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Percutaneous Transendocardial Delivery of Self-complementary Adeno-associated Virus 6 Achieves Global Cardiac Gene Transfer in Canines

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Achieving efficient cardiac gene transfer in a large animal model has proven to be technically challenging. Previous strategies have used cardiopulmonary bypass or dual catheterization with the aid of vasodilators to deliver vectors, such as adenovirus, adeno-associated virus (AAV), or plasmid DNA. Although single-stranded AAV (ssAAV) vectors have shown the greatest promise, they suffer from delayed expression, which might be circumvented using self-complementary vectors. We sought to optimize cardiac gene transfer using a percutaneous transendocardial injection catheter to deliver adeno-associated viral vectors to the canine myocardium. Four vectors were evaluated—ssAAV9, self-complementary AAV9 (scAAV9), scAAV8, scAAV6—so that comparison could be made between single-stranded and self-complementary vectors as well as among serotypes 9, 8, and 6. We demonstrate that scAAV is superior to ssAAV and that AAV 6 is superior to the other serotypes evaluated. Biodistribution studies revealed that vector genome copies were 15–4,000 times more abundant in the heart than in any other organ for scAAV6. Percutaneous transendocardial injection of scAAV6 is a safe, effective method to achieve efficient cardiac gene transfer.

INTRODUCTION

Gene therapy has great therapeutic potential. Adeno-associated virus (AAV)-mediated gene therapy has been described extensively in the literature resulting in efficient cardiac gene transfer in small animal models, including mice,[1–8] rats,[9] and hamsters,[10] and from these reports it appears that serotype 9 (AAV9) is superior to other serotypes in the heart.[3,4,7,8] In addition to being efficient, AAV-mediated transgene expression is also stable, an advantage over other commonly used vectors such as adenovirus or plasmid DNA.[6] However, AAV vectors have the significant disadvantage of delayed expression, taking nearly 1 month to reach full expression,[11] which would limit their usefulness in a rapidly progressing cardiomyopathy. This limitation might be overcome by using the recently developed self-complementary AAV (scAAV) vectors, which package a double-stranded genome and thus bypass the need for complementary strand synthesis. This offers the advantage of faster onset of expression that may also be more efficient than traditional single-stranded AAV (ssAAV) vectors, albeit at the cost of halving the possible size of the expression cassette.[12–16]

Although cardiac gene transfer has been extremely successful in small animal models, delivery to the heart in large animal models and humans has proven to be technically challenging. Several delivery methods have been investigated with varying degrees of success using AAV, adenovirus, or plasmid DNA as vectors. Pericardial instillation of vector results in gene transfer that is restricted to the epicardium.[17,18] Direct, transepicardial injection of vector after left thoracotomy allows delivery throughout the left ventricular free wall (LVFW); however, this is highly invasive and cannot target the interventricular septum (IVS).[19–23] Infusion of vector into the coronary arteries can lead to efficient gene transfer, but optimal transfer often requires highly invasive cardiopulmonary bypass[24–28] or dual catheterization of a coronary artery and vein[29–33] using potentially dangerous pharmacological vasodilators. Promising preclinical and phase I/II results have been obtained using NOGA left ventricular electromechanical mapping to guide transendocardial injections of either plasmid DNA or adenovirus via a percutaneously inserted injection catheter.[34–37] Although this method is relatively noninvasive, it requires creation of a 3D map of the heart using very specific and expensive equipment before injection. In addition, the gene transfer vectors used in these studies have been associated with inflammation and unstable expression in the case of adenovirus, and low efficiency and unstable expression in the case of plasmid DNA.[38]

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Our goal in this study was to optimize cardiac gene delivery in a large animal model. We focused on AAV vectors because we believe that their low immunogenicity and stable, efficient expression makes them the ideal gene therapy vector for myocardial diseases. We hypothesized that we could achieve safe, efficient, global cardiac gene transfer in the canine model with a novel injection catheter to deliver scAAV vectors under fluoroscopic guidance. We report (i) that percutaneous transendocardial delivery of scAAV6 can be used to achieve highly efficient, global gene transfer to the canine heart with rapid onset of expression and (ii) that AAV6 is superior to other serotypes tested.

