Short Hairpin RNA Interference Therapy for Ischemic Heart Disease

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Background—During hypoxia, upregulation of hypoxia inducible factor-1 alpha transcriptional factor can activate several downstream angiogenic genes. However, hypoxia-inducible factor-1 alpha is naturally degraded by prolyl hydroxylase-2 (PHD2) protein. Here we hypothesize that short hairpin RNA (shRNA) interference therapy targeting PHD2 can be used for treatment of myocardial ischemia and this process can be followed noninvasively by molecular imaging.

Methods and Results—PHD2 was cloned from mouse embryonic stem cells by comparing the homolog gene in human and rat. The best candidate shRNA sequence for inhibiting PHD2 was inserted into the pSuper vector driven by the H1 promoter followed by a separate hypoxia response element-incorporated promoter driving a firefly luciferase reporter gene. This construct was used to transfect mouse C2C12 myoblast cell line for in vitro confirmation. Compared with the control short hairpin scramble (shScramble) as control, inhibition of PHD2 increased levels of hypoxia inducible factor-1 alpha protein and several downstream angiogenic genes by >30% (P<0.01). Afterward, shRNA targeting PHD2 (shPHD2) plasmid was injected intramyocardially following ligation of left anterior descending artery in mice. Animals were randomized into shPHD2 experimental group (n=25) versus shScramble control group (n=20). Bioluminescence imaging detected plasmid-mediated transgene expression for 4 to 5 weeks. Echocardiography showed the shPHD2 group had improved fractional shortening compared with the shScramble group at Week 4 (33.7%±1.9% versus 28.4%±2.8%; P<0.05). Postmortem analysis showed increased presence of small capillaries and venules in the infarcted zones by CD31 staining. Finally, Western blot analysis of explanted hearts also confirmed that animals treated with shPHD2 had significantly higher levels of hypoxia inducible factor-1 alpha protein.

Conclusions—This is the first study to image the biological role of shRNA therapy for improving cardiac function. Inhibition of PHD2 by shRNA led to significant improvement in angiogenesis and contractility by in vitro and in vivo experiments. With further validation, the combination of shRNA therapy and molecular imaging can be used to track novel cardiovascular gene therapy applications in the future. (Circulation. 2008;118[suppl 1]:S226–S233.)

Key Words: hypoxia inducible factor ■ ischemic heart disease ■ molecular imaging ■ prolyl hydroxylases ■ RNA interference

Coronary artery disease is the leading cause of morbidity and mortality in the Western world. Conventional treatment for coronary artery disease consists of medical therapy as the first-line strategy followed by percutaneous coronary intervention or coronary artery bypass graft. However, a significant number of patients will still have refractory angina despite these treatments. For such patients, the alternative approach of delivering potent angiogenic factors to stimulate new vessel growth has undergone intense investigation over the past decade. With the use of various gene transfer techniques, it is now possible to modify cardiac cells to overexpress beneficial proteins or inhibit pathological proteins and achieve desired therapeutic effects. The field has expanded tremendously from preclinical studies in the early 1990s to large randomized clinical trials in the early 2000s. Although initial Phase 1 trials in patients with myocardial ischemia provided encouraging results, recent Phase 2 randomized trials (AGENT, VIVA, KAT) yielded only modest benefits. These inconsistencies have been attributed to the unclear role of single therapeutic genes such as vascular endothelial growth factor or fibroblast growth factor as well as the inability to monitor gene transfer in vivo. Newer approaches based on the upstream transcriptional factor hypoxia inducible factor-1 alpha (HIF-1α) may be a more natural choice. HIF-1α is known to control the expression of over 60 genes that affect cell survival and

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metabolism in adverse conditions, including vascular endothelial growth factor, fibroblast growth factor, insulin-like growth factor, erythropoietin, and nitric oxide synthase among others. Unfortunately, HIF-1α has a biological half-life of only approximately 5 minutes under normoxic conditions. This is because during normoxic condition, HIF-1α is hydroxylated by oxygen-dependent prolyl hydroxylase-2 (PHD2), ubiquitinated, and subsequently degraded. In this article, we demonstrated that the inhibition of HIF-1α degradation through short hairpin RNA (shRNA) knockdown of PHD2 in the ischemic heart represents a novel angiogenic therapy approach. At the same time, we tracked the shRNA vector in vivo through molecular imaging technology.

