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HIF-Prolyl Hydroxylase 2 Inhibition Enhances the Efficiency of Mesenchymal Stem Cell-Based Therapies for the Treatment of Critical Limb Ischemia

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ABSTRACT

Upregulation of hypoxia-inducible transcription factor-1α (HIF-1α), through prolyl-hydroxylase domain protein (PHD) inhibition, 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. We aimed to unravel the effect of specific PHD2 isoform silencing in cell-based strategies designed to promote therapeutic revascularization in patients with critical limb ischemia (CLI). PHD2 mRNA levels were upregulated whereas that of HIF-1α were downregulated in blood cells from patients with CLI. We therefore assessed the putative beneficial effects of PHD2 silencing on human bone marrow-derived mesenchymal stem cells (hBM-MSC)-based therapy. PHD2 silencing enhanced hBM-MSC therapeutic effect in an experimental model of CLI in Nude mice, through an upregulation of HIF-1α and its target gene, VEGF-A. In addition, PHD2-transfected hBM-MSC displayed higher protection against apoptosis in vitro and increased rate of survival in the ischemic tissue, as assessed by Fluorescence Molecular Tomography. Cotransfection with HIF-1α or VEGF-A short interfering RNAs fully abrogated the beneficial effect of PHD2 silencing on the proangiogenic capacity of hBM-MSC. We finally investigated the effect of PHD2 inhibition on the revascularization potential of ischemic targeted tissues in the diabetic pathological context. Inhibition of PHD-2 with shRNAs increased postischemic neovascularization in diabetic mice with CLI. This increase was associated with an upregulation of proangiogenic and proarteriogenic factors and was blunted by concomitant silencing of HIF-1α. In conclusion, silencing of PHD2, by the transient upregulation of HIF-1α and its target gene VEGF-A, might improve the efficiency of hBM-MSC-based therapies. STEM CELLS *2014;32:231–243*

INTRODUCTION

Critical Limb ischemia (CLI) is the most severe form of peripheral arterial disease and is characterized by ischemia that gives rise to non-healing ulcers and tissue loss. Of particular interest, insufficient neovascularization leading to tissue hypoperfusion is an integral component of adverse tissue remodeling in patients with CLI. This muted response is likely due to aging, diabetes, and other cardiovascular risk factors leading to impaired adaptive vascular response after ischemia and low availability and dysfunction of stem/progenitor cells from different origins [1–7]. Thus, efforts need to be directed toward the development of new and more effective strategies for revascularization of ischemic limb that may circumvent the defective adaptive vascular response and improve stem/progenitor cells related-actions.

The hypoxia-inducible transcription factor-1 (HIF-1) is a master regulator of postischemic neovascularization and controls the expression of multiple angiogenic cytokines, mobilization of angiogenic and arteriogenic cells, maintenance of tissue viability, and recovery of limb perfusion following femoral artery ligation [8–10]. Of interest, HIF-1 expression and the cellular response to hypoxia are depressed by cardiovascular risk factors, including diabetes [11]. Therefore, endogenous or exogenous modulation of HIF-1 has been used to promote therapeutic revascularization in pathophysiological settings. Ectopic expression of HIF-1 using adenovirus leads to a significant improvement in postischemic neovascularization in old animals and in diabetic mice [8, 12]. Pharmacological treatments increasing HIF expression also show promising result in

Table 1. Baseline characteristics of the study population

Characteristic	Value
CLI patients, *n*	13
Mean age, year ± SD	65 ± 10
Diabetes (%)	46.2
Hypertension treatment (%)	61.6
Coronary heart disease (%)	15.4
Hyperlipidemia (%)	61.6
Lipid-lowering treatment (%)	53.8
Heart failure history (%)	15.4
Cerebrovascular disease (%)	7.7
Renal failure, creatinine clearance <60 (%)	23.0
Current smoker (%)	15.5
Former smoker (%)	69

different models of angiogenesis [13, 14]. A synergistic effect of HIF-1α gene therapy and injection of HIF-1α-activated bone marrow (BM)-derived cells have been reported in CLI [15]. In this line, hypoxic preconditioned cells display a marked proangiogenic and protherapeutic potential in mouse models of cerebral ischemia [16], CLI [17], and myocardial infarction [18, 19]. Recently, we and others have developed a new strategy to upregulate and stabilize endogenous HIF levels using shRNA directed against prolyl-hydroxylase domain proteins (PHDs). We demonstrated that the inhibition of PHD1, PHD2, or PHD3 triggers vessel growth and remodeling in control mice with hind limb ischemia [9]. PHD2 inhibition appears critical and also shows beneficial effects on cardiac function in a mouse model of myocardial infarction [20].

In this study, we investigated the efficiency of PHD2 inhibition on the promotion of therapeutic revascularization in cell-based therapies. We demonstrated that PHD2 inhibition was efficient to enhance stem/progenitor cells function and to skew human BM-derived mesenchymal stem cells (hBM-MSC) toward a proangiogenic phenotype to increase their therapeutic potential. Furthermore, PHD2 silencing restored diabetes-induced impairment of adaptive vascular response in a type I diabetic mouse model of CLI and PHD2 downregulation significantly enhanced postischemic neovascularization, through HIF-1α-dependent mechanisms.

Materials and Methods

Study Population

The OPTIPEC clinical trial (http://clinicaltrials.gov/ct2/show/NCT00377897) was a phase I nonrandomized study (Table 1). Patients were eligible for the protocol if they had CLI associated with limited gangrene or a nonhealing ischemic ulcer and if they were ineligible for surgical revascularization or percutaneous angioplasty, or if such a procedure carried little chance of success. BM aspirates were collected as described elsewhere [21]. The cell therapy protocol was similar to the one published initially by Tateishi-Yuyama et al. [22]. Briefly, BM-mononuclear cells (MNCs) were collected and isolated on buffy coat. Three hours after cell isolation, 40 injections of 0.75 ml each were made in the gastrocnemius muscle of the ischemic limb. All the patients were examined each week during the first month, and then every month for 1 year. One week before injection, mRNA whole blood sample was collected in Paxgene tube from each patient, and blood was processed within 2 hours of collection. mRNA extraction was performed according to manufacturer's instruction (Qiagen, Courtaboeuf/France, www.qiagen.com). Peripheral blood MNCs were also isolated by ficoll gradient from whole blood samples from healthy volunteer (HV) or CLI patients, stained with anti-CD14 FITC antibody (BD Biosciences, Le Pont de Claix/France, www.bdbiosciences.com), and then sorted by flow cytometry. mRNA extraction was performed according to manufacturer's instruction (Qiagen). CLI patients (median age 65 years) were compared with a group of healthy subjects of 60–70 years who were free of cancer and cardiovascular diseases or its risk factors (http://clinicaltrials.gov/ct2/show/NCT01038700). Tissue samples from limb amputation specimens of OPTIPEC patients were submitted to a standardized protocol as described [23]. Paraffin sections were stained with antibodies directed against PHD2 (1:100, Novus Biotechnology, Interchim, Montluçon/France, www.interchim.com) or an irrelevant antibody, using a three-step avidin-biotin immunoperoxydase method.

Cell Culture

hBM-MSCs were obtained from nine different HV donors. MSCs were isolated from BM-MNCs collected from ficoll gradients and cultured as previously described [24]. Cells were used between passage 4 and passage 7. In vitro, experiments were performed with hBM-MSC from two to three different donors. In vivo, experiments were performed with hBM-MSC from one donor as hBM-MSC from three different donors displayed similar proangiogenic potential in our experimental condition (Supporting Information Fig. S1).