RESULTS

Study design

In this canine study, we compared the cardiac transduction efficiency of four different AAV vectors 7–10 days after endomyocardial injection via a percutaneously inserted, steerable injection catheter. Forty injections were performed to distribute the vector globally throughout the LVFW and IVS. Each AAV vector was designed to express the enhanced green fluorescent protein (EGFP) reporter gene under control of the chicken β-actin promoter with cytomegalovirus enhancer (AAV-CB-EGFP). The vectors evaluated were ssAAV9, scAAV9, scAAV8, and scAAV6 ($n = 4$ dogs per group, 16 total dogs). An additional dog received saline-only injection to serve as a negative control, and three dogs were injected with AAV6 empty capsid for long-term (6 months) safety evaluation. By analyzing these vectors, we were able to compare ssAAV with scAAV (both AAV9) as well as serotypes 9, 8, and 6 (all scAAV).

A subset of canines ($n = 8$), all of which received parvovirus vaccine, was screened for the presence of pre-existing antibodies for AAV9, 8, and 6. Antibody titer was undetectable for serotypes 8 and 9, and either undetectable ($n = 2$) or borderline detectable [1:20 serum dilution ($n = 4$) or 1:40 serum dilution ($n = 2$)] for AAV6.

scAAV9 versus ssAAV9 in the canine myocardium

Based on the reports that AAV9 is the most efficient AAV serotype for cardiac gene transfer in mice,[3,4,7,8] we first chose to assess the efficiency of AAV9-mediated cardiac gene transfer in the canine model. In addition, because the newly developed scAAV vectors have been used to achieve expression levels 1–2 logs greater than traditional ssAAV vectors,[12–15] we compared the gene transfer efficiency of ssAAV9 with scAAV9. A lower dose of scAAV9 was used [5×10^{11} genome copies (gc/kg)] compared with ssAAV9 (2×10^{13} gc/kg) because we believed that the potentially higher efficiency of this scAAV vector would allow us to achieve significant gene transfer while minimizing viral load in the subject. scAAV9 mediated 50-fold greater cardiac gene transfer expression than ssAAV9 at a 40-fold lower dose ($P = 0.001$), suggesting an efficiency advantage of ~3 logs (**Figure 1a–d**). However, only ~7% of the myocardium was transduced in the scAAV9 group (positive for GFP expression) (**Figure 1e**).

