Recombinant Adeno-Associated Virus Serotype 9 Leads to Preferential Cardiac Transduction In Vivo

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Abstract—Heart disease is often the end result of inherited genetic defects, which may potentially be treatable using a gene-transfer approach. Recombinant adeno-associated virus (rAAV)-mediated gene delivery has emerged as a realistic method for the treatment of such disorders. Here, we demonstrate and compare the natural affinity of specific AAV serotype capsids for transduction of cardiac tissue. We compared the previously accepted optimal rAAV serotype for transduction of skeletal muscle, rAAV2/1, with rAAV2/8 and the newer rAAV2/9 vectors carrying the CMV-lacZ construct in their respective abilities to transend vasculature and transduce myocardium following intravenous delivery of 1×10^{11} vector genomes in neonatal mice. We found that both rAAV2/8 and rAAV2/9 are able to transduce myocardium at ≈20- and 200-fold (respectively) higher levels than rAAV2/1. Biodistribution analysis revealed that rAAV2/9 and rAAV2/8 demonstrate similar behavior in extracardiac tissue. Vector genome quantification showed an increase in genome copy numbers in cardiac tissue for several weeks following administration, which corresponds to expression data. In addition, we intravenously administered 1×10^{11} vector genomes of rAAV2/9-CMV-lacZ into adult mice and achieved an expression biodistribution profile similar to that found following delivery to newborns. Although higher doses of virus will be necessary to approach those levels observed following neonatal injections, adult myocardium is also readily transduced by rAAV2/9. Finally, we have demonstrated physiological disease correction by AAV9 gene transfer in a mouse model of Pompe disease via ECG tracings and that intravenous delivery of the same vector preferentially transduces cardiac tissue in nonhuman primates. (Circ Res. 2006;99:e3-e9.)

Key Words: AAV9 • gene therapy • gene transfer • adeno-associated virus • cardiomyopathy

Cardiovascular disease accounts for 38.5% of all deaths in the United States per year.1 A number of the cardiac-related disorders that contribute to this statistic are the consequence of an inherited genetic predisposition or a congenital metabolic defect. Common heart problems such as myocardial infarction, atherosclerosis, stroke, and dilated cardiomyopathy, in addition to rare disorders such as ion channel abnormalities, structural protein defects, or enzymatic deficiencies, may all potentially be ameliorated by gene therapy. In addition, there are other inherited diseases, such as those found in the muscular dystrophy family, that affect both cardiac and skeletal muscle and will ultimately require global gene delivery to prevent or correct disease.

Recent advances in a variety of areas have turned the once-daunting task of establishing a clinically relevant gene therapy approach into a near-term reality. A wide array of both viral vectors such as retrovirus, lentivirus, herpes simplex virus, recombinant adeno-associated virus (rAAV), and adenovirus (Ad), in addition to nonviral gene delivery systems such as naked plasmid DNA and lipid vesicles, are currently being tested for various applications. For the majority of potential heart-related therapies, the ideal gene-transfer vehicle will be nonpathogenic, provide for sustained transgene expression throughout the myocardium, and be easily transferable using a clinically feasible delivery route. When taking into consideration those characteristics desirable in a vehicle for cardiac gene delivery, the (4.7 kb) nonpathogenic parvovirus rAAV has emerged as an attractive choice, mainly because of its small size and proven ability to persist for long periods of time in infected cells.2-7

rAAV is a single-stranded DNA virus that requires a helper, such as herpes virus or adenovirus, to replicate. With recent discoveries of novel serotypes of rAAV, it has become possible to select those with the most advantageous tropisms to target and/or evade tissues as desired for specific applications. The most optimal gene-delivery system for any therapeutic application will combine a clinically advantageous physical delivery route with the rAAV serotype that has the highest natural affinity for a specifically targeted tissue of interest.
Our goal in this study was to identify a serotype with the ability to achieve high transduction levels of myocardium in vivo. The serotypes that have been most extensively studied for this application are rAAV2/1 and rAAV2 which are both capable of the transduction of cardiac tissue to some extent. Whereas in vitro and in vivo evidence has suggested that rAAV2 is better able to transduce younger, undifferentiated cardiomyocytes, studies suggest that rAAV2/1 is more efficient at the transduction of mature, adult hearts.8

