Inhibition of Prolyl Hydroxylase Domain Proteins Promotes Therapeutic Revascularization

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Background—The hypoxia-inducible transcription factor (HIF) subunits are destabilized via the O_2-dependent prolyl hydroxylase domain proteins (PHD1, PHD2, and PHD3). We investigated whether inhibition of PHDs via upregulating HIF might promote postischemic neovascularization.

Methods and Results—Mice with right femoral artery ligation were treated, by in vivo electrotransfer, with plasmids encoding for an irrelevant short hairpin RNA (shRNA) (shCON [control]) or specific shRNAs directed against HIF-1α (shHIF-1α), PHD1 (shPHD1), PHD2 (shPHD2), and PHD3 (shPHD3). The silencing of PHDs induced a specific and transient downregulation of their respective mRNA and protein levels at day 2 after ischemia and, as expected, upregulated HIF-1α. As a consequence, 2 key hypoxia-inducible proangiogenic actors, vascular endothelial growth factor-A and endothelial nitric oxide synthase, were upregulated at the mRNA and protein levels. In addition, monocyte chemotactic protein-1 mRNA levels and infiltration of Mac-3–positive macrophages were enhanced in ischemic leg of mice treated with shPHD2 and shPHD3. Furthermore, activation of HIF-1α–related pathways was associated with changes in postischemic neovascularization. At day 14, silencing of PHD2 and PHD3 increased vessel density by 2.2- and 2.6-fold, capillary density by 1.8- and 2.1-fold, and foot perfusion by 1.2- and 1.4-fold, respectively, compared with shCON ($P<0.001$). shPHD1 displayed a lower proangiogenic effect. Of interest, coadministration of shHIF-1α with shPHD3 abrogated shPHD3-related effects, suggesting that activation of endogenous HIF-1–dependent pathways mediated the proangiogenic effects of PHD silencing.

Conclusions—We demonstrated that a direct inhibition of PHDs, and more particularly PHD3, promoted therapeutic revascularization. Furthermore, we showed that activation of the HIF-1 signaling pathway is required to promote this revascularization. **(*Circulation*. 2009;120:50-59.)**

Key Words: angiogenesis ■ hypoxia ■ inflammation ■ ischemia

Four principal processes, vasculogenesis, angiogenesis, arteriogenesis, and collateral growth, contribute to tissue repair and remodeling during acute and chronic ischemic vascular diseases.[1] Thus, therapeutic neovascularization represents an alternative treatment modality for patients with advanced ischemic coronary or peripheral artery occlusive disease. Numerous animal studies have established that angiogenesis of ischemic tissue can be enhanced with exogenous growth factors or vascular progenitor cells, and clinical studies have been initiated. However, clinical trials to date are negative or give conflicting results.[2–4] It is clear that collateral growth, angiogenesis, and vasculogenesis are parts of the same process in the succession of events leading to neovascularization, and it is likely that they may complement each other. Hence, to advance the therapeutic objective, strategies should focus on the promotion of neovascularization in general rather than targeting collateral growth or angiogenesis specifically.

Clinical Perspective on p 59

The hypoxia-inducible transcription factor (HIF) is a master regulator controlling genes involved in several processes that promote neovascularization, making modulation of HIF activity an attractive approach for the treatment of ischemic disease.[5,6] HIF consists of 1 of the 3 O_2-regulated HIF-α subunits (HIF-1α, HIF-2α, and HIF-3α) and the constitutively expressed HIF-β subunit. In normoxia, HIF-α is hydroxylated at 2 critical prolines (402 and 564 in the human

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HIF-1α) by the prolyl hydroxylases domain proteins (PHDs).[7,8] This hydroxylation allows the binding of the von Hippel–Lindau tumor suppressor protein, which forms the recognition component of an E3 ubiquitin ligase complex, leading to ubiquitylation of HIF-1α and subsequently degradation by the proteasome.[5] In contrast, under acute hypoxia, the PHD activity is compromised, allowing the stabilization and translocation of HIF-α in the nucleus, dimerization with its constitutive nuclear partner protein HIF-β, and their binding to hypoxia-responsive elements in regulatory regions of target genes. Among these HIF-induced targets are key angiogenic genes such as vascular endothelial growth factor-A (VEGF-A), angiopoietin-2, Notch ligands, endothelial nitric oxide synthase (eNOS), and platelet-derived growth factor.[9] Therefore, hypoxia-induced HIF upregulation is a major driving force for postischemic angiogenesis within the surrounding tissue. HIF may also control the inflammatory reaction. Activation of HIF-1α is essential for myeloid cell infiltration and activation in vivo through regulation of glycolytic capacity.[10,11] Alternatively, inflammatory cytokines may control HIF-1–dependent signaling. Interleukin-1β strongly increases HIF-1α activity in cultured human hepatoma cells, emphasizing a possible role of HIF-1α as a trans-acting factor in the inflammatory process as well.[12] Of interest, inflammation has been shown to drive collateral growth.[13] Hence, it is likely that upregulation of HIF-1 can be thought of as a master switch that coordinates the expression of a wide repertoire of genes involved in regulating vascular growth and remodeling. In this line, ectopic expression of HIF-1α enhances neovascularization in models of rabbit hindlimb ischemia[14] or rat myocardial infarctus.[15] Pharmacological approaches with the use of PHD inhibitors, such as dimethyloxalylglycine, L-Mim, and others, have also been developed with promising results.[16,17] More recently, specific PHD silencing emerges as an innovative strategy to upregulate HIF-α. In this context, intraperitoneal injection of small interfering PHD (siPHD) upregulates cardiac inducible nitric oxide synthase expression, increases ventricular function, and reduces infarct size in a mouse model of myocardial ischemia/reperfusion injury.[18,19] In the present study, we used short hairpin RNAs (shRNAs) to downregulate murine PHD1, PHD2, or PHD3 levels and analyzed their efficiency as a therapeutic strategy in a model of mice hindlimb ischemia.

