery. In general, the blood supply of axial pattern flaps is superior to that of random pattern flaps.[1] Although microsurgical free flaps and perforator flaps are newer techniques, random pattern flap reconstruction remains the most commonly used method for defect coverage. The main drawback to the random pattern flap is the potential for distal flap necrosis. Flap necrosis usually occurs at its distal portion, and thus, the defect area remains partially uncovered, leading to delayed healing and the occasional need for a second operation. Augmentation of flap perfusion is very important in random pattern skin flap reconstruction. Various pharmacological approaches have been reported to improve skin flap survival. For example, therapeutic angiogenesis with

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basic fibroblast growth factor,[2] vascular endothelial growth factor (VEGF),[3] and other pharmacological agents can enhance flap perfusion and survival.[4–8]

Hypoxia-inducible factors (HIFs) directly regulate the major angiogenic cytokines.[9] Furthermore, HIFs are the key transcriptional factors in the cellular adaptive response to ischemia.[10] Activated HIF-1α and HIF-2α accumulate in the nucleus and heterodimerize with HIF-1β. These complexes bind to cis-acting hypoxia-response elements present in the promoter sequences of hypoxia-responsive genes. In our previous study, we demonstrated that transient activation of the HIF signaling pathway via single systemic dimethyloxalylglycine (DMOG) treatment upregulated antiapoptotic pathway and enhanced neovascularization in a number of bone marrow–derived progenitor cells.[11]

HIF-1α seems to be expressed in most cell types, whereas HIF-2α mRNA is detected most prominently in embryonic vascular endothelial cells (ECs), kidney mesangial cells, neural crest–derived sympathetic ganglia, and ECs of the developing lung.[12–15] A recent study showed that ablation of Int6 gene expression upregulated HIF-2α and promoted the function of blood vessels, resulting in acceleration of wound healing in the skin of mice.[16] The present study investigated the hypothesis that elevation of HIF-2α in ECs promotes vascularization and improves skin flap survival by characterizing a mouse random pattern skin flap model with specific overexpression of HIF-2α in ECs.

MATERIALS AND METHODS

Ethics Statement

All experiments using mice were performed with the consent of the Animal Experimentation Committee of the University of Tokushima. The study did not involve human experiments.

Animal Study

The transgenic mouse strain was generated by pronuclear microinjection of the transgene into fertilized C57BL/6 oocytes. One transgenic line, *pBROAD3-LSL-FmHIF2α-IRES-tomato* (Acc. No. CDB0488T: http://www.cdb.riken.jp/arg/TG%20mutant%20mice%20list.html), was selected because transgene integrity was complete. For the generation of compound mice, these transgenic mice, the ROSA26-regulated loxP-stop-loxP activatable Hif-2α mice containing internal ribosome entry site–dependent translation of td Tomato fluorescent protein gene (Fig. 1A), were crossed with vascular endothelial-cadherin (VE-Cadherin)-Cre transgenic mice.[17,18] EC-specific HIF-2α conditional overexpression transgenic mice (Tg mice) and wild-type littermate control mice (male, C57BL/6J background, 8–12 weeks old, 24.62 ± 1.39 g body weight) were used in this study. Two experimental groups of each mouse strain ($n = 6$) were subjected to skin flap surgery. A cranially based random pattern skin flap measuring 1.0 × 3.0 cm was elevated under the panniculus carnosus layer on the dorsum of each mouse. The lateral thoracic vessels were ablated intraoperatively to establish the severely ischemic skin flap model. After flap elevation, the flap was sutured in place with 4-0 nylon and then covered with Tegaderm (3M, Maplewood, MN). Seven days after surgery, the mice were anesthetized and the area of skin flap survival was measured.

Flap Survival Evaluation

Pictures of the dorsal flaps were obtained using a digital camera (PowerShot A2300; Canon, Tokyo, Japan) on postoperative day 7. The necrotic area of the flap was determined grossly by the presence of scab formation, alopecia, and loss of elasticity. The survival area was calculated as a percentage of the total flap area using Image J Software.