The physical administration of rAAV to the heart has been previously performed using a variety of techniques. Direct intramyocardial injections into adult mice using either a basic thoracotomy surgical procedure followed by an intracardiac injection or catheter-based cardiac injection systems generally result in regional distribution of gene transfer.9 Other techniques have been developed involving coronary injections or infusions of various vector-containing formulations to enhance diffusion of the gene-delivery vehicle across the pericardial membrane.10 Each of these methods requires an invasive surgery, which adds to the cumulative risk of the potential human therapy. More recently, investigators have shown that intravenous delivery of rAAV2/1 into 1-day-old neonatal mice results in broad distribution of expression and successful disease correction in a mouse model for Pompe disease.11 Here, we report results using the intravenous route of administration in combination with other rAAV serotypes that are more readily able to cross the vasculature and efficiently transduce cardiac tissue. This approach results in overall higher levels of in vivo transgene expression in cardiomyocytes when compared with rAAV2/1.

Materials and Methods

Virus Production

pRep2Cap9 was kindly provided by James Wilson and Guangping Guo (University of Pennsylvania). Recombinant AAV vectors were generated, purified, and titered at the University of Florida Powell Gene Therapy Center Vector Core Laboratory as previously described.12

Intravenous Injections

All animal procedures were performed in accordance with the University of Florida Institutional Animal Care and Use Committee (IACUC) guidelines (mice) or the University of California (UC) Davis IACUC (monkeys; see below). One-day-old mouse pups were injected via the superficial temporal vein as previously described.13 Briefly, mice were anesthetized by induced hypothermia. A 29.5-gauge tuberculin syringe was used to deliver vector in a total volume of 35 μL directly into the left temporal vein. Two-month-old adult mice were injected via the jugular vein. Mice were first anesthetized using a mixture of 1.5% isoflurane and O2 (1 to 2 L). A 0.5-cm incision was made to expose the jugular vein. A 29-gauge sterile needle and syringe were then used to deliver virus in a volume of 150 μL. Hemostasis was obtained; the skin was approximated and held secure with Vetbond (3M, St Paul, Minn).

β-Galactosidase Detection

Tissue lysates were assayed for β-galactosidase enzyme activity using the Galacto-Star chemiluminescence reporter gene assay system (Tropix Inc, Bedford, Mass). Protein concentrations for tissue lysates were determined using the Bio-Rad DC protein assay kit (Hercules, Calif).

Nonhuman Primate Studies

Studies with monkeys were conducted in the Center for Fetal Monkey Gene Transfer for Heart, Lung, and Blood Diseases located at the California National Primate Research Center (UC Davis). Gravid rhesus monkeys (n = 6) were monitored during pregnancy by ultrasound, and newborns delivered by cesarean section at term using established techniques.14 Within an hour of birth, newborns were injected intravenously with vector (~1 mL) via a peripheral vessel. Infants received either rAAV2/1-CMV-hGaa (n = 3) or rAAV29-CMV-hGaa (n = 3). Infants were nursery reared and monitored for 6 months and then euthanized by an overdose of pentobarbital and complete tissue harvests performed (one per group) using established methods.15 Specimens from control animals of a comparable age were made available through the Center for Fetal Monkey Gene Transfer. GAA activity was measured from tissues harvested 6 months postinjection as previously described16 and background activity from noninjected controls was subtracted to yield the results in Figure 5A. Genomic DNA (gDNA) was extracted from tissues according to the protocol of the manufacturer (Qiagen; DNeasy tissue kit). Resulting DNA concentrations from the extraction procedure were determined using an Eppendorf Biophotometer (Model 6131; Eppendorf, Hamburg, Germany). One microgram of extracted gDNA was used in all quantitative PCRs according to a previously used protocol,17 and reaction conditions (recommended by Perkin-Elmer/Applied Biosystems) included 50 cycles of 94.8°C for 40 seconds, 37.8°C for 2 minutes, 55.8°C for 4 minutes, and 68.8°C for 30 seconds. Primer pairs were designed to the CMV promoter as described18 and standard curves established by spike-in concentrations of a plasmid DNA containing the same promoter.19 DNA samples were assayed in triplicate. The third replicate was supplemented with CBATDNA at a ratio of 100 copies/μg of gDNA. If at least 40 copies of the spike-in DNA were detected, the DNA sample was considered acceptable for reporting vector DNA copies.