Human endothelial progenitor cells (hEPCs) were derived from cord-blood MNCs, as previously described [25]. Cells were grown in rat-tail type I collagen-coated flasks in ECBM supplemented with Supplement Pack Endothelial Cell Growth Medium (Promocell, Heidelberg/Germany, www.promocell.com), 10% Foetal Calf Serum, and antibiotics.

Transfection Protocol

PHD2 (siPHD2), HIF-1α (siHIF-1α), VEGF-A (siVEGF-A), and nontargeting control (siCON) siRNAs (Smartpool) were purchased from Dharmacon (Abgene, Thermo Fisher Scientific, St Leon-Rot/Germany, U.K., www.thermoscientific.com/onebio). Transfection for hBM-MSC was performed according to the manufacturer's instructions. Briefly, cells were grown on six-well plates until 70% confluence. The siRNA solution was mixed with serum-free and antibiotic-free cell culture medium (EBM-2, Lonza, Saint-Beauzire/France, www.lonza.com) containing DharmaFECT1 siRNA transfection reagent. The culture medium was aspirated and replaced with 1,600 μl fresh antibiotic-free medium containing 5% fetal calf serum; 400 μl of the transfection mix was added to each well to achieve a final siRNA concentration of 50 nM. Transfected cells were incubated at 37°C for 30 hours, medium was then replaced with fresh EBM-2 medium containing 5% serum during 18 additional hours. Protein and RNA expressions were analyzed either by Western blot or RT-qPCR 48 hours after transfection.

Animals

All experiments were performed in accordance with the European Community guidelines for the care and use of laboratory animals (No. 07430). Ten-week-old males C57Bl/6 and Nude mice (Janvier,

St Berthevin/France, www.janvier.europe.com) were used. To induce diabetes, C57BL/6 mice were injected intraperitoneally with 60 mg/kg streptozotocin in sodium citrate buffer (0.05 mol/l, pH 4.5) daily for 5 days. Three days after the fifth injection, blood glucose levels were measured. Mice with glucose levels $<$300 mg/dl were excluded from the study. In control groups, mice were injected intraperitoneally with sodium citrate buffer (0.05 mol/l, pH 4.5). Hind limb ischemia was performed on C57BL/6 diabetic mice at 9 weeks after constant hyperglycemia was observed in animals [26, 27].

Mouse Hind Limb Ischemia Model and Neovascularization Analysis

Mouse hind limb ischemia was induced by right femoral artery ligation. Postischemic neovascularization was assessed by three independent approaches: microangiography, capillary density analysis, and laser foot Doppler perfusion imaging as described previously [9].

Twenty-four hours after ligation, 1×10^5 hBM-MSCs treated with or without siRNAs were injected in phosphate buffer saline into the gastronecmius muscle. Day 0 is the day of induction of ischemia and Day 1, the day of injection of cells. Viability of hBM-MSC was assessed by trypan blue exclusion staining and was superior to 95%. For in vivo shRNAs treatment, 6 hours after ligation, 25 μg of expression plasmids encoding for shRNAs directed against PHD2 (shPHD2), HIF-1α (shHIF-1α), and an irrelevant shRNA (shCON) was injected and electrotransferred into both tibial anterior and gastrocnemius muscles of the anesthetized mouse, as described previously [9].

Analysis of Protein Expression

To prepare total protein extracts, hBM-MSCs 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 dodecylsulfate 0.1%, with protease inhibitor cocktail).

To prepare nuclear protein extracts from hBM-MSC, cells were homogenized in 20 mmol/l HEPES, 4.2 mmol/l NaOH, 10 mmol/l KCl, 1 mmol/l EDTA, 0.2% Nonidet P40, and 10% glycerol supplemented with protease inhibitor cocktail and incubated for 30 minutes on ice. After centrifugation (18,000*g* for 6 minutes at 4°C), supernatants were removed and pellets were resuspended in 20 mmol/l HEPES, 4.2 mmol/l NaOH, 10 mmol/l KCl, 1 mmol/l EDTA, 350 mmol/l NaCl, 10% glycerol, and protease inhibitor cocktail. After sonication, the pellet was incubated for 30 minutes on ice and then centrifuged (18,000*g* for 3 minutes at 4°C).

Proteins were resolved in 10% or 12% denaturing gel electrophoresis and blotted onto nitrocellulose sheets (Bio-Rad, Marnes-la-coquette/France, www.bio-rad.com). Antibodies against HIF-1α, PHD2, and PHD3 (1:500; Novus Biological or Bethyl Laboratories, Interchim) 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), and specific chemiluminescent signal was detected as described previously [9].

RNA Extraction and Quantitative Reverse Transcription Polymerase Chain Reaction

Total RNA from cells was extracted with RNeasy Micro kit (Qiagen). cDNA synthesis was performed with QuantiTectReverse Transcription Kit (Qiagen). Polymerase chain reaction was performed on an ABI Prism 7700 with the use of Power SYBRGreen PCR Master Mix (Applied Biosystems, Life Technologies, Saint-Aubin/France, www.lifetechnologies.com/fr/fr/home/brands/applied-biosystems.html). GAPDH was used to normalize sample amplification. The following primers were used for quantification of human PHD2 forward: 5′-GCC CGG CTG CGA AAC CA-3′, reverse: 5′-TGC CCG GAT AAC AAG CAA CCA-3′; human HIF-1α forward: 5′-TGC CCC AGA TTC AGG ATC AGA-3′, reverse: 5′-AGG GCT TGC GGA ACT GCT TT-3′; and human GAPDH forward, 5′-GAA GGT GAA GGT CGG AGT C-3′, 5′-GAA GAT GGT GAT GGG ATT TC-3′ reverse.

Enzyme-Linked Immuno-Absorbent Assay

Seventy-two hours after siRNA transfection, culture supernatants were harvested from hBM-MSC grown in EBM-2 medium with 5% Foetal Calf Serum and centrifuged at 1,000*g* during 10 minutes at 4°C. ELISA for human VEGF-A was purchased from R&D Systems, Lille/France, www.rndsystems.com.

Apoptosis Assay

Forty-eight hours after transfection, hBM-MSCs cultured in four-well chamber slides were incubated in Hanks' balanced saline solution (HBSS) (control) or HBSS with H_2O_2 75 μM and $FeSO_4$ 100 μM (H_2O_2) for 14 hours. After incubation, cells were washed, fixed in Paraformaldehyde 4%, and permeabilized in Phosphate Buffer Saline/Bovine Serum Albumin 5%/0.1% Triton X-100. Cells were then stained with TUNEL technology (Roche Diagnostics, Meylan/France, www.roche-applied-science.com) and incubated with antibody directed against cleaved caspase 3 (1:400, Cell Signaling, Ozyme, Saint Quentin Yvelines/France, www.ozyme.com). The percentage of apoptotic cells was determined by counting the number of TUNEL or cleaved caspase 3-positive cells and the total number of cells stained by DAPI on five random fields per well at a ×10 magnification on a Axioimager Carl Zeiss Microscope using Image J software.

hBM-MSC Survival Imaging In Vivo

For in vivo imaging of hBM-MSC survival, labeled 1×10^5 hBM-MSC treated with siRNAs were injected into the gastronecmius muscle 24 hours after ligation (Day 1). Labeling of hBM-MSC was performed right before injection with the VivoTag S 680 dye (Perkin Elmer, Courtaboeuf / France, http://www.perkinelmer.com/FR/). Briefly, 1×10^6 cells were incubated 30 minutes at 37°C in a humidified incubator with 100 μM of VivoTag S 680. Cells were then washed extensively with PBS and injected into the ischemic limb. Nude mice were then placed in the imaging cartridge and introduced into the fluorescence molecular tomography (FMT) system (Perkin Elmer). 3D FMT dataset was reconstructed in which fluorescence per voxel was expressed in picomoles.

