Double Knockdown of Prolyl Hydroxylase and Factor-Inhibiting Hypoxia-Inducible Factor With Nonviral Minicircle Gene Therapy Enhances Stem Cell Mobilization and Angiogenesis After Myocardial Infarction

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Background—Under normoxic conditions, hypoxia-inducible factor (HIF)-1α is rapidly degraded by 2 hydroxylases: prolyl hydroxylase (PHD) and factor-inhibiting HIF-1 (FIH). Because HIF-1α mediates the cardioprotective response to ischemic injury, its upregulation may be an effective therapeutic option for ischemic heart failure.

Methods and Results—PHD and FIH were cloned from mouse embryonic stem cells. The best candidate short hairpin (sh) sequences for inhibiting PHD isoenzyme 2 and FIH were inserted into novel, nonviral, minicircle vectors. In vitro studies after cell transfection of mouse C2C12 myoblasts, HL-1 atrial myocytes, and c-kit+ cardiac progenitor cells demonstrated higher expression of angiogenesis factors in the double-knockdown group compared with the single-knockdown and short hairpin scramble control groups. To confirm in vitro data, shRNA minicircle vectors were injected intramyocardially after left anterior descending coronary artery ligation in adult FVB mice (n=60). Functional studies using MRI, echocardiography, and pressure-volume loops showed greater improvement in cardiac function in the double-knockdown group. To assess mechanisms of this functional recovery, we performed a cell trafficking experiment, which demonstrated significantly greater recruitment of bone marrow cells to the ischemic myocardium in the double-knockdown group. Fluorescence-activated cell sorting showed significantly higher activation of endogenous c-kit+ cardiac progenitor cells. Immunostaining showed increased neovascularization and decreased apoptosis in areas of injured myocardium. Finally, western blots and laser-capture microdissection analysis confirmed upregulation of HIF-1α protein and angiogenesis genes, respectively.

Conclusions—We demonstrated that HIF-1α upregulation by double knockdown of PHD and FIH synergistically increases stem cell mobilization and myocardial angiogenesis, leading to improved cardiac function. (Circulation. 2011; 124[suppl 1]:S46–S54.)

Key Words: prolyl hydroxylase ■ hypoxia-inducible factor 1 ■ RNA interference ■ DNA minicircles ■ myocardial ischemia ■ stem cells ■ laser capture microdissection

Coronary artery disease is the leading cause of morbidity and mortality in the Western world.1 Despite conventional treatments, a significant number of patients still have refractory angina.2 Cytokine agents are a promising therapeutic option, which can be used as adjunctive treatment to revascularization or cell therapy. Cytokine therapy may act directly on the myocardium (eg, inhibiting apoptosis and stimulating proliferation) or indirectly by mobilization of stem cells from the systemic circulation.3 However, randomized clinical trials have shown mixed results, which may be partly due to the limited benefits of single gene therapy.4 A better option may be to administer hypoxia-inducible factor (HIF)-1α, an upstream transcriptional factor that regulates >100 genes and protects the myocardium from ischemic injury.5,6

Unfortunately, HIF-1α has a biological half-life of only ≈5 minutes under normoxic conditions. Its rapid proteosomal degradation results from hydroxylation of HIF-1α at Pro402 or Pro564 by a set of HIF prolyl hydroxylase (PHD) isoenzymes (1 to 3) that mediate its recognition by von Hippel-Lindau...
ubiquitin ligase complex. Concurrently, factor-inhibiting HIF-1 (FIH) also catalyzes the hydroxylation of HIF-1α Asn803, which blocks its interaction with the transcriptional coactivator p300. In contrast, during hypoxia, the lack of hydroxylase activities enables HIF-1α to escape proteosomal destruction and allows it to bind with the HIF-1β subunit to become transcriptionally active.

In this study, we used short hairpin (sh) sequences inserted into a novel, nonviral, minicircle vector to inhibit PHD isoenzyme 2 (shPHD2) and FIH (shFIH), which then prevented HIF-1α degradation. We found that upregulation of HIF-1α enhances stem cell mobilization (through molecular imaging and fluorescence-activated cell sorting [FACS]) and increases myocardial angiogenesis (through laser-capture microdissection [LCM] and immunohistochemistry), leading to significant improvement in cardiac function after myocardial infarction (MI).

### Methods

#### RNA Interference of Mouse PHD2 Gene in Culture Cell

Mouse PHD2 and FIH genes were cloned from mouse embryonic stem cells (SV129 line). We used the PHD2 isoenzyme because a previous study showed higher expression of PHD2 in the heart compared to PHD1 or PHD3 isoenzymes. We designed 4 sequences of RNA interference sites for the PHD2 gene and 8 for the FIH gene. The targeting sequences are shown in online-only Data Supplement Figure I. The optimal knockdown fragment was inserted after the H1 promoter in the minicircle parental backbone vector.

#### Cell Culture, shRNA Transfection, and Hypoxia Exposure

Mouse C2C12 myoblasts were cultured in DMEM medium (high glucose)+10% FBS. Mouse HL-1 atrial myocytes were cultured in 10% FBS (BioWhittaker), Life Technologies), 50 µg/mL endothelial cell growth supplement (Upstate Biotechnology), 1 µmol/L retinoic acid (Sigma), 10 µmol/L norepinephrine (Sigma), 100 µU/mL penicillin, 100 µg/mL streptomycin (Life Technologies), and an additional 1% nonessential amino acids (Life Technologies) on 0.01% gelatin-coated plate. Mouse c-kit+ cardiac progenitor cells (CPCs) were isolated based on a previously described protocol. CPCs were cultured in 10% embryonic stem cell-qualified FBS (Gibco), DMEM, Ham F-12 medium, insulin-transferrin-selenium, 10 ng/mL leukemia inhibitory factor, 10 ng/mL basic fibroblast growth factor (bFGF), 20 ng/mL epidermal growth factor, and penicillin-streptomycin-glutamine. Minicircle vectors with shPHD2 and shFIH driven by H1 promoter were cotransfected into mouse C2C12, HL-1, and CPC using lipofectamine 2000 (Invitrogen), with penicillin-streptomycin-glutamine. Minicircle shRNA transfection, cells were cultured for 1 day before being subjected to the hypoxia condition with 5% CO2, 1% O2, and 94% N2, at 37°C for 48 hours.