Results

We have directly compared rAAV2/111 with 2 less-characterized serotypes (rAAV2/8 and rAAV2/9) in their abilities to transduce myocardium in vivo. These recombinant or pseudotyped vectors are created by inserting a transgene of interest flanked by the inverted terminal repeats (ITRs) of AAV2 into the capsid of another serotype. We delivered 1×1011 vector genomes (vg) of each of 3 different serotypes (rAAV2/1, rAAV2/8, or rAAV2/9) carrying the CMV-lacZ construct (cytoplasmic lacZ) by the systemic venous route to 1-day-old mice (5 neonates per group) in an injection volume of 35 μL (Figure 1). Hearts from the injected mice were harvested at 4 weeks postinjection and 5-bromo-4-chloro-3-indolyl β-d-galactoside (X-gal) staining was performed on frozen cryosections to visualize the extent of β-galactosidase expression biodistribution across the myocardium (Figure 1A through 1C). In addition, β-galactosidase activity was determined to quantify LacZ expression (Figure 1D). The SYBR green quantitative PCR technique was also performed on these hearts to compare the relative amounts of vector genomes present. We found that 0.19, 16.12, and 76.95 vg per diploid cell were present in the hearts of mice injected with rAAV2/1, rAAV2/8, and rAAV2/9, respectively. Calculations for vector genomes per cell were determined as previously described19.

ECG Analysis

ECG tracings were acquired using standard subcutaneous needle electrodes (MLA1203, 1.5 mm Pin 5; AD Instruments) in the right shoulder, right forelimb, left forelimb, left hindlimb, and tail and a Power Laboratory Dual BioAmp instrument. Five minutes of ECG tracings from each animal were analyzed using AD Instrument Chart software.
Our results show that of those serotypes compared in this work, systemic venous delivery of AAV2/9 results in broad and even distribution of vector and transgene product in the myocardium without selective cardiac administration. The level of gene expression was shown to result in a 200-fold greater level of expression than that observed for rAAV2/1. rAAV2/8 provides exceptional transduction of myocardium at levels ~20-fold greater than those obtained using rAAV2/1; however, there is also significant transduction of hepatocytes with this serotype. X-Gal-stained cryosections demonstrated that both rAAV2/8 and rAAV2/9 provide a broad and even distribution of transgene expression throughout the entire heart. In contrast, those hearts injected with rAAV2/1 showed far less overall expression. Additionally, we performed immunohistochemistry with a cardiac troponin antibody (Santa Cruz Biotechnology; data not shown) and found that the cells expressing β-galactosidase were cardiomyocytes. Also, our preliminary studies demonstrated that rAAV2/9 transduced cardiomyocytes more efficiently than myoblasts in vitro.

The β-galactosidase enzyme detection assay was then performed on other tissues from these same animals to characterize the biodistribution of lacZ expression. We found that rAAV2/8 and rAAV2/9 are both capable of transduction of skeletal muscle to some degree (Figure 2A). In general, rAAV2/8 has the ability to provide an overall broad and even biodistribution of expression across muscle in addition to the heart, whereas rAAV2/9-delivered transgene expression is far greater in the heart than any other tissue.

The β-galactosidase assay was also performed on non-cardiac, nonskeletal muscle tissue samples from these mice (Figure 2B). These results showed that whereas rAAV2/8 and rAAV2/9 are able to transduce tissues such as brain, lung and kidney, there is less transduction of spleen and small intestine. Once we established that rAAV2/9 displayed the highest natural affinity for myocardium, we further characterized rAAV2/9 activity in vivo.

We chose to use the CMV promoter for these studies because this expression cassette was appropriate in size and expression profile in the target tissue of interest. SYBR green

Figure 1. Intravenous delivery of rAAV2/9 results in high-level transduction of the heart. One-day-old C57BL6/129SvJ mouse neonates (n=5) were injected with 1×10^11 vg of rAAV pseudotypes AAV2/1, AAV2/8, and AAV2/9 carrying the CMV-lacZ construct via the previously described temporal vein delivery route. At 4-weeks postinjection, the β-galactosidase enzyme detection assay was performed to quantify lacZ expression levels. A through C, X-Gal–stained cryosections from hearts injected with AAV2/1 (A), AAV2/8 (B), and AAV2/9 (C). D, β-Galactosidase enzyme levels in hearts (n=5).
quantitative PCR was performed on heart, liver, and quadriceps tissue specimens from mice that were injected with rAAV2/9 to compare the relative amounts of vector genomes present in these tissues. These results showed that there were \( \approx 76.95 \) vg/cell (vector genomes per diploid cell) in myocardium and 2.89 vg/cell and 11.47 vg/cell present in liver and quadriceps, respectively. The clinical implication of these findings is that even when using an AAV capsid, which displays a high natural affinity for a specific tissue, the use of a tissue-specific promoter will be critical to ultimately ensure restricted transgene expression to the area of interest.