[^{3}H] Thymidine Incorporation Assay

Thirty hours after transfection with siCON or siPHD2 or siHIF-1α or siPHD2 and siHIF-1α, cells were arrested in G0/G1 phase by serum deprivation for 15 hours. Cells were cultured at 37°C for 24, 48, and 72 hours in complete EGM-2 medium (Lonza) and pulsed 18 hours with 1 μCi [methyl 3H]

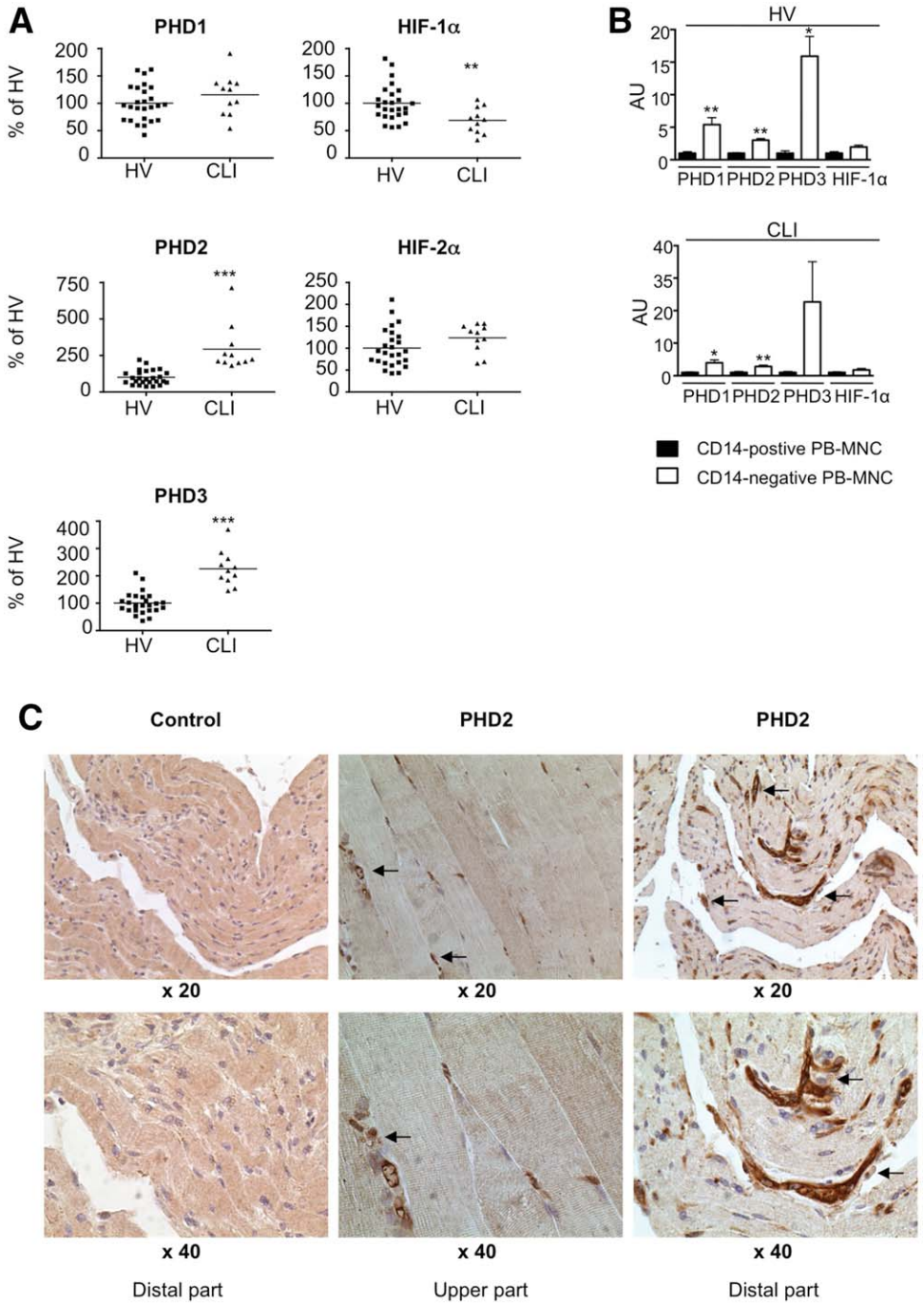

Figure 1. Regulation of PHDs and HIF-1α levels in patients with CLI. **(A):** mRNA levels of PHD1, PHD2, PHD3, HIF-1α, and HIF-2α were measured in blood samples from patients with CLI. mRNA levels are expressed as percentage of mRNA of healthy volunteers. Values are mean; $n = 26$ for HV, $n = 11$ for patients with CLI (CLI). *, $p < .05$; **, $p < .01$; ***, $p < .001$ versus HV. **(B):** PHD1, PHD2, PHD3, and HIF-1α are mainly expressed by CD14-negative cells. RT-qPCR analysis was performed on CD14-positive or CD14-negative cells isolated from PB-MNC from HV or CLI patients. Results are expressed as arbitrary units normalized to the levels of each mRNAs in the CD14-positive fraction. $n = 5$ patients per group. *, $p < .05$; **, $p < .01$, versus CD14-negative PB-MNC. **(C):** Representative photomicrographs of PHD2 immunostaining in the upper part and the distal part of the ischemic legs of patients with CLI receiving BM-MNCs as a cell therapy product are shown. Arrows point to area of interest. Abbreviations: CLI, critical limb ischemia; HIF-1α, hypoxia-inducible transcription factor-1α; HV, healthy volunteer; PB-MNC, peripheral blood mononuclear cells; PHD, prolyl-hydroxylase domain.

thymidine. Thymidine incorporation was assessed using a Topcount NXT scintillation counter (Perkin Elmer). Results are presented as percentage of proliferation of siCON-hBM-MSC after 24 hours.

Statistical Analysis

Results were expressed as mean ± SEM. One-way analysis of variance was used to compare each parameter when there were three or more independent groups. Comparisons