#### Quantitative Polymerase Chain Reaction Analysis of Angiogenesis Genes

At the end of the 48-hour hypoxia treatment, cells were harvested for immediate RNA extraction (n=3 biological replicates). Quantitative polymerase chain reaction (qPCR) was used to compare the expression of angiogenic genes (fibroblast growth factor b [bFGF], vascular endothelial growth factor [VEGF], vascular endothelial growth factor receptor 1 [VEGFR1], vascular endothelial growth factor receptor 2 [KDR], transforming growth factor [TGF], and plasminogen activator inhibitor-1 [PAI-1]) in transfected cells under normoxic versus hypoxic conditions. Total RNA was prepared from C2C12 cells with Trizol reagent (Invitrogen) according to the manufacturer’s protocol. The probe sets used in the amplification reaction were obtained from Applied Biosystems. PCR reactions were performed on the ABI 7900HT system.

#### Angiogenesis Cytokine Array After Hypoxia Exposure

At the end of the hypoxic treatment, the supernatant was collected for angiogenesis cytokine array (Panomics). In 2 separate experiments (duplicate spots, 2 biological replicates), the arrays were hybridized and imaged together. Expression intensities were calculated by adding the total pixel intensity for each spot. Interarray normalization was performed by using positive control spots (8 per array) on each array. Protein expression levels were normalized to PBS controls so that changes in protein expression could be easily assessed.

#### Animal Surgery To Induce MI

Ligation of the mid-left anterior descending coronary artery was performed in adult female FVB mice (Charles River Laboratories; Wilmington, MA) by a single experienced microsurgeon (Y.G.). Survival rate for each surgery group was between 80% to 90%. MI was confirmed by myocardial blanching and ECG changes. Animals were randomized into shFIH (n=15), shPHD2 (n=15), shPHD2+shFIH (n=15), and shScramble control (n=15) groups. Animals were injected intramyocardially with 25 µg of shRNA or shScramble minicircle vector using a 31-gauge Hamilton syringe. In all groups, the volume of injection was 25 µL in 3 different spots near the perinfarct zone. Study protocols were approved by the Stanford Animal Research Committee.

#### Activation of Endogenous c-kit+ CPCs With shRNA Therapy

For immunostaining, frozen heart sections (n=5 mice per group) were deparaffinized, and antigen retrieval was performed using 10 mmol/L citrate (pH 6.0). Slides were blocked in TNB (PerkinElmer) for 1 hour after which goat antimiatus c-kit antibody (R&D Systems) was added (1:100) overnight at 4°C. Slides were washed in 1 mol/L Tris/NaCl followed by secondary antibody incubation for 2 hours at room temperature. For FACS of c-kit+ CPCs in the heart (n=5 mice per group), we excised the perinfarct zone and digested it using 0.1% collagenase IV into single-cell suspension. FACS was performed using c-kit (allophycocyanin-Cy7 conjugated; BD Biosciences) antibody as previously described.

#### Creation of Bone Marrow Chimeric Mouse Model

To determine whether c-kit+ cells found in the heart were endogenous or donor-derived bone marrow-derived cells, we generated a hematopoietic chimeric mouse model that we termed the mouseinduced mouse model. Using a strategy described previously, we used lineage-negative bone marrow cells (BMCs) from C57BL/6 mice (major histocompatibility complex [MHC] class I haplotype, H2b) to reconstitute NOD/SCID IL-2Rg (-/-) mice (MHC class I haplotype, H2g), a severely immunocompromised strain that lacks T cells, B cells, and NK cells. Briefly, 2- to 3-day-old NOD/SCID IL-2Rg (-/-) pups received myeloablative treatment consisting of 100 cGy gamma irradiation. Twelve hours later, pups received an intrahepatic injection of 1×10^6 lineage-negative BMCs isolated from adult C57BL/6 mice. Six to 8 weeks later, hematopoietic engraftment was confirmed by detecting engraftment of donor-derived (H2b^+^) multilineage immune cells in peripheral blood of mousenized mice treated with FACS. After donor cell engraftment was confirmed, the mousenized mice underwent left anterior descending coronary artery ligation followed by injection of shPHD2+shFIH versus shScramble into the perinfarct zone (n=3 mice per group). Cells harvested from the perinfarct zone were stained with allophycocyanin-conjugated c-kit and R-phycocerythrin-conjugated H2b antibodies. Donor-derived c-kit+ cells were identified by FACS as c-kit^+ and H2b^+.
Analysis of Angiogenesis Genes Using LCM and RNA Extraction

Murine hearts were removed after perfusion with 20 mL PBS, embedded in optimal cutting temperature, and immediately frozen in liquid nitrogen. Seven thick tissue sections of left ventricle were prepared on polyethylene naphthalate membrane-coated slides (MicroDissect GmbH). For LCM, slides were thawed briefly and air dried 5 minutes before dissection. Green fluorescence observed under laser microscopy was used as a landmark for microdissection. Specific green fluorescence tissues, as well as normal cardiac tissues without green fluorescence, were independently dissected out by applying Leica LCM Systems (MicroDissect GmbH). The dissected tissues were placed on the caps of microcentrifuge tubes with 5 M lysis enhanced buffer. After dissection, tissues were collected by centrifugation at 8000g for 5 minutes. Total RNA extraction and reverse transcription of these samples were performed using a commercial 1-step kit (Invitrogen) (n=5 mice per group).