Materials and Methods

RNA Interference of Mouse Prolyl Hydroxylase-2 Gene in Culture Cell

Mouse PHD2 gene was cloned from mouse embryonic stem cell after comparing human and rat homolog genes. We designed 4 sequences of RNA interference sites. The targeting sequences are shown in Figure 1A. Sequence for the short hairpin scramble (shScramble) antisense is TGTGAGGAACTTGAGATCT (control). Construction of the H1 promoter driving sense and antisense, respectively, was performed as described. The fragment No 2 knocking down site was inserted after H1 promoter in the vector pSuper as described in the Oligoengine manual.

Cell Culture, Short Hairpin RNA Transfection, and Hypoxia Exposure

Mouse C2C12 myoblasts were cultured in DMEM medium (high glucose) supplemented with 10% fetal bovine serum as described in the ATCC protocol. The sense and antisense fragments of mouse PHD2 driven by the H1 promoter were cotransfected into C2C12 with the plasmid pCMV-firefly luciferase (Fluc) as control for equal transgene expression for that mouse on that particular day. Animal Surgery to Induce Myocardial Infarction

Ligation of the mid left anterior descending artery was performed in adult female FVB mice (Charles River Laboratories, Wilmington, Mass) by a single experienced surgeon (G.H.). Myocardial infarction was confirmed by myocardial blanching and electrocardiographic changes. After waiting for 10 minutes, animals were then injected intramyocardially with 25 μg of shRNA plasmid at the peri-infarct zone (n=20) or 25 μg of shScramble plasmid (n=20) as control. In both groups, the volume of injection was 50 μL using a 31-gauge Hamilton syringe. Study protocols were approved by the Stanford Animal Research Committee.

Optical Bioluminescence Imaging of Plasmid Gene Expression

Cardiac bioluminescence imaging was performed with the Xenogen In Vivo Imaging System (Alameda, Calif). After intraperitoneal injection of the reporter probe D-luciferin (200 mg/kg body weight), animals were imaged for 1 to 10 minutes. The same mice were scanned repetitively for 4- to 10-week period according to the specific study design. Bioluminescence signals were quantified in maximum photons per second per centimeter squared per steradian (p/s/cm2/sr) as described. Briefly, after anesthetic induction with 2% isoflurane, reporter probe D-luciferin (Promega) was injected into the peritoneal cavity. The animals were immediately placed in a light-tight chamber and baseline gray-scale body-surface images were taken. Afterward, photons emitted from Fluc-luciferin photochemical reaction within the animal were acquired repetitively (1- to 10-minute acquisition time per image, 5 to 15 images per animal) until peak value was confirmed. We then averaged the 3 images with the highest p/s/cm²/sr values and used that to represent the Fluc transgene expression for that mouse on that particular day.

Validation of In Vivo Bioluminescence Imaging With Ex Vivo Enzyme Assays

A subset of the animals (n=5) were injected with varying doses of the shPHD2 plasmid (5, 10, 15, 20, and 25 μg). Animals were euthanized immediately after bioluminescence imaging. Different organs (heart, lungs, liver, kidney, and spleen) were excised and placed in 6-well plastic dishes containing D-luciferin (100 μmol/L). Ex vivo bioluminescence counts were determined. Afterward, these organs were homogenized and luciferase enzyme assays performed using a luminometer (Turner Design-20/20) as previously described.

Analysis of Left Ventricular Function With Echocardiogram

Echocardiography was performed before (Day −7) and after (Week 2, Week 4, Week 8) the left anterior descending artery ligation. The
Siemens-Acuson Sequoia C512 system equipped with a 14.7-MHZ transducer was used by an investigator (Z.L.) blinded to group designation. Left ventricular end diastolic diameter and end systolic diameter were measured and used to calculate left ventricular fractional shortening by the formula: LVFS=[(EDD−ESD)/EDD], in which LVFS is left ventricular fractional shortening, EDD is end diastolic diameter, and ESD is end systolic diameter.

