Noninvasive Imaging of Hypoxia-Inducible Factor-1 α Gene Therapy for Myocardial Ischemia

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Abstract

Hypoxia-inducible factor-1 alpha (HIF-1 α) gene therapy holds great promise for the treatment of myocardial ischemia. Both preclinical and clinical evaluations of this therapy are underway and can benefit from a vector strategy that allows noninvasive assessment of HIF-1 α expression as an objective measure of gene delivery. We have developed a novel bidirectional plasmid vector (pcTnT-HIF-1 α -VP2-TSTA-fluc), which employs the cardiac troponin T (cTnT) promoter in conjunction with a two-step transcriptional amplification (TSTA) system to drive the linked expression of a recombinant HIF-1 α gene (HIF-1 α -VP2) and the firefly luciferase gene (fluc). The firefly luciferase (FLuc) activity serves as a surrogate for HIF-1 α -VP2 expression, and can be noninvasively assessed in mice using bioluminescence imaging after vector delivery. Transfection of cultured HL-1 cardiomyocytes with pcTnT-HIF-1 α -VP2-TSTA-fluc led to a strong correlation between FLuc and HIF-1 α -dependent vascular endothelial growth factor expression ($r^2=0.88$). Intramyocardial delivery of pcTnT-HIF-1 α -VP2-TSTA-fluc into infarcted mouse myocardium led to persistent HIF-1 α -VP2 expression for 4 weeks, even though it improved neither CD31+ microvessel density nor echocardiographically determined left ventricular systolic function. These results lend support to recent findings of suboptimal efficacy associated with plasmid-mediated HIF-1 α therapy. The imaging techniques developed herein should be useful for further optimizing HIF-1 α -VP2 therapy in preclinical models of myocardial ischemia.

Introduction

PAST EFFORTS IN GENE THERAPY for myocardial ischemia have primarily focused on the development of a single therapeutic gene capable of inducing therapeutic angiogenesis (Henry *et al.*, 2007; Giacca and Zacchigna, 2012). Recent evidence, however, suggests that overexpression of a single angiogenic factor alone may not be sufficient for the functional revascularization of ischemic tissue (Banfi *et al.*, 2012). Newer approaches based on hypoxia-inducible factor-1 alpha (HIF-1 α), a transcriptional activator of multiple genes involved in angiogenesis, have shown promise in reducing infarct size and increasing myocardial perfusion in animal models of myocardial ischemia (Shyu *et al.*, 2002; Heintz-Green *et al.*, 2005; Huang *et al.*, 2008). As HIF-1 α continues to be tested in the laboratory setting, as well as in clinical trials, the development of an imaging technique to noninvasively

monitor the locations, magnitude, and persistence of HIF-1 α expression should greatly facilitate and objectify the assessment of HIF-1 α gene therapy.

Currently, no single imaging probe exists for *direct imaging* of HIF-1 α transgene expression in living subjects. However, we have previously validated various *indirect imaging* approaches for monitoring transgene expression after vector delivery (Hiona and Wu, 2008). These techniques rely on building a vector construct capable of coupling the expression of the therapeutic gene to that of a reporter gene (e.g., firefly luciferase, fluc), whose expression can be feasibly imaged using the appropriate imaging probe and modality (e.g., bioluminescence imaging [BLI]). Semi-quantitative imaging of reporter activity, therefore, allows accurate inference on the therapeutic gene expression.

Two coupling strategies, namely, the internal ribosomal entry site (IRES)-based and the dual promoter-based

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strategies, have been validated for indirect imaging of transgene expression in the myocardium (Chen *et al.*, 2004; Wu *et al.*, 2004). Their utility, however, is for the most part limited by their potential to lead to uneven expression of the two linked genes (Yu *et al.*, 2003). Recently, we have developed a novel bidirectional two-step transcriptional amplification (TSTA) vector strategy, which can achieve high levels of balanced, correlated transgene expression, even when a weak tissue-specific promoter is used for transcriptional regulation (Ray *et al.*, 2008). Such a vector strategy maximizes both therapeutic efficacy and the sensitivity for indirect gene imaging, and has the potential to mediate transcriptionally targeted gene therapy as previously described (Chen *et al.*, 2010).

In the present study, we report the first validation of this vector strategy for the delivery and noninvasive imaging of HIF-1 α therapy in mouse models of myocardial ischemia. We specifically constructed and characterized a bidirectional vector (pcTnT-HIF-1 α -VP2-TSTA-fluc), which uses the cardiac troponin T (cTnT) promoter in conjunction with a TSTA system to simultaneously enhance the expression of a recombinant, constitutively active HIF-1 α (HIF-1 α -VP2) (Gheysens *et al.*, 2011) gene and the fluc gene (Fig. 1). Using BLI, we indirectly assessed the kinetics of myocardial HIF-1 α -VP2 expression after vector delivery, as well as the persistence of HIF-1 α signal transduction via HIF-1 α -VP2's binding to the hypoxia response element (HRE). By performing serial BLI and echocardiography, as well as postmortem immunohis-

tochemical (CD31) staining on myocardial tissue, we aimed to investigate the effects of HIF-1 α -VP2 transgene expression on both left ventricular systolic function and local angiogenesis after experimental myocardial infarction (MI) and pcTnT-HIF-1 α -VP2-TSTA-fluc vector delivery.