Additional studies were performed to evaluate a time-course assay of rAAV2/9-CMV-\( \beta \)-galactosidase expression in cardiac and skeletal muscle. One-day-old mouse neonates were injected with \( 5 \times 10^9 \) vg, and cardiac and skeletal muscles were harvested at 1, 7, 14, 28, and 56 days postinjection (Figure 3A). The results show that the onset of transgene expression in both tissues occurred between 1 and 7 days following administration of vector. The amount of expression in skeletal muscle increased gradually over the first 28 days, then leveled off and sustained a constant level out to at least 56 days. The amount of transgene expression in cardiac tissue was consistently higher than that in skeletal muscle and continued to steadily increase throughout the duration of the experiment (56 days).

We next performed SYBR green quantitative PCR on these tissues to determine whether the increase of transgene expression in cardiac tissue was attributable to an increase in \( \beta \)-galactosidase protein stability in cardiac tissue as compared with skeletal muscle tissue (Figure 3B). We found that vector genome copy number increased in cardiac tissue but not skeletal muscle tissue. Additionally, we isolated RNA from these tissues and found that RNA transcript numbers also increased over the duration of the experiment (Figure 3C).

The cellular receptor for AAV9 is not currently known; however, the preferential cardiac transduction warrants further evaluation of cardiac ligands, which are bound by AAV. Our data suggest that the AAV9 capsid may not be absorbed by other tissues as easily as previously studied serotypes because of its inability to bind to a more ubiquitous receptor located throughout the body, such as the heparin sulfate proteoglycan receptor. Therefore the AAV9 capsid could require more time to reach cardiac tissue. An additional explanation for the increase in vector genome concentration over the course of our experiment is that there may be a delay in the double-strand synthesis of the delivered transgene in the heart that could potentially account for a doubling of vector genomes.

Next, we performed a study in adult mice to determine whether the rAAV9 behavior we observed in neonates is similar in adult animals. rAAV2/9-CMV-\( \beta \)-galactosidase (\( 1 \times 10^1 \) vg) was administrated to 3-month-old mice using an intravenous delivery route via the jugular vein (Figure 4B). Tissues were harvested at 4 weeks postinjection, and the level of transgene expression was determined for both cardiac and skeletal muscle. Our results show that rAAV2/9 does transduce cardiac and skeletal muscle in adult mice, although in comparison with the same dose administered to neonates, the expression levels were far lower (Figure 4A). Incidentally, the rAAV2/9-delivered expression level in adults was comparable to that observed following intravenous delivery of the same dose of rAAV2/1-CMV-\( \beta \)-galactosidase to neonates. Lower overall rAAV2/9 transduction in adults in comparison to the same dose in neonates is not unexpected because of the reduced dose per kilogram of body weight. These data demonstrate, however, that a similar biodistribution profile is observed whether rAAV2/9 is intravenously delivered to adults or neonates and provide further evidence that rAAV2/9 preferentially transduces cardiac tissue.

We have used a model of inherited cardiomyopathy to assess a gene-transfer approach to this condition. Pompe disease is a form of muscular dystrophy and metabolic myopathy caused by mutations in the acid \( \alpha \)-glucosidase (\( Gaa \)) gene. An insufficient amount of the GAA enzyme leads to the accumulation of glycogen in lysosomes and consequent cellular dysfunction. In human patients, there is a direct correlation between the amount of GAA produced and the severity of disease. Without treatment, cardiorespiratory failure typically occurs in the early-onset patients within the first year of life.21,22