Mice Genotyping

Tail tissues were harvested from 4- to 5-week-old mice. Isopropanol was used for the extraction of DNA. PCI (phenol/chloroform/isoamyl alcohol)/ethanol was used for purification of DNA. For the polymerase chain reaction–based genotyping, the following primers were used for the analyses: *td tomato* forward (5′-AGCAAGGGCGAGGAGGTCATC-3′), *td tomato* reverse (5′-CCTTGGAGCCGTACATGAACTGG-3′), *LSL-368* forward (5′-AAGTTATGTCGACCTCGATCG-3′), and *HIF2-116* reverse (5′-TGAGCCAACTCATAGAAGACC-3′), for detection of the *td tomato* fragment (200 bp) and the *set3* fragment (208 bp), respectively, in Figure 1A.

Western Blot Analysis

Protein levels were assessed by Western blot analysis. After mice had been bred for 12 hours under low oxygen conditions (10% oxygen, 90% nitrogen), whole pulmonary tissue was collected. The ischemic flap (measuring 1 × 3 cm) was divided into 3 sections (proximal, middle, and distal parts). The proximal portion (measuring 1 × 1 cm) was defined

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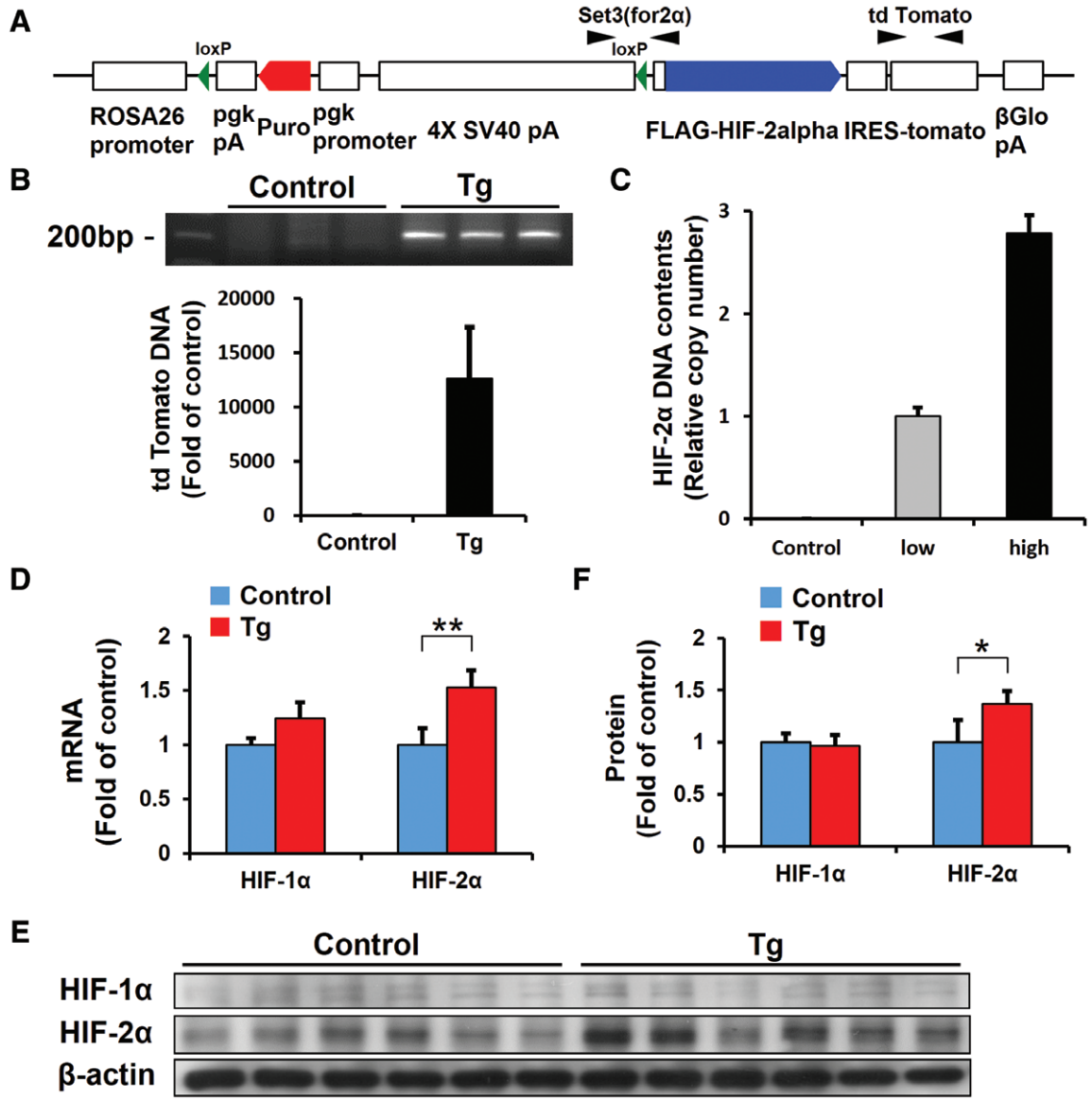