Materials and Methods

Construction of plasmid vectors

pcDNA3.1(+) (Invitrogen, Carlsbad, CA), pCEP4/HIF-1 α (ATCC, Johns Hopkins Special Collection, Manassas, VA), pIRES-EGFP (Clontech, MountainView, CA), pCMV-hRL (Promega, Madison, WI), pBCVP2-G5L (Zhang *et al.*, 2002), pcTnT-bid-TSTA (Chen *et al.*, 2010), and p5X-HRE-fluc (Huang *et al.*, 2008) were used as starting vectors for the construction of other vectors. An intermediate vector containing a cytomegalovirus (CMV) promoter driving the expression of two tandem repeats of herpes simplex VP16 transcriptional activator (VP2) was constructed by PCR amplifying VP2 from pBCVP2-G5L, followed by inserting it into the *Bam*HI/*Xho*I sites of pcDNA3.1(+) downstream of CMV. A truncated human HIF-1 α gene (amino acids 1–390) was then PCR-amplified from the commercially available pCEP4/HIF-1 α vector using sense and antisense primers containing *Nhe*I and *Bam*HI, respectively, and ligated to the *Nhe*I/*Bam*I-digested pCMV-VP2 to yield another immediate vector, pCMV-HIF-1 α -VP2. pcTnT-HIF-1 α -VP2-TSTA-fluc

FIG. 1. Schematics of the bidirectional TSTA plasmid vector (pcTnT-HIF-1 α -VP2-TSTA-fluc) for indirect imaging of HIF-1 α -VP2 expression. The experimental vector, pcTnT-HIF-1 α -VP2-TSTA-fluc, uses the cTnT promoter to drive the expression of the GAL4-VP2 fusion protein, which can bind to any of the eight tandem repeats of a GAL4-binding sequence (8xGAL4bs) on the same vector, leading to recruitment of RNA polymerase, followed by enhanced bidirectional transcription of a recombinant hypoxia-inducible factor-1 α (HIF-1 α -VP2) gene and the firefly luciferase (fluc) gene. The translated HIF-1 α -VP2 can subsequently stimulate angiogenesis by activating the expression of downstream angiogenic genes (e.g., VEGF) via binding to their HRE in the promoter region. Given a strong correlation between the expression of HIF-1 α -VP2 and the firefly luciferase enzyme (FLuc), the HIF-1 α -VP2 expression can be inferred from the FLuc enzyme activity, which can be imaged with BLI using a systemically delivered probe, D-Luciferin. Upon entering the cytosol of a transfected cell (yellow background), D-Luciferin interacts with FLuc to emit bioluminescence, which can be detected and quantified using an ultra-sensitive cooled CCD camera. BLI, bioluminescence imaging; cTnT, cardiac troponin T; E4TATA, adenovirus E4 minimal promoter; HRE, hypoxia response element; PA, SV40 poly(A) tail; TSTA, two-step transcriptional amplification; VEGF, vascular endothelial growth factor; VP2, 2 tandem repeats of herpes simplex virus VP16 transcriptional activator. Color images available online at www.liebertpub.com/hgtb

was constructed by PCR-amplifying a fragment containing HIF-1 α -VP2 and BGH poly(A) tail from pCMV-HIF-1 α -VP2 and subcloning it into the *Bgl*II/*Bsu*36I sites of pcTnT-Bid-TSTA in the same orientation as the cTnT promoter. pcTnT-EGFP-TSTA-fluc (a control vector for pcTnT-HIF-1 α -VP2-TSTA-fluc that expresses enhanced green fluorescent protein [EGFP]) was similarly constructed by PCR-amplifying a fragment containing EGFP and SV40 Poly(A) from pIRES-EGFP and subcloning it into the *Bgl*II/*Bsu*36I sites of pcTnT-Bid-TSTA. p5X-HRE-hRLuc was constructed by inserting a *Bgl*II/*Sall*-digested fragment from pCMV-hRL into *Bgl*II/*Sall*-digested p5X-HRE-fluc. pCMV- β -gal (a CMV promoter-driven vector expressing β -galactosidase [β -GAL]) was obtained from Invitrogen.

