Vigilant Vector: Heart-Specific Promoter in an Adeno-Associated Virus Vector for Cardioprotection

M. Ian Phillips, Yi Tang, Kai Schmidt-Ott, Keping Qian, Shuntaro Kagiyama

Abstract—Repeated bouts of ischemia in the heart lead to fibrosis and eventually to heart failure. Although certain genes, such as SOD or hemoxygenase and antisense to AT_1R, ACE, and (β_1-AR can provide short-term protection of the heart from ischemia, there is no known mechanism for constantly responding to repeated incidences of ischemia. We hypothesized that a "vigilant vector," designed to be expressed specifically in the heart and switch on therapeutic genes only during hypoxia, would provide cardioprotection. To attain cardiac specificity, we inserted an MLC2v promoter into an adeno-associated virus (AAV) designed to deliver AS to AT_1R and gfp. In in vitro experiments in cardiomyocytes (H9C2 cells), the MLC2v-AAV-gfp drove gene expression in all cells at levels comparable to a cytomegalovirus (CMV) promoter. In in vivo experiments, the rAAV-MLC2v-gfp was injected intravenously into mice or rats. Green fluoresence protein (GFP) DNA was located in kidney, heart (right and left ventricle), lung, adrenal and spleen. GFP mRNA, however, was expressed only in the heart and absent in other tissues. To switch on the rAAV transgene during ischemia, we inserted a hypoxia response element (HRE). This upregulates transcription when O_2 levels are low. Thus, there are 4 components to the vigilant vector; a gene switch (HRE), a heart-specific promoter (MLC2v), a therapeutic gene (AS-AT_1R) and a reporter gene (gfp). To silence or lower basal level of expression while retaining specificity, we have reduced the length of the MLC2v promoter from 3 kb to 1775 bp or 281 bp. The truncated promoter is equally effective in heart specific expression. Preliminary studies with the rAAV-HRE-gfp in vitro show an increased expression in 1% O_2 in 4 to 6 hours. By adding additional hypoxia-inducible factor (HIFα) (5 μg), the MLC2v-gfp expression is increased by 4-fold in 1% O_2. Further amplification of the gene to 400-fold in 1% O_2 can be achieved with a double plasmid. The construct may serve as a prototype "vigilant vector" to switch on therapeutic genes in specific tissue with physiological signals. **(*Hypertension*. 2002;39[part 2]:651-655.)**

Key Words: ischemia ■ cardiac function ■ hypoxia ■ myosin ■ gene therapy ■ adeno–associated virus

The human heart can be subject to repeated bouts of hypoxia, which leads to silent or overt myocardial tissue damage.[1] Cumulatively, this can lead to heart failure. In an attempt to combat this with gene therapy we are proposing the development of a "vigilant vector," inactive until switched on by hypoxia, that would protect the heart during ischemia with therapeutic genes. This concept requires the engineering of a stable vector that would contain 4 elements (Figure 1): (1) a safe vector that could reach the heart by systemic injection and show stable expression of the gene in the heart; (2) a therapeutic gene for cardioprotection against ischemia; (3) a tissue-specific promoter to drive the transgene to express mRNA in the heart only; and (4) a gene switch that would switch on the tissue-specific promoter in response to hypoxia and that would switch off in response to normoxia.

