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Chapter 7

SHORT HAIRPIN RNA GENE SILENCING OF PROLYL HYDROXYLASE-2 WITH A MINICIRCLE VECTOR IMPROVES NEOVASCULARIZATION OF HINDLIMB ISCHEMIA

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ABSTRACT

Aim: In this study, we target the hypoxia inducible factor-1 alpha (HIF-1-alpha) pathway by short hairpin RNA (shRNA) interference therapy targeting prolyl hydroxylase-2 (shPHD2). We use the minicircle (MC) vector technology as an alternative for conventional nonviral plasmid (PL) vectors in order to improve neovascularization after unilateral hindlimb ischemia in a murine model.

Methods and Results: Gene expression and transfection efficiency of MC and PL, both in vitro and in vivo, were assessed using bioluminescence imaging (BLI) and firefly Luciferase (Luc) reporter gene. C57Bl6 mice underwent unilateral electro-coagulation of the femoral artery and gastrocnemius muscle injection with MC-shPHD2, PL-shPHD2, or PBS as control. Blood flow recovery (BFR) was monitored using Laser Doppler Perfusion Imaging (LDPI), and collaterals were visualized by immunohistochemistry and angiography. MC-Luc showed a 4.6-fold higher in vitro BLI signal compared to PL-Luc. BLI signals in vivo were $4.3 \times 10^5 \pm 3.3 \times 10^5$ (MC-Luc) versus $0.4 \times 10^5 \pm 0.3 \times 10^5$ (PL-Luc) at day 28 ($p=0.016$). Compared to PL-shPHD2 or PBS, MC-shPHD2 significantly improved BFR, up to 50% from day 3 until day 14 after ischemia-induction. MC-shPHD2 significantly increased collateral density and capillary density, as monitored by alpha-smooth muscle actin (α -SMA) expression and CD31⁺ expression, respectively. Angiography data confirmed the histological findings. Significant downregulation of PHD2 mRNA levels by MC-shPHD2 was confirmed by quantitative PCR (qPCR). Finally, Western blot analysis confirmed significant higher levels of HIF-1-alpha protein by MC-shPHD2, compared to PL-shPHD2 and PBS.

Conclusions: This study provides initial evidence of a new potential therapeutic approach for peripheral artery disease (PAD). The combination of HIF-1-alpha pathway targeting by shPHD2 with the robust nonviral MC plasmid improved post-ischemic neovascularization, making this approach a promising potential treatment option for critical limb ischemia.

INTRODUCTION

Critical limb ischemia (CLI) affects up to 2% of the patients diagnosed with peripheral artery disease (PAD), with some 1,000 new cases of CLI diagnosed per 1 million Europeans or North Americans every year [1]. Unfortunately, many patients are ineligible for treatment, which consists of revascularization either percutaneously or surgically, with the latter resulting in a 25% amputation rate.

Therapeutic angiogenesis represents an alternative treatment modality for these patients. However, despite the numerous positive results in animal studies, clinical trials using a single angiogenic factor by either recombinant protein or gene-based formulations have resulted in either negative or conflicting outcomes [25]. To increase the success of gene-based therapeutic angiogenesis in the clinical situation, various gene transfer techniques have undergone intense investigation in recent years. Nonviral plasmid DNA is commonly used in clinical trials due to safety concerns with viral vectors, but these conventional plasmids also may provoke negative side effects that affect the gene expression. For instance, the bacterial backbone may lead to unwanted heterochromatin formation that changes eukaryotic gene expression or induces immune responses to CpG sequences [6-8]. To resolve these issues, an attractive gene transfer tool (nonviral minicircle (MC) plasmid) was designed. MCs are supercoiled DNA molecules that are smaller compared to conventional plasmids and lack a bacterial origin of replication or an antibiotic resistance gene, features that greatly improve the transgene expression both in terms of expressed levels of gene expression as well as in the persistence of the gene expression [9, 10].