Fig. 1. A, Schematic representation of the *LSL-HIF-2α* transgenic mouse gene. *Set3* (for2α) and *td Tomato* (solid triangles) indicate the sites of PCR primers used for mouse genotyping. B, Quantitative RT-PCR-based genotyping for *td Tomato*. *Y* axis shows relative mRNA level. PCR was performed with primers td Tomato. C, Quantitative RT-PCR-based genotyping for HIF-2α. *Y* axis shows relative copy number of *HIF-2α* to *Arnt* gene. PCR was performed with primers *Set3* (for2α). Transgene copy number in 2 transgenic mice lines was compared. D, *HIF-1α* and *HIF-2α* mRNA levels in the lung. High-copy *HIF-2α* transgenic lines maintained under normoxic (21% O_2) conditions. Data in bar graphs are presented as means ± SEM ($n = 6$). $^{**}P < 0.01$. E and F, Western blot analysis of HIF-1α and HIF-2α protein in the lung. High-copy HIF-2α transgenic lines maintained under hypoxic (10% O_2, 90% N_2) conditions. β-Actin was used as a loading control. Values are means ± SEM ($n = 6$). $^{*}P < 0.05$. RT-PCR indicates reverse transcriptase-polymerase chain reaction; Tg, endothelial cell–specific HIF-2α conditional overexpression transgenic mice.

as a location adjacent to the pedicle of flap. Tissue samples from the proximal parts were harvested on postoperative day 7. The methods of protein extraction and Western blot analysis were described in detail previously.[11] Semiquantitative analysis of immunoblot band densitometry was performed using Image J software.

Quantitative Reverse Transcriptase-Polymerase Chain Reaction

Total RNA was isolated from whole pulmonary tissues, dorsal skin taken preoperatively, and from the proximal parts of the skin flaps collected on postoperative days 1 and 7 using ISOGEN (Nippon Gene, Tokyo, Japan). One microgram of the total RNA was

subjected to reverse transcriptase-polymerase chain reaction using a PrimeScript RT-PCR Kit with Oligo dT Primer (Takara Bio, Shiga, Japan). The following primers were used for the analyses: *HIF-1α* forward (5′-CCTGCACTGAATCAAGAGGTTGC-3′), *HIF-1α* reverse (5′-CCATCAGAAGGACTTGCTGGCT-3′), *HIF-2α* forward (5′-GGACAGCAAGACTTTCCTGAGC-3′), *HIF-2α* reverse (5′-GGTAGAACTCATAGGCAGAGCG-3′), *VEGF* forward (5′-GTAACGATGAAGCCCTGGAGTG-3′), *VEGF* reverse (5′-TGAGAGGTCTGGTTCCCGAAAC-3′), *prolyl hydroxylase domain* (*PHD*) *1* forward (5′-ATGGCTCACGTGGACGCAGTAA-3′), *PHD1* reverse (5′-CATTGCCTGGATAACACGCCAC-3′), *PHD2* forward (5′-TAAACGGCCGAACGAAAGC-3′), *PHD2* reverse (5′-GGGTTATCAACGTGACGGACA-3′), *PHD3* forward (5′-CTATGTCAAGGAGCGGTCCAA-3′), and *PHD3* reverse (5′-GTCCACATGGCGAACATAACC-3′).

Immunohistochemistry

For vascular studies, mice on preoperative and postoperative day 7 were anesthetized (3 mg pentobarbital/100 g body weight) and intravenously injected with 100 μl of 1 mg/ml rhodamine and lectin (Vector Laboratories, Burlingame, CA) in phosphate-buffered saline via the tail vein, after which mice were perfused with phosphate-buffered saline intracardially, as previously described.[19] Tissues from the proximal portions were fixed in 4% paraformaldehyde and embedded in paraffin. Samples were cut into 5-μm sections. Sections were deparaffinized in xylene, rehydrated in a series of ethanol washes, and incubated with Hoechst33342 (Wako, Osaka, Japan) for 30 minutes. Immunohistochemistry methods were described in detail previously.[11] For analysis of angiogenesis, the lectin-positive vessels were counted in 5 high-power fields (×400) by confocal laser scanning microscopy in each group.