For the vector, the adeno-associated virus (AAV) is proving to be a stable, nonpathological vector.[2,3] There are several genes that could be considered for protection of the heart during ischemia. In a previous study[4] we had found that the angiotensin II type 1 receptor (AT_1-R) antisense (AS) protected rat hearts from ischemia-reperfusion. Dzau et al[5] have recently shown that transgenic mice with hemoxygenase are protected from cardiac ischemia. Superoxide dismutase protects against super oxide radicals generated during ischemia or reperfusion.[6] Thus, these genes are good choices for cardioprotective transgenes in the vector. For tissue-specific expression of AAV in the heart, we have studied the ventricular form of myosin light chain (MLC-2v).[7,8] MLC-2v expression is important in the development of the heart during embryogenesis, and alterations in the MLC-2v expression produce cardiac defects.[8] In humans, cardiomyopathy is associated with point mutations in MLC-2v.[9] MLC-2v seems to be highly specific for hearts, both during embryonic development and in post-natal development and maturity. The MLC-2v promoter is 3.0 kb, but the sequences that give it the property of heart specificity are within 250 bp, close to the TATA box.[8,9–13] We tested the specificity of a 1700 kb and a 281 bp MLC-2v promoter in AAV delivered in vitro and in vivo. To switch on the vector, we tested a hypoxia-regulatory element (HRE) which is activated by transactivat-

Received October 1, 2001; first decision November 2, 2001; accepted November 13, 2001.

From the Department of Physiology and Functional Genomics, University of Florida, Gainesville, FL.

Correspondence to Dr M. Ian Phillips, Physiology and Functional Genomics, University of Florida, College of Medicine, Box 100274, Gainesville, FL 32610-0274. E-mail MIP@ufl.edu

Hypertension **is available at http://www.hypertensionaha.org**

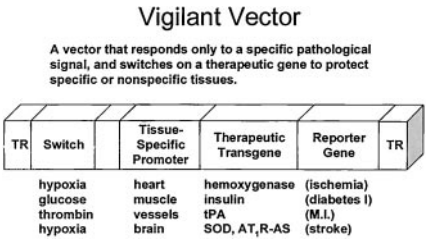

Figure 1. General design for a "vigilant vector" that could be applied for constant protection against cardiac ischemia, diabetes type 1, stroke, heart attack, cancer, or even bioterrorism. The main elements are (1) a safe, stable vector; (2) a gene switch; (3) a tissue-specific promoter; and (4) therapeutic genes (with a reporter gene to monitor its activity).

ing hypoxia inducible factor (HIF-1) in response to a reduction in oxygen.[14,15] Under normoxic conditions, the HIF-1α subunit is undetectable because it is degraded by proteosomes,[16,17] but during hypoxia HIF-1α is no longer degraded; it accumulates exponentially as cellular hypoxia increases.[18] Although we have not completed and tested all components of a vigilant vector, we present the results of a study on the heart specificity of MLC-2v and its interaction with HRE and HIF-1α.

Methods

Construction of Plasmid and Recombinant AAV

The linear, single-stranded AAV-derived vector can be adapted for several genes and promoters between the inverted terminal repeats (ITRs) at each end (Figure 1). We inserted a reporter gene, green fluorescent protein (GFP), and a rat 1.7 kb MLC-2v promoter (pMLC-2v-GFP). Methods to prepare recombinant AAV (rAAV) have been described previously.[19] The pMLC-2v-GFP was packaged into AAV-2 (rAAV-MLC-2v-GFP).

A 281 bp (−264 to +17, Genebank: U26708) fragment of MLC-2v promoter was amplified by a polymerase chain reaction (PCR) from pMLC-2v-GFP with the primer pair designed with 5′ XhoI or 3′ HindIII sites on the ends. The MLC-2v fragment was digested by XhoI and HindIII and ligated to XhoI/HindIII-digested plasmid gene luciferase (pGL)-SV40 (Promega) to generate pGL-MLC.

Based on Semenza et al,[14] a 68bp human enolase (ENO) 1 HRE sequence (-416 to -349, Genebank: X16287) was inserted into 5′ flank of the MLC-2v promoter in the pGL-MLC to generate pGL-HRE/MLC.

pCEP4/HIF-1α, which contains human HIF-1α cDNA sequence downstream of a cytomegalovirus promoter, was a kind gift from Dr Semenza (Johns Hopkins University).