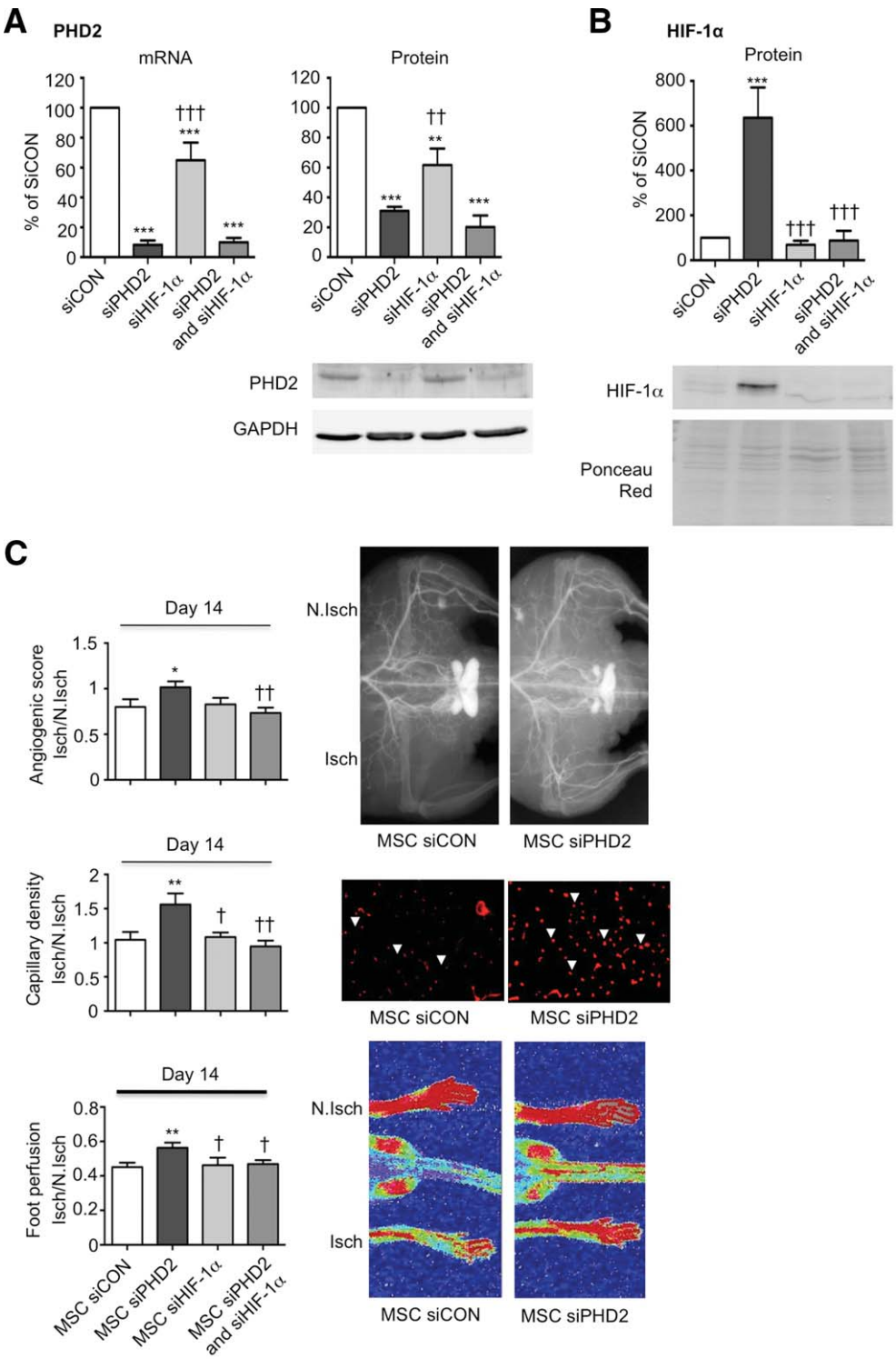

Figure 2. PHD2 inhibition increases human bone marrow (hBM)-MSC therapeutic potential. **(A)** mRNA (left) and protein (right) levels of PHD2 and **(B)** HIF-1α were quantified 2 days after treatment with siCON or siPHD2 or siHIF-1α or siPHD2 and siHIF-1α in hBM-MSC. Results are expressed as percentage of siCON-treated-hBM-MSC (siCON). Values are mean ± SEM; $n = 3$–6 independent experiments. ***, $p < .001$ versus siCON; ††, $p < .01$; †††, $p < .001$ versus siPHD2-treated-hBM-MSC (siPHD2). Representative pictures of Western blot of PHD2 and HIF-1α after 2 days of treatment are shown. **(C):** Microangiography analysis, capillary density, and foot perfusion were analyzed in Nude mice after intramuscular injection of siCON or siPHD2 or siHIF-1α or siPHD2 and siHIF-1α-treated-hBM-MSC. Results are expressed as ratio of ischemic to nonischemic (Isch/NIsch) legs. Values are mean ± SEM; $n = 8$–19 mice per group. *, $p < .05$; **, $p < .01$ versus MSC siCON; †, $p < .05$; ††, $p < .01$ versus MSC siPHD2. Representative photomicrographs of microangiography analysis, capillary density, and foot perfusion are shown. Abbreviations: HIF-1α, hypoxia-inducible transcription factor-1α; MSC, mesenchymal stem cell; PHD, prolyl-hydroxylase domain.

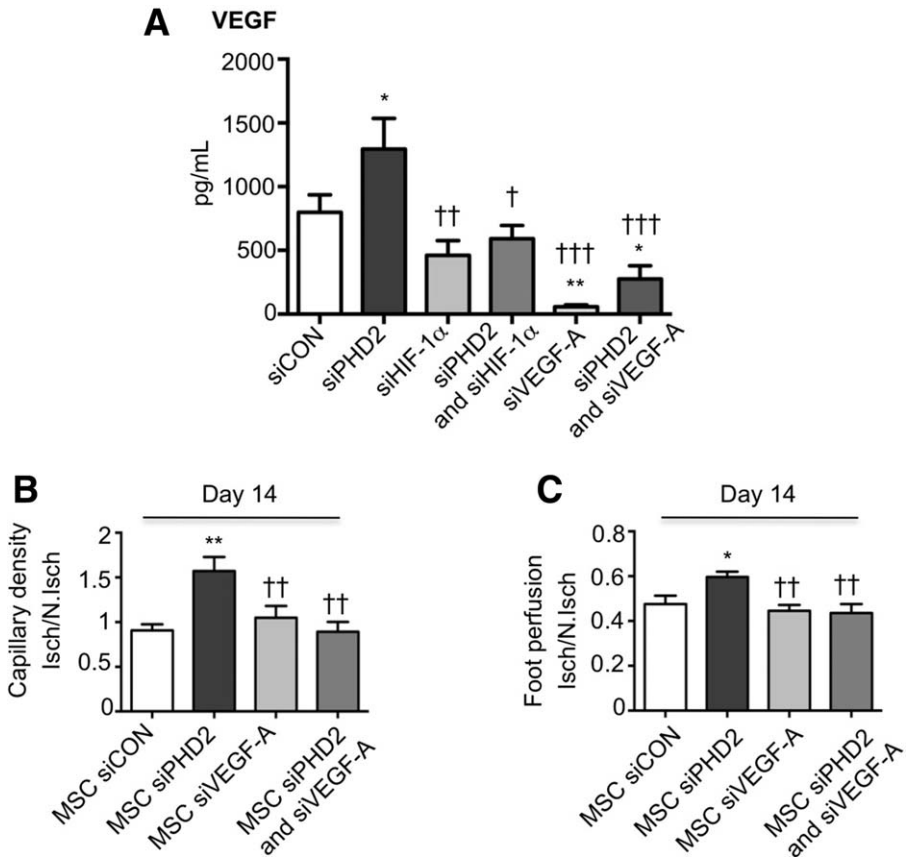

Figure 3. PHD2 inhibition increases the therapeutic potential of human bone marrow (hBM)-MSC through HIF-1α-induced VEGF-A upregulation. **(A):** VEGF-A content was quantified by ELISA in conditioned medium from treated hBM-MSC with siCON or siPHD2 or siHIF-1α or siPHD2 and siHIF-1α or siVEGF-A or siPHD2 and siVEGF-A 3 days after cell transfection. Values are mean ± SEM of VEGF-A content in pg/ml from three different hBM-MSC donors. *, $p < .05$; **, $p < .01$ versus siCON; †, $p < .05$; ††, $p < .01$; †††, $p < .001$ versus siPHD2. Capillary density **(B)** and foot perfusion **(C)** were analyzed in Nude mice after intramuscular injection of siCON or siPHD2 or siVEGF-A or siPHD2 and siVEGF-A-treated-hBM-MSC. Results are expressed as ratio of ischemic to nonischemic (Isch/NIsch) legs. Values are mean ± SEM; $n = 8$–10 mice per group. *, $p < .05$; **, $p < .01$, MSC siCON; ††, $p < .01$ versus MSC siPHD2. Abbreviations: HIF-1α, hypoxia-inducible transcription factor-1α; MSC, mesenchymal stem cell; PHD, prolyl-hydroxylase domain.

between groups were performed using post hoc Fisher LSD when the analysis of variance test was statistically significant. A p value $<.05$ was considered significant.