Methods

Plasmids

Sequences previously validated by transient transfection in NIH3T3 cells and thus knowing to specifically target each of the 3 PHD isoforms were cloned into the pTER vector as described previously.[20]

Hindlimb Ischemia

All the experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (No. 07430). C57BL/6 mice (12 weeks old; Charles River, L'Arbresle, France) (n=8 per group) underwent surgical ligation of the proximal part of the right femoral artery, as described previously.[21,22] After ligation, 25 μg of expression plasmids encoding for shRNAs directed against PHD1 (shPHD1), PHD2 (shPHD2), PHD3 (shPHD3), HIF-1α (shHIF1α), and the irrelevant shCON (control) were injected into both tibial anterior and gastrocnemius muscles of the anesthetized mouse, as described previously.[21,22] Then transcutaneous electric pulses (8 square-wave electric pulses of 200 V/cm, 20 ms each, at 2 Hz) were delivered by a PS-15 electropulsator (Jouan) with 2 stainless steel plate electrodes placed 4.2 to 5.3 mm apart, at each side of the leg. The left leg was not ligated or electrotransferred and was used as an internal control.

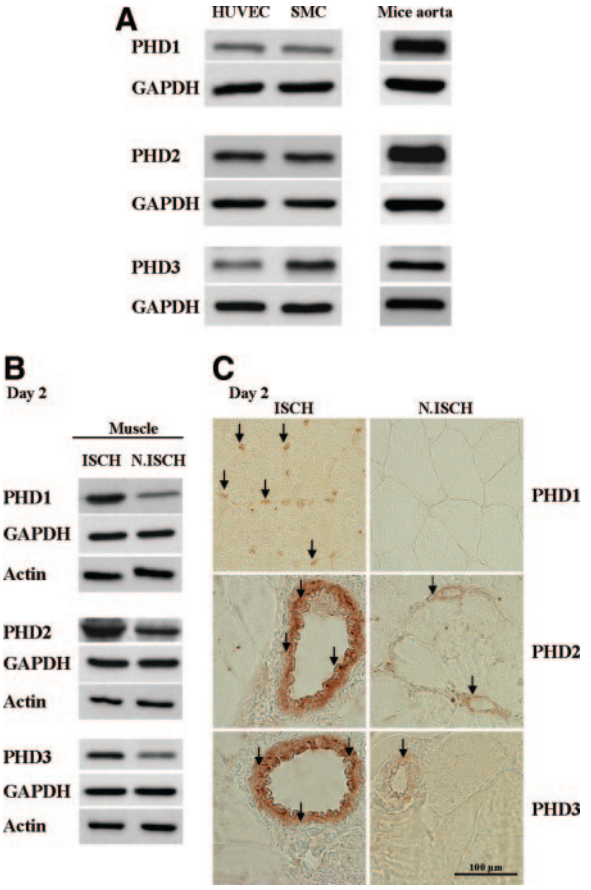

Figure 1. Representative Western blot of PHDs in human umbilical vein endothelial cells (HUVEC), human smooth muscle cells (SMC), and mice aorta (A) and ischemic (ISCH) or nonischemic (N.ISCH) hindlimbs of wild-type mice (B) 2 days after the onset of ischemia. C, Representative photomicrographs of PHD immunostaining in ischemic and nonischemic muscle of wild-type mice 2 days after the onset of ischemia.

Analysis of Neovascularization

Fourteen days after ischemia, postischemic neovascularization was evaluated by 3 different methods, as described previously.[21,22]

Microangiography

Mice were anesthetized (pentobarbital), and longitudinal laparotomy was performed to introduce a polyethylene catheter into the abdominal aorta and inject contrast medium (barium sulfate, 1 g/mL). Angiography of hindlimbs was then performed, and images (2 per animal) were acquired with the use of a high-definition digital x-ray transducer. Images were assembled to obtain a complete view of the hindlimbs. The number of pixels occupied by vessels was measured in the quantification area with the use of Primedangio software (Trophy System, Paris, France). Area of quantification was limited by placement of the ligature on the femoral artery, the knee, the edge of the femur, and the external limit of the leg. The results were then expressed as a ratio of ischemic to nonischemic leg.

Capillary Density Analysis

Sections (7 μm) were first incubated for 30 minutes in phosphate-buffered saline containing 5% bovine serum albumin at room temperature and 1 hour with rabbit polyclonal antibody directed

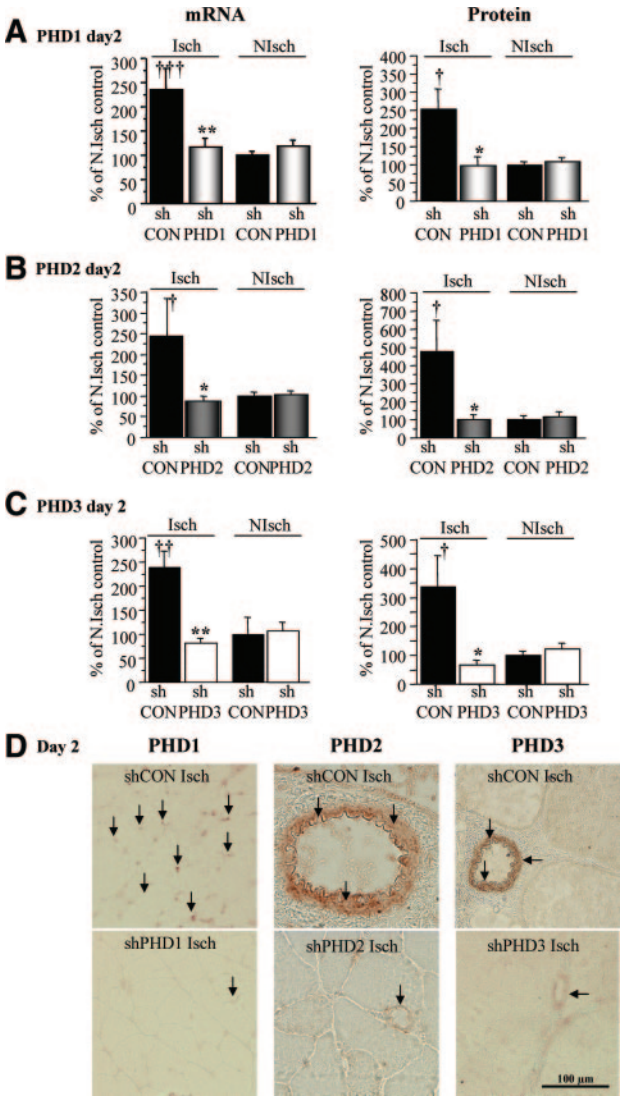

Figure 2. Quantification of PHD1 (A), PHD2 (B), and PHD3 (C) mRNA (left) and protein levels (right) in shPHD- and shCON-treated mice after 2 days of treatment. D, Representative photomicrographs of PHD immunostainings in ischemic muscle of mice treated with shCON, shPHD1, shPHD2, or shPHD3. Values are mean±SEM; n=8 per group. *$P<0.05$, **$P<0.01$ vs shCON ischemic (Isch); †$P<0.05$ vs shCON nonischemic (N.Isch).