In Vitro Transfection

Rat embryonic cardiac myoblast cell line,[20] H9c2 (ATCC: CRL1446), or glioma cells C6 (ATCC: CCL-107) were grown in DMEM supplemented with sodium pyruvate, 10% fetal bovine serum (FBS), or 5% FBS in an incubator (Quene Systems, Inc) with a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Hypoxia conditions were achieved using hypoxia chambers (Oxygen Sensors) by evacuation and gassing with 1% O_2/5% CO_2/94% N_2 repeatedly, tightly sealing the chambers, and then incubating them at 37°C.

To examine the MLC-2v promoter specificity in cells, both H9c2 and C6 cells were transfected with pGL-MLC (1 μg/well) cand internal control plasmid pRL-TK (50 ng/well, Promega) by using Lipofectamine (Invitrogen) in 6-well plates. Twenty-four hours after transfection, cell lysates were prepared. Luciferase assays were performed with the dual luciferase assay system (Promega) and quantified with a Monolight 3010 luminometer (Pharmingen). Results are expressed as a ratio of firefly luciferase activity over *Renilla* luciferase activity.

For cotranfection experiments with pCEP4/HIF-1α, H9c2 was transfected with 2 μg/well pGL-HRE/MLC, 100 ng/well control plasmid pRL-TK, various amounts of pCEP4/HIF-1alpha, and empty vector so that all cells received a total of 6 μg plasmid in 60 mm dishes. Twenty-four hours after transfection, the medium was changed and duplicate plates were incubated at 1% or 20% O_2 for 24 hours before preparation of lysates.

Expression of AAV in Vivo

All animals were kept in a temperature-controlled room on a 12-hour day/night cycle with free access to food and water. The Institutional Animal Care and Use Committee at the University of Florida approved all experimental procedures.

AAV Expression in Adult Animal

Adult male BALB/c mice (n=6) were obtained from Harlan (Indianapolis, Ind) and anesthetized with pentobarbital (80 mg/kg). 10^{10} infectious particles of rAAV-MLC-2v-GFP (100 μL) were injected intravenously. After 2 to 8 weeks, animals were deeply anesthetized with pentobarbital (120 mg/kg). Samples of spleen, liver, lung, kidney, left ventricle, testis, and brain were dissected and frozen on dry ice.

AAV Expression in Young Animal

Five-day-old male Sprague Dawley rats (n=3) were obtained accompanied by their dam from Harlan. They were kept with their dam until 21 days of age. At 6 days of age, the pups were anesthetized with Metofane injected intracardiacally with 10^{10} infectious particles of rAAV-MLC-2v-GFP (25 μL) or the same volume of saline as a control. Four weeks later, rats were deeply anesthetized with ketamine, xylazine, and acepromazine (30, 6, and 1 mg/kg, respectively, subcutaneously) and perfused with ice-cold saline via the left ventricle. Samples of spleen, liver, lung, kidney, left ventricle, testis, heart, and brain were dissected and frozen on dry ice for DNA, RNA, and GFP protein measurements.

Detection of GFP

Total RNA and DNA was isolated using TRIZOL reagent. Expression of green fluorescent protein (GFP) was analyzed by nested PCR. The GFP specific primers used in the first amplification were 5′-CAGCGGAGAGGGTGAAGGTG-3′(sense) and 5′-CAGGGCAGACTGGGTGGACA-3′ (antisense). The GFP specific primers used in the second amplification were 5′-GCCACATACGGAAAGCTCAC-3′ (sense) and 5′-ATGGTTGTCTGG GAGGAGCA-3′(antisense).

RT-PCR

Twenty μg of total RNA were digested by DNase I in a 40 μL reaction mixture consisting of 40 U DNase I and 33 U RNase inhibitor. Reverse transcription (RT) and first amplification were performed in a single tube. Four μL of the RNA (2 μmg) pretreated with DNase I were added to 20 μml final volume of the PCR reaction. The first amplification was performed in the following conditions: 60 minutes at 37°C (RT); 4 minutes at 94°C; 35 cycles of 1 minute at 94°C; 1 minute at 58°C (annealing); 1 minute at 72°C; and a final extension period of 7 minutes at 72°C in PE DNA Thermal Cycles 480. One μL product from the first amplification was added to 25 μL final volume of the PCR reaction. The conditions of second amplification were the same as the first with the exception of the addition of 30 cycles with annealing at 60°C.