Results

Increase of Circulating and Vascular PHD2 Expression in CLI Patients

We first analyzed PHDs and HIF mRNA levels in blood samples from patients with CLI of the OPTIPEC Clinical Trial. Table 1 shows baseline characteristics of patients [23, 28, 29]. Of note, 46.2% of patients had a history of diabetes and 69% of them were former smokers. We found a significant increase of PHD2 and PHD3 expression and a downregulation of HIF-1α compared to healthy subjects. In contrast, PHD1 and HIF-2α expressions were comparable (Fig. 1A). In addition, we showed that PHD1, PHD2, PHD3, and HIF-1α mRNA levels were mainly detected in CD14-negative cells in both HV and CLI patients (Fig. 1B). PHD2 expression was then explored in distal tissues from amputation specimens from patients with CLI who had received local therapeutic injections of BM-MNCs [23, 28, 29]. Immuno-histochemistry analysis on tissue samples from these patients showed a strong PHD2 expression around and within the capillary structures of the ischemic muscles in the distal part of the lower limb amputation specimen (Fig. 1C). PHD2 levels were lower in the upper part of the lower limb, which is less ischemic, than that observed in the distal part.

PHD2 Silencing in hBM-MSC Upregulates HIF-1α

These findings prompted us to investigate the outcome of PHD-2 inhibition in cell-based therapies. Previous studies demonstrated that PHD2 inhibition alone was sufficient to stabilize HIF-1α expression in several cell lines [30]. To address this issue, we used hBM-MSC from HVs donors, as we have recently shown that the hBM-MSCs isolated from the BM of HVs are able to induce blood flow recovery in vivo to the same degree as CLI patients [24]. Moreover, PHD2 is the major PHD isoforms expressed in hBM-MSCs whereas PHD3 is almost undetectable (Supporting Information Fig. S2), as previously described [31]. Forty-eight hours after transfection, treatment with siPHD2 significantly decreased PHD2 mRNA and protein levels by 92% and 70%, respectively (Fig. 2A). Meanwhile, PHD2 inhibition increased HIF-1α protein expression by 6.5-fold (Fig. 2B).

PHD2 Silencing in hBM-MSC Enhances Their Therapeutic Potential

We then analyzed the therapeutic efficiency of siPHD2-treated-hBM-MSC in an in vivo model of CLI (Fig. 2C). siPHD2-treated-hBM-MSC increased significantly the angiographic score, foot perfusion, and capillary density at Day 14 after the onset of ischemia when compared with siCON-treated-hBM-MSC. Cotransfection with siHIF-1α blunted the siPHD2-induced activation of postischemic revascularization.

PHD2 Silencing Increases hBM-MSC Therapeutic Potential Through Their Paracrine Potential

In order to gain insight into the molecular pathways triggered by PHD2 silencing, we analyzed the expression of proangiogenic factors by hBM-MSC. Indeed, one of the main mechanism involved in the therapeutic effect of hBM-MSC relies on their ability to release proangiogenic factors such as VEGF-A [32, 33]. It is also well known that HIF-1α is implicated in the regulation of VEGF-A gene transcription in hypoxic condition [34]. To study whether the release of VEGF-A levels was affected by PHD2 inhibition, we performed an ELISA assay on hBM-MSC conditioned medium, 3 days after siPHD2 transfection. The protein levels of VEGF-A were significantly increased in the cultured supernatant of siPHD2-treated hBM-MSC compared to siCON-treated hBM-MSC. This effect was abrogated after siPHD2 and si-HIF1α cotransfection, suggesting that VEGF-A increase was mediated by HIF1α upregulation (Fig. 3A). To further confirm that siPHD2-treated-hBM-MSC triggered postischemic neovascularization through VEGF-A, we abolished VEGF-A expression using specific siRNAs. Three days after transfection, the protein levels of VEGF-A were downregulated in the cultured supernatant of siVEGF-A-treated hBM-MSC. Concomitant silencing of VEGF-A and PHD2 also significantly decreased VEGF-A protein levels compared to siCON-treated hBM-MSC and siPHD2-treated-hBM-MSC. Then, we analyzed the effect of VEGF-A silencing on the therapeutic potential of siPHD2-treated-hBM-MSC in an in vivo model of CLI. Cotransfection with siVEGF-A blunted the siPHD2-induced activation of postischemic revascularization suggesting that PHD2 inhibition significantly potentiated hBM-MSC therapeutic potential, at least in part, through HIF-1α-induced VEGF-A upregulation (Fig. 3B, 3C).

PHD2 Silencing Increases hBM-MSC Survival In Vitro and In Vivo

A key step for cell therapy is the improvement of cell survival of transplanted cells. Indeed, cell viability after transplantation is usually poor [35]. Upregulation of HIF-1α through either culture under hypoxia or, in presence of the prolyl-hydroxylase inhibitor dimethyloxalylglycine (DMOG), has been shown to promote cell survival and reduce apoptosis [17, 36, 37]. First, to assess whether PHD2 silencing could increase cell survival in vitro, we induced apoptosis by H_2O_2 treatment of siCON-treated hBM-MSC, siPHD2-treated hBM-MSC, siHIF-1α-treated hBM-MSC, and siPHD2 and siHIF-1α-treated hBM-MSC. The percentage of apoptotic cells, evaluated by TUNEL and cleaved caspase 3 stainings, was decreased by 70% and 80%, respectively, after siPHD2 treatment compared to siCON treatment. This effect was reduced when siPHD2 and siHIF-1α were simultaneously transfected, suggesting that PHD2 treatment protects hBM-MSC against H_2O_2-induced apoptosis, through a HIF-1α-dependent mechanism (Fig. 4). Then, to further assess whether PHD2 silencing could increase cell survival in vivo, we injected fluorescent-labeled siCON or siPHD2 or siHIF-1α or siPHD2 and siHIF-1α-treated hBM-MSC in the ischemic muscle. At Day 1, the same amount of cells was injected in the four conditions. siCON-treated-hBM-MSC fluorescence