Statistical Analysis

Values are given as means ± standard error of mean (SEM) for 3 separate experiments. Data were analyzed using a one-way analysis of variance to determine significant differences among groups, after which a modified *t* test with the Bonferroni correction was used for comparison between groups. *P* values of <0.05 were considered significant.

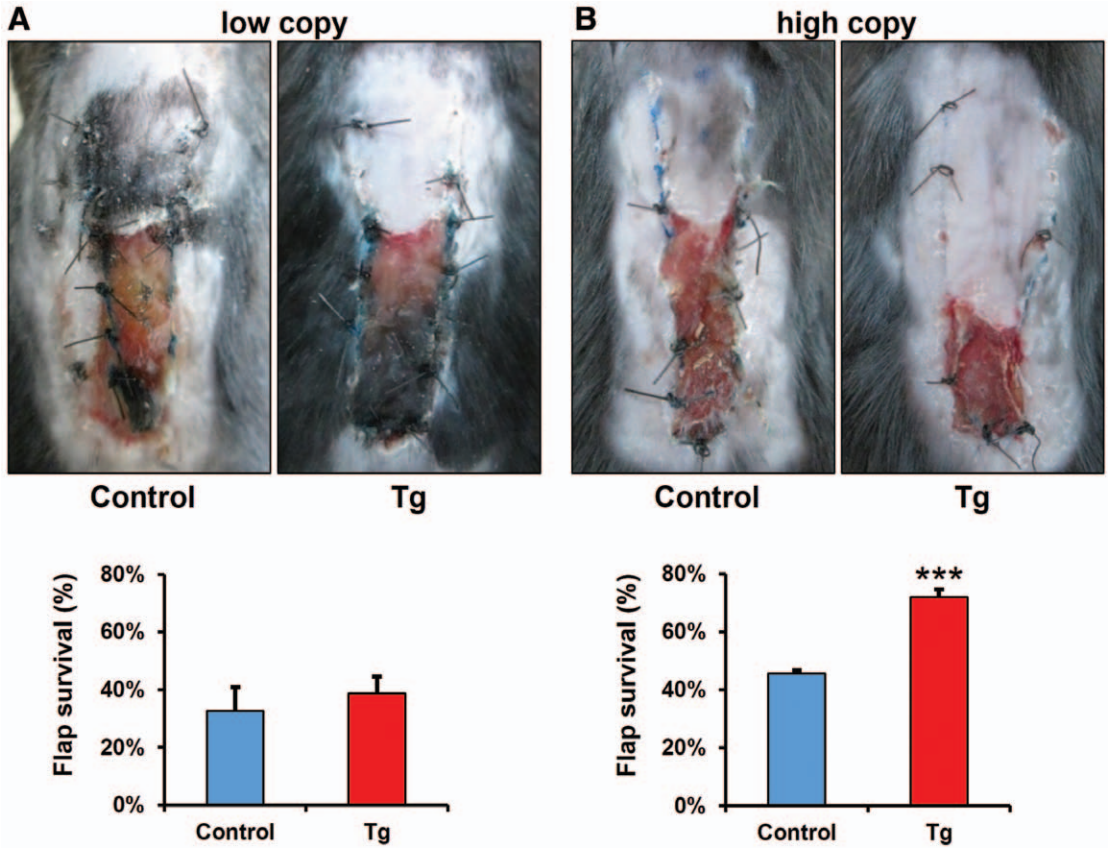

Fig. 2. Comparison of flap viability between the control mice and the Tg mice. A, Low-copy *HIF-2α* transgenic lines. B, High-copy *HIF-2α* transgenic lines. Flap survival was evaluated on postoperative day 7. Data in bar graphs are presented as percentages of flap survival ± SEM ($n = 6$). ***$P < 0.001$.