PCR

One μg of DNA was amplified by nested PCR to detect GFP expression. The procedures were the same with GFP detection in RNA (RT-PCR) except DNase I digestion and Reverse transcription (RT) were omitted.

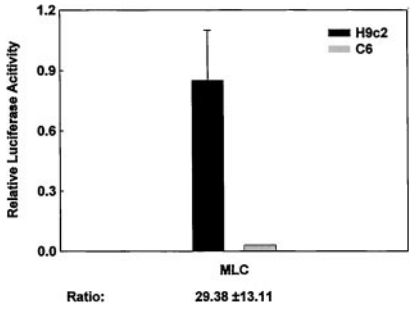

Figure 2. The expression of luciferase activity (relative to control) in cardiac (H9c2 cells) versus glioma (C6) cells after treatment with pGL-MLC. Myocardial cells specifically expressed the transgene. Cells were transfected with control pRL-TK (50 ng/well) and pGL-MLC (1 μg/well) plasmids. Duplicate plates were incubated at 20% O_2 for 24 hours (mean±SD, n=3 independent experiments).

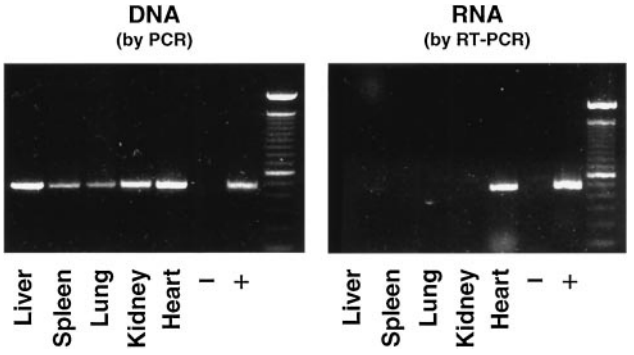

Figure 3. Top, Expression of GFP DNA in various tissues of the young rat detected by PCR. Bottom, Expression of GFP mRNA in the same tissues, detected by RT-PCR. Analysis was made of DNA and RNA extracted from the tissues 4 weeks after a single systemic injection of rAAV-MLC-2v-GFP.

Electrophoresis

Amplification products were analyzed on 1% agarose stained with ethidium bromide. The expected product size was 489 bp.

Immunofluorescence Staining

Tissues were incubated in Zamboni's solution overnight and cryosectioned at 20 μm thickness. The sections were blocked with blocking buffer (10 mmol/L TBS, 1.5% normal goat serum and 1% BSA) for 1 hour and incubated in primary antibody (0.1% anti-GFP, rabbit IgG) overnight at 4°C. After washing with TBS, the sections were incubated with 0.5% anti-rabbit IgG FITC in the dark at room temperature for 1 hour. The sections were washed and put on slides. The slides were covered by slips with fluoromount G when dry. GFP was detected within 3 hours by confocal microscopy.

Results

In Vitro

The pGL-MLC was specifically expressed in cardiomyocytes. Figure 2 shows the luciferase activity of pGL-MLC-2v in cardiomyocytes (H9c2) and a lack of expression in a nonmyocardial cell line (C6). The relative luciferase activity ratio of H9c2 cells to C6 cells was 29.38±13.11. The uptake efficiency in both cell types was 90%.

In Vivo

PCR of DNA showed the transduction of rAAV-MLC-2v-GFP in many tissues at 4 weeks after a systemic injection. The tissue-specific expression of GFP under MLC-2v promoter was examined by RT-PCR of RNA in the adult mouse tissues and young rats (Figure 3). GFP DNA was detected in the spleen, liver, lung, kidney, and heart. However, GFP mRNA was detected only in the heart.