against total fibronectin (dilution 1:50; Abcys, Paris, France) to identify capillaries. Capillaries were then revealed with a fluorescent fluorescein isothiocyanate anti-rabbit antibody (dilution 1:10; Amersham, Orsay, France). Capillary densities were calculated in 5 randomly chosen fields of a definite area with the use of Optilab/Pro software. Analyses were performed in a blinded manner by 2 independent investigators. The capillary-to-myocytes ratio was then determined in both ischemic and nonischemic legs. Results were expressed as ischemic to nonischemic ratio.

Laser Doppler Perfusion Imaging

Briefly, excess hairs were removed by depilatory cream from the limb, and mice were placed on a heating plate at 37°C to minimize temperature variation. Nevertheless, to account for variables, including ambient light, temperature, and experimental procedures, perfusion was calculated in the foot and expressed as a ratio of ischemic to nonischemic leg.

Analysis of Protein Expression

To prepare total protein extracts, tibial anterior muscles from ischemic and nonischemic hindlimbs, aorta, human umbilical vein

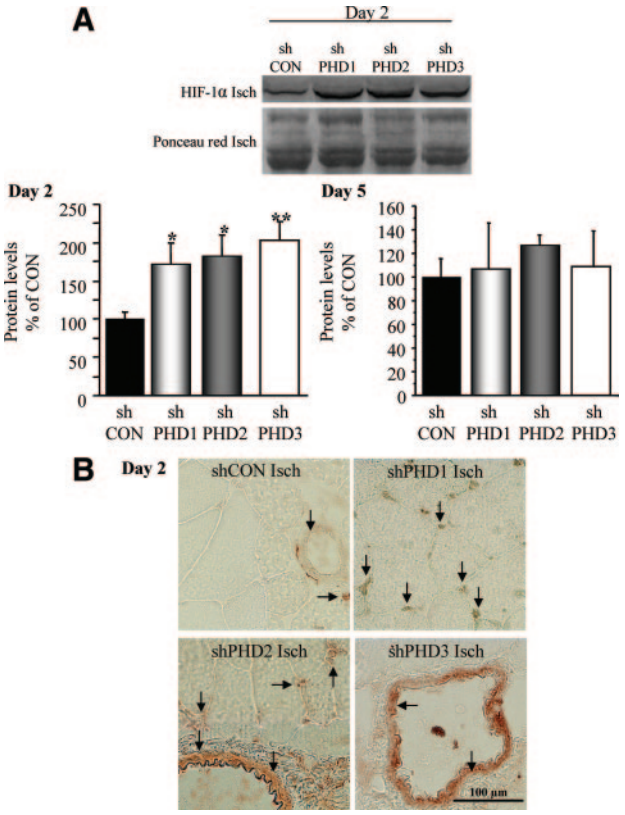

Figure 3. A, Quantification and representative Western blot of HIF-1α protein levels in nuclear extract of ischemic calf muscles in shPHD- and shCON-treated animals at days 2 and 5 after treatment. B, HIF-1α immunostaining in ischemic calf muscle of shCON- and shPHD-treated animals 2 days after the onset of ischemia. Values are mean±SEM; n=8 per group. *$P<0.05$, **$P<0.01$ vs shCON ischemic (Isch).

endothelial cells, and smooth muscle cells were homogenized in buffer RIPA (Tris-HCl 50 mmol/L, pH 7.4, NaCl 150 mmol/L, EDTA 1 mmol/L, Triton X-100 1%, deoxycholate 1%, sodium dodecyl sulfate 0.1%, with protease and phosphatase inhibitors). To prepare nuclear protein extracts, tibial anterior muscles from ischemic and nonischemic hindlimbs were homogenized in 10 mmol/L Tris-HCl, pH 7.8, 1.5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L dithiothreitol, 1 mmol/L sodium orthovanadate, supplemented with 1× protease inhibitor cocktail. After centrifugation (4500*g* for 5 minutes at 4°C), pellets were resuspended at 4°C for 30 minutes in 20 mmol/L Tris-HCl, pH 7.8, 1.5 mmol/L $MgCl_2$, 420 mmol/L KCl, 20% glycerol, supplemented with 1× protease inhibitor cocktail, and centrifuged (10 000*g* for 30 minutes at 4°C). Supernatants were dialyzed with Slide-a-Lyzer MINI Dialysis Units (Pierce Biotechnology) for 2 hours, twice at 4°C, in 20 mmol/L Tris-HCl, pH 7.8, 1.5 mmol/L $MgCl_2$, 100 mmol/L KCl, 0.2 mmol/L EDTA, and 20% glycerol. Proteins were resolved in 7%, 9%, or 12% denaturing gel electrophoresis and blotted onto nitrocellulose sheets (Bio-Rad, Marnes-la-Coquette, France; 0.2 μm). Antibodies against HIF-1α, PHD1, PHD2, PHD3, carbonic anhydrase IX (1:1000; Novus Biological or Bethyl Laboratories, Interchim), VEGF-A (1:1000; Santa Cruz Biotechnology, Santa Cruz, Calif), and eNOS (1:1000; Becton Dickinson) were used for immunoblotting. As a protein loading control, membranes were stripped, stained with ponceau red, or incubated with a monoclonal antibody directed against GAPDH (1:10 000; Abcyss), and specific chemiluminescent signal was detected as described previously.[23]

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA from gastrocnemius muscle was extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen,