scAAV6 versus scAAV8 versus scAAV9 in the canine myocardium

A vector capable of gene transfer to only 7% of the myocardium would have low therapeutic potential. Therefore, we next evaluated
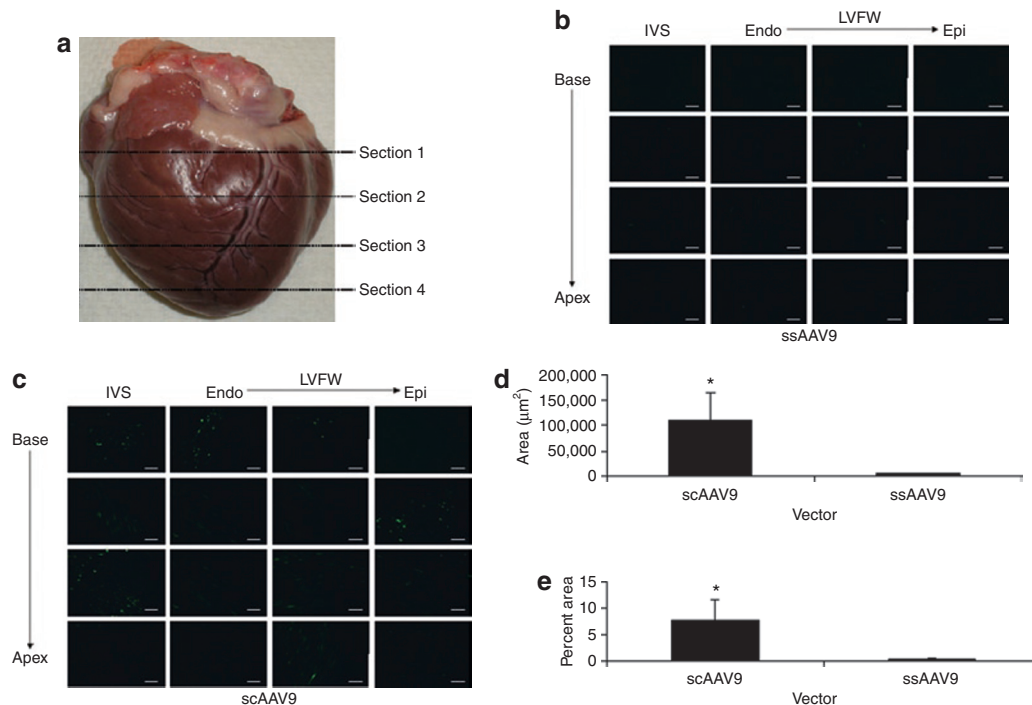