Four weeks after intracardiac injection of rAAV-MLC-2v-GFP, the presence of GFP in various tissues of rats was further examined by immunofluorescence staining (Figure 4). The green epifluorescence of the protein was clearly apparent in the heart and absent in the control (no GFP). GFP was undetectable in the kidney and liver of the same animals and undetectable in controls.

Hypoxia did not induce an increase in transgene expression of the pGL-HRE/MLC (Figure 5). However, hypoxia induces a 3 to 4-fold increase in transgene expression when the HRE-MLC-2v enhancer/promoter complex is in the presence of 0.5 to 4 μg of HIF-1α in H9c2 cells (Figure 5).

Discussion

The results demonstrate that the MLC-2v promoter incorporated into a rAAV vector can drive a reporter gene specifically in the heart. rAAV-MLC-2v-GFP was injected systemically, either through direct injection into the heart or via the jugular vein. Measurements of DNA showed that the vector was taken up into multiple tissues, including liver, lung, kidney, heart, and spleen. Although the rAAV-MLC-2v-GFP was taken up into many tissues after a single injection, the transgene (gfp) was only expressed in heart tissue. This was found in both mice and in rats. In two animals we found low-level expression in the liver but not in the kidney or other tissues. As AAV has limited loading capacity, we tested two truncated forms of MLC-2v. We used the 1700 bp length for the promoter in vivo and 250 bp in vitro to attempt to reduce basal levels without losing specificity. Both lengths contain the heart-specific *cis* regulatory elements[8] that endow the MLC-2v with its heart-specific responsiveness.[9,10] In glioma cells (C6) there was no expression of luciferase, although there was comparable uptake efficiency with or without the MLC-2v promoter.

Attaching HRE to the MLC-2v with luciferase (Luc), as the transgene, did not alter basal expression in vitro at 20% O_2. However, the HRE plus MLC-2v did not respond to 1% O_2. With an HRE-SV40 promoter-Luc plasmid in heart cells, we have shown elsewhere that HRE will drive the promoter up to 7-fold under hypoxia within 4 to 6 hours.[21] We considered that the HRE/MLC-2v complex may have reduced the accessibility of the HIF-1α binding. To test this, we used an additional plasmid expressing the hypoxia inducible factor-1α (HIF-1α).[14–17] When we cotransfected a plasmid containing HIF-1α cDNA with pGL-HRE/MLC and exposed the cells to 1% oxygen we noted a 4-fold increase in Luc expression. Thus, the results indicate that MLC-2v can be used as a specific promoter for heart tissue, and HIF-1α plus HRE (but not HRE alone) will cause the MLC-2v to increase transgene expression in vitro by at least 4-fold in response to hypoxia. This is not a major limitation because dual vectors overcome the vector size limitation[22] and increase gene