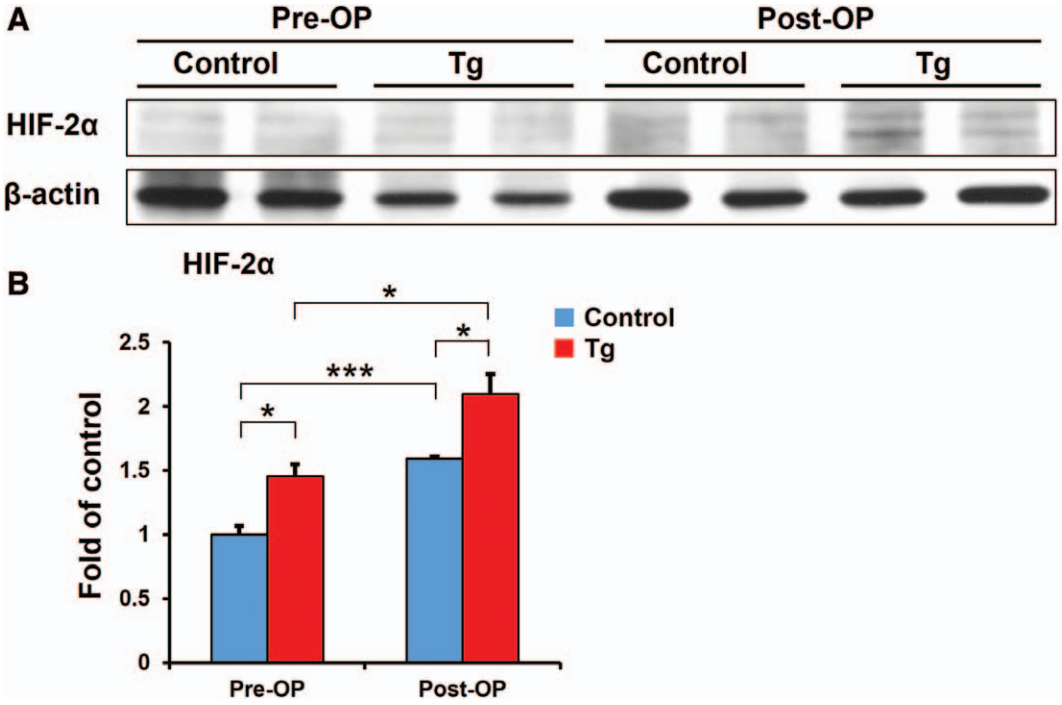

Fig. 3. Expression of HIF-2α protein in a mouse skin flap model. A and B, Western blot analyses were performed on tissue lysates from dorsal skin taken preoperatively and from the proximal parts of the skin flaps on postoperative day 7. High-copy *HIF-2α* transgenic lines and wild-type littermate control mice were subjected to analysis. β-Actin was used as a loading control. Values are means ± SEM ($n = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

RESULTS

Characterization of Transgenic Mice

The *td Tomato* DNA was only detected in the transgenic mice (Fig. 1B). Relative copy number of the *HIF-2α* transgene was compared between 2 transgenic mouse lines. We defined mice with a greater number of copies of the transgene as the "high copy line." Gene expression of *HIF-2α* in the high copy line was 2.8-fold higher than that in the low copy line (Fig. 1C).

Levels of *HIF-2α* mRNA in the lung tissue derived from the high copy line were significantly higher, even under normoxic conditions (Fig. 1D). Consistent with this result, the level of HIF-2α protein also increased significantly under hypoxic conditions (Figs. 1E, F) but not under normoxic conditions. By contrast, in the low copy line, the level of HIF-2α protein was not increased even under hypoxic condition when compared with the control mice (data not shown).

Overexpression of HIF-2α Results in Improved Wound Healing

Skin flap necrosis occurred in the distal parts of the flap in all animals. At postoperative day 7, the flap survival area was significantly larger in the high copy line than in the wild-type littermate control mice. Quantitative analysis showed that the flap survival area was 72.0% ± 2.7% of the total area in the high copy lines but only 45.7% ± 1.1% of the total area in the control mice ($P < 0.001$; Fig. 2B). There were no significant differences in the flap survival area between the low copy lines and controls (32.7% ± 8.1% vs 38.8% ± 5.8%, Fig. 2A). Therefore, we used only high copy line for the following study.