Statistical Analysis

For calculation of relative gene expression, the expression level of each specific gene was divided by the expression level of GAPDH. For statistical analyses of treatment groups, a Box-Cox transformation was used to achieve approximate normality for analysis by ANOVA (1 tail, equal variance) with post hoc testing. ANOVA and repeated-measures ANOVA. ANOVA indicates basic fibroblast growth factor; FIH, factor-inhibiting HIF-1; HIF-1α, hypoxia-inducible factor-1α; PAI-1, plasminogen activator inhibitor-1; PHD, prolyl hydroxylase; sh, short hairpin; TGFα, transforming growth factor-α; TIMP-1, tissue inhibitor of metalloproteinase-1; TNF, tumor necrosis factor; VEGF, vascular endothelial growth factor.*P<0.001 vs shScramble. **P<0.05 vs shScramble.

Results

In Vitro Characterization of shFIH and shPHD2 Double Knockdown

We measured activation of HIF-1α and subsequent upregulation of angiogenesis genes after hydroxylase inhibition using shRNAs under normoxic and hypoxic conditions. Plasmid hypoxia response element pHRE-SV40-FLuc is a hypoxia-sensing 5xHRE-SV40 promoter driving FLuc cassette. The 5 copies of HRE derived from the erythropoietin gene are activated through binding of the HIF-1 complex, which allowed us to monitor the efficacy of the upstream shRNA knockdown compared to shScramble control (Figure 1A). In the normoxic condition, cells transfected with shPHD2+shFIH (5.32×10¹⁵±3.23 electric photons/s per cm²/steradian [p/s per cm²/ster]) had significantly higher FLuc bioluminescence signals than cells transfected with shPHD2 (3.41×10¹⁵±57 184 p/s per cm²/ster), shFIH (4.48×10¹⁵±4513 p/s per cm²/ster), and shScramble control (2.86×10¹⁵±1934 p/s per cm²/ster), indicating increased binding of 5xHRE-SV40 promoter by HIF-1α after double shRNA knockdown. A similar, but more robust trend was observed when the cells were exposed to hypoxic conditions. This is an expected finding given that HIF-1α acts by binding to the HREs to drive the expression of FLuc under hypoxic conditions. Western blot confirmed that higher levels of HIF-1α expression are present under hypoxic conditions, as shown in online-only Data Supplement Figure II.

To quantify luciferase activity, we lysed the cells and determined the luminescence activity normalized to protein concentration (online-only Data Supplement Figure III). The luminescence activity was highest in the double-knockdown group under both normoxic (2495±55 luminescence activity/mg protein) and hypoxic conditions (5232±100 luminescence activity/mg protein). To confirm similar effects in different cell types, mouse HL-1 atrial myocytes and mouse...
c-kit+ CPCs also were transfected by minicircle shRNA and pHRE-SV40-FLuc. Comparable results were observed in these 2 cell types (online-only Data Supplement Figure IV).

To confirm the pHRE-SV40-FLuc imaging signals, mRNA was isolated and qPCR performed for detection of HIF-1α and downstream angiogenesis genes. As shown in Figure 1B, relative expression of 6 genes related to angiogenesis (eg, bFGF, VEGF, Flk, KDR, TGF, PAI-1) were increased by 28.8±5.3% and 54.3±8.6% after treatment with shPHD2 and double knockdown, respectively. HIF-1α mRNA levels were not changed, which is expected because shRNA affects HIF-1α at the protein and not at the mRNA level. HIF-1α protein can activate several downstream genes responsible for stimulation of angiogenesis.15 To examine whether upregulation of HIF-1α protein through shRNA knockdown of PHD2 and FIH also can exert similar effects, supernatant from transfected C2C12 cells was used for angiogenesis assays. Figure 1C and 1D show significant upregulation of several angiogenesis activators (eg, FGFα, IL-6, leptin, VEGF, tumor necrosis factor-α, and TGF-α) after double knockdown.

Interestingly, both IFN-γ and tissue inhibitor of metalloproteinase-1 (TIMP-1) were also upregulated in the double-knockdown group (Figure 1D). IFN-γ is a soluble cytokine, which has antiviral, immunoregulatory, and antitumor properties.16 In contrast, TIMP-1 is a 28-kDa protein that inhibits the function of metalloproteinases and has been associated with cell growth promotion, matrix binding, apoptosis induction, and angiogenesis regulation.17 Previous studies have shown that HIF-1α regulates IFN-γ and TIMP-1.18–22 To confirm this, we measured the relative expression of TIMP-1 after transfection of C2C12 cells with a minicircle carrying the following genes: (1) HIF-1α (positive control), (2) an inhibitor of HIF-1α (siHIF-1α), (3) shFIH and shPHD2 (double knockdown), and (4) blank vector (negative control). We found that siRNA inhibition of HIF-1α led to significantly lower TIMP-1 and IFN-γ expression levels. In contrast, overexpression of minicircle HIF-1α and minicircle shPHD2+shFIH led to significantly higher levels of TIMP-1 (P<0.001 for both) and IFN-γ (P<0.05 for both) compared with control (online-only Data Supplement Figure V). Consistent with these findings, Yang et al23 demonstrated that shRNA inhibition of HIF-1α can dramatically decrease the expression of TIMP-1 mRNA expression and protein levels. Taken together, these data confirm that similar to physiological hypoxia response, PHD2+FIH double knockdown can effectively stabilize HIF-1α protein and induce HIF-1α-dependent gene activation in vitro.