Cell culture and plasmid transfection

HL-1 mouse cardiomyocytes were cultured in Claycomb Medium (JRH Biosciences, Lenexa, KS) as previously described (Chen *et al.*, 2010). Using lipofectamine 2000 (1 μ l/1 μ g total DNA; Invitrogen), cells were cotransfected with increasing doses of pcTnT-HIF-1 α -VP2-TSTA-fluc (0–1 μ g), a fixed dose of p5X-HRE-hRLuc (1 μ g), as well as pCMV- β -gal (0.4 μ g) and pcDNA3.1(+) (0–1 μ g) to correct for transfection efficiency and total DNA, respectively. Thirty-six hours after transfection, the cell medium was assayed for vascular endothelial growth factor (VEGF), whereas the cells lysed with 1 $\times$ Passive Lysis Buffer (Promega) were assayed for FLuc, *Renilla* luciferase (RLuc), and β -GAL enzyme activities. Cytotoxicity was additionally assessed at 36 hr post-transfection in a repeat transfection experiment.

In vitro protein, reporter, enzyme-linked immunosorbent, and cytotoxicity assays

Cell lysates were assessed for protein content by mixing them with Bio-Rad protein assay reagent (Bio-Rad Laboratories, Hercules, CA) and measuring the light absorbance at 595 nm with Beckman DU640 Spectrometer (Beckman Coulter, Fullerton, CA). FLuc and RLuc activities were assayed by mixing the cell lysates with the appropriate substrates (LARII for FLuc [Promega]; Coelenterazine for RLuc [NanoLight Technology, Pinetop, AZ]) and measuring light emission in relative light unit per minute (RLU/min) using a TD 20/20 luminometer (Turner Designs, Sunnyvale, CA) as previously described (Chen *et al.*, 2010). β -GAL activity was assessed using the β -galactosidase Enzyme Assay (Promega) according to the manufacturer's protocol, with the exception that 15 μ g of the lysate was used per reaction and the substrate incubation time was extended from 30 min to 1 hr. Both FLuc and RLuc activities were normalized to total protein and β -GAL activity (light absorbance at 420 nm), and represented herein as RLU/min/ μ g/ β -GAL activity. Secretory VEGF was quantified using the Mouse VEGF Quantikine ELISA Kit (R&D, Minneapolis, MD) according to the manufacturer's protocol. Cytotoxicity was assessed using the alamarBlue Cell Viability Reagent (Invitrogen) following the manufacturer's protocol.

Animals and study design

All animal studies were approved by the Stanford Institutional Animal Care and Use Committee (IACUC). Five

groups of 7–8-week-old female Balb/c mice ($n=39$ total) were obtained from the Stanford In-house Breeding Colony. Group 1 ($n=5$) underwent intramyocardial injection of a DNA mixture containing 80 μ g pcTnT-HIF-1 α -VP2-TSTA-fluc and 80 μ g p5X-HRE-hRLuc, followed by serial BLI for 4 weeks to assess both FLuc expression and HIF-1 α -VP2-mediated induction of RLuc expression. Group 2 ($n=5$) underwent the same procedures as group 1 except that a mixture of 80 μ g pcDNA3.1(+) and 80 μ g p5X-HRE-hRLuc was injected. Group 3 ($n=9$) underwent intramyocardial injection of a DNA mixture containing varying doses (80, 60, and 40 μ g) of pcTnT-HIF-1 α -VP2-TSTA-fluc, 80 μ g p5X-HRE-hRLuc, and enough empty vector pcDNA3.1(+) to control for the total amount of plasmid injected. Afterward, mice were euthanized on day 2, with their explanted hearts assayed for FLuc and RLuc enzyme activities. Group 4 ($n=11$) underwent induction of MI by permanent ligation of the left anterior descending (LAD) artery, followed by three injections of pcTnT-HIF-1 α -VP2-TSTA-fluc (total 240 μ g; experimental vector) into the peri-infarct regions, and then serial BLI afterward for 4 weeks to track FLuc expression. Group 5 ($n=9$) underwent the same procedures as group 4 except with injections of pcTnT-EGFP-TSTA-fluc (total 240 μ g; control vector). Echocardiography was performed for groups 4 and 5 at baseline (3 days before MI) and on days 3, 14, and 28 post-MI to assess left ventricular fractional shortening (LVFS%) and left ventricular ejection fraction (LVEF%). After the last echocardiographic or BLI scan, five mice each from groups 4 and 5 were euthanized, with their hearts explanted for histological analysis.

Animal surgery, MI induction, and plasmid vector delivery

Mice were anesthetized with 3% isoflurane, orotracheally intubated, and ventilated with 2% isoflurane using a Harvard mouse ventilator (Harvard Apparatus, Holliston, MA). After left thoracotomy and opening of the pericardium, mice in groups 1, 2, and 3 received two 15 μ l injections of DNA plasmids at one site near the base of the left ventricle, whereas mice in groups 4 and 5 underwent LAD ligation just distal to the level of the left atrium to induce MI as previously described (Li *et al.*, 2007), followed by three 15 μ l injections of DNA plasmids at three separate sites in the peri-infarct regions to maximize tissue coverage. Afterward, the chest was closed in layers, with pneumothorax evacuated by suction using a 16G angiocath (BD Biosciences, San Jose, CA). Mice received buprenorphine (0.1 mg/kg) SQ for postoperative analgesia.