To demonstrate the ability of the rAAV2/9 pseudotype to deliver a therapeutic transgene to correct and/or prevent the onset of a disease phenotype, we have treated the \( Gaa^{-/-} \) mice...
Because of the rAAV2/9 marker gene results, we anticipated that a lower therapeutic dose than is typically necessary would be sufficient to provide correction in a mouse model of cardiomyopathy. Therefore, doses per neonate of either 4×10^5 or 4×10^6 vg of rAAV2/9-CMV-hGaa were administered to Gaa^-/- mice at 1 day of age using the intravenous delivery route. At 3 months postinjection, ECGs were performed on each dosage group of treated mice and noninjected, age-matched Gaa^-/- and healthy wild-type (B6/129) controls. Similar to the human form of this disease, untreated Gaa^-/- mouse ECGs display a shortened PR interval as compared with healthy B6/129 controls (PR=33.41±1.35 ms, or 26% shorter than wild type [PR=44.95±1.58 ms]). The mice that were treated with the low dose (4×10^5 vg) of rAAV2/9-CMV-hGaa displayed a PR interval of 36.76±1.12 ms, or only 18% shorter than wild-type, age-matched controls (P=0.062). The dosage group treated with 4×10^6 vg displayed a PR interval of 39.38±2.42 ms, or only 12% shorter than B6/129 age-matched controls (P=0.058). Essentially, at these low doses, we observe a lengthened PR interval that may increase as time progresses.

Although the mouse is a generally well-accepted model for gene therapy studies, behavior of the various AAV capsids in humans may be quite different. Therefore, long-term experiments are presently being performed in nonhuman primates to assess the expression over time in an animal model more phylogenetically similar to humans. Preliminary results from this ongoing study show that at 6 months following intravenous delivery via a peripheral vessel (at birth) of rAAV2/9-CMV-hGaa or rAAV2/1-CMV-hGaa to infant rhesus macaques, the expression profile between serotypes is similar to what we have observed in mice with rAAV2/9, providing ~4-fold more GAA expression than rAAV2/1 (Figure 5A). The vector genome biodistribution profile observed in these nonhuman primate tissues was also similar to what we had found in mouse tissue (Figure 5B) with rAAV2/9, demonstrating a dramatic preference for cardiac tissue over skeletal muscle. For both the expression and vector genome analysis of nonhuman primate heart specimens, numbers were averaged between right and left heart including the atria and ventricles. Biodistribution of expression and vector genomes appeared to be even throughout the heart.

The long-term goal of this project is to develop a clinically relevant gene therapy approach for the treatment of genetic diseases affecting the heart. The ability to pseudotype or cross-package genes flanked by the ITRs of AAV2 into alternative AAV capsids possessing tropisms for different tissues has inspired us to investigate which of the current serotypes is the most optimal for cardiac gene delivery. Here we have characterized the behavior of the rAAV9 capsid in its ability to transduce myocardium and found that in comparison with the other capsids analyzed, it is the best suited for gene delivery to the heart. Based on a protein blast comparison, the AAV8 and AAV9 capsid amino acid sequences are 82% and 83% homologous, respectively, with AAV1 and 85% homologous with one another. As demonstrated in our study, these small sequence differences between the various AAV capsid serotypes dramatically alter their behavior in vivo.

The increase in transduction we have observed using rAAV2/9 is most likely attributable to the presence of receptors or ligands located on the outside of the viral capsid,
which, although unidentified as of yet, display a strong affinity for cardiac tissue. The effect may be magnified by an increase in the kinetics of double-stranded DNA synthesis and/or annealing or variations in protein-trafficking pathways as a result of a more rapid capsid uncoating mechanism. Many of the answers to these basic virology questions are currently being pursued but have yet to be elucidated.

Overall, the focus of this work was to identify the AAV capsid best suited for gene delivery to the heart. Our studies demonstrate the ability of a previously uncharacterized pseudotyped rAAV capsid rAAV2/9 to successfully pass through the vasculature and transduce cardiomyocytes following intravenous administration of a marker gene. To gain further insight into the transduction efficiency in vivo, we performed cell counts of β-galactosidase–positive cells and expressed total positive cells per high-power field by light microscopy. In assessing multiple sections from 5 animals injected with rAAV2/9-CMV-lacZ, we found that 21% of cardiomyocytes were transduced at 4 weeks postadministration of 1 × 10¹¹ vg. At later time points or at higher doses, it is possible to transduce the entire myocardium by this approach.