**Improvement of Left Ventricular Function and Perfusion After Dual shRNA Therapy**

To examine whether double shRNA knockdown therapy can synergistically improve cardiac function, cardiac MRI was performed. At weeks 4 and 8 after MI, significant improvement in left ventricular ejection fraction (LVEF) was seen in the double-knockdown group compared to the other groups (Figure 2). The shPHD2 knockdown group also had improvement in LVEF compared with the shScramble group at weeks 4 and 8. These findings also were confirmed by serial ECGs (online-only Data Supplement Figure VI). In addition, invasive hemodynamic parameters showed that the increase in LVEF was associated with lower left ventricular end-diastolic volume and left ventricular end-systolic volume in double shRNA knockdown compared with shScramble (online-only Data Supplement Figure VII). Finally, shPHD2+shFIH treatment improved myocardial perfusion as determined by measuring the distribution of fluorescent microspheres within myocardial tissues after injection into the left ventricular cavity (online-only Data Supplement Figure VIII). These results also coincide with observed differences in HIF-1α expression at weeks 4 and 8 in the shPHD2+shFIH group compared with the other groups (online-only Data Supplement Figure IX).
higher presence of c-kit

mobilization in the infarction area. To confirm kines have been shown to improve the cardiac stem cell analyze the source of c-kit

demonstrated more GFP

(Figure 3A and 3B). GFP and troponin T double staining also showed the highest neovascularization in the shPHD2 + shFIH group (179 ± 23 vessels/mm²) compared with the other 2 treated groups (shPHD2, 478 ± 36 vessels/mm²; shFIH, 231 ± 18 vessels/mm²), with the lowest neovascularization in the shScramble group (23 vessels/mm²) (P < 0.05 compared with shFIH and shScramble; P < 0.01 compared with shPHD2) (Figure 5C). To analyze the in vivo transfection efficiency of the minicircle vector, we injected mimicircle GFP into the periinfarct zone of murine hearts. Cells were harvested 1 week later and stained with mouse cardiac troponin T and CD31 antibody for cardiac and endothelial cells, respectively. FACs analysis indicated that there were 10.1 ± 1.4% GFP+/troponin T+ cells and 2.7 ± 0.4% GFP+/CD31+ cells (data not shown). Overall, these data demonstrate that minicircle can efficiently transfect cardiac cells in vivo, which is consistent with the in vitro transfection data on HL-1, H9c2, and CPC shown in Figure 1A and online-only Data Supplement Figure IV.

Double Knockdown Activates c-kit+ CPCs

Next, we assessed whether double knockdown also can induce proliferation of c-kit+ CPCs. Immuno staining showed higher presence of c-kit+ CPCs near the periinfarct area in the shPHD2 + shFIH group (Figure 4A). To validate the histological data, we then performed FACs analysis for c-kit+ CPCs after excision of the periinfarct tissue and digestion into single-cell suspension. FACs confirmed that the number of c-kit+ CPCs was significantly greater in the shPHD2 + shFIH group than in shScramble control (Figure 4B and 4C). To analyze the source of c-kit+ cells, a bone marrow chimeric mouse model (mousenized mouse model) was created. The hematopoietic system of NOD/SCID IL-2Rg (−/−) mice (H2g−) was reconstituted using lineage-negative BMCs from C57BL/6 murine (H2k+). BMCs from C57BL/6 strain were identified by the expression of H2k+ MHC class I haplotype. After injection with shPHD2 + shFIH versus shScramble into the mousenized mice, FACs analysis demonstrated a greater percentage of bone marrow recruitment (H2k+/c-kit+) in the treatment group than in control group (2.46 ± 0.18% versus 1.05 ± 0.06%; P < 0.01). Similarly, there was greater endogenous cell activation (H2k−/c-kit+) in the treatment group than in the control group (1.06 ± 0.06% versus 0.50 ± 0.09%, P < 0.05) (online-only Data Supplement Figure XI). Of the number of c-kit+ cells isolated from the treatment group, a greater percentage of isolated cells originated from the recipient (H2k−) than the donor (H2k+) (69.3 ± 3.1% versus 30.9 ± 4.3%). Overall, the data suggest that both processes (endogenous activation and bone marrow recruitment of c-kit+ cells) are involved with double-knockdown therapy.

Ex Vivo Histological Analysis Confirmed In Vivo Functional Data

After imaging, animals were euthanized and their hearts explanted. Hematoxylin-eosin staining showed thicker heart wall size for the shPHD2 + shFIH group than for the shScramble control (Figure 5A), confirming the positive functional data seen by MRI, echocardiography, and pressure-volume loop. Minicircle vector containing shPHD2 + shFIH significantly decreased left ventricular scar ring compared with shFIH and shScramble control (Figure 5B). Immunohistochemistry of the periinfarct region by CD31 staining also showed the highest neovascularization in the shPHD2 + shFIH group (615 ± 57 vessels/mm²) compared with the other 2 treated groups (shPHD2, 478 ± 36 vessels/mm²; shFIH, 231 ± 18 vessels/mm²), with the lowest neovascularization in the shScramble group (179 ± 23 vessels/mm²) (P < 0.05 compared with shFIH and shScramble; P < 0.01 compared with shPHD2) (Figure 5C). To analyze the in vivo transfection efficiency of the minicircle vector, we injected mimicircle GFP into the periinfarct zone of murine hearts. Cells were harvested 1 week later and stained with mouse cardiac troponin T and CD31 antibody for cardiac and endothelial cells, respectively. FACs analysis indicated that there were 10.1 ± 1.4% GFP+/troponin T+ cells and 2.7 ± 0.4% GFP+/CD31+ cells (data not shown). Overall, these data demonstrate that minicircle can efficiently transfect cardiac cells in vivo, which is consistent with the in vitro transfection data on HL-1, H9c2, and CPC shown in Figure 1A and online-only Data Supplement Figure IV.