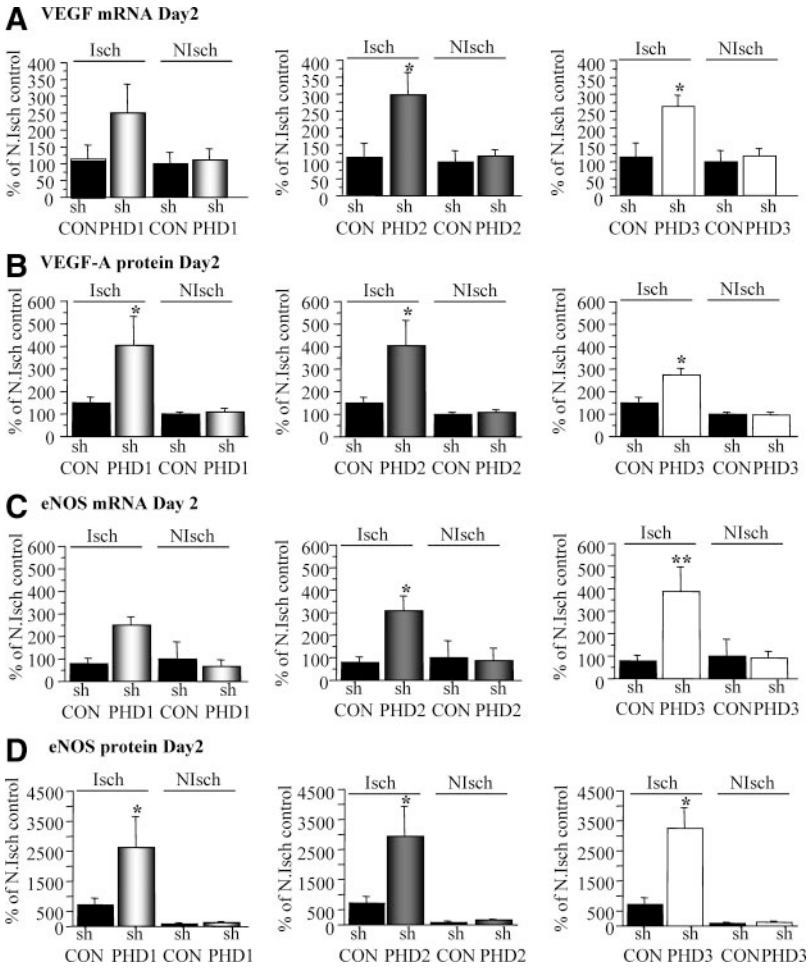

Figure 4. Quantification of VEGF mRNA (A), VEGF-A protein (B), eNOS mRNA (C), and eNOS protein (D) levels in mice treated with shPHD or shCON after 2 days of treatment. Values are mean±SEM; n=8 per group. *$P<0.05$, **$P<0.01$ vs shCON ischemic (Isch), ††$P<0.01$versus shCON nonischemic (NIsch).

Paris, France). cDNA synthesis was performed with QuantiTect Reverse Transcription Kit (Qiagen). Polymerase chain reaction was performed on an ABI Prizm 7700 with the use of Power SYBR Green PCR Master Mix (Applied Biosystems). Mouse GAPDH and α-actin were used to normalize sample amplification. The following oligonucleotides (Applied Biosystems, Courtaboeuf, France) served as primers: GAPDH forward: 5′-CGTCCCGTAGACAAAATGGTGAA-3′, reverse: 5′-GCCGTGAGTGGAGTCATACTGGAACA-3′; HIF-1α forward: 5′-CGGCGAGAACGAGAAGAAAAAGA-3′, reverse: 5′-TGGGGAAGTGGCAACTGATGAGCA-3′; PHD1 forward: 5′-CGCAGCATTCGTGGGGACCAGATT-3′, reverse: 5′-CATCGCCGTGGGGATTGTCAACAT-3′; PHD2 forward: 5′-AACTCAAGCCCAATTCAGTCAGCA-3′, reverse: 5′-CCCCACAGTACAGTCCCAGCAGAT-3′; PHD3 forward: 5′-GGCACCTGCGAGGCGACCAGAT-3′, reverse: 5′-TGGCGAACATAACCTGTCCCATTT-3′; eNOS forward: 5′-CGCCCACCCAGGAGAGATCCAC-3′, reverse: 5′-GCATCGGCAGCCAAACACCAAAGT-3′; monocyte chemotactic protein-1 (MCP-1) forward: 5′-CCCCACTCACCTGCTGCTA-3′, reverse: 5′-TTACGGGTCAACTTCACATTCAAA-3′; VEGF forward: 5′-CGAAGCTACTGCCGTCCGATTGAGA-3′, reverse: 5′-TGGTGAGGTTTGATCCGCATGATCTG-3′; α-actin, skeletal muscle forward: 5′-TCGCGACCTCACTGACTACCTGAT-3′, reverse: 5′-CCCCCTGACATGACGTTGTTG-3′.

Immunohistochemistry

Frozen tissue sections (7 μm) from gastrocnemius muscle were stained with antibodies directed against PHD1, PHD2, PHD3, HIF-1α (1:20; Santa Cruz Biotechnology), and Mac-3 (1:50, BD PharMingen, Le Pont-de-Claix, France). Tissue sections were then analyzed with the use of antibody peroxidase visualization systems (1:200; Jackson ImmunoResearch). Mac-3–positive cells were counted in randomly chosen fields with the use of Histolab software.

Statistical Analysis

Results were expressed as mean±SEM. Kruskal-Wallis ANOVA was used to compare each parameter. Post hoc Mann-Whitney *U* test with Bonferroni correction was then performed to identify which group differences account for the significant overall Kruskal-Wallis value. A value of $P<0.05$ was considered significant.

Results

Expression of PHD

We first analyzed the expression of PHD in vascular tissues. PHD1, PHD2, and PHD3 protein levels were detected in human umbilical vein endothelial cells, human smooth muscle cells, and mice aorta (Figure 1A). PHD1, PHD2, and PHD3 were also expressed in mice hindlimb muscle (Figure 1B and 1C). PHD1 could be detected in nucleus of myofibers, whereas PHD2 and PHD3 were expressed in endothelial and smooth muscle cells of a number of capillaries and arterioles (Figure 1C). Finally, PHD1, PHD2, and PHD3 protein levels were upregulated by ischemia (Figure 1B and 1C).