As indicated above, the results of clinical trials using a single angiogenic (growth) factor were disappointing [11, 12]. This suggests that the expression of a single angiogenic factor such as vascular endothelial growth factor (VEGF) alone may not be sufficient to improve neovascularization [13]. Newer therapeutic approaches designed to circumvent this problem utilize the upstream transcriptional factor, hypoxia inducible factor-1 alpha (HIF-1-alpha). Factors expressed by the HIF pathway include vascular endothelial growth factor, fibroblast growth factor, insulin-like growth factor, erythropoietin, and nitric oxide synthase, among others [14, 15]. However, the HIF-1-alpha protein has a limited biological half-life as HIF-1-alpha is degraded in normoxic conditions by oxygen-dependent prolyl hydroxylase-2 (PHD2) [16]. PHD2 mediates the interaction of HIF-1-alpha with von Hippel-Lindau protein

(pVHL) ubiquitin ligase complex, which leads to proteasomal degradation of HIF-1- α [17]. Inhibition of HIF-1- α degradation through short hairpin RNA (shRNA) knockdown of PHD2 has been shown to significantly improve neovascularization, which in turn improved cardiac function in a mouse model of myocardial infarction (MI) [18]. Moreover, inhibition of PHD by shRNA provides a promising pro-angiogenic therapeutic approach, as in vivo electrotransfer of plasmids with either shPHD2 or shPHD3 has been shown to significantly improve post-ischemic neovascularization in a mouse model of PAD [19].

In the present study, we combine the advantages of MC with the angiogenic effects of the HIF-1- α pathway, through shRNA knockdown of PHD2 in a murine hindlimb ischemia model. We demonstrate that the combination of these two strong concepts offers a promising new avenue for improving post-ischemic neovascularization in the future.

METHODS

Construction of MC plasmids and MC DNA

The MC plasmids are constructed and produced as described earlier [6, 18]. Briefly, for the production of MC, ubiquitin promoter-driving firefly luciferase (Luc) is amplified with Luc forward and Luc reverse primers using pUbiquitin-Luc as a template. The mouse PHD2 gene is cloned, as described earlier, with TGTGAGGAACTTGAGATCT as the short hairpin scramble (shScramble) antisense sequence, and with the fragment No 2 knockdown site inserted after H1 promoter in the vector pSuper [18]. The minicircle DNA plasmid used here (a gift from Dr. Mark Kay, Stanford University) was produced as described previously [6, 20].

Cell culture and transfection

Mouse C2C12 cells were cultured in DMEM containing 10% fetal bovine serum [18]. All cells were maintained in a 5% CO₂ incubator. For transfection, cells were seeded with a density of 2×10^5 cells/well in 6-well flat-bottom microassay plates (Falcon Co., Franklin Lakes, NJ) 24 hr before transfection. At 70%-80% confluence, cells were transfected with 4 μ g of plasmids carrying the Luc reporter gene (PL-Luc) or equimolar 2 μ g of MC carrying the Luc reporter

gene (MC-Luc), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. C2C12 cells show rapid proliferation with a doubling time of approximately 19 hr in vitro [21]. In order to more closely resemble the in vivo conditions of slower proliferating cells, C2C12 cells were exposed to 9000 Rad 3 hr before transfection, resulting in an optimal proliferation pattern (*Supplemental Figure 1*). Proliferation of cells was quantified by using a 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) proliferation assay according to the manufacturer's protocol. As a control, non-irradiated mouse C2C12 myoblast cells were used. Noninvasive bioluminescence imaging to assess the duration of reporter gene expression. To compare the duration of gene expression in vivo, 25 μ g of PL-Luc or equimolar 12.5 μ g of MC-Luc were injected into normal gastrocnemius muscles of C57Bl6 mice, with PL-Luc injected on the right side and MC-Luc injected on the left side. Due to the well-known divergent neovascularization capacities of various mice strains [22], the duration of gene expression in C57Bl6 mice in the present study was closely examined. The same mouse strain was used in the hindlimb ischemia experiments as described in the next paragraph. Bioluminescence imaging (BLI) was performed with the Xenogen In Vivo Imaging System (Alameda, California) on days 1, 3, 5, and 7, and weekly thereafter until the endpoint on day 28, by an investigator blinded to study conditions (E.J.B.), as previously described [23, 24]. BLI signals were quantified in maximum photons per second per centimeter squared per steradian (p/s/cm²/sr). To compare the duration of gene expression in vitro, irradiated C2C12 cells were imaged using BLI at 3, 24, and 48 hr after transfection.