BLI of HIF-1 α -VP2 expression in living mice

Mice underwent BLI of either RLuc or FLuc expression using the *In Vivo* Imaging System (IVIS 100; Xenogen, Alameda, CA) as previously described (Chen *et al.*, 2010). Briefly, after anesthesia with 2% isoflurane, mice injected with coelenterazine IV (2.5 mg/kg) were imaged immediately afterward for 4 min to assess for RLuc expression, whereas mice injected with D-Luciferin IP (375 mg/kg; Biosynth International, Naperville, IL) received four to six 5 min scans (until the imaging signal peaks) to assess for FLuc expression. Region of interest (ROI) analysis using Living Image 2.50 (Xenogen) was performed by placing a fixed-size,

default ROI over the heart region to quantify the peak FLuc signal in terms of total flux (photons per second; p/sec) and the RLuc signal in terms of maximum radiance (photons per second per centimeter squared per steradian; p/sec/cm²/sr).

Echocardiographic assessment of MI and left ventricular systolic function

Echocardiographic examination was performed by an independent, experienced operator (Z.L.) blinded to the study using a multifrequency (8–14 MHz) 15L8 transducer coupled to the Siemens-Acuson Sequoia C512 system (Siemens, Malvern, PA). Both short-axis and parasternal long-axis images were obtained, and analyzed using the Siemens built-in image analysis software. M-mode traces were obtained at the papillary muscle level. LVFS% was calculated from the LV dimensions averaged over three consecutive cardiac cycles in a short-axis view as previously described (Li *et al.*, 2007). LVEF% was calculated from the LV dimensions obtained in both short-axis and parasternal long-axis views using the Bullet method as previously described (Nemoto *et al.*, 2002).

Tissue harvest and ex vivo reporter enzyme assays

Whole hearts with aortopulmonary vessels disconnected and atrial appendages removed were rinsed in phosphate buffered saline, immersed in 1 ml of 1×Passive Lysis Buffer (Promega), and homogenized using a Kontes Dual tissue grinder (Fisher Scientific, Pittsburgh, PA). Supernatants obtained by centrifugation were assessed for protein content, FLuc activity, and RLuc activity as previously described for cell lysates. Tissue FLuc and RLuc enzyme activities were normalized to total protein and expressed in units of RLU/min/μg.

Immunohistochemical CD31 staining of peri-infarct microvasculature

Explanted hearts were embedded in optimal cutting temperature (OCT; Sakura Finetek, Torrance, CA) and rapidly frozen in nitrogen-cooled isopentane (Sigma-Aldrich, St. Louis, MO). Ten-micrometer frozen sections obtained from nine consecutive, equally spaced (50 μm) levels spanning the entire infarct zone were stained for microvasculature using rat anti-mouse CD31 primary antibody (1:200; BD Biosciences, San Diego, CA), biotinylated goat anti-rat secondary antibody (1:500; Jackson ImmunoResearch Laboratories, West Grove, PA), followed by AlexaFluor594-conjugated streptavidin (Invitrogen) for visualization with fluorescence microscopy. All images were captured at high-power field (HPF; 200×) using an integrated fluorescence/brightfield microscope (Axiophot; Carl Zeiss, Thornwood, NY) connected to a digital camera (AxioCam MRC; Carl Zeiss, Bernried, Germany). Microvessel density was assessed using ImageJ (NIH, Bethesda, MD) by counting the number of CD31-positive microvessels per HPF, averaged over 27 randomly selected fields in the peri-infarct regions (3 fields/level).

Statistical analysis

All data are presented as mean ± standard error of mean (SEM). The unpaired, two-tailed Student's *t*-test was used to compare two measurements. Linear regression analysis was

performed to assess the linearity between two variables. The strength of correlation was quantified in terms of the square of Pearson product moment coefficient (r^2). The significance of correlation was calculated by performing the Student's *t*-test against the null hypothesis that the correlation coefficient (*r*) is zero. For *in vivo* BLI experiments involving animal groups 1 and 2, differences in FLuc and RLuc signals over time were tested by performing two-factor mixed-effects random-intercept regressions on the log of the bioluminescence signal, with fixed factors of group, day, day squared, and random factor of animal. For BLI experiments involving mouse groups 4 and 5, the equivalence of bioluminescence signal between MI groups receiving different vectors (pooled over all days) was tested by conducting a Westlake equivalence test of means using the log of the bioluminescence signal. All statistical analyses were performed with either GraphPad Prism (GraphPad Software, San Diego, CA) or Stata Release 9.2 (StataCorp LP, College Station, TX). *p*-values less than 0.05 were considered statistically significant.