PHD Gene Silencing

We next evaluated the in vivo silencing of the endogenous PHDs. At day 2 of treatment, intramuscular electrotransfer of the shPHD1-expressing construct reduced by 50% ($P<0.01$) and 61% ($P<0.05$) PHD1 mRNA and protein levels, respectively, compared with mice treated with control plasmid (Figure 2A and Figure IA in the online-only Data Supple-

ment). Accordingly, expression of PHD1 was decreased in nucleus of myofibers by PHD1 gene silencing (Figure 2D). Similarly, shPHD2 decreased by 64% ($P<0.05$) and 78% ($P<0.05$) PHD2 mRNA and protein levels, respectively, compared with mice treated with shCON (Figure 2B and Figures IA and IIB in the online-only Data Supplement). Expression of PHD2 was also abrogated in arterioles of shPHD2-treated animals (Figure 2D). Finally, PHD3 gene silencing reduced by 66% ($P<0.01$) and 80% ($P<0.05$) PHD3 mRNA and protein levels, respectively, compared with the shCON group (Figure 2C and Figures IA, IIIA in the online-only Data Supplement). Expression of PHD3 was decreased in arterioles of shPHD3-treated animals (Figure 2D). Interestingly, PHD mRNA and protein contents returned to basal levels as early as day 5 of treatment (Figures IIA, IIB, and IIIA in the online-only Data Supplement). Specific PHD gene silencing did not affect PHD levels in the left nonischemic leg. We also confirmed the lack of impact of each shPHD construct on the expression of the 2 other PHDs (data not shown). Thus, the observed PHD silencing resulted from specific and effective targeting of PHDs by their corresponding small interfering RNA (siRNA) duplexes.

Effect of PHD Silencing on the HIF-Dependent Signaling Pathway

HIF-α prolyl hydroxylation by PHDs triggers proteasome targeting and thus protein degradation.[24,25] Accordingly, decrease in PHD activity is associated with HIF-α accumulation.[26,27] Thus, to further characterize the mechanism triggered by PHD silencing, we analyzed HIF-1α levels (Figure 3A). Silencing of each of the 3 PHD isoforms led to HIF-1α accumulation compared with the control animals. shPHD1 raised HIF-1α levels in nucleus of myofibers and in some capillaries, whereas HIF-1α upregulation was observed mainly in endothelial and smooth muscle cells of a number of capillaries and arterioles after shPHD2 and shPHD3 treatments. HIF-1α protein contents returned at basal levels as early as day 5, whatever the treatment (Figure 3).

Effect of PHD Silencing on Proangiogenic Factors: VEGF-A and eNOS

We next analyzed the effect of PHD silencing and subsequently HIF-1α upregulation on the regulation of 2 key HIF-dependent proangiogenic factors, VEGF-A and eNOS. Femoral ligation and electrotransfer with the use of any of the shPHD-expressing plasmids enhanced VEGF mRNA levels as measured by quantitative reverse transcription polymerase chain reaction at day 2 after treatment (Figure 4A). Accordingly, VEGF-A protein levels were also upregulated (Figure 4B and Figures IB, IIIB, and IV in the online-only Data Supplement). PHD silencing increased eNOS mRNA expression at day 2 and blocked its later upregulation observed in control animals. Similarly, at day 2, eNOS protein levels were increased in shPHD-silenced mice, whereas no differences between PHD-silenced and control animals were detected from day 5 until day 14 of treatment (Figure 4C and 4D and Figures IB, V, and VIA in the online-only Data Supplement).

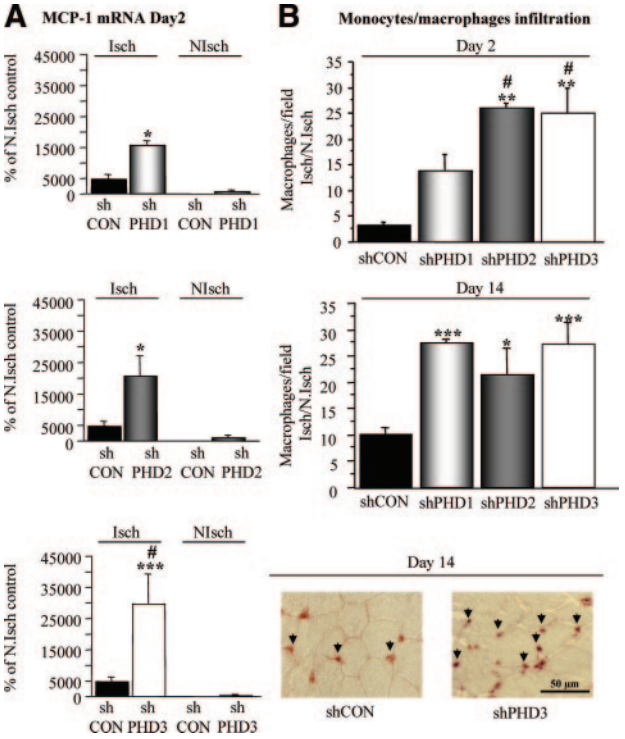

Figure 5. A, Quantitative evaluation of MCP-1 mRNA levels in mice treated with shPHD1, shPHD2, shPHD3, or shCON at 2 days of treatment. B, Quantitative evaluation and representative photomicrographs of Mac3-positive cells in mice treated with shPHD or shCON. Results were expressed as a ratio of ischemic (Isch) to nonischemic (NIsch) legs. Values are mean±SEM; n=8 per group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs shCON Isch; #$P<0.05$ vs shPHD1.

Effect of PHD Silencing on Proarteriogenic Factors: Chemokines and Inflammation

Sequential recruitment of monocytes to the ischemic area requires coordinated orchestration by a family of small, discrete chemotactic proteins called chemokines. In particular, monocyte infiltration has been shown to depend on MCP-1 and its cognate receptor CCR2[28,29] but also on fractalkine and Rantes, the ligands of CX3CR1 and CCR5, respectively.[30,31] In addition, retention of monocytes in close proximity to angiogenic vessels is mediated by stromal cell–derived factor-1/CXCR4 signaling pathways.[32,33] We therefore analyzed MCP-1, stromal cell–derived factor-1, fractalkine, and Rantes regulation in our experimental conditions.