Double Knockdown Promotes Angiogenesis and HIF-1α Stabilization in Myocardial Tissues

To further elucidate the mechanisms of shPHD2 and shFIH therapy, we harvested the periinfarct area tissue by LCM and performed qPCR of angiogenesis genes at 2 weeks after MI (Figure 6A). The remote nonischemic tissue of the same heart was used as internal controls. FGF2, VEGF, FRT1, KDR, TGF, and PAI-1 genes showed significantly higher levels among shPHD2 + shFIH and shPHD2 groups than the shFIH and shScramble groups (Figure 6B). To confirm the LCM
qPCR results, we also performed western blots of HIF-1α protein levels. Infarcted hearts were harvested at weeks 1, 4, and 8 after single- and double-shRNA therapy. Quantitative analysis of the western blots demonstrated that HIF-1α protein levels were significantly higher in shPHD2 and shPHD2+shFIH-treated hearts compared to shScramble and shFIH alone starting at week 1 (data not shown). As the minicircle vector decayed over time, protein levels also decreased progressively from week 1 to week 8 as expected (online-only Data Supplement Figure IX). This may explain why there is no significant difference between LVEF measured at weeks 4 and 8. However, LVEF was significantly higher in both week 4 and week 8 in the double-knockdown group than in the shScramble group. At 14 days postinfarction, the double-knockdown group also had a significantly lower percentage of TUNEL-positive apoptotic cells in the periinfarct area than the shPHD2, shFIH, and shScramble groups (online-only Data Supplement Figure XII). Finally, 1 week after minicircle shRNA delivery, the double-knockdown group had higher HIF-2α protein expression in the periinfarct area (online-only Data Supplement Figure XIII). HIF-2α is another transcription factor that is stabilized in hypoxic tissue. Similar to HIF-1α, HIF-2α complex binds to HREs in the promoters of many genes involved in adaptation to the hypoxic environment. In addition, several endothelial cell-specific genes (Tie-2 and Flk-1) are exclusively regulated by HIF-2α.

Discussion
In this study, we describe a novel minicircle vector that mediates double shRNA knockdown of PHD2 and FIH, resulting in upregulation of the HIF-1α protein in a murine model of MI. The major findings can be summarized as follows: shRNA targeting PHD2+FIH (1) increases BMC homing to the myocardium; (2) activates endogenous c-kit+ CPCs; (3) promotes myocardial neoangiogenesis; (4) de-
creases cellular apoptosis; and, importantly, (5) improves cardiac function after MI. We demonstrated stable and efficient double knockdown of 2 hydroxylases using dual shRNAs inserted into novel, nonviral, minicircle vectors, resulting in upregulation of HIF-1α/HIF-1β. Minicircle has several advantages over both viral-based and conventional plasmid vectors. Compared with viral vectors, minicircle has a better safety profile and allows larger expression cassette and possibly easier clinical translation because of simple, good-manufacturing practices. Compared with regular plasmid vectors, minicircle has a significantly higher level and longer duration of transgene expression both in vitro and in vivo.

HIF-1α is a master transcriptional activator that mediates the physiological response to hypoxia. In response to cardiac hypoxia, BMCs in the peripheral blood home to the site of injury, and CPCs that reside in the myocardium are activated.13-16 These recruited cells can regenerate damaged tissue by differentiating into endothelial cells, smooth muscle cells, and cardiac myocytes.17 These cells also secrete angiogenic or antiapoptotic factors, which can improve the recovery of ischemic myocardium and the function of nonischemic regions.18,19 Mobilization of these cells is controlled by several genes, including VEGF, granulocyte macrophage colony-stimulating factor, FGF, insulin-like growth factor, erythropoietin, and stromal cell-derived factor-1; all of which are regulated by HIF-1α.20-22 For example, previous studies have shown that exogenous administration of granulocyte macrophage colony-stimulating factor mobilized endothelial progenitor cells from peripheral blood to the areas of ischemic injury in the hindlimb and myocardium, resulting in neovascularization.23,24 Similarly, activation of CPCs by injection of insulin-like growth factor has been shown to regenerate myocytes and induce vessel growth, leading to improvement in cardiac structure and function.25 Consistent with these findings, we demonstrated that higher levels of HIF-1α through double shPHD2 and shFIH knockdown improved BMC homing and survival as well as enhanced activation of endogenous c-kit+ CPCs in the injured myocardium, resulting in increased angiogenesis and improved cardiac function. We also monitored the spatiotemporal kinetics of exogenously administered BMC recruitment to the ischemic heart after dual shRNA therapy, which to our knowledge has not been previously reported.