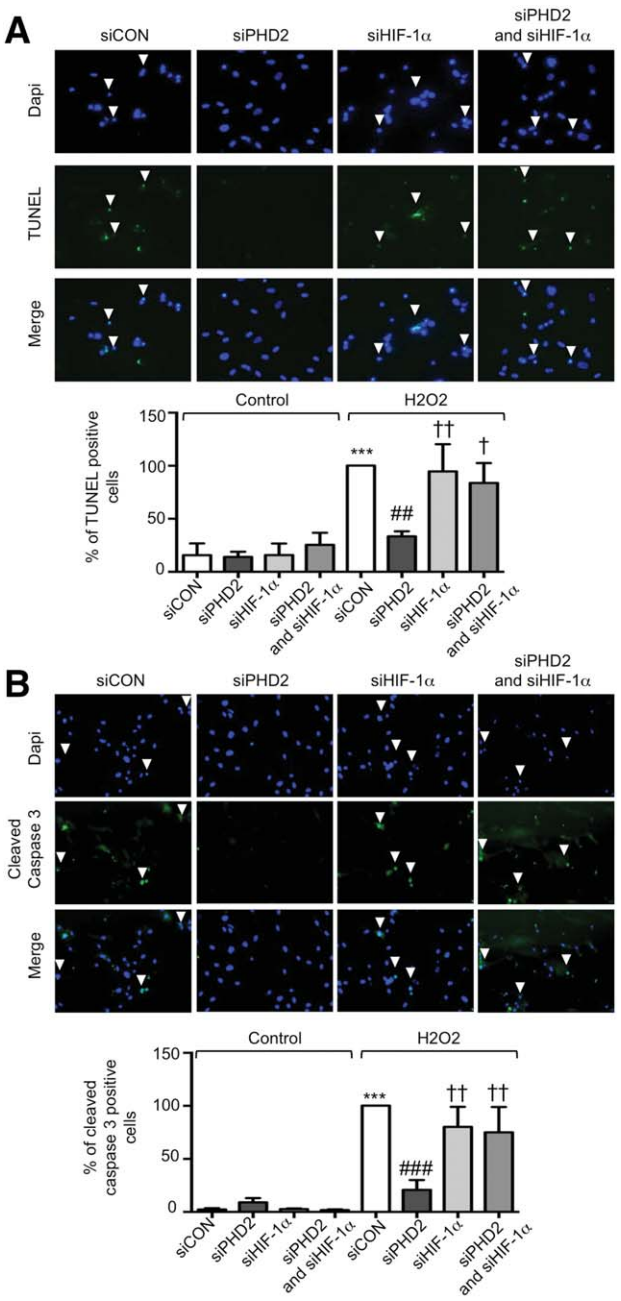

Figure 4. PHD2 inhibition protects human bone marrow-derived mesenchymal stem cell (hBM-MSC) against H_2O_2-induced apoptosis in vitro. Apoptotic cells number was evaluated with TUNEL **(A)** and anticleaved caspase 3 **(B)** stainings. Results are expressed as percentage of H_2O_2-treated MSC siCON. Values are mean ± SEM from three different hBM-MSC donors. ***, $p < .001$ versus control MSC siCON; ##, $p < .01$; ###, $p < .001$ versus H_2O_2-treated MSC siCON; ††, $p < .01$; †††, $p < .001$ versus H_2O_2-treated MSC siPHD2. Abbreviations: HIF-1α, hypoxia-inducible transcription factor-1α; PHD, prolyl-hydroxylase domain.

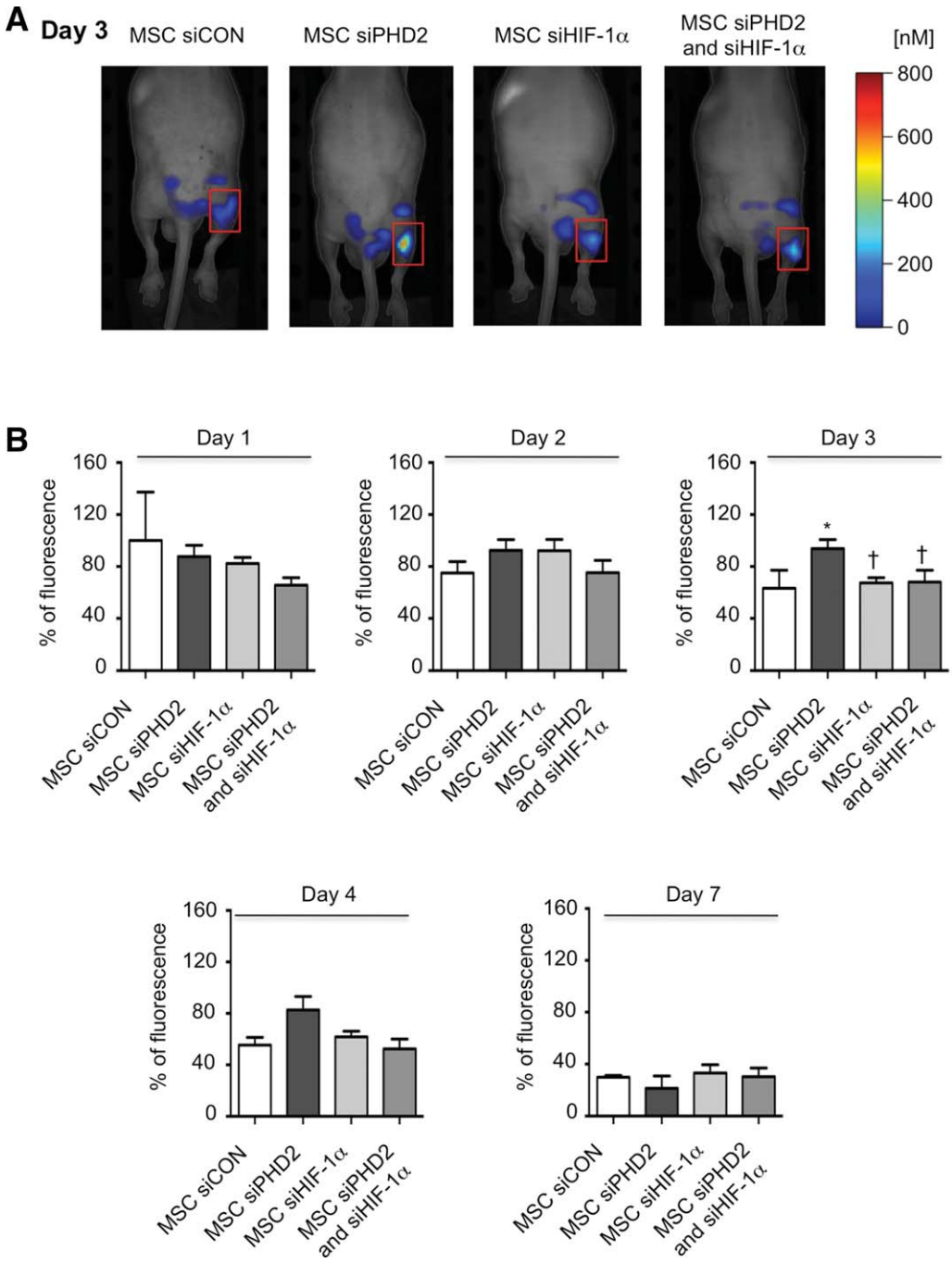

Figure 5. PHD2 inhibition increases human bone marrow (hBM)-MSC survival in the ischemic limb. **(A):** Fluorescence intensity was evaluated in Nude mice after intramuscular injection of siCON or siPHD2 or siHIF-1α or siPHD2 and siHIF-1α-treated-hBM-MSC labeled with VIVOTag S 680 at Day 1 (day of the injection), Day 2, Day 3, Day 4, and Day 7. **(A):** Representative pictures of fluorescence in the ischemic limb of Nude mice at Day 3 after injection of labeled siCON or siPHD2 or siHIF-1α or siPHD2 and siHIF-1α-treated-hBM-MSC. Red rectangles point to the site of injection. **(B):** Quantitative analysis. Results are expressed as percentage of fluorescence of MSC siCON (siCON-treated hBM-MSC). Values are mean ± SEM; $n = 3$–11 mice per group. *, $p < .05$ versus MSC siCON; †, $p < .05$ versus MSC siPHD2. Abbreviations: HIF-1α, hypoxia-inducible transcription factor-1α; MSC, mesenchymal stem cell; PHD, prolyl-hydroxylase domain.

decreased by 10% on Day 3 compared to Day 2. Similarly, siHIF-1α-treated-hBM-MSC and siPHD2 and siHIF-1α-treated-hBM-MSC fluorescence decreased by 23% and 8%, respectively, on Day 3 compared to Day 2. By contrast, siPHD2-treated-hBM-MSC fluorescence did not change and was significantly higher on Day 3 compared to the other treatments (Fig. 5). As the amount of cells correlates with the fluorescence observed (Supporting Information Fig. S3), this result suggests that siPHD2-treated-hBM-MSC survival in the ischemic muscle is higher than siCON-treated hBM-MSC. On Day 7, hBM-MSC fluorescence has almost disappeared whatever the experimental conditions be (Fig. 5). Finally, we performed a thymidine

proliferation assay and found no significant difference in hBM-MSCs proliferation 24, 48, and 72 hours after starvation (Supporting Information Fig. S4).