Histological Examination
Explanted hearts from study and control groups were embedded into OCT compound (Miles Scientific, Elkhart, Ind). Frozen sections (5 μm thick) were processed for immunostaining. To detect microvascular density in the peri-infarct area, a rat anti-CD31 (BD Pharmingen) was used. The number of capillary vessels was counted by a blinded investigator (X.X.) in 10 randomly selected areas using a light microscope (×200 magnification). Additional samples were used to examine the infarction size by Masson’s trichrome staining.

Statistical Analysis
Analysis of variance and repeated-measures analysis of variance with post hoc testing as well as the 2-tailed Student t test were used. Differences were considered significant at probability values of <0.05. Unless specified, data are expressed as mean±SD.

Statement of Responsibility
The authors had full access to and take full responsibility for the integrity of the data. All authors have read and accept the manuscript as written.

Results

Mouse Prolyl Hydroxylase-2 Gene Isolation and Knocking Down in Culture Cells
Based on the reported nucleotide sequence of PHD2 gene in rats and humans (www.genebank), we isolated the PHD2 DNA clone from mouse embryonic stem cell (Sv129 line). We designed 4 small interfering RNA sites (Figure 1A) using commercially available web-based software (www.ambion.com). To determine the site that possesses the optimal knocking-down efficiency, we cloned the sense and antisense downstream of the H1 promoter, respectively, by polymerase chain reaction (Supplement Figure 1A). These 4 shRNA constructs were used to transfet C2C12 myoblasts in 6-well plates along with pCMV-luciferase plasmid used to confirm for equal transfection efficiency (data not shown). After 48 hours of cell culture, mRNA levels of PHD2 within C2C12 cells were measured by reverse transcriptase–polymerase chain reaction. Using the densitometric analysis software, Site 2 and Site 3 inhibition could degrade 50% to 60% of the mouse PHD2 mRNA, which were significantly better than Site 1 (15% to 25%) and Site 4 (20% to 30%; Supplement Figure 1B).

In Vitro Characterization of Short Hairpin Prolyl Hydroxylase-2 Under Nomoxia and Hypoxia Conditions
To achieve in vivo inhibition by nonviral transfection, we constructed plasmid targeting PHD2 (shPHD2) by inserting the short hairpin structure downstream of the H1 promoter in a pSuper vector. A hypoxia sensing 5×hypoxia response element (HRE)-SV40 promoter driving Fluc cassette was also inserted into the backbone of pSuper vector. The 5 copies of HRE derived from the erythropoietin gene are activated through binding of the HIF-1 complex and thus allow us to monitor the efficacy of the upstream shPHD2 knockdown compared with the upstream shScramble control (Figure 1B). In the normoxic condition, cells transfected with shPHD2 had significantly higher Fluc bioluminescence signals compared with cells transfected with shScramble control, indicating increased binding of 5×HRE-SV40 promoter by HIF-1α after shPHD2 knockdown (Figure 2A). As expected, a similar but more robust trend was observed when the cells were exposed to the hypoxic condition. To confirm the imaging signals, nuclear extracts were isolated and Western blot analysis performed for detection of HIF-1α protein. As shown in Figure 2B, robust HIF-1α stabilization was observed after exposure to hypoxia in shPHD2 transfected cells. The protein level was increased up to 50% after shPHD2 transfection. Upregulation of the HIF-1α pathway has been shown to activate several downstream genes responsible for stimulation of angiogenesis. To examine if upregulation of HIF-1α by shRNA knockdown of PHD2 can exert similar effects, total RNAs were extracted from C2C12 cells transfected with shPHD2. As shown in Figure 2C, 6 common genes related to angiogenesis were increased by approximately 30% after shPHD2 treatment. Thus, both physiological hypoxia and PHD2 knockdown can effectively stabilize HIF-1α and induce HIF-1α-dependent gene activation in cell cultures.

Correlation of Imaging Signals With Cell Numbers, Enzyme Assays, and Reverse Transcriptase–Polymerase Chain Reaction
To determine the validity of in vivo bioluminescence imaging with more conventional ex vivo assays, we first transfected different numbers of mouse C2C12 myoblasts (0.375×10⁶ to 6×10⁶) with 4 μg of shRNA plasmid in 6-well plates. As shown in Figure 3A, the bioluminescence signals correlated robustly with in vitro Fluc enzyme activity (r²=0.99) expressed as relative light unit per microgram protein. Next, a subset of the animals (n=5) were injected with different doses of the shPHD2 plasmid (5 to 25 μg). At 1 week, bioluminescence signals were detectable in the heart, which also correlated robustly with the ex vivo Fluc enzyme activity (r²=0.96). Finally, to determine the plasmid biodistribution after intramyocardial delivery, we explanted different organs from these animals. With this experimental setup, both bioluminescence imaging and reverse transcriptase–polymerase chain reaction analysis demonstrate the presence of Fluc transgene expression in the heart, liver, and lung but not spleen and kidney (Figure 3C).