At day 2 of treatment, shPHD1, shPHD2, and shPHD3 increased by 3.4-, 4.5-, and 6.4-fold, respectively, MCP-1 mRNA levels compared with shCON (Figure 5A). Although 5 days after treatment, MCP-1 mRNA was still increased in shPHD2-silenced mice, its level already returned to basal levels in the shPHD1- and shPHD3-treated groups. In contrast, at day 14, PHD-silenced and control animals showed similar MCP-1 mRNA levels (Figure VIB in the online-only Data Supplement). At day 2 of treatment, shPHD2 and shPHD3 also increased by ≈12-fold stromal cell–derived factor-1 mRNA levels compared with shCON (Figure VIII in the online-only Data Supplement). Stromal cell–derived factor-1 gene expression returned to basal levels 14 days after the onset of treatment. Fractalkine and Rantes mRNA levels

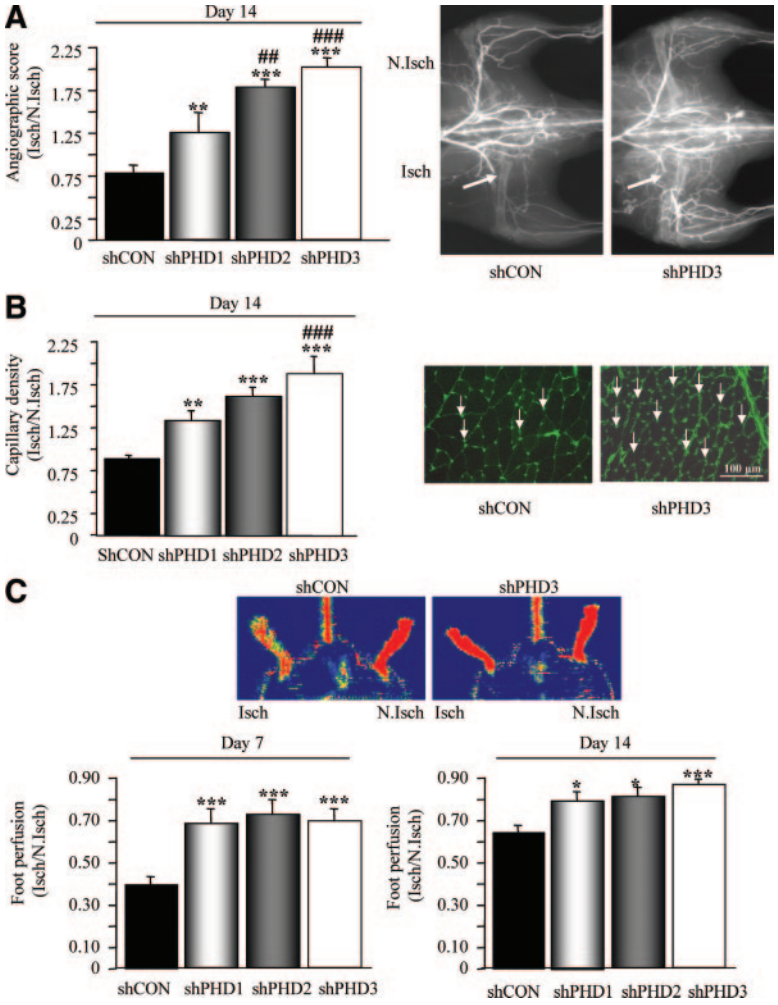

Figure 6. Quantitative evaluation and representative photomicrographs of microangiography (A), capillary density (B) (arrows indicate representative examples of fibronectin-positive capillaries appearing in green), and foot perfusion (C) in shPHD- and shCON-treated groups. Isch indicates ischemic; N.Isch, nonischemic. Values are mean±SEM; n=8 per group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs shCON; ##$P<0.01$, ###$P<0.001$ vs shPHD1.

were also upregulated as early as day 2 of treatment but were still increased after 14 days of treatment (Figure VIII in the online-only Data Supplement).

Chemokine upregulation should be associated with an infiltration of monocytes/macrophages in ischemic leg. Thus, we analyzed macrophage infiltration by measuring the number of Mac-3–positive cells. After 2 days of treatment, the number of Mac-3–positive cells increased by 7.4- and 7.2-fold in shPHD2- and shPHD3-treated groups, respectively, compared with the shCON group (Figure 5B). PHD1 silencing tended to increase the quantity of Mac-3–positive cells, but this did not reach statistical significance. Interestingly, differences in the number of PHD gene silencing–induced monocyte/macrophage infiltrations remained throughout the treatment from day 2 until day 14.

Effect of PHD Silencing on Postischemic Neovascularization

We next evaluated the effect of PHD silencing on therapeutic neovascularization at day 14 after treatment by using 3 complementary and independent approaches. The angiographic score (ischemic/nonischemic ratio) showed a significant increase of 1.6- ($P<0.01$), 2.2- ($P<0.001$), and 2.6-fold ($P<0.001$) in shPHD1-, shPHD2-, and shPHD3-treated groups, respectively, compared with the control group (Figure 6A). Results obtained with microangiography were confirmed by capillary density evaluation. Indeed, silencing of PHDs was associated with an increase in capillary density. More precisely, the ratio of ischemic to nonischemic leg capillary density was 1.5- ($P<0.01$), 1.8- ($P<0.001$), and 2.1-fold ($P<0.001$) higher in shPHD1-, shPHD2-, and shPHD3-treated mice, respectively, compared with control mice (Figure 6B). The improvement in both angiographic score and capillary density was also correlated with a significant increase in foot perfusion, as shown in Figure 6C. Taken together, all of these results clearly demonstrate that in vivo PHD silencing triggers postischemic neovascularization.

Involvement of HIF-1α in PHD3 Silencing–Induced Postischemic Neovascularization

The impact of PHD3 gene silencing in postischemic neovascularization was higher than that observed after inhibition of PHD1 or PHD2. We then focused on PHD3 inhibition to further investigate whether activation of HIF-dependent signaling is required in this process. Thus, we analyzed the effect of simultaneous electrotransfer of shRNAs directed against HIF-1α and PHD3 in our mouse model.