HIF-2α Protein Levels But Not HIF-1α Are Increased in the Skin Flaps from Tg Mice

HIF-2α protein levels on postoperative day 7 were significantly higher in Tg mouse skin flaps than in skin flaps from control mice [Figs. 3A (above), B], whereas there was no significant difference in the HIF-1α protein levels when comparing these 2 groups (data not shown).

Angiogenesis-related Gene Expression Is Increased in Tg Skin Flaps

HIF-2α and *VEGF* mRNA levels were significantly increased in the Tg mice when compared with the controls on postoperative days 1 and 7 (Figs. 4B, C). In addition, *prolyl hydroxylase domain (PHD)-1* and *PHD-2* were markedly expressed on postoperative day 7 (Fig. 4C). There was no difference in the angiogen-

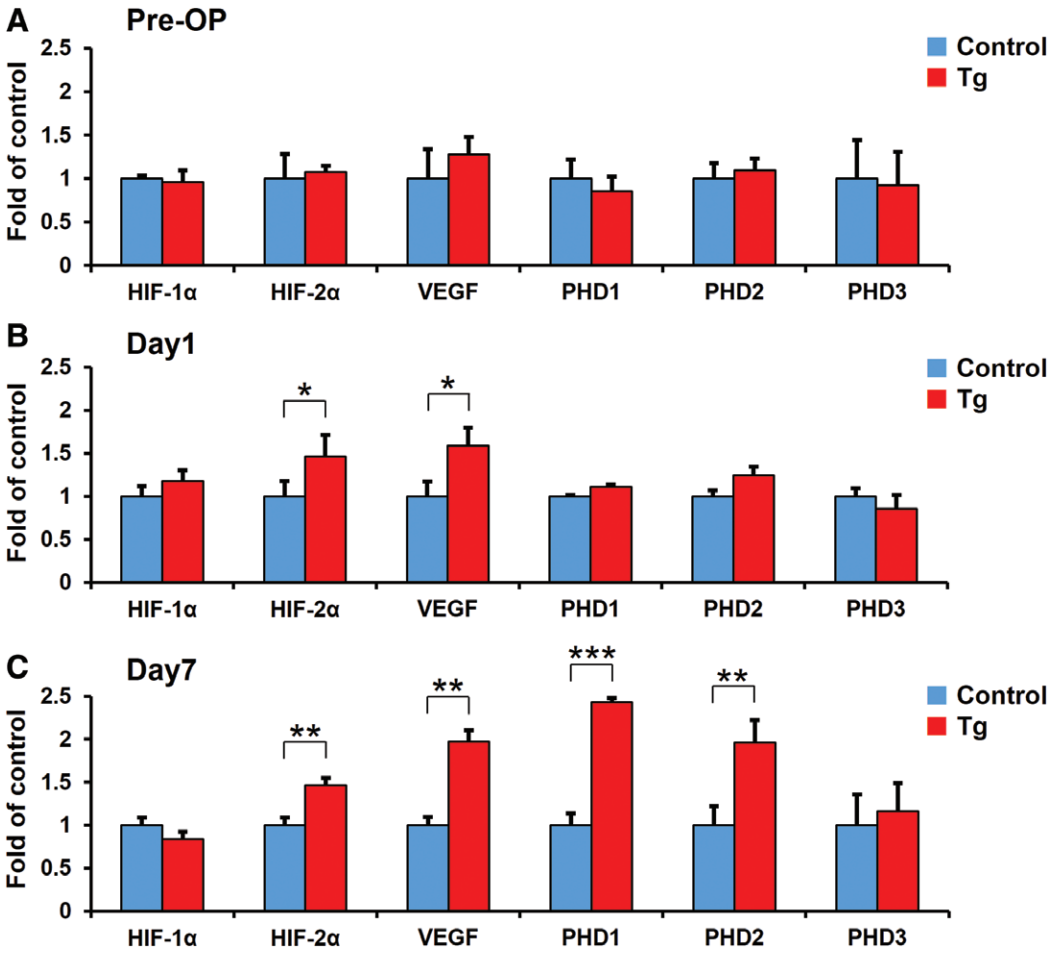

Fig. 4. *HIFs*, *VEGF*, and *PHD* mRNA levels in the mouse skin flap model. Transcript levels of angiogenesis-related factors were assessed by quantitative RT-PCR analysis. A, Total RNA was isolated from dorsal skin taken before operation. Values are means ± SEM ($n = 6$). B and C, Total RNA was isolated from the proximal parts of the skin flaps on postoperative day 1 and 7. Data in bar graphs are presented as means ± SEM ($n = 6$). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. RT-PCR indicates reverse transcriptase-polymerase chain reaction.

esis-related gene mRNA level when comparing control and Tg mice in preoperative (Pre-OP) (Fig. 4A).