In summary, our results demonstrate that PHD2 silencing in hBM-MSC promotes the expression of VEGF-A and increases cell survival through HIF-1α upregulation, leading to activation of hBM-MSC therapeutic potential in a mouse model of CLI.

PHD2 Silencing in Diabetic Mice Enhances Postischemic Neovascularization Through the Local and Transient Upregulation of Proangiogenic and Proarteriogenic Factors

Part of the beneficial action of stem/progenitor cell-based therapy may also rely on the interaction with the ischemic tissue and mainly in the capacity of this latter to restore the angiogenic program despite the deleterious macroenvironment and microenvironment. To mimic the clinical settings of patients with CLI, we used our experimental model of CLI in diabetic animals and analyzed PHDs expression in gastrocnemius muscle of these diabetic mice. PHD1 was observed in the nucleus of skeletal myocytes, whereas PHD2 and PHD3 were mainly localized in vascular structures (Fig. 6A).

We then investigated the impact of specific PHD2 inhibition on postischemic neovascularization in diabetic mice subjected to femoral artery ligation. Six hours after ligation, shCON or shPHD2 was transfected into the ischemic muscle of streptozotocin-induced diabetic mice. Two days after transfection, we evaluated the efficiency of our strategy by analyzing HIF-1α and PHD2 mRNA and protein levels. shPHD2 treatment significantly decreased PHD2 mRNA and protein levels by more than 60% and upregulated that of HIF-1α in diabetic tissue (Supporting Information Fig. S5). We next evaluated the role of PHD2 silencing on postischemic neovascularization in diabetic mice. The angiographic score showed a significant increase of 3.5-fold in shPHD2-treated diabetic mice compared to shCON-treated diabetic mice (Fig. 6B). Results obtained by microangiography were confirmed by capillary density and foot perfusion analysis. The ratio of ischemic to nonischemic leg was increased by 3.0- and 2.5-fold for capillary density (Fig. 6B) and foot perfusion (Fig. 6B), respectively, in the shPHD2-treated diabetic mice compared to shCON-treated diabetic mice. These effects were blunted by cotransfection with shHIF1-α suggesting that PHD2 inhibition significantly triggered postischemic neovascularization through HIF-1α upregulation in ischemic leg of diabetic mice.

Subsequently, we analyzed the expression of two HIF-dependent proangiogenic factors, VEGF-A and e-NOS. Treatment with shPHD2 significantly enhanced VEGF-A expression both at the mRNA and protein levels (Supporting Information Fig. S6A). Cotreatment with shPHD2 and shHIF-1α abrogated this effect. Similar results were obtained with e-NOS levels (Supporting Information Fig. S6B). In addition, we evaluated the proarteriogenic effect of PHD2 silencing which is mainly driven by monocytes/macrophages accumulation into the ischemic area. The recruitment of monocytes is under the control of chemoattractant molecules including, CCL2 [38, 39], CX3CL1, CCL5 [39, 40], and CXCL12 [41]; and the levels of these chemokines are modulated by HIF-1α [42, 43]. We therefore analyzed the expression of CX3CL1, CXCL12, CCL2, and CCL5 into the ischemic muscle, 2 and 14 days after the treatment with the different shRNAs (Supporting Information Fig. S7A). Two days after the treatment, the expression of CX3CL1, CXCL12, and CCL2 was significantly increased in the ischemic limb of shPHD2-treated

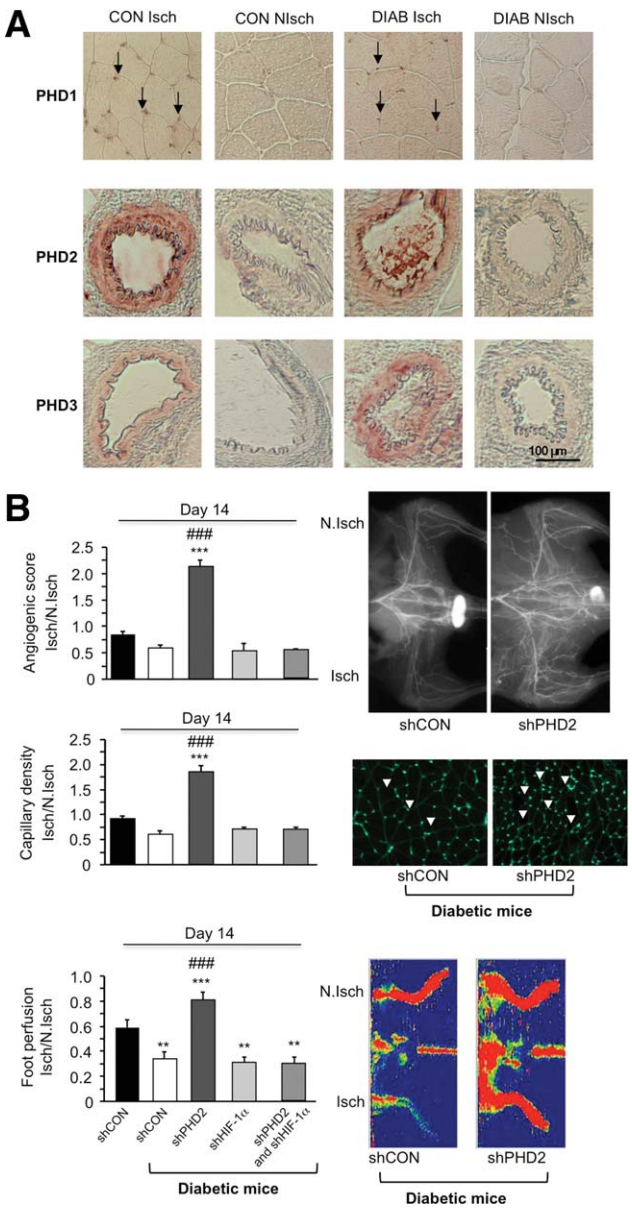

Figure 6. PHD2 inhibition increases postischemic neovascularization in diabetic mice. **(A):** Immunostainings of PHD1, PHD2, and PHD3 were performed on frozen tissue sections from ischemic (Isch) and nonischemic (NIsch) legs of nondiabetic mice (CON) and ischemic and nonischemic legs of diabetic mice (DIAB). Representative images are shown. Arrows point to area of interest. **(B):** Microangiography analysis, capillary density, and foot perfusion were analyzed in shCON-treated nondiabetic and shCON or shPHD2 or shHIF-1α or shPHD2 and shHIF-1α-treated diabetic mice. Results are expressed as ratio of ischemic to nonischemic (Isch/NIsch) legs. Values are mean ± SEM; $n = 5$–8 mice per group. **, $p < .01$; ***, $p < .001$ versus shCON in nondiabetic mice; ###, $p < .001$ versus shCON in diabetic mice. Representative photomicrographs of microangiography analysis, capillary density, and foot perfusion are shown. Abbreviations: HIF-1α, hypoxia-inducible transcription factor-1α; PHD, prolyl-hydroxylase domain. Cont, Control mice; Diab, Diabetic mice; Isch, ischemic leg; NIsch, Non ischemic leg; shCON, mice treated with shRNAs scramble, shPHD2, mice treated with shRNAs directed against PHD2, shHIF-1α, mice treated with shRNAs directed against HIF1α.

diabetic mice compared to shCON-treated diabetic mice. The addition of shHIF-1α abrogated the PHD2 inhibition-induced chemokine levels upregulation. In contrast, CCL5 contents were unaffected by PHD2 inhibition. We next assessed macrophages number into the ischemic limb and demonstrated that the number of macrophages was upregulated in shPHD2-treated diabetic mice compared to shCON-treated diabetic mice, in a HIF-1α-dependent manner (Supporting Information Fig. S7B).