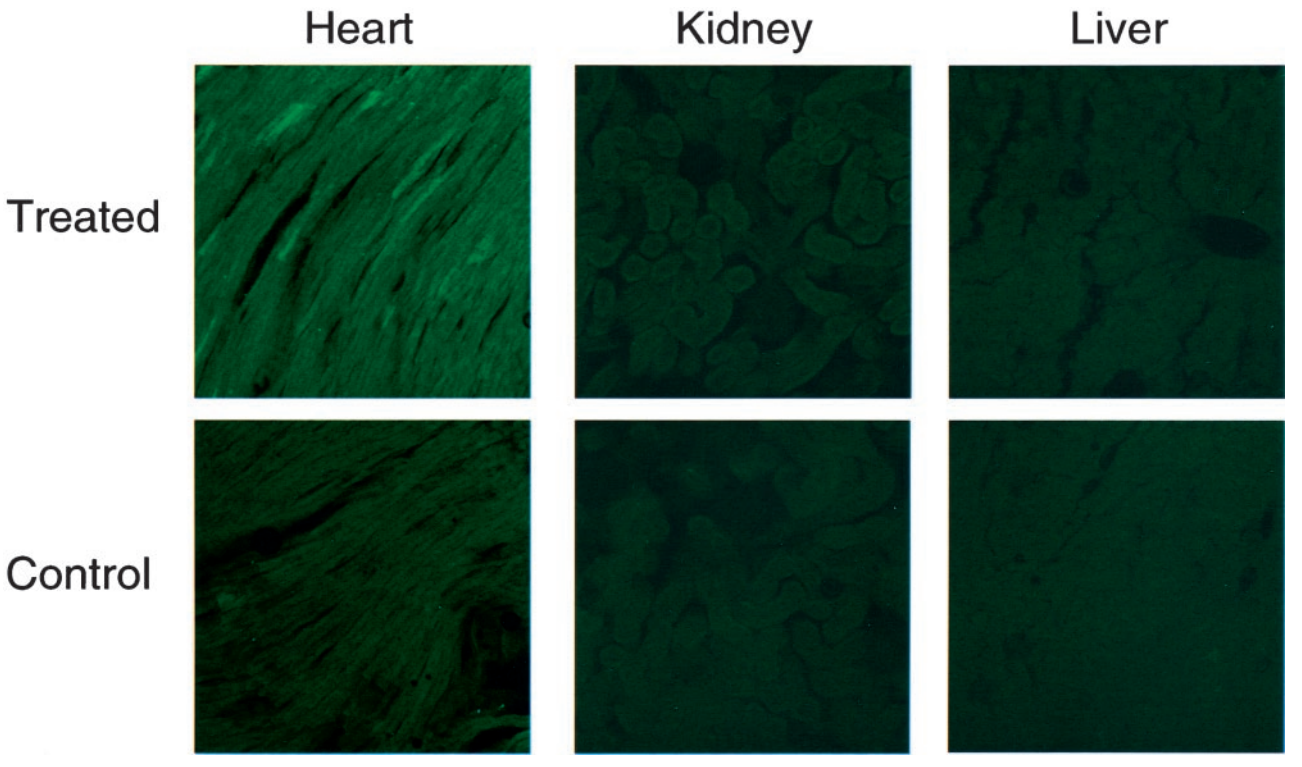

Figure 4. Expression of rAAV-MLC-2v-GFP was present in the heart, but absent in the liver and kidney at 4 weeks after transduction in young rat. Control: rAAV without GFP. The immunofluorescence staining with an antibody to green fluorescent protein reveals intense expression in heart only, which matches the expression of mRNA from the same animal in Figure 3.

expression.[23] We are not yet satisfied that a 4-fold increase is sufficient to provide a cardioprotective effect with a therapeutic gene. A double plasmid approach that produces a powerful chimeric transcription factor consisting of the yeast transactivator factor GAL4 DNA binding domain and the p65 transactivation domain[24,25] is being tested.[21] Incorporating the HRE in this double plasmid system with SV40 promoter increased Luc gene expression by 400-fold when activated by hypoxia.[21]

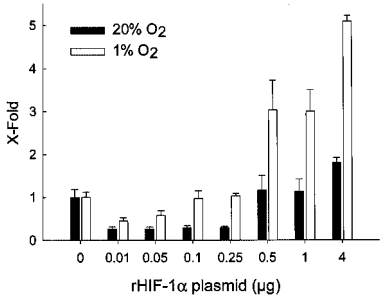

Figure 5. Hypoxia induces a 3- to 4-fold increase in transgene expression when the HRE/MLC enhancer/promoter complex is in the presence of rHIF-1alpha. H9c2 cells were cotransfected with 2 μg/well pGL-HRE/MLC (281 bp) and 100 ng/well pRL-TK, in the absence of rHIF-1α or in the presence of various amount of rHIF-1α plasmid, respectively. Twenty-four hours after transfection, duplicates were exposed to 1% or 20% O_2 for another 24 hours. The ratio of firefly luciferase/*Renilla* luciferase activity was normalized to the result obtained for cells transfected with pGL-HRE/MLC (281 bp) and exposed to 20% O_2 (X-Fold). Expression at 1% relative to 20% O_2 was calculated to determine the hypoxia induction ratio. (mean$\pm$SD, n=3 independent experiments).