Expression of HIF-2α Is Increased in Skin Flaps from Tg Mice

We evaluated the number of vessels in the proximal portions of the skin flaps preoperatively and on postoperative day 7. There were no differences in lectin-positive vessels when comparing Tg mice and controls preoperatively (11.8±0.7 vs 13.8±1.4, Figs. 5A, C). However, on postoperative day 7, the number of vessels was increased in the Tg mice when compared with control mice (27.0±1.9 vs 38.0±1.9, $P < 0.001$; Figs. 5B, C).

DISCUSSION

We previously reported that transient activation of HIF signaling pathways in both ischemic skin tissue and bone marrow cells by a single systemic administration of a PHD inhibitor upregulated antiapoptotic pathways and reconstituted the vasculature in ischemic skin flaps and preserved ischemic tissue viability in HIF-deficient mouse model.[11,17] HIF-2α is mainly expressed in ECs and is essential for the function and maintenance of ECs and the vasculature. A recent report revealed that Int6 suppresses HIF-2α, even during normoxia, and that knock-down of the *int6* gene leads to upregulated expression HIF-2α, thereby promoting blood vessel function.[16] In addition, a single injection of *int6* siRNA promoted angiogenesis through upregulation of HIF-2α-related angiogenic factors and restored function of the ischemic hindlimb in a rat model of peripheral artery disease.[20] Another report suggested that endothelial HIF-2α regulates vascular function and tumor angiogenesis in a mouse model.[19,21] Therefore, this pres-