Results

pcTnT-HIF-1α-VP2-TSTA-fluc led to robust correlated FLuc and HIF-1α-VP2-dependent VEGF expression in cell culture

To characterize pcTnT-HIF-1α-VP2-TSTA-fluc in cell culture by directly assessing its FLuc expression and the ability of the expressed HIF-1α-VP2 to activate downstream angiogenic gene expression (e.g., VEGF) via binding to HRE, we co-transfected HL-1 cardiac cells with increasing doses of pcTnT-HIF-1α-VP2-TSTA-fluc and a fixed dose of the reporter plasmid p5X-HRE-hRLuc, which contains five tandem repeats of HRE upstream of a minimal promoter to drive the expression of RLuc. pCMV-β-gal and pcDNA3.1(+) were also co-transfected during the experiment to correct for transfection efficiency and total plasmid dose, respectively. Expectedly, both FLuc and RLuc activities of the cell lysates 36 hr post-transfection increased linearly with plasmid dose up to 1 μg (FLuc: $r^2 = 0.94$, $p < 0.002$ [Fig. 2A]; RLuc: $r^2 = 0.98$, $p < 0.0002$ [Fig. 2B]). Similarly, the VEGF concentration of cell medium exhibited a linear, dose-dependent increase over the same range of plasmid dose ($r^2 = 0.93$, $p < 0.002$; Fig. 2C), reaching 1.5-fold over baseline at 1 μg plasmid while maintaining a strong correlation with the FLuc activity ($r^2 = 0.88$, $p < 0.006$; Fig. 2D).

To assess whether the overexpression of the VP16 transactivation domain from both HIF-1α-VP2 and Gal4-VP2 can lead to cytotoxicity, the same dose-escalating transfection experiment described above was repeated. Thirty-six hours after transfection, no adverse impact on cell proliferation, as assessed by the alamarBlue assay, was observed over the dose range of pcTnT-HIF-1α-VP2-TSTA-fluc tested ($p > 0.05$; Fig. 2E).

Significant HIF-1α-VP2 expression and signaling were achieved after intramyocardial delivery of pcTnT-HIF-1α-VP2-TSTA-fluc

To study the kinetics of HIF-1α-VP2 expression and the activation of the HIF-1α/HRE pathway after intramyocardial injection of pcTnT-HIF-1α-VP2-TSTA-fluc, we performed serial BLI for 4 weeks on experimental mice co-injected with

FIG. 2. *In vitro* characterization of pcTnT-HIF-1 α -VP2-TSTA-fluc. HL-1 cells co-transfected with increasing doses of pcTnT-HIF-1 α -VP2-TSTA-fluc, fixed doses of both p5X-HRE-hRLuc and pCMV- β -gal, as well as appropriate doses of pcDNA3.1(+) (empty vector) were assayed for FLuc, RLuc, and β -GAL activities 36 hr later. Cytotoxicity, in terms of cell proliferation at 36 hr post-transfection, was additionally assessed in a repeat transfection experiment. (A) FLuc and (B) RLuc activities normalized by total protein and transfection efficiency (β -GAL activity) are plotted for the plasmid dose indicated. VEGF from cell medium is plotted against (C) plasmid dose and (D) FLuc activity at the corresponding plasmid dose. (E) Cell proliferation, expressed as a percentage of that at 0 μ g plasmid, is plotted for the plasmid dose shown. The error bars represent SEM for triplicate determinations. SEM, standard error of mean.

pcTnT-HIF-1 α -VP2-TSTA-fluc and p5X-HRE-hRLuc, as well as control mice co-injected with pcDNA3.1(+) and p5X-HRE-hRLuc. The kinetics of both FLuc heart signal (surrogate of HIF-1 α -VP2 expression; Fig. 3A and B) and RLuc heart signal (surrogate of HIF-1 α /HRE signaling; Fig. 3C and D) over the entire study period was found to be statistically different between the two mouse groups ($p < 0.0001$ for both FLuc and RLuc). For both FLuc and RLuc imaging, the signal in the experimental group first increased and then decreased over the study period, whereas the signal in the control group stayed relatively constant. However, the difference in signal between experimental and control mice was greater for FLuc than for RLuc. Although FLuc signal did not correlate as well as expected with the RLuc signal over this time span ($r^2 = 0.33$, $p < 0.002$; data not shown), when additional mice were co-injected with varying doses of pcTnT-HIF-1 α -VP2-TSTA-fluc and a fixed dose of p5X-HRE-hRLuc, the degree of correlation between the postmortem cardiac tissue

FLuc activity and the postmortem cardiac tissue RLuc activity on day 2 was found to be excellent at $r^2 = 0.90$ ($p < 0.0001$; Fig. 3E). The plasmid dose of pcTnT-HIF-1 α -VP2-TSTA-fluc also correlated well with the postmortem cardiac tissue FLuc activity ($r^2 = 0.89$, $p < 0.002$).