In the ischemic leg, VEGF-A and eNOS mRNA and protein levels were reduced in mice cotreated with siHIF-1α and shPHD3 compared with the shPHD3-treated group (Fig-

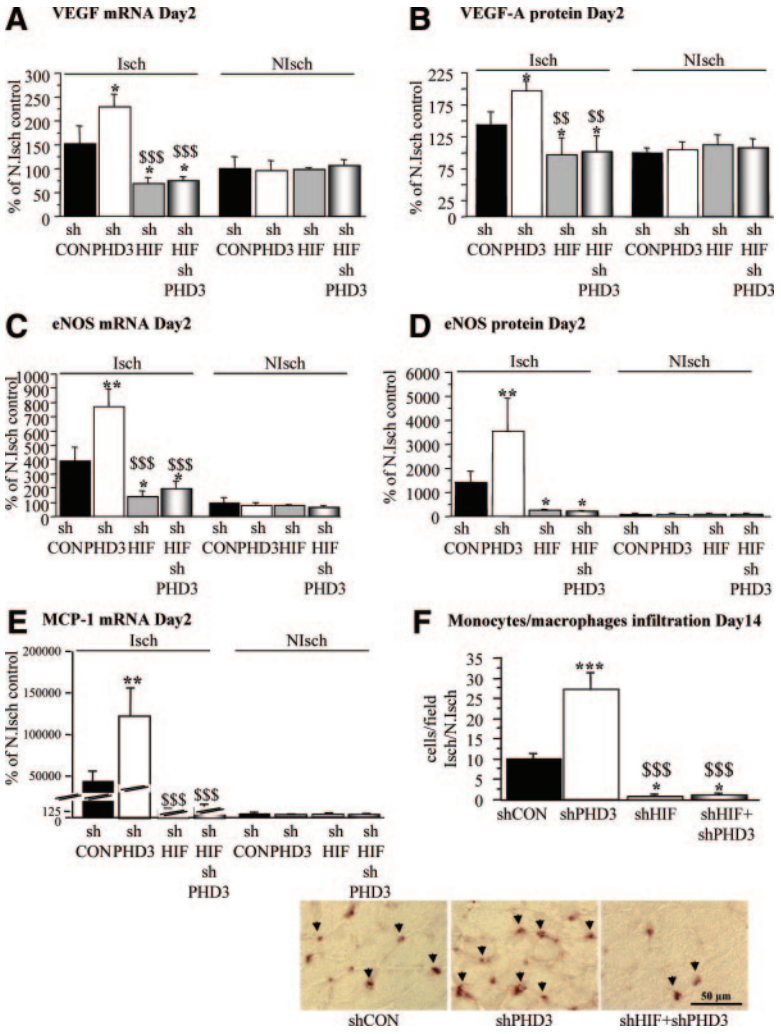

Figure 7. Quantitative evaluation of VEGF mRNA (A), VEGF-A protein (B), eNOS mRNA (C), eNOS protein (D), and MCP-1 mRNA (E) levels after 2 days of treatment. F, Quantitative evaluation and representative photomicrographs of Mac3-positive cell number after 14 days of treatment. N.Isch indicates nonischemic. Values are mean±SEM; n=8 per group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ vs shCON; \$\$$P<0.01$, \$\$\$$P<0.001$ vs shPHD3.

ure 7A through 7D). No significant differences were observed in the nonischemic leg (data not shown). Furthermore, in accordance with previous results suggesting the contribution of inflammatory cell infiltration to postischemic neovascularization, silencing of HIF-1α abrogated the shPHD3-induced chemokine upregulation and monocyte/macrophage infiltration (Figure 7E and 7F and Figures VII and IX in the online-only Data Supplement). In support of our initial hypothesis, angiographic score, capillary density, and foot perfusion were reduced in mice cotreated with both shRNAs compared with shPHD3-treated mice (Figure 8A through 8C). Taken together, these results strongly suggest that upregulation of endogenous HIF-1α levels and the subsequent activation of target genes mediate the proangiogenic and proarteriogenic effects of PHD silencing.

Discussion

Insufficient activation of the HIF complex precludes revascularization and tissue repair and may participate to the progression of ischemic disease.[34] The hypoxia-triggered and HIF-dependent induction of the PHD2 and PHD3 protein could at least partially account for this insufficient HIF activation, despite the low oxygen availability, because, as we demonstrated previously, this negative feedback mechanism mediates the extremely short half-life of HIF-α.[35] Therapeutic strategies leading to upregulation of HIF-1α may counterbalance the endogenous degradation of the protein and constitute an alternative approach in the treatment of ischemic disease. Previously, HIF-based gene therapy has been reported in models of rabbit hindlimb ischemia[14] or rat myocardial infarctus.[15] The safety and efficacy of modified constitutively active forms of HIF-1α have even been tested in the lower extremity of patients with critical limb ischemia.[36] However, this approach implies the risk of using virus-based therapies and needs to be tested in appropriately powered clinical trials. Inhibitors of prolyl hydroxylation, such as dimethyloxalylglycine, L-Mim, and others, are also capable of inducing HIF-α and HIF target genes in vitro and in vivo and induce adaptive responses to hypoxia, including angiogenesis.[16,17] Nevertheless, the use of these kinds of inhibitors can trigger toxic effects associated with the marked and sustained upregulation of endogenous HIF-1α.[37]

More recently, specific PHD2 silencing has been shown to enhance ventricular function and reduce infarct size in a mouse model of myocardial ischemia/reperfusion injury.[18,19] In the present study, we have developed a therapeutic strategy that promotes the local and transient inhibition of PHD1, PHD2, or PHD3. We demonstrated that PHD gene silencing