Tracking Short Hairpin Prolyl Hydroxylase-2 Vector Using Bioluminescence Imaging in Living Animals
Previously, Natarajan and colleagues have demonstrated the feasibility of small interfering RNA therapy for attenuating myocardial ischemia reperfusion injury. However, subsequent analysis showed that the actual knockdown target was to murine procollagen prolyl 4-hydroxylase-2 rather than HIF prolyl 4-hydroxylase-2. Here we confirmed our selection target with the GenBank database. Instead of using small interfering RNA fragments, which are only stable in vivo for
72 hours, we selected shRNA plasmid. However, at present, the duration of shRNA-mediated expression is unknown. Thus, we incorporated the 5×HRE-SV40-driving Fluc gene to track the shRNA expression activity. To evaluate the pharmacokinetics of shRNA in vivo, we injected the 2 shRNA plasmids into mice with myocardial infarction and followed their gene expression by Fluc bioluminescence imaging (Figure 4A). As expected, mice injected with shPHD2 plasmid (bottom row) had significantly higher Fluc activity compared with mice injected with shScramble plasmid (top row). This can be attributed to the efficient knockdown of PHD2, resulting in more HIF-1α protein binding to the 5×HRE-Sv40 promoter site. For control animals injected with shScramble, endogenous activation of HIF-1α after myocardial infarction led to visible but lower Fluc signals. Quantitative analyses of Fluc activities for both groups are shown in Figure 4B. Overall, infarcted animals had significantly higher activation of Fluc compared with noninfarcted animals during the first 2- to 4-week period.

Injection of Short Hairpin Prolyl Hydroxylase-2 Plasmid Improved Left Ventricular Ejection Function

To examine whether shPHD2 therapy can also improve cardiac function after myocardial infarction, echocardiography was performed before (Day −7) and after (Week 2, Week 4, Week 8) the left anterior descending artery ligation. At Day −7, there was comparable LVFS between the shPHD2 group and shScramble control group. After left anterior descending artery ligation, the shPHD2 group had significantly higher LVFS ($p=0.03$) compared with the
shScramble control group at Week 2 and Week 4 (Supplement Figure IIA). However, this beneficial effect was no longer maintained by week 8 (shPHD2: 38.3 ± 3.8% versus shScramble: 36.8 ± 2.1%; P = 0.23). This is likely due to the limited short-term expression of plasmid-mediated shRNA expression within the first 4 weeks only, as shown by our imaging results (Supplement Figure IIB). To confirm the functional imaging data, trichrome staining showed less infarction size for the shPHD2 group compared with the shScramble group at Week 4. Immunohistochemistry of the peri-infarct region by CD31 staining also showed more neovascularization for the shPHD2 group compared with the shScramble group (Supplement Figure III).

**Short Hairpin Prolyl Hydroxylase-2 Knockdown Mediates Hypoxia Inducible Factor-I Alpha Upregulation in Myocardial Tissues**

To further confirm the in vivo imaging data, we assayed for HIF-1α protein expression of explanted hearts at Day 1, Day 4, Day 7, Day 14, and Day 28 after shPHD2 plasmid therapy (Figure 5A). Quantitative analysis of the Western blot indicates that HIF-1α proteins were significantly higher in the shPHD2-treated hearts compared with shScramble-treated hearts starting at Day 1. Protein levels peaked at Day 14 and returned back to baseline levels by Week 4 (Figure 5B).