Sustained myocardial expression of pcTnT-HIF-1 α -VP2-TSTA-fluc improved neither angiogenesis nor left ventricular systolic function after experimental MI

To investigate whether HIF-1 α -VP2 therapy delivered via pcTnT-HIF-1 α -VP2-TSTA-fluc can lead to improvement in left ventricular systolic function after experimental MI, we performed serial BLI and echocardiography on mice that had undergone both permanent LAD ligation and intramyocardial injection of either pcTnT-HIF-1 α -VP2-TSTA-fluc (experimental vector) or pcTnT-EGFP-TSTA-fluc (control vector). Longitudinal BLI, followed by ROI analysis,

FIG. 3. HIF-1 α -VP2 expression and HIF-1 α signaling after intramyocardial delivery of pcTnT-HIF-1 α -VP2-TSTA-fluc. Experimental mice co-injected with pcTnT-HIF-1 α -VP2-TSTA-fluc and p5X-HRE-hRLuc underwent serial BLI imaging for 4 weeks. Mice co-injected with pcDNA3.1(+) (empty vector) and p5X-HRE-hRLuc served as negative controls. **(A)** The mean FLuc signal (surrogate for HIF-1 α -VP2 expression) and **(C)** the mean RLuc signal (indicator of HIF-1 α /HRE pathway activation) of the experimental (black solid line; EXP) and control (gray solid line; CNTRL) mouse groups are plotted for the days indicated. The error bars represent SEM of five mice. Representative **(B)** FLuc and **(D)** RLuc images of an experimental mouse (EXP) and a control mouse (CNTRL) are displayed for the selected days indicated, with the heart signal and the background hepatic RLuc signal indicated by the red and black arrows, respectively. **(E)** Mice that received intramyocardial co-injections of varying amounts of pcTnT-HIF-1 α -VP2-TSTA-fluc and a fixed amount of p5X-HRE-hRLuc were sacrificed on day 2, with their hearts assayed for both FLuc and RLuc enzyme activities, which are plotted against each other. Color images available online at www.liebertpub.com/hgtb

revealed robust, steady FLuc signal for 4 weeks after vector delivery for both experimental and control mouse groups (Fig. 4A and B). The kinetics (sloping) of the Fluc signal over time was statistically different between the two groups ($p < 0.0001$), although this difference across the study period was very small (6%; $p = 0.012$). At day 28, microscopic examination of the peri-infarct myocardium in both the experimental and the control mice revealed a similar pattern of microvasculature (as indicated by CD31 staining) (Fig. 4C). Image-based quantification of microvessel density in the peri-infarct regions showed no statistical difference between the two groups ($p = 0.2$; Fig. 4D). Serial echocardiography performed at baseline, immediately post-MI (day 3), as well as 2 and 4 weeks post-MI revealed no statistical difference in terms of both LVFS% and LVEF% at any time point between the two mouse groups ($p > 0.08$; Fig. 5A and B). In both groups, both measures of cardiac function declined signifi-

cantly after MI induction ($p < 0.0001$) and recovered minimally for the remainder of the study period.

Discussion

This study represents our ongoing effort to develop a generalizable vector strategy for monitoring therapeutic gene expression after vector delivery. Many of our previous studies have focused on identifying a coupling strategy for indirect imaging that is capable of achieving an optimal balance between the therapeutic gene expression and the linked reporter gene expression (Wu *et al.*, 2004). We have recently validated a bidirectional TSTA strategy capable of performing this task with the added potential benefit of targeting transgene expression to the myocardium using the cTnT promoter (Chen *et al.*, 2010). Here we demonstrate the first successful use of this robust strategy to noninvasively

FIG. 4. Sustained HIF-1 α -VP2 expression failed to improve long-term microvessel density. Mice that underwent experimental MI, followed by intramyocardial injection of either pcTnT-HIF-1 α -VP2-TSTA-fluc (experimental vector) or pcTnT-EGFP-TSTA-fluc (control vector), were serially imaged with BLI for 4 weeks to assess vector expression by inferring from the FLuc-mediated BLI signal. **(A)** The mean FLuc signal over time is plotted for both the experimental (solid black line; EXP) and the control (solid gray line; CNTRL) mouse groups. The error bars represent SEM for 11 experimental mice and 9 control mice. **(B)** Representative bioluminescence images of a control mouse (CNTRL) and an experimental mouse (EXP) are displayed for the selected days indicated, with the heart signals indicated by the red arrows. **(C)** For both mouse groups, the postmortem CD31 immunohistochemical staining of the peri-infarct regions at day 28 highlighted the microvessels (bright red) within the host myocardium (darker red background). **(D)** The mean microvessel density quantified using ImageJ is plotted for both mouse groups. The error bars represent SEM for five mice. Color images available online at www.liebertpub.com/hgtb

image the long-term expression of an angiogenic gene (HIF-1 α -VP2) in a true therapy application.