The concept of a vigilant vector for cardioprotection can be applied generally to a number of other disease states. For example, in diabetes type 1, glucose would be the gene switch and insulin and its necessary enzymes would be the transgenes. The tissue specificity could be limited to the pancreas or to muscle. In cancer, tumor markers could be the gene switch, and the transgenes could be tumor suppressors. In heart attacks the switch would again be hypoxia or a protein marker and the transgene tPA. Similarly in stroke, hypoxia could be the switch and GFAP the tissue-specific promoter with hemoxygenase or superoxide dismutase or AT_1-R-antisense as the therapeutic genes. For the vector, the rAAV seems to have the most desirable qualities of being safe and stable for a very long time.[2,3] Obviously each vigilant vector has to be designed and thoroughly tested, both in vivo and in vitro. Basal levels times of response, tissue specificity and amplification of signals are all challenges to be met. The present results represent promising new data for the development of a vigilant vector for long-term protection of cardiac performance during exposure to hypoxia.

Acknowledgments

This work was supported by the NIH MERIT Award HL# 27339 to M.I.P., and fellowship from the American Heart Association, Florida affiliate to Y.T. and S.K.

References

1. Bleske BE, Shea MJ. Current concepts of silent myocardial ischmiea. *Clin Pharm*. 1990;9:339–357.
2. Song S, Embury J, Laipis PJ, Berns KI, Crawford JM, Flotte TR. Stable therapeutic serum levels of human alpha-1 antitrypsin (hAAT) after