Figure 1 Delivery of single-stranded adeno-associated virus 9 (ssAAV9) and self-complementary AAV9 (scAAV9) to the canine heart via needle-tip injection catheter. (**a**) For analysis, the heart was divided into four equal sections through the short axis from base to apex. Green fluorescent protein (GFP) expression in representative cryosections of the heart 7–10 days after injection of either (**b**) 2×10^{13} genome copies (gc)/kg of ssAAV9-CB-EGFP or (**c**) 5×10^{11} gc/kg of scAAV9-CB-EGFP. Endo, endocardium; Epi, epicardium; IVS, interventricular septum; LVFW, left ventricular free wall. Bar = 200 μm. Quantitative analysis of GFP expression reported as either (**d**) total area positive for GFP or (**e**) percent cardiomyocyte area positive for GFP. Note that scAAV9 is superior to ssAAV9 even at a 40-fold lower dose (*$P = 0.001$). Error bars represent mean ± SD.
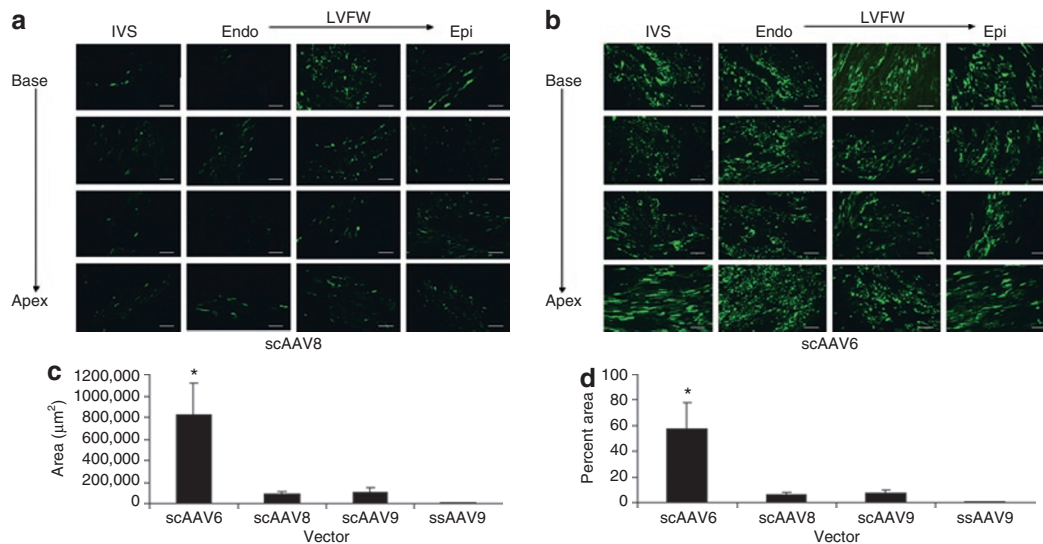
Figure 2 Delivery of self-complementary adeno-associated virus 8 (scAAV8) and scAAV6 to the canine heart via needle-tip injection catheter. Green fluorescent protein (GFP) expression in representative cryosections of the heart 7–10 days following injection of either 5×10^{11} gc/kg of (**a**) scAAV8-CB-EGFP or (**b**) scAAV6-CB-EGFP. Endo, endocardium; Epi, epicardium; IVS, interventricular septum; LVFW, left ventricular free wall. Bar = 200 μm. Quantitative analysis of GFP expression reported as either (**c**) total area positive for GFP or (**d**) percent cardiomyocyte area positive for GFP. Note that scAAV6 is superior to the other serotypes examined (*$P = 0.0001$). Error bars represent mean ± SD.
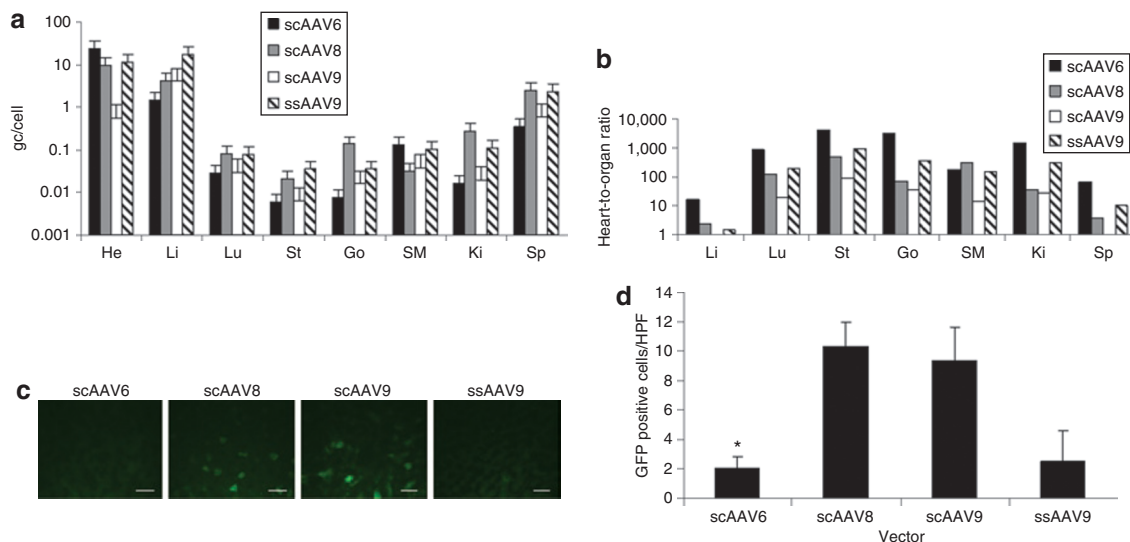

Figure 3 Biodistribution of adeno-associated virus (AAV) vectors 7–10 days after delivery to the canine heart via needle-tip injection catheter. (**a**) Biodistribution in several organs examined. Go, gonad; He, heart; Ki, kidney; Li, liver; Lu, lung; SM, skeletal muscle; Sp, spleen; St, stomach. (**b**) Tissue tropism of vectors evaluated reported as ratio of vector detected in heart to vector detected in organ of choice. Note that self-complementary AAV6 (scAAV6) is highly tropic for cardiac tissue when compared with the other vectors. (**c**) GFP expression in cryosections of the liver 7–10 days after treatment. Bar = 40 μm. (**d**) Quantitative analysis of GFP expression in liver. Note that there are significantly fewer positive cells in the scAAV6 group compared with other scAAV serotypes (*$P = 0.002$). HPF (high power field) (×40