Theoretically, imaging of HIF-1 α -VP2 expression can be performed either directly using a targeted imaging probe or indirectly via a molecular construct capable of linking the expression of HIF-1 α -VP2 and a surrogate reporter gene. The former approach could not be performed in this study because of the lack of an existing imaging probe for HIF-1 α -VP2, as well as the extended timeframe (months to years) needed to develop one. Instead, the indirect imaging approach was undertaken because of the shorter timeframe (weeks to months) required for the construction of a plasmid vector and the potential for a vector strategy to be generalizable for use with any gene/promoter of choice. The caveat for indirect imaging lies in the requirement for a strong coupling between the expression of HIF-1 α -VP2 and the reporter gene, so that the latter can serve as a surrogate for the former. Such a coupling, however, could not be directly assessed in this study using Western blot analysis because of the lack of a commercial antibody against the HIF-1 α portion of HIF-1 α -VP2. Probing of the VP2 moiety using polyclonal anti-VP16 antibody was attempted, but was found to be suboptimal because of its limited specificity. Nevertheless, using VEGF expression as a surrogate for HIF-1 α -VP2 expression, we were able to demonstrate a strong correlation between the expression of FLuc and VEGF in cell culture

($r^2=0.94$; Fig. 2D). A robust correlation observed between the myocardial tissue FLuc activity and the activation of the HIF-1 α /HRE pathway after pcTnT-HIF-1 α -VP2-TSTA-fluc delivery ($r^2=0.90$; Fig. 3E) further justified the use of our vector for indirect monitoring of HIF-1 α -VP2 expression in living subjects.

The greatest strength of noninvasive molecular imaging, as compared with postmortem histology, lies in its ability to repetitively interrogate molecular events (e.g., transgene expression) in the same subjects. This attribute is useful in the clinical setting when therapeutic vector expression needs to be assessed to determine if further action needs to be taken (e.g., re-injection at a higher dose of vector). Tomographic imaging modalities such as positron emission tomography (PET) and magnetic resonance imaging (MRI) can be useful for delineating the precise three-dimensional location of therapeutic gene expression to ensure successful vector delivery (Genove *et al.*, 2005; Wu and Yla-Herttuala, 2005). In our studies, BLI was likewise useful for assessing the magnitude and duration of HIF-1 α -VP2 transgene expression, and for confirming the presence of HIF-1 α -VP2 signal transduction via the HIF-1 α /HRE pathway (Fig. 3). Using FLuc expression as an indicator for vector expression, we were able to show a small overall difference (6%) in vector expression between the experimental (pcTnT-HIF-1 α -VP2-TSTA-fluc) and the control MI groups (Fig. 4A), which

FIG. 5. Serial echocardiographic assessment of cardiac function pre- and post-MI or pcTnT-HIF-1 α -VP2-TSTA-fluc delivery. (A) The LVFS% (top graph) and the LVEF% (bottom graph) of the experimental (black bar; EXP) and the control (gray bar; CNTRL) MI mouse groups injected with pcTnT-HIF-1 α -VP2-TSTA-fluc and pcTnT-EGFP-TSTA-fluc, respectively, are shown for baseline (3 days before MI) and days 3, 14, and 28 post-MI or vector injection. Error bars represent SEM for 11 experimental mice and 9 control mice. (B) Representative M-mode strips acquired at the levels indicated by the short-axis images displayed above are shown for both a control mouse (top row, CNTRL) and an experimental mouse (bottom row, EXP) at 3 days (left column) and 4 weeks (right column) after MI. Color images available online at www.liebertpub.com/hgtb

partially contributed to the lackluster results on left ventricular systolic function after pcTnT-HIF-1 α -VP2-TSTA-fluc delivery. Although very high levels of VP16 transactivator expression (in the forms of HIF-1 α -VP2 and GAL4-VP2) can theoretically cause transcriptional suppression of FLuc or cytotoxicity in a known “squenching” phenomenon (Gill and Ptashne, 1988), such an occurrence is less likely in our case because our version of the VP16 domain (amino acids 413–454) is known to squelch less effectively than the intact VP16 activation domain (Iyer *et al.*, 2001) and that no gross cytotoxicity was observed in our *in vitro* dose-escalating transfection experiments. Whether the overexpression of HIF-1 α -VP2 can incur significant immune response *in vivo* so as to compromise the long-term expression of pcTnT-HIF-1 α -VP2-TSTA-fluc will need further exploration.

Other reasons for the lack of significant LV functional improvement at 2 and 4 weeks after pcTnT-HIF-1 α -VP2-TSTA-fluc delivery could include the following: First, plasmid-mediated gene delivery might not have been sufficient to induce therapeutic levels of angiogenic gene expression. This was supported by our immunohistochemical CD31 staining, which showed no significant difference in microvessel density between the experimental (pcTnT-HIF-1 α -VP2-TSTA-fluc-injected) and the control groups. A porcine study investigating the efficacy of HIF-1 α -VP16 in chronic ischemia likewise showed no improvement in LV function at 3 weeks for plasmid-mediated gene delivery, further corroborating with our results (Heinl-Green *et al.*, 2005). Second, the therapeutic efficacy of gene therapy in the setting of acute