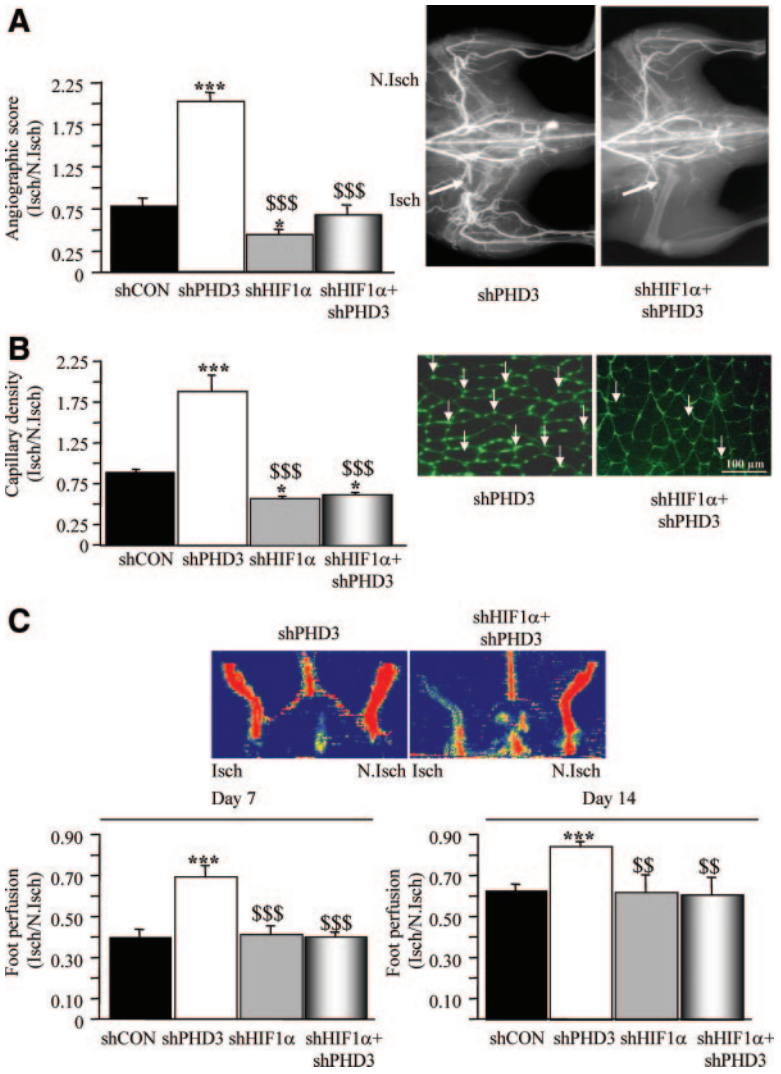

Figure 8. Quantitative evaluation and representative photomicrographs of microangiography (A), capillary density (B), and foot perfusion (C) in mice treated with shRNAs directed against nonspecific sequences (shCON), PHD3 (shPHD3), HIF-1α (shHIF1α), and both HIF-1α and PHD3 (shHIF1α+shPHD3) after 14 days of treatment. Isch indicates ischemic; N.Isch, nonischemic. Values are mean±SEM; n=8 per group. ***$P<0.001$ vs shCON; $$$P<0.01$, $$$$P<0.001$ vs shPHD3.

promoted therapeutic revascularization in mice with hindlimb ischemia, with PHD3 attenuation leading to a better effect than similar reduction of PHD2 or PHD1. Similarly, in different cell types, isoform-specific patterns of PHD induction by hypoxia and hormones alter both the relative abundance of the PHDs and their relative contribution to the regulation of HIF.[27] In addition, inhibition of the PHD2 isoform with siRNA, but not inhibition of PHD1 or PHD3, was sufficient to upregulate HIF-1α in endothelial cells.[26] Furthermore, PHDs manifest specificity for different prolyl hydroxylation sites within each HIF-1α subunit and a degree of selectively between HIF-1α and HIF-2α isoforms, indicating that differential PHD inhibition has the potential to selectively alter the characteristics of HIF activation.

PHD inhibition improved HIF-1α protein levels, and PHD3 silencing–induced neovascularization was fully blunted by cotreatment with HIF-1α siRNA, demonstrating that HIF-1α upregulation mediated the proangiogenic effect of PHD inhibition. It is noteworthy that PHD attenuation also upregulated HIF-1α at the mRNA levels (data not shown). Interestingly, proinflammatory cytokines have been shown to control HIF-1 gene expression, suggesting that the postischemic inflammatory response may contribute to increase HIF-1 gene expression in our experimental conditions.[12,38]

By virtue of its ability to upregulate VEGF-A and eNOS, 2 genes intimately involved in postischemic neovascularization, HIF-1 upregulation likely promotes angiogenesis in ischemic tissue. In addition, PHD silencing–induced HIF-1 upregulation leads to activation of inflammation-dependent vessel growth. As inflammation and hemodynamic factors drive collateral growth, it is noteworthy that strategies based on PHD inhibition triggered neovascularization in general.

Alternatively, HIF-1 upregulation may activate additional pathways involved in postischemic revascularization. In this view, HIF-1 has been shown to regulate directly chemokine stromal cell–derived factor-1, allowing the recruitment of vascular progenitor cells that express CXCR4 in the ischemic tissue.[34,39] In addition, inhibition of PHD may affect skeletal muscle metabolism. Hence, loss of PHD1 lowers oxygen consumption in skeletal muscle by reprogramming glucose metabolism from oxidative to more anaerobic ATP production. This metabolic adaptation to oxygen conservation impairs oxidative muscle performance in healthy conditions, but it provides acute protection of myofibers against lethal ischemia.[40]

In conclusion, our study reveals, for the first time, that silencing of PHDs by the transient and local upregulation of endogenous HIF-1α improves vessel growth in ischemic hindlimb. This study also paves the way for future strategies based on administration of siRNAs directed against PHD to promote therapeutic revascularization.

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Disclosures

None.

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CLINICAL PERSPECTIVE

Therapeutic neovascularization represents an alternative treatment modality for patients with advanced ischemic coronary or peripheral artery occlusive disease. Numerous animal studies have established that angiogenesis of ischemic tissue can be enhanced with exogenous growth factors or vascular progenitor cells, and clinical studies have been initiated. However, clinical trials to date are negative or give conflicting results. The hypoxia-inducible transcription factor (HIF) is a master regulator controlling genes involved in several processes that promote neovascularization, making modulation of HIF activity an attractive approach for the treatment of ischemic disease. In normoxia, HIF-α is hydroxylated by the prolyl hydroxylases domain proteins (PHDs), leading to ubiquitylation of HIF-1α and subsequently degradation by the proteasome. In contrast, under acute hypoxia, the PHD activity is compromised, allowing the stabilization and translocation of HIF-α in the nucleus and upregulation of wide repertoire of genes involved in vessel growth. In the present study, we used short hairpin RNAs to downregulate murine PHD1, PHD2, or PHD3 levels and analyzed their efficiency as a therapeutic strategy in a model of mice hindlimb ischemia. We demonstrated that a direct inhibition of PHDs, and more particularly PHD3, promoted therapeutic revascularization via specific activation of proangiogenic molecules, including vascular endothelial growth factor-A and endothelial nitric oxide synthase, and proarteriogenic proinflammatory pathways. Therefore, silencing of PHDs by the transient and local upregulation of endogenous HIF-1α improves vessel growth in ischemic tissue. This study also paves the way for future strategy based on administration of small interfering RNAs directed against PHD to promote therapeutic revascularization in ischemic disease.