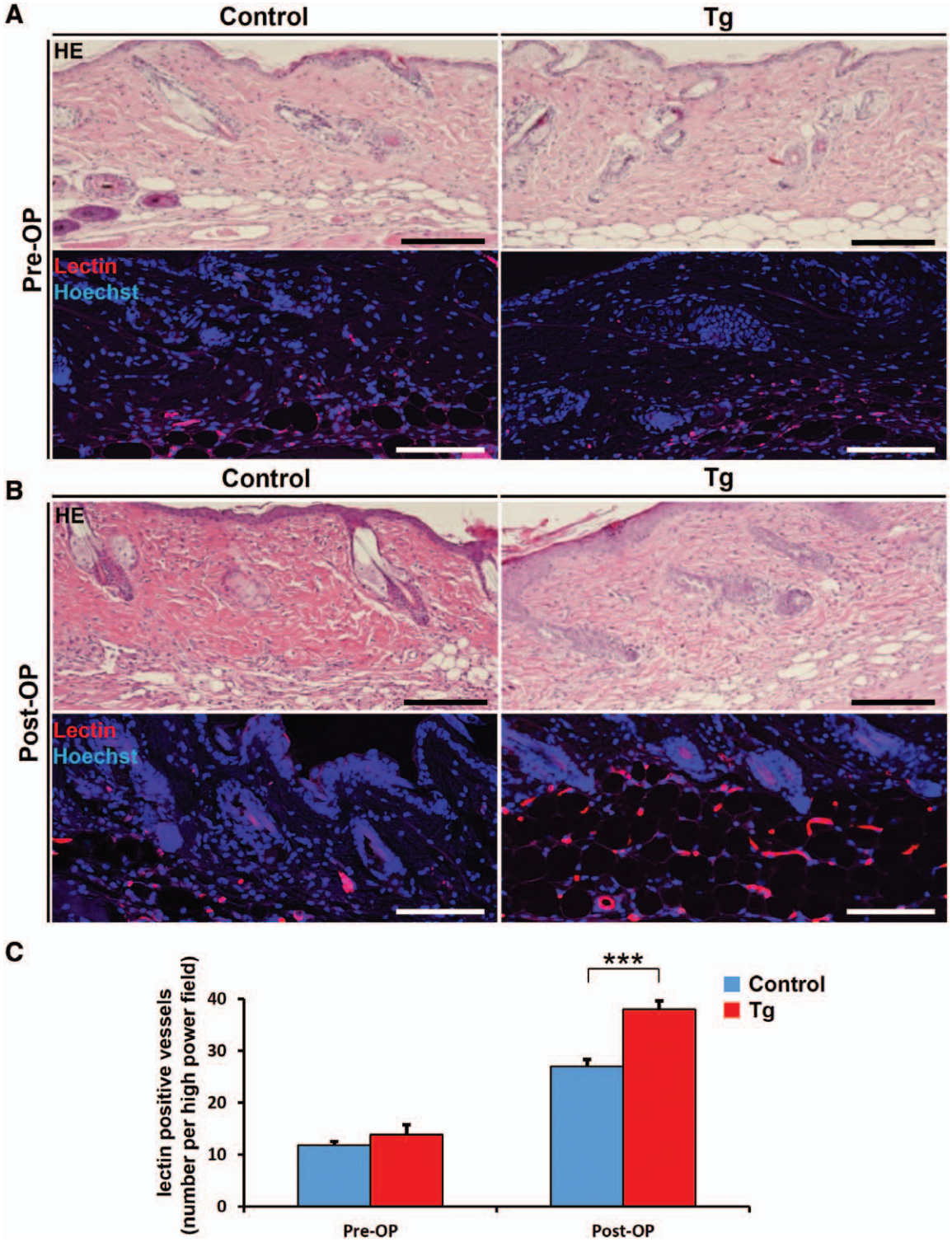

Fig. 5. Effect of specific HIF-2α overexpression in ECs on postoperative angiogenesis and vasculature in mouse subcutaneous plexus. Section of proximal portions of skin flaps preoperatively (A) and on postoperative day 7 (B) were stained with hematoxylin and eosin (HE) and rhodamine-lectin. C, The number of lectin-positive (red) vessels was counted per high-power field. Scale bar indicates 200 μm. ***$P < 0.001$.

ent study analyzed the function of HIF-2α in ECs through the investigation of transgenic mice overexpressing HIF-2α.

Under normoxic condition, HIF-2α subunit is hydroxylated at their proline residues by PHD, allowing their recognition and ubiquitination by the von Hippel-Lindau tumor suppressor protein, a component of E3 ubiquitin ligase complex, which labels them for rapid degradation by the proteasomes. Once hypoxic condition occurs, HIF-2α proteins are stabilized via decrease of PHD activation. In the present study, the skin flap survival area was significantly increased in the high copy line but not in the low copy line. In addition, HIF-2α protein levels in the lungs were significantly increased under normoxic condition only in the high copy line as shown in Figures 1E, F. These data suggest that a threshold copy number of transgenes is necessary for increase in a functional HIF-2α expression and that the copy number in low copy line is not enough to produce the functional HIF-2α proteins due to their degradation.

We also demonstrated that HIF-2α overexpression in ECs induced neovascularization in ischemic skin flaps. *HIF-2α* (but not *HIF-1α*) mRNA and protein levels were significantly increased in skin flaps from Tg mice. Moreover, *VEGF* mRNA levels were significantly increased. These results indicate that the expression of VEGF and the improvement of the vascularization were due to HIF-2α overexpression in ECs. In addition, HIF-2α protein in skin was expressed constantly in the Tg mice. Further study is required to determine how endothelial HIF-2α enhances skin flap survival via an increase in angiogenesis.

Both HIF-1α and HIF-2α play an important role in vascularization. We previously described the utility of the DMOG treatment for ischemic preconditioning. In the present study, PHD-1 and PHD-2 were expressed, and DMOG might be similarly useful. However, systemic DMOG treatment might cause adverse effects. Local coexpression of HIF-1α and HIF-2α can produce very strong angiogenic activity without adverse effects. The present study investigated whether therapeutic angiogenesis was induced by specific HIF-2α overexpression in ECs in a mouse model, and we evaluated the clinical importance of HIF-2α as a key transcription factor in skin flap ischemia. This study also paves the way for future clinical strategies based on expression of HIFs in ECs to promote skin flap survival.

CONCLUSIONS

Overexpression of HIF-2α in ECs induced neovascularization and increased random pattern skin flap survival in vivo in a mouse model. Further research using these Tg mice as a model of wound healing would be of benefit.

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