**Discussion**

In this article, we describe a novel shRNA therapy method, which can also be tracked by noninvasive molecular imaging in a murine model of myocardial infarction. The major findings can be concluded as follows: (1) shRNA can be expressed consistently with two H1 promoters driving sense and antisense fragment, respectively. The sense and antisense fragment anneal automatically in cytoplasm to exert their knocking down effects; (2) downregulation of the mouse PHD2 gene by plasmid-mediated shRNA interference (shPHD2) leads to activation of downstream angiogenic genes and proteins involved in the hypoxia response pathway...
as assessed by both in vitro and in vivo assays; (3) direct injection of shRNA targeting PHD2 can improve ventricular function and enhance neoangiogenesis in a mouse model of myocardial infarction during 4-week follow-up; (4) importantly, the pharmacokinetics of shRNA plasmid delivery can be monitored noninvasively in living subjects by a novel 5×HRE-SV40 binding site driving Fluc reporter gene; (5) intramyocardial delivery of plasmid can lead to extracardiac leakage and expression of Fluc transgene activity in other organs such as the liver and lung; and (6) finally, a time-dependent decrease of Fluc signal activity was observed within a 4-week period due to plasmid degradation, which likely explains the loss of cardiac functional recovery at 8-week follow-up.

RNA interference is an innate biological phenomenon that has evolved during mammalian evolution.15 Biologically, RNA interference has an important role for the transient and long-term blocking protein expression. It is achieved by
loading the RNA interference silencing complex with a short single-stranded antisense RNA that is complementary to a target mRNA. In this study, we selected PHD2 as the knockdown target. PHD2 is an upstream negative regulatory gene in HIF-1 pathway. During hypoxia, when HIF-1α is stabilized, HIF-1 mediates transcriptional responses by binding to HREs present on a series of target genes involved in metabolic adaptation, hematopoiesis, angiogenesis, and apoptosis. Under normal oxygenated conditions, HIF-1α is hydroxylated on 2 conserved proline residues, proline 402 or proline 564, by a family of prolyl-4-hydroxylases. Several studies have demonstrated that prolyl-4-hydroxylase inhibition recapitulates various cellular and physiology responses to hypoxia or preconditioning stimuli. These include HIF-1α stabilization, the induction of hypoxia inducible genes (eg, HO-1 and GLUT-1), stimulation of angiogenesis, and protection against metabolic stress. Importantly, recent evidence suggests that the expression of a single angiogenic factor such as vascular endothelial growth factor alone may not be sufficient for the functional revascularization of ischemic tissues. Based on those previous studies, HIF-1 plays a critical role in a variety of physiological processes, and upregulation of HIF-1α through PHD2 knockdown represents a potentially new target in the field of cardiovascular gene therapy.

In this study, we were able to track the HIF-1α upregulation through a novel noninvasive molecular imaging approach, avoiding the sampling biases and errors that may occur when different groups of animals are euthanized at different time points. Five×HRE-SV40 promoter was inserted in front of the Fluc reporter gene. This hypoxia sensing construct can reflect the effects of shRNA plasmid expression through HIF-1α binding to the HRE element. For in vivo imaging signals, the plasmid expression reached peak activities between Week 1 and Week 2 (Figure 4A). These results concur with the Western blot data of explanted hearts shown in Figure 5A, which indicate that the HIF-1α activity (up-regulated by shPHD2 knockdown) also increased during Week 1 to Week 2 and became degraded by Week 4. Furthermore, the echocardiographic data showed improvement of heart function within the first month, confirming the Western blot and molecular imaging results. However, we also observed a time-dependent decrease of bioluminescence signal activity within this time period, indicating a loss of the shRNA plasmid. Thus, adoption of newer vectors that are less immunogenic such as minicircles or adeno-associated virus may prolong gene expression and provide a more persistent functional recovery.

In summary, nonviral gene therapy through shRNA is a rapidly evolving area of investigation. With further validation, knocking down one or more regulatory factors involved in angiogenesis pathways as described here could provide a new avenue for treating myocardial ischemia. Furthermore, we believe molecular imaging can be a valuable tool in monitoring the localization and activity of the shRNA vectors used for cardiovascular therapy. The in vivo information gathered is already generating useful insights and will enable better understanding of shRNA activity and mechanism in living subjects.

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Disclosures
None.

References


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Supplement Figure 1

A

Tandem Type

H1 promoter  Sense  TTTTT

H1 promoter  Antisense  TTTTT

Transaction, Hybridization

Sense

Antisense

siRNA duplex

B

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