Discussion

To our knowledge, our study demonstrates for the first time that HIF-1α-related pathways are hampered in circulating cells from CLI patients, which can be potentiated by the inhibition of PHD2. Similarly, impaired HIF-1α signaling has been found in circulating angiogenic cell derived from myeloid subpopulation in patients with pulmonary hypertension [44, 45] suggesting that BM abnormalities and function can modify vessel forming ability. In CLI, we found an imbalance in PHD-2 and HIF-1α expression in circulating cells. It is noteworthy that PHDs and HIF-1α are mainly expressed in CD14-negative MNCs. Hence, alteration of HIF-1α signaling in these subpopulations of circulating cells could participate to vascular disease associated with CLI. These results might seem confusing as one could expect a significant increase in HIF-1α expression due to the ischemic situation [46]. However, it is not obvious that the chronic ischemic situation should be associated with an increase in HIF-1α. Indeed, chronic hypoxia promotes HIF-1α protein degradation through not only an increase in the pool of PHDs but also an overactivation of the three PHD isoforms. This overactivation appears to be mediated by an increase in intracellular O_2 availability consequent to the inhibition of mitochondrial respiration [47]. In addition, it is unlikely that, in these clinical settings, HIF-1α is only under the control of oxygen levels and all the cardiovascular risk factors associated with CLI can also affect HIF-1α levels. In this line, hyperglycemia prevents HIF-1α stabilization under hypoxia and this is in part related to PHDs [1, 48]. Hyperglycemia also increases reactive oxygen species and the glyoxalase 1 substrate methylglyoxal levels leading to a reduction in HIF-1α levels [49, 50]. Another mechanism that might explain HIF-1α downregulation is the exposure to cigarette smoke (69% of CLI patients were former smokers). Cigarette smoke has been shown to enhance Von Hippel Lindau protein and PHD2 levels and to decrease HIF-1α expression in mouse skeletal muscles [51, 52].

In the French OPTIPEC trial (Clinical trial registration: NCT00377897), we have shown, the development of a proliferative angiogenic process in the distal part of the legs in amputation specimens from patients with CLI who had received local therapeutic injections of BM-MNCs [23, 28, 29]. BM-MNCs contain a complex assortment of angiogenic cells, including hematopoietic progenitors as well as MSC. We have previously shown that MSC administration promoted the infiltration of murine CD31-positive cells, in agreement with the concept of a paracrine angiogenic effect of MSC on local murine endothelial cells [24]. In this line, results from FMT likely suggest that hBM-MSCs do not differentiate into specific cell types such as pericytes but rather produce proangiogenic factors [53]. Similarly, previous findings established that cell-free conditioned media from MSC had proangiogenic effect and improved postischemic remodeling in model of myocardial infarction [32, 33]. MSC can produce HGF [54], bFGF, IGF-1, and VEGF [55], all of which could participate to the regenerative process. Of note, preconditioning of MSC under hypoxic conditions increased in vitro secretion of VEGF [56] and their beneficial effects in a mouse model of CLI [17]. In accordance with these results, we observed that silencing of PHD2 triggers HIF-1α-induced-VEGF-A release that might account, in part, for the increase of the beneficial therapeutic effect of hBM-MSC. An important issue in cell-based therapy is cell homing and cell survival. Several studies showed very poor if no recruitment of intravenously injected cells at the site of the ischemic insult [15, 57]. To avoid this effect, we performed intramuscular injection of hBM-MSC. Nevertheless, fluorescent molecular tomography showed that hBM-MSC remained primarily at the site of injection, during the first week after injection and decreased thereafter. In addition, we demonstrated that siPHD2-treated-hBM-MSC survival was increased compared to siCON-treated-hBM-MSC. An important drawback of cell labeling dyes is that apoptotic- or necrotic-injected cells could be engulfed by other cells types, such as macrophages. However, we confirmed these results in vitro as we demonstrated that siPHD2-treated-hBM-MSCs were protected against apoptosis compared to siCON-treated-hBM-MSC and that this effect was abrogated by HIF-1α silencing. Administration of hBM-MSCs, to the ischemic limb of mice, led to the persistence of the transplanted cells in the tissue, few days only after the ischemic event. Beyond that, the number of these cells decreased markedly, whereas vessel growth was still increased at Day 14 after cell administration. It is well known that part of the beneficial effect of stem/progenitor cells therapy relies on their ability to interact with the host tissue. In this view, exogenous administration of different types of stem/progenitor cells has been shown to induce endogenous expression of proangiogenic factors, mobilization of BM-derived cells as well as activation of the local immuno-inflammatory reaction [27, 58, 59].

Our study aimed to develop an autologous, safe, and efficient cell therapy product for a clinical translation. In this line, it is noteworthy that PHD2 inhibition also improves the therapeutic efficiency of other sources of stem/progenitor cells including cord-blood-derived hEPCs (Supporting Information Fig. S8). Additionally, modulation of PHD2 might be used to increase the synergic effect between injected stem/progenitor cells and the ischemic host tissue. Our study underscores the efficiency of PHD2 shRNAs mediated inhibition in a type I diabetic mouse model of CLI. After the ischemic insult, new vessel formation and collateral growth are impaired in diabetes due to, at least in part, defect in recruitment of progenitor cells and in situ proliferation [60, 61], endothelial dysfunction, alterations in VEGF and VEGF receptor 2 signaling [62, 63], increased levels of the antiangiogenic and proapoptotic proteins [9, 64], changes in inflammation-pathways [65], overproduction of reactive oxygen species [7, 62], accumulation of glycation end products [26], and upregulation of microRNA-503 [66]. In addition, impaired bioavailability of NO may significantly contribute to the impaired neovascularization response to ischemia in diabetes [6]. We confirmed and extended these previous

findings since we showed that HIF-1α was downregulated in ischemic tissues of diabetic mice and patients with CLI. In addition, PHD2 ShRNAs-induced endogenous upregulation of HIF-1α was sufficient to trigger proangiogenic and collateralization-related pathways and restore efficient vascular adaptive response in this setting. Of note, we obtained similar results with PHD3 inhibition, in line with our previous study [9] (Supporting Information Fig. S9). Finally, it is noteworthy that in contrast to strategies overexpressing HIF-1α by adenovirus or plasmid administration, our approach allows the transient and local activation of multiple proangiogenic and prosurvival genes, and thus prevents the potential deleterious effect of a sustained activation of HIF signaling [47]. In our experimental conditions, we modulated PHD2 expression and subsequently HIF-1α activity using siRNA/shRNA, but endogenous upregulation of HIF-1α might also be achieved by chemical inhibitors, such as DMOG or Desferrioxamine, that interfere with the catalytic activity of these enzymes. However, none of these inhibitors exhibit absolute specificity for PHDs subtypes or FIH [67] and due to the wide effects of PHDs and HIF-related pathways, new specific inhibitors are highly desirable before this strategy could be used for therapeutic purpose.

Conclusion

The major finding of this study is that PHD2 inhibition enhances the therapeutic potential of hBM-MSC cell-based therapies in CLI, through activation of HIF1α/VEGF-A-related pathways. Further experiments need to be performed in order to assess the potential of PHDs inhibition in cells from patients with impaired vascular response. However, our study paves the way for future strategies, based either on injection of PHD2-modified cells or direct PHD2 inhibition in the ischemic muscle of patients with CLI or both.

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Author contributions

K.-Y.H.W.Y.: collection and/or assembly of data, data analysis and interpretation, and manuscript writing; C.L. and W.B.: collection and/or assembly of data, data analysis and interpretation; C.L.G., J.V., C.D'A., L.M., and P.B.: collection of data; J.E.: provision of study material or patients; B.L.: final approval of manuscript; J.P.: provision of study material; D.M.S. and J.-S.S.: conception and design and manuscript writing.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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