Unlike other approaches using single-gene therapy (eg, VEGF, FGF, insulin-like growth factor), HIF-1α upregulation can harness more cardioprotective components of the ischemic reperfusion response. The shRNA dual therapy alters the posttranslational modification of HIF-1α, which prevents its degradation in normoxic environments and results in enhanced stem cell mobilization and increased angiogenesis. In addition to promoting potential myocyte regeneration and new vessel growth, HIF-1α may alter tissue metabolism and protect the myocardium against ischemic injury.26 These potential benefits have been demonstrated in

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**Figure 5.** Histological analysis of minicircle-based shRNA therapy. A, Representative histology of infarcted hearts injected with shScramble, shFIH, shPHD2, and shPHD2 + shFIH at week 4 (hematoxylin-eosin, magnification ×5). B, Quantitative analysis of ventricular wall showed significant improvement in the double-knockdown group compared with shScramble control group. *P<0.05. **P<0.01 vs shScramble. C, Immunostaining for CD31 marker indicated significantly increased neovascularization in the double-knockdown group (staining with FITC and PI, magnification 100×). Five mice per group. Abbreviations as in Figure 1.
leading to improvement of cardiac function after MI. Taken
enhanced BMC homing, activation of endogenous CPCs,
early expression of HIF-1 protein, which in turn resulted in

Studies using HIF-1α/VP16 hybrid gene and HIF-1α activator
proteins. 39,40

In summary, we demonstrate a novel therapeutic approach
to preserve the myocardium by harnessing the normal phys-
ological response to hypoxia. Inhibition of HIF-1α degra-
dation by dual shPHD2 and shFIH therapy produced a robust
early expression of HIF-1 protein, which in turn resulted in
enhanced BMC homing, activation of endogenous CPCs,
increased neoangiogenesis, and decreased cardiac apoptosis,
leading to improvement of cardiac function after MI. Taken

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None.

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Figure 6. Activation of myocardial angiogenesis genes after dual
shRNA therapy. A. Frozen slides for laser capture microdissec-
tion showing the experimental and control areas used to assess
levels of angiogenic gene activation after shRNA therapy (no
staining, magnification 50×). B. Quantitative polymerase chain
reaction showed significant increase in expression of angiogenic
genes in the double-knockdown group compared with the
single-knockdown and shScramble control groups. Five mice
per group. Abbreviations as in Figure 1.


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Double Knockdown of Prolyl Hydroxylase and Factor-Inhibiting Hypoxia-Inducible Factor With Nonviral Minicircle Gene Therapy Enhances Stem Cell Mobilization and Angiogenesis After Myocardial Infarction
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SUPPLEMENTAL MATERIAL
SUPPLEMENTAL METHODS

Minicircle vectors carrying shRNA knockdown of PHD2 and FIH. Minicircles (MC) are the product of site-specific intramolecular recombination between the attB and attP sites driven by bacteriophage ΦC31 integrase. By addition of arabinose at 32°C, the phage ΦC31 integrase performs a site-specific intramolecular recombination of sequences between attB and attP recognition sites, splitting minicircle parental plasmid into two super-coiled circular DNAs: the minicircle with its transgene of interest and the “junk” bacterial backbone. After adjustment of the temperature to 37°C, the bacterial backbone plasmid was then linearized by the induced I-SceI and degraded by bacterial exonucleases. Only the minicircle containing the H1 promoter driving shRNA in our study remained intact as an episome formation in the bacterial cytosol, which was then isolated for subsequent usage.

Measurement of left ventricular contractility with cardiac magnetic resonance imaging (MRI). We randomly selected five mice per group for evaluation of LV function. Imaging was performed on a Signa 3.0T Excite HD scanner (GE Healthcare Systems, Milwaukee, Wisconsin; http://www.gehealthcare.com/euen/mri/index.html) with a Mayo Clinic T/R MRI coil (Mayo Clinic Medical Devices, Rochester, Minnesota). Mice were anesthetized with 1.5% isoflurane with oxygen (1 ml/min) and placed in the prone position for imaging. A small animal electrocardiogram and respiratory gating system (Small Animal Instruments, Stony Brook, New York) was used to acquire images as previously described. Gradient recalled echo (GRE) and fast spoiled GRE (FSPGR) sequences were used to acquire sequential short-axis slices spaced 1 mm apart from apex to base of the mouse heart. For each sequence, 20 cine frames encompassing 1 cardiac cycle were obtained with the following sequence parameters: TR = 10
ms, TE = 4.6 ms, number of excitations (NEX) = 10, field of view (FOV) = 40 × 49 mm, matrix = 256 × 256, flip angle (FA) = 45°, and slice thickness 1.5 mm. A contouring program, Osirix Version 3.81, was used to trace the endocardial border of the left ventricle (LV) for each slice of the heart over the entire cardiac cycle in order to determine ejection fraction (EF), end-diastolic (EDV) and end-systolic volumes (ESV).

**Analysis of left ventricular function with echocardiogram and pressure-volume (PV).** We randomly selected five mice per group to measure LV function by echocardiography and PV loops. Echocardiography was performed before (day -7) and after (week 2, 4, and 8) the LAD ligation. The Siemens-Acuson Sequioa C512 system equipped with a multi-frequency (8-14 MHZ) 15L8 transducer was used by an experienced operator (SH) blinded to the group designation. Analysis of M-mode images was performed using Siemens built-in software. Left ventricular volume at end of diastolic diameter (EDD) and end-systolic diameter (ESD), and volume blood at end of diastolic (BED) were measured and used to calculate left ventricular ejection fraction by the formula: $\text{LVFS} = \frac{\text{EDD}-\text{ESD}}{\text{BED}}$. At the end of the study (week 8), invasive hemodynamic measurements were performed as previously described.³ Briefly, after midline neck incision, a 1.4 conductance catheter (Millar Instruments, Houston, Texas) was introduced into the left ventricle through the right carotid artery. After stabilization, the signals were continuously recorded at a sampling rate of 1,000/s using PV conductance system coupled to a PowerLab/4SP analog and to a digital converter (AD Instruments, Colorado Springs, Colorado). Data were analyzed by using a cardiac PV analysis program (PVAN 3.4, Millar Instruments) and Chart/Scope Software (AD Instruments).
**Measurement of myocardial perfusion using fluorescent beads.** We randomly selected 10 mice per group to perform myocardial perfusion imaging. Myocardial blood flow (BF) was measured using fluorescent microspheres of 0.02μm in diameter (Invitrogen, Paisley, UK). Anesthetized mice were intubated and a polyethylene catheter (PE10) was inserted into the right carotid artery and connected to a syringe pump for collection of reference BF. The chest was then opened and microspheres were injected into the LV cavity at the rate of 0.15ml/min (200μl of total volume). Animals were sacrificed 2 minutes later and the heart removed and the LV separated. To verify homogenous distribution of microspheres into the bloodstream, both kidneys were also collected and analyzed as internal controls. Each sample was weighted, cut in small pieces, and digested in 10ml of 2M ethanolic KOH at 60°C for 48 hours. Finally, microspheres were collected and fluorescence intensity determined by a fluorometer. Regional absolute BF was calculated and expressed in ml/min/g of tissue.