MI may be limited by the kinetics of vector uptake and transgene expression. Unlike percutaneous coronary interventions that could provide instant flow to ameliorate myocardial ischemia, the use of gene therapy has an intrinsic delay in action with angiogenic gene expression peaking around days 2–5 (Fig. 4), before which the myocardium may continue to experience the deleterious effects of acute ischemia. This could explain why HIF-1 α therapy would be effective in the setting of chronic ischemia (Heinl-Green *et al.*, 2005), when the requirement for instant revascularization is more relaxed. Nevertheless, to thoroughly test these hypotheses, more studies using immunohistochemistry and neutron-activated microbubbles are needed to study the temporal changes in angiogenesis and myocardial perfusion during HIF-1 α -VP2 therapy for acute myocardial ischemia. Additionally, noninvasive PET assessment of angiogenesis using radiolabeled RGD peptides or VEGF as imaging probes could help better characterize the precise kinetics of stimulated angiogenesis (Niu and Chen, 2009). Lastly, recent unpublished studies have suggested HIF-1 α -VP16 to be less potent than VEGF when administered in equal doses (Risänen and Ylä-Herttuala, 2007), even though HIF-1 α -VP16 is known to have a broader action in its ability to activate multiple angiogenic genes, including VEGF. Although our version of the recombinant, constitutively active (i.e., insensitive to oxygen tension) HIF-1 α (HIF-1 α -VP2) is at least twofold more potent in activating VEGF than the HIF-1 α -VP16 reported in the literature (data not shown), its ability to upregulate angiogenesis has not been directly compared

with VEGF. A head-to-head comparison of HIF-1 α -VP2 with VEGF or other angiogenic genes should help determine whether the suboptimal therapy results reported herein were related to a suboptimal angiogenic potency associated with HIF-1 α -VP2.

This study has some limitations worth discussing. First, plasmid-mediated gene delivery was performed in this study to demonstrate the utility of the bidirectional TSTA vector strategy for indirect monitoring of HIF-1 α -VP2 expression. This mode of gene transfer, however, has been shown to be less efficient than viral-mediated gene delivery (Wright *et al.*, 2001). This was supported by the fact that we were not able to detect significantly more myocardial VEGF expression after pcTnT-HIF-1 α -VP2-TSTA-fluc delivery using enzyme-linked immunosorbent assay (data not shown), even though we were able to detect significant FLuc expression or activation of the HIF-1 α /HRE pathway (via the RLuc reporter) using the more sensitive reporter enzyme assays or BLI. Furthermore, because of the limited half-life of plasmid DNA in blood (<5 min) (Lew *et al.*, 1995), biodistribution studies that are typically done for viral vector-mediated gene delivery were not useful here for assessing the transcriptional targeting capability of our bidirectional TSTA vector because of inefficient plasmid transfection in most noncardiac tissues (e.g., liver, lungs, spleens, kidneys) after intramyocardial vector delivery (e.g., <1% of myocardial transfection in our preliminary studies; data not shown) (Son *et al.*, 2005). Therefore, to both improve the therapeutic efficacy of our vector and allow for a better assessment of transcriptional targeting, we will need to incorporate our vector design into a high-capacity adenoviral vector for more efficient gene delivery. Second, *in vivo* imaging of myocardial RLuc expression was associated with less imaging sensitivity and a smaller dynamic range in signal (Fig. 3C), compared with FLuc imaging, because of the suboptimal blue-shifted wavelength photons emitted by RLuc when coelenterazine is used as its substrate (λ_{peak} =480 nm). This limitation made quantification of RLuc signal relatively more difficult and could explain why the correlation between the *in vivo* FLuc and RLuc signals was worse than that between the *ex vivo* FLuc and RLuc enzyme activities (Fig. 3E). To overcome this limitation, we could either use a mutant RLuc (RLuc/M185V) with a threefold greater light output than RLuc (Chen *et al.*, 2010) or a red-shifted RLuc (λ_{peak} =547 nm) in conjunction with a red-shifted coelenterazine analog (λ_{peak} =588 nm) to try to achieve the utmost sensitivity for imaging *in vivo* RLuc activity (Loening *et al.*, 2007).

In conclusion, the numerous limitations of current cardiac gene therapy dictate the use of a highly versatile gene therapy vector such as the bidirectional TSTA vector validated in this study (pcTnT-HIF-1 α -VP2-TSTA-fluc). Such a vector provides a general platform by which any therapeutic gene of interest can be delivered into the myocardium via either a viral or a nonviral vehicle, so that the magnitude, duration, and locations of the therapeutic gene expression can be serially monitored by noninvasive imaging. Any novel promoter of interest deemed more robust than cTnT can also be incorporated into the vector for transcriptional regulation. Further modification of the vector by replacing fluc with a PET reporter gene (e.g., herpes simplex virus type 1 thymidine kinase or human mitochondrial thymidine kinase) (Ponomarev *et al.*, 2007; Willmann *et al.*, 2009) or an MRI

reporter gene (e.g., ferritin) (Lee *et al.*, 2012) should allow noninvasive imaging of therapeutic gene expression in large animals or humans using an existing clinical PET or MRI scanner.

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