Our data also show that although AAV2/9 has a high natural affinity for cardiac tissue, it is able to transduce several other tissues at levels similar to what we observe using AAV2/8. In addition, our time-course assay shows that the level of expression in myocardium from a transgene delivered by AAV2/9 continues to increase up to at least 56 days postadministration in newborn mice, whereas transgene expression levels in skeletal muscle appears to plateau at ∼28 days postinjection. Studies reported here also indicate that rAAV2/9 is able to transduce adult cardiac and skeletal muscle when delivered using an intravenous administration route.

One of the advantages of using a rAAV pseudotype, which is able to transduce the heart so efficiently, is that if the lowest corrective dose with a less-potent AAV capsid has been established, an ∼200-fold lower dose of rAAV2/9 would be required to achieve the same effect at 4 weeks postadministration, using an identical delivery route, disease model, and transgene. One benefit of a lower established effective dose per kilogram of body weight is that less virus would be required for therapeutic effect in humans. This is

Figure 4. A, β-Galactosidase expression level analysis of heart and skeletal muscle (4-weeks postadministration) from mice that were injected with 1 × 10¹¹ vg of rAAV2/9-CMV-lacZ as 1-day old neonates. B, β-Galactosidase expression level analysis of heart and skeletal muscle (4 weeks postadministration) from mice that were injected with 1 × 10¹¹ vg of rAAV2/9-CMV-lacZ via the jugular vein at 3 months of age (n=3).

Figure 5. A, Graph of GAA activity in tissue specimens from the hearts of rhesus macaques intravenously injected at birth with either rAAV2/1-CMV-hGaa or rAAV2/9-CMV-hGaa. Y-axis shows total GAA activity measured as previously described,¹⁶ minus background activity from noninjected controls per vector genome delivered. B, Graph demonstrating the vector genome biodistribution profile between heart and skeletal muscle tissue from rhesus macaque intravenously injected at birth with rAAV2/9-CMV-hGaa. All data are at 6 months post–vector administration.
particularly significant because although efforts for developing methods to scale up the production of rAAV are in progress, it has proven to be a challenging endeavor. An additional benefit to a lower effective dose is that the overall safety of the therapy increases as a result of the fact that less viral capsid protein would be available to potentially elicit a primary immune response. Finally, although integration of rAAV genomes is a rare event, a reduction in the total amount of transgene delivered will further reduce the likelihood of host genome integration and add to the overall safety and clinical utility of AAV vectors. Overall, these experiments provide strong preclinical data supporting future human studies.

Using our Gaa<sup>−/−</sup> mouse model for Pompe disease, we have shown that the delivery of rAAV2/9-CMV-hGaa can improve the characteristically shortened PR interval. These noninvasive studies are ongoing to fully elucidate whether this will continue to improve further and/or how long this improvement can be maintained.

Safety is a principal concern in all gene-transfer studies, and, importantly, we observed no adverse events or signs of illness in mice or primates following systemic gene delivery of any of the AAV serotypes used in this study. Additionally, hematoxylin/eosin staining was performed on all tissues harvested from these animals, and there were no signs of inflammation in these samples at the time points studied. We anticipate that these ongoing studies will lead to clinically relevant gene-transfer strategies for cardiovascular disease and offer new therapeutic options to patients with conditions amenable to this approach.

Acknowledgments

We thank Mark Potter and the University of Florida Powell Gene Therapy Center (PGTC) Vector Core Laboratory for the production of rAAV vectors used in this study and the PGTC National Gene Vector Laboratories Toxicology Core for vector genome quantification. The authors also thank the technical staff members, including Alyssa Lepley and Christine Mall in the Center for Fetal Monkey Gene Transfer for NHLBI and technical staff at the Primate Center.

Sources of Funding

This work was supported by grants from the NIH (National Heart, Lung, and Blood Institute grant PO1 HL59412; National Institute of Diabetes and Digestive and Kidney Diseases grant PO1 DK58327; AT-NHLBI-U01 HL69748; National Center for Research Resources base operating grant RR00169 to the Primate Center, A.F.T.); the American Heart Association, Florida and Puerto Rico Affiliate (to C.A.P.); and the AHA National Center (to C.S.M.).

Disclosures

The Johns Hopkins University, the University of Florida, and B.J.B. could be entitled to patent royalties for inventions described in this article.

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_Circ Res._ 2006;99:e3-e9; originally published online July 27, 2006; doi: 10.1161/01.RES.0000237661.18885.f6

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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