**Analysis of bone marrow cell (BMC) homing to the injured myocardium.** We randomly selected five animals per group to undergo bioluminescence imaging after BMC injection. BMCs were harvested from the long bones of male FVB transgenic mice with constitutive ubiquitin promoter driving fusion green fluorescent protein and firefly luciferase (GFP-FLuc) reporter genes. Isolated BMCs were centrifuged in a density cell separation medium (Ficoll-Hypaque; GE Healthcare) as previously described. One million BMCs were injected via tail vein at 24 hours following MI. Bioluminescence imaging was performed on days 0, 1, 7, 21, and 28 using the Xenogen In Vivo Imaging System (Caliper) as previously described.
**Histological examination.** Explanted hearts from study and control groups were embedded into OCT compound (Miles Scientific, Elkhart, IN). Frozen sections (5 µm thick) were processed for immunostaining. To quantify the left ventricle (LV) infarct size, H&E staining was performed in shScramble, shFIH, shPHD2, and shFIH + shPHD2 (n=5 mice per group). For each heart, eight to ten sections from apex to base (1.2 mm apart) were analyzed. Images were taken for each section to calculate the ventricular and septal wall thickness. The NIH Image J software was used to quantify the infarct zones. To detect microvascular density (MVD) in the peri-infarct area, a rat anti-CD31 (BD Pharmingen) was used. A blinded investigator (FJ) counted the number of capillary vessels in ten randomly selected areas, using fluorescent microscopy (x100 magnification). Goat anti-mouse c-kit antibody was used to detect c-kit⁺ cardiac progenitor cells (for endogenous activation of CPC experiment) and GFP antibody was used to detect GFP⁺ bone marrow cells (for exogenously administered BMC homing experiment). A typical green color vessel was selected as a sample after opening one picture randomly by Image J software. The process was repeated 10 times in different peri-infarct areas to calculate vessel numbers at 1 mm² scale. Additional samples were used to examine the infarction size by H&E staining.

**Analysis apoptosis cells in infarction area by histology.** We randomly selected five mice per group for analysis of apoptosis. The formalin-fixed and paraffin-embedded explanted hearts were used for terminal deoxynucleotidyl Transferase-mediated dUtp Nick End Labeling (TUNEL) assay. Nuclei undergoing apoptosis were stained with the Dead End Fluorometric TUNEL System (Promega). The TUNEL positive nuclei were counted and calculated as a percentage of all nuclei according to the manufacturer’s protocol.
**Western blot of mouse hearts to assess HIF-1α and HIF-2 levels.** In order to determine the extent of HIF-1α activation following different shRNA minicircle injections after LAD ligation, we randomly selected 5 mice in each of the 4 experimental groups. Hearts from these animals were assayed for HIF-1α levels using Western blots on week 1, 4, and 8. After excising the peri-infarct region of the left ventricle under a 10X microscope, we isolated tissue protein by Ripa buffer (Sigma). Protein blots were analyzed with rat anti-mouse HIF-1α (1/500 dilution, Novus) primary antibody followed by sheep anti-rat IgG whole antibody-HRP secondary antibody (1/3000 dilution, Amersham) and were developed using ECL assay (Amersham).
SUPPLEMENTAL FIGURE LEGEND

Supplemental Figure 1. Strategy for double shRNA knock down. (A) Schema of the sense and antisense sequences for shScramble, shPHD2, and shFIH. (B) Schema of the non-viral minicircle vectors carrying shRNA. The H1 promoter drives the expression of a hairpin structure in which the sense and antisense strands of the siRNA are connected by a 9-bp long loop sequence. In addition, a separate 5xHRE-SV40 promoter driving firefly luciferase is used to track shRNA activity in vitro. 5x HRE, 5 repeat of hypoxia response elements.

Supplemental Figure 2. Western blot analysis of HIF-1α expression under normoxia and hypoxia conditions in vitro. Western blot analysis of C2C12 cells in culture showed higher levels of HIF-1α expression in the double knock group compared to single knockdown and shScramble groups under both normoxic and hypoxic conditions. N=3 biological replicates.

Supplemental Figure 3. Quantification of luciferase activity in vitro. The bar graph shows luminescence activity normalized by mg protein after minicircle shRNA transfection in C2C12 cells under normoxic and hypoxic conditions. Luciferease activity is highest in the double knockdown group under hypoxic conditions. *P<0.05, **P<0.01 vs. shScramble. N=3 biological replicates.