protein vein injection of recombinant adeno-associated viral (rAAV) vectors. *Gene Therapy*. 2001;8:1299–1306.
3. Phillips MI, Mohuczy-Dominiak D, Coffey M, Galli SM, Kimura B, Wu P, Zelles T. Prolonged reduction of high blood pressure with an in vivo, nonpathogenic, adeno-associated viral vector delivery of AT_1-R mRNA antisense. *Hypertension*. 1997;29:374–380.
4. Yang BC, Phillips MI, Ambuehl PEJ, Shen LP, Mehta P, Mehta JL. Increase in angiotensin II type 1 receptor expression immediately after ischemia-reperfusion in isolated rat hearts. *Circulation*. 1997;96: 922–926.
5. Yet SF, Tian R, Layne MD, Wang ZY, Maemura K, Solovyeva M, Ith B, Melo LG, Zhang L, Ingwall JS, Dzau VJ, Lee ME, Perella MA. Cardiac-specific expression of heme oxygenase-2 protects against ischemia and reperfusion injury in transgenic mice. *Circ Res*. 2001;89:205–107.
6. Li Q, Bolli R, Qui Y, Tang XL, Murphree SS, French BA. Gene therapy with extracellular superoxide dismutase attenuates myocardial stunning in conscious rabbits. *Circulation*. 1998;98:1438–1448.
7. Lee KJ, Ross RS, Rockman HA, Harris AN, O'Brien TX, Van Bilsen M, Shubeita HE, Kandolf R, Brem G, Price J, Evans SM, Zhu H, Franz WM, Chien KR. Myosin light chain-2 luciferase transgenic mice reveal distinct regulatory program for cardiac and skeletal muscle-specific expression of a single contractile protein gene. *J Biol Chem*. 1992;267:15875–15885.
8. Henderson SA, Spencer M, Sen A, Kumar C, Siddiqui MAQ, Chien KR. Structure, organization, and expression of the rat cardiac myosin light chain-2 gene. Identification of a 250-based pair fragment which confers cardiac-specific expression. *J Biol Chem*. 1989;264:18142–18148.
9. Geisterfer-Lawiance AAT, Kass S, Tanigawa G, Vosberg HP, McKenna W, Seidman CE, Seidman JG. Molecular basis for familial hypertrophic cardiomyopathy: a cardiac myosin heavy chain gene missense mutation. *Cell*. 1990;12:999–1006.
10. Zhu H, Garcia AV, Ross RS, Evans SM, Chien KR. A conversed 28-base-pair element (HF-1) in the rat cardiac myosin light-chain-2 gene confers cardiac-specific and alpha-adrenergic-inducible expression in cultured neonatal rat myocardial cells. *Mol Cell Biol*. 1991;11: 2273–2281.
11. Lee KJ, Hickey R, Zhu H, Chien KR. Positive regulatory elements (HF-1a) and HF-1b) and a novel negative regulatory element (HF-3) mediate ventricular muscle-specific expression of myosin light chain 2-luciferase fusion genes in transgene mice. *Mol Cell Biol*. 1994;14: 1220–1229.
12. Griscelli F, Gilardi-Hebenstreit P, Hanania N, Franz WM, Opolon P, Perricaudet M, Ragot T. Heart-specific targeting of beta-galactosidase by the ventricle-specific cardiac myosin light chain 2 promoter using adenovirus vectors. *Hum Gene Ther*. 1998;9:1919–1928.
13. Franz WM, Rothmann T, Frey N, Katus HA. Analysis of tissue-specific gene delivery by recombinant adenoviruses containing cardiac-specific promoters. *Cardiovasc Res*. 1997;35:560–566.
14. Semenza GL, Jiang BH, Leung SW, Passantino R, Concordet JP, Maire P, Giallongo A. Hypoxia response elements in the aldolase A, enolase 1, and lactate dehydrogenase A gene promoters contain essential binding sites for hypoxia-inducible factor 1. *J Biol Chem*. 1996;271:32529–32537.
15. Wang GL, Semenza GL. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. *Proc Natl Acad Sci U S A*. 1993;90:4304–4308.
16. Salceda S, Caro J. Hypoxia-inducible factor 1α (HIF-1α) protein is rapidly degraded by the ubiquitin-proteasome system under normoxic conditions. Its stabilization by hypoxia depends on redox-induced changes. *J Biol Chem*. 1997;272:22642–22647.
17. Huang LE, Gu J, Schau M, Bunn HR. Regulation of hypoxia-inducbile factor 1α is mediated by an O2-dependent degradation domain via the ubiquitin-proteasome pathway. *Proc Natl Acad Sci U S A*. 1998;95: 7987–7992.
18. Jiang BH, Semenza GL, Bauer C, Marti HH. Hypoxia-inducible factor 1 levels vary exponentially over a physiologically relevant range of O_2 tension. *Am J Physiol*. 1996;271:C2272–C1180.
19. Phillips MI. Antisense inhibition and adeno-associated viral vector delivery for reducing hypertension. *Hypertension*. 1997;29:177–187.
20. Kimes BW, Brandt BL. Properties of a clonal muscle cell line from rat heart. *Exp Cell Res*. 1976;98:367–381.
21. Tang Y, Jackson M, Qian K, Phillips MI. Hypoxia inducible double plasmid system for myocardial ischemia gene therapy. *Hypertension* 2001 (in press).
22. Sun L, Li J, Xiao X. Overcoming adeno-associated virus vector size limitation through viral DNA heterodimerization. *Nat Med*. 2000;6: 599–602.
23. Duan D, Yue Y, Yan Z, Engelhardt JF. A new dual-vector approach to enhance recombinant adeno-associated virus-mediated gene expression through intermolecular cis activation. *Nat Med*. 2000;6:595–598.
24. Wang Y, O'Malley BW, Jr, Tsai SY, O'Malley BW. A regulatory system for use in gene transfer. *Proc Natl Acad Sci U S A*. 1994;91:8180–8184.
25. Ornitz DM, et al. Binary system for regulating transgene expression in mice: targeting int-2 gene expression with yeast GAL4/UAS control elements. *Proc Natl Acad Sci U S A*. 1991;88:698–702.