Experimental animals

Ten-week old C57Bl6 mice (Jackson laboratory) were used (10 mice per group). All animal experiments were performed after approval of the relevant authorities at both Institutes (Stanford Animal Research Committee and the Leiden University Animal Research Committee).

Surgical procedure to induce hindlimb ischemia

C57Bl6 mice possess a well-known fast recovery after hindlimb ischemia induction [25]. Mice injected with MC-shPHD2 were expected to have enhanced blood flow recovery as compared to controls. Double electro-coagulation of

both the common femoral artery and the popliteal artery distally was performed because it could provide a larger therapeutic window [25]. This enables a better analysis of the enhancing effects of MC-shPHD2 on arteriogenesis. Before surgery, mice were anesthetized with an intraperitoneal injection of a combination of midazolam (5 mg/kg, Roche), medetomidine (0.5 mg/kg, Orion), and fentanyl (0.05 mg/kg, Janssen). A skin incision was made unilaterally from the left inguinal region to the knee. For a double coagulation model such as that used in this study, both the common femoral artery and the popliteal artery were dissected from the nerve and vein as described before [25]. An electro-coagulation of the common femoral artery was performed and followed by an electro-coagulation of the popliteal artery. After surgery, the skin was closed with 6-0 Ethilon sutures.

Laser Doppler perfusion imaging

To compare the neovascularization effects with equimolar concentrations, 25 µg of PL-shPHD2 and 12.5 µg of MC-shPHD2 were injected into the left gastrocnemius muscles of C57Bl6 mice (n=10/group) 24 hr after the induction of hindlimb ischemia. Additional mice (n=10) were injected with PBS (50 µl) as a control. Measurements of paw perfusion were performed of the mouse hindlimbs before, directly after, biweekly until day 14, and weekly until 4 weeks after the surgical procedure with Laser Doppler Perfusion Imaging (LDPI) (Moor Instruments). Perfusion was expressed as a ratio of the left (ischemic) to right (non-ischemic) paw, as previously described [26].

Histological analysis

Additional animals were sacrificed 10 days after ischemia induction followed by injection of MC-shPHD2, PL-shPHD2, or PBS, with their calf and adductor muscles removed and fixed with 4% formaldehyde and paraffin-embedded. Serial 5 µm cross-sections were generated. Sections were re-hydrated and endogenous peroxidase activity was blocked for 20 minutes in methanol containing 0.3% hydrogen peroxide. Capillaries and collaterals were visualized using antibodies recognizing CD31 on endothelial cells or α -smooth muscle actin (SMA) in smooth muscle cells, as previously described [26, 27]. Quantification of labeled tissue sections was performed using ImageJ (9 sections per mouse were analyzed to obtain the mean per animal, and 5 animals per group were

measured).

Quantitative polymerase chain reaction (qPCR). For real-time reverse transcriptase–polymerase chain reaction (RT–PCR), we used inventoried assay on demand primers sets (Applied Biosystems). Oligonucleotides for PHD2 (Applied Biosystems, EGLN1, Mm00459770_m1) were used to analyze PHD2 mRNA levels, and mouse HPRT (Applied Biosystems, HPRT, Mm00446969_m1) was used to normalize sample amplification. Polymerase chain reaction was performed on a 7500 Fast Real Time PCR System (Applied Biosystems). mRNA expression levels of PHD2 were corrected for expression of HPRT and displayed as relative expression values (delta Ct) and analyzed as previously described [28]. RNA extraction was performed as described in the supplementary methods section.

Protein extraction and Western blot analysis

Protein from gastrocnemius muscle (n=14) was extracted and analyzed as previously described [18, 29]. Cellular lysed homogenates in radioimmunoprecipitation assay buffer were isolated for protein concentration determination with a bicinchoninic acid assay (Thermo Scientific, Rockford, IL, USA). Equal amounts of protein were loaded per well (20µg of protein per well). Samples were extracted with a sample buffer (Biorad, Hercules, CA, USA) containing 50 mM dithiothreitol as a reducing agent for 5 minutes at 95°C, resolved by polyacrylamide gel electrophoresis, and transferred to polyvinylidene fluoride membranes. The membranes were then blocked with 5% milk/Tris-buffered saline-Tween (TBST) for 1 hr at room temperature, incubated with the appropriate primary antibody at 4°C overnight, and washed with TBST. Primary antibodies used were HIF-1-alpha (1:200, NB100-479, Novus, Littleton, CO, USA) and actin as control (1:1000, SC 1615, Santa Cruz Biotech, Santa Cruz, CA, USA). The appropriate horseradish peroxidase-conjugated secondary antibody, diluted in 5% milk/TBST, was applied for 1 hr at room temperature. After washing with TBST, immunoblots were visualized and quantified using the Super Signal West Dura Extended Duration Substrate (Perbio Science), LabWorks 4.6 software, and a luminescent image workstation (UVP), as previously described [30, 31]. Hif-1-alpha expression levels were corrected for expression of actin and displayed as relative expression values.

Statistical analysis

Results are expressed as mean $\pm$ sem. Comparisons between means were performed using an independent T-test. For repeated measures, 1-way repeated measures ANOVA with post-hoc Bonferroni-Holm's correction was used. P-values <0.05 were considered statistically significant. All calculations were performed in SPSS 19.0.

RESULTS

Evaluation of minicircle vectors versus regular plasmid vectors *in vitro*. To assess the transfection efficiency, MC-Luc and PL-Luc were used in equimolar amounts to transfect mouse C2C12 cells. Luc was evaluated by BLI. MC-Luc showed a higher Luc expression compared to PL-Luc, with a 4.6-fold higher BLI signal at 24 hr and a 44-fold higher BLI signal at 48 hr in irradiated C2C12 cells (Figure 1). As a control, in non-irradiated cells MC-Luc showed 2.5-fold higher Luc expression compared to PL-Luc at 24 hr after transfection (Supplemental Figure 2).

Comparison of MC versus regular plasmids *in vivo*. Equimolar amounts of MC-Luc and PL-Luc were injected into the left or right mouse gastrocnemius muscles, respectively, to determine expression levels in C57Bl6 mice *in vivo*. Gene expression was followed by BLI from day 0 until day 28. MC-Luc injection showed a significantly higher Luc activity compared to PL-Luc from day 3 until the end point at day 28, with BLI of $6.9 \times 10^3 \pm 1.7 \times 10^3$ versus $3.6 \times 10^3 \pm 0.46 \times 10^3$ p/s/cm²/sr at day 1 (p=ns); $3.2 \times 10^5 \pm 2.4 \times 10^5$ versus $0.4 \times 10^5 \pm 0.3 \times 10^5$ p/s/cm²/sr at day 7 (p=0.002); $3.5 \times 10^5 \pm 2.8 \times 10^5$ versus $0.5 \times 10^5 \pm 0.3 \times 10^5$ p/s/cm²/sr at day 21 (p=0.004); and $4.3 \times 10^5 \pm 3.3 \times 10^5$ versus $0.4 \times 10^5 \pm 0.3 \times 10^5$ p/s/cm²/sr at day 28 (p=0.016) (Figure 2).

Injection of MC encoding shPHD2 improves post-ischemic blood flow recovery. To examine whether MC-shPHD2 could improve post-ischemic neovascularization *in vivo* as compared to PL-shPHD2 or PBS, hindlimb ischemia was performed in C57Bl6 mice followed by injection of MC-shPHD2, PL-shPHD2, or PBS, respectively. After double electro-coagulation of both the common femoral artery and the popliteal artery, blood flow decreased to

less than 5% in all mice. Mice injected with MC-shPHD2 showed significantly improved blood flow recovery, up to 50% from day 3 until day 14 after ischemia induction as compared to mice injected with PL-shPHD2 or PBS (Figure 3). Injection of PL-shPHD2 did not improve blood flow recovery significantly as compared to PBS injection.

Increase in collateral and capillary density in post-ischemic skeletal muscle after MC-shPHD2 injections. To study collateral artery formation at the tissue level, ischemic skeletal muscles were harvested 10 days after hindlimb ischemia induction, and immunohistochemical staining was performed. Mice injected with MC-shPHD2 showed a significant increase in collateral density, as measured by SMA expression, in the post-ischemic adductor muscle as compared to mice injected with PL-shPHD2 or PBS (3.5 ± 0.5 vs. 1.9 ± 0.2 and 1.0 ± 0.1 collaterals per mm²) (Figure 4A). Furthermore, collaterals were significantly larger in mice injected with MC-shPHD2 as compared to mice injected with PL-shPHD2 or PBS (850.5 ± 397.9 μm^2 vs. 258.8 ± 91.7 μm^2 and 196.2 ± 33.6 μm^2) (Figure 4B). In addition, injection of MC-shPHD2 significantly increased the CD31+ capillary density of the post-ischemic calf muscles of mice as compared to injection of PL-shPHD2 or PBS (40.3 ± 4.9 vs. 18.1 ± 5.2 and 11.8 ± 3.4 capillaries per mm²) (Figure 4D). To illustrate collateral growth in the post-ischemic hindlimb, angiographs were made 10 days after hindlimb ischemia induction. Collaterals were observed in the post-ischemic adductor muscles of all mice, indicating that C57Bl6 mice do have the capacity to form collaterals after hindlimb ischemia. Mice injected with MC-shPHD2 showed more typical corkscrew-like collateral arteries in the ischemic adductor muscle area as compared to mice injected with PL-shPHD2 or PBS. These angiographic-results confirm the results of the SMA-staining (Figure 5).

Quantitative PCR analysis of PHD2 mRNA expression. Additional C57Bl6 mice were subjected to hindlimb ischemia followed by injection of MC-shPHD2, PL-shPHD2, or PBS, to assess PHD2 mRNA and HIF-1-alpha protein expression at 7 days after surgery. MC-shPHD2 injection significantly decreased the mean PHD2 mRNA expression in gastrocnemius muscles, with delta Ct levels of 0.28 ± 0.022 , compared to PL-shPHD2 and PBS (0.35 ± 0.016 and 0.36 ± 0.026 , respectively; p<0.05) (Figure 6).

Western blot analysis of HIF-1-alpha protein expression. Western blot

analysis of HIF-1- α protein expression in the gastrocnemius muscles at day 7 after surgery showed a significantly increased mean relative HIF-1- α protein level in the MC-shPHD2-treated group of 1.0 ± 0.058 , compared to PL-shPHD2 treatment (0.80 ± 0.058) and PBS treatment (0.67 ± 0.12) ($p < 0.05$ for both) (Figure 7).

DISCUSSION

To our knowledge, this is the first study that combines the advantages of stimulating the angiogenic potential of the HIF-1- α pathway by shRNA interference of PHD2 with the nonviral minicircle vector technology. Our results indicate that MC-shPHD2 improves post-ischemic neovascularization in a hindlimb ischemia mouse model, making this approach an attractive potential treatment option for critical limb ischemia.

Functional revascularization capacity of MC-shPHD2 was studied by repeated blood flow perfusion measurements after hindlimb ischemia induction in mice treated with MC-shPHD2, PL-shPHD2, or PBS. Mice treated with MC-shPHD2 showed significantly improved blood flow recovery up to 50% as compared to treatment with PL-shPHD2 or PBS. A significant increase in collateral and capillary density in the post-ischemic skeletal muscles of mice treated with MC-shPHD2 confirmed its beneficial effects on neovascularization. These results demonstrate the potential of MC-shPHD2 as a valuable target for therapeutic neovascularization.

Therapeutic neovascularization is a promising strategy for treatment of ischemic vascular disease that uses angiogenic growth factors or genes encoding these proteins to stimulate neovascularization. Single recombinant growth factors such as fibroblast growth factor (FGF) [32] and vascular endothelial growth factor (VEGF) [33] have been studied in preclinical studies, but their use seemed to be restricted by the limited half-life. For instance, phase I clinical trials achieved promising results using plasmid DNA encoding human growth factors in patients with PAD [12, 34, 35], but phase III clinical trials have failed to demonstrate unambiguous success that a single growth factor could benefit patients with CLI [36]. Newer approaches based on upregulation of an upstream transcriptional factor as HIF-1- α may provide better alternatives.

Additionally, the inconsistent benefits of gene therapy using a single growth factor might also be attributable to the lack of ideal vectors and limited

transfection efficiencies. To assess transfection efficiency *in vitro*, MC-Luc and PL-Luc were used in equimolar amounts to transfect irradiated mouse C2C12 cells. To correct for the rapid division of non-irradiated cells, we used irradiated cells that more closely resemble *in vivo* situation of slower proliferating cells. The present study reported up to 4.6-fold higher gene expression, which was even higher than the transfection efficiency shown in our control experiment with non-irradiated cells. Transfection efficiency *in vivo* was determined by the injection of MC-Luc and PL-Luc in the gastrocnemius muscles of C57Bl6 mice. Up to a 10-fold higher gene expression of MC-Luc during 28 days as compared to PL-Luc in the mouse hindlimb was reported in this study. This was in line with a recent report that compared gene expression of MC-Luc with PL-Luc in gastrocnemius muscles of FVB/N mice [6]. These results confirm the same beneficial transfection efficiency of MCs in two different mice strains, which were found to have different neovascularization reactions on hindlimb ischemia [37]. The mechanism of enhanced transgene expression of MCs is unclear, but may result from the elimination of the unnecessary plasmid sequences, in particular CPG islands, which can lead to transcriptional gene silencing *in vivo*. In addition, the smaller size of MCs may confer better extracellular and intracellular bioavailability and therefore improve gene delivery properties [8].

Previous studies on therapeutic angiogenesis have demonstrated regression of neovessels as time progressed due to a drop in expression after gene transfer [38-41]. By using minicircle as a vector, we and others have shown that gene expression levels are high up to 4 weeks after transfection [6]. Additionally, previous studies have shown that the therapeutic window ranges from 1 to 3 weeks, with complete blood flow recovery in untreated control animals after these time points [25]. Therefore, we used a single administration to analyze the effect on neovascularization. No significant neovessel regression was seen in our study. MC-shPHD2 treatment resulted in 100% blood flow recovery 3 weeks after ischemia induction. A non-significant decrease in blood flow was seen at 28 days in this group. This might be explained by the relative decrease in HIF-1- α levels in the normoxic paws. This is in concordance with the previously mentioned studies in which gene expression tends to vanish in the absence of an angiogenic stimulus. However, we believe that restoring the blood flow to the downstream tissue, and thus resolving the ischemia in the affected areas, is also an important factor that may explain the small decline in perfusion. After formation of sufficient new collaterals and consequently repair of the blood flow to the ischemic tissue, part of the newly formed collaterals

will become redundant and will disappear, a process called pruning, whereas other collaterals will fully mature [42, 43]. For future larger animal studies that require a longer therapeutic window, repeated transfection using minicircle vectors is feasible with robust transgene activities, with lower host cellular and humoral immune responses as seen with repeated injections of viral vectors [6]. In this study, we selected PHD2 as the target gene by downregulation of the mouse PHD2 gene by MC-mediated shRNA interference, which leads to activation of downstream angiogenic genes and proteins. In line with our results, a recent report showed that downregulation of PHD2 by shRNA enhanced neoangiogenesis in a mouse model of myocardial infarction [18]. A different study evaluated the effects of shPHD1, shPHD2, and shPHD3 on neovascularization in a mouse hindlimb ischemia model [19]. Electroporation of plasmid vectors was used and silencing of PHDs triggered post-ischemic neovascularization, with shPHD2 and shPHD3 having the most robust effects. In the current study, we chose PHD2 as a target because it is the most abundant isoform [44]. Both in vivo reports have provided a better understanding of PHD2 downregulation with shRNA in human subjects. Importantly, recent evidence suggests that downregulation of PHD2 promotes tumor growth [45].

In this study, Bordoli et al. showed that PHD2 functions as a tumor suppressor in xenografted tumors derived from breast carcinoma. In this respect, as PHD inhibitors have been developed for the treatment of PAD, there are concerns over possible side effects of these inhibitors in tumor progression. More encouragingly, others have shown that genetic inactivation of PHD2 increased the delivery of chemotherapeutics to the tumor and thereby their anti-tumor and anti-metastatic effect [46]. This study also showed that PHD2 haplodeficiency prevented oxidative damage, organ failure, and tissue loss. In addition, a randomized, double-blind, placebo-controlled study using adenoviral HIF-1-alpha showed no evidence of promotion of tumor growth [47].

Finally, a recent randomized study studied the effect of adenoviral administration of HIF-1-alpha in a group of 289 patients with claudication [48]. No significant differences in claudication onset time, ankle-brachial index, or quality-of-life measurements between the placebo and HIF-1-alpha groups were found. Possible explanations for the negative results include the limited biological activity of HIF-1-alpha in patients with claudication and the possible limited efficacy of gene transfer with an adenovirus vector, due to immunological responses. Hence, shPHD2 treatment in combination with the minicircle vector might circumvent these issues. Although PAD is a chronic

progressive disease, triggering the collateral formation and neovascularization might be very beneficial in restoring the blood flow to the ischemic limbs [49, 50]. MC-shPHD2 would therefore be an interesting alternative treatment strategy for therapeutic angiogenesis in the future.

In conclusion, the present study provides encouraging initial evidence for a new potential therapeutic approach for triggering the collateral formation and neovascularization in PAD. Using a nonviral minicircle vector carrying shPHD2, HIF-1-alpha is upregulated through PHD2 knockdown, resulting in an improved post-ischemic neovascularization in a hindlimb ischemia mouse model.

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SUPPLEMENTAL METHODS

Angiography. Post mortem angiography of both hindlimbs was performed using Microfil contrast agent (Flow tech, Carver, MA, USA) according to the manufacturer's protocol. After thoracotomy, contrast fluid was injected into the left ventricle of the mouse heart. Five minutes before contrast injection, mice were intravenously injected with papaverine (50 mg/ml) for vasodilatation. The skin of both hindlimbs was removed and X-rays were made. **RNA extraction.** To confirm that MC-shPHD2 could decrease PHD2 mRNA levels and could increase HIF-1-alpha protein levels in vivo as compared to PL-shPHD2 or PBS, additional C57BL6 mice were subjected to hindlimb ischemia followed by injection of MC-shPHD2, PL-shPHD2, or PBS. Gastrocnemius muscles (n=27) were harvested 7 days after surgery for further analysis. Total RNA was extracted with Trizol reagent according to the manufacturer's instructions (Invitrogen). RNA was reverse-transcribed into cDNA using the High Capacity RNA to cDNA kit (Applied Biosystems, Foster City, CA, USA) to assess the mRNA expression levels of PHD2.

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