Supplemental Figure 4. Confirmation of transfection in different cell types. Bioluminescence imaging showed both HL-1 and CPC cells have significantly robust signals after transfection with double shRNA knockdown under both normoxic and hypoxic conditions. N=3 biological replicates.
Supplemental Figure 5. Relative gene expression of IFNγ and TIMP1 after minicircle transfection. The bar graph shows lower IFN-γ and TIMP1 expression after HIF-1α inhibition by transfection with minicircle carrying siRNA (siHIF-1α). In contrast, over-expression of minicircle HIF1α and minicircle shPHD2 + shFIH led to significantly higher levels of both IFN-γ and TIMP1. *P<0.05, **P<0.01 vs. HIF-1α siRNA. N=3 biological replicates.

Supplemental Figure 6. Evaluation of heart function after minicircle shRNA injection by M-mode echo. (A) Echocardiogram (M-mode) of mice with LAD ligation following minicircle injections at day 2, week 4, and week 8. (B) Animals injected with shPHD2 + shFIH showed significant improvement in left ventricular ejection fraction (LVEF) compared to shScramble, shFIH alone, and shPHD2 alone after 8 weeks. *P<0.01 vs. shFIH and shScramble. **P<0.05 vs. shPHD2. N=5 mice per group.

Supplemental Figure 7. PV-loop analysis of heart function after shRNA injection. (A) Representative pressure volume (P-V) loops measured from shScramble, shFIH, shPHD2, and shPHD2 + shFIH. Curvilinear end-systolic P-V relations in shPHD2 + shFIH and shPHD2 mice were shifted to the left, indicating enhanced contractility. (B-C) Invasive hemodynamic assessment of ejection fraction (EF), end-systolic pressure (ESP), end-diastolic pressure (EDP), end-systolic volume (ESV), and end-diastolic volume (EDV) in the indicated mice at day 28 after shScramble operation or ischemia-reperfusion injury. *P < 0.05 vs. sham. N=5 mice per group.
Supplemental Figure 8. Myocardial perfusion using fluorescent beads after shRNA injection. The bar graph shows left ventricle (LV) absolute blood flow was highest in the double knockdown group at 14 days post-surgery. Data are expressed as mean±SEM. *P<0.05, **P<0.01 vs. shScramble. N=10 mice per group.

Supplemental Figure 9. Robust HIF-1α expression after shPHD2 + shFIH treatment. Western blots of explanted hearts after injection with shScramble, shFIH, shPHD2, or shPHD2 + shFIH showed significant up-regulation of HIF-1α in the double knockdown group at week 1. N=5 mice per group.

Supplemental Figure 10. Immunostaining of cell homing experiment. Immunostaining for GFP and troponin markers confirmed the presence of more systemically delivered GFP+ BMCs in the double knockdown group. *P<0.05, **P<0.01 vs. shScramble. N=5 mice per group.

Supplemental Figure 11. Double knockdown therapy activates endogenous progenitor cells and recruits stem cells from the bone marrow. Following injection with shPHD2 + shFIH versus shScramble into the mousenized mice, FACs analysis demonstrated a greater percentage of endogenous activation (H2k+/-c-kit+) and bone marrow recruitment (H2k+/c-kit+) in the treatment group compared to control. N=3 mice per group.

Supplemental Figure 12. Decreased cell apoptosis in the peri-infarct area after shPHD2 + shFIH injection. Double shRNA group (27.5 ± 6.5 tunnel number/mm²) had significantly lower Tunnel positive cells compared to shPHD2 group (59.8 ± 12.2 tunnel number/mm²), shFIH
group (111.5 ± 4.3 27.5 ± 6.5 tunnel number/mm²), and shScramble group (130.4 ± 7.5 27.5 ± 6.5 tunnel number/mm²). *P<0.05, **P<0.01 vs. shScramble. N=5 mice per group.

Supplemental Figure 13. Western blot analysis of HIF-1α and HIF-2α expression in the peri-infarct area. Western blot analysis performed 1 week after minicircle delivery showed higher up-regulation of both HIF-1α and HIF-2α in the double knockdown group. Both HIF-1α and HIF-2α activate genes involved in the adaptive response to hypoxia. N=5 mice per group.
REFERENCES


Supplemental Figure 1
**Supplemental Figure 2**

Supplemental Figure 2 illustrates the expression levels of HIF-1α under normoxia and hypoxia conditions across different conditions:

- **HIF-1α (Normoxia)**
- **HIF-1α (Hypoxia)**
- **β-actin**

The bands represent the levels of expression for each condition, with shScramble, shFIH, shPHD2, and shPHD2 + shFIH indicated at the top.
Supplemental Figure 3

Luminescence Activity / mg Protein

shScramble         shFIH            shPHD2           shPHD2 + shFIH

- Nomoxia
- Hypoxia

* *
Supplemental Figure 4
Supplemental Figure 5

Relative Gene Expression (Gene/GADPH)

- blank
- HIF-1α
- siHIF-1α
- shPHD2 + shFIH

Comparison of gene expression levels under different conditions.

* and ** indicate statistical significance.
Supplemental Figure 6
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<th>Week 1</th>
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<tbody>
<tr>
<td>shScramble</td>
<td>shFIH</td>
<td>shPHD2</td>
<td>shPHD2 + shFIH</td>
<td>shScramble</td>
<td>shFIH</td>
<td>shPHD2</td>
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**Supplemental Figure 9**
Supplemental Figure 12

A

B

shScramble  shFIH  shPHD2  shPHD2 + shFIH

Tunnel Number/mm²

0  50  100  150

shScramble  shFIH  shPHD2  shPHD2 + shFIH

*  **
Supplemental Figure 13

- **shScramble**
- **shPHD2**
- **shFIH**
- **shPHD2 + shFIH**

**Protein bands:**
- **HIF-1α**
- **HIF-2α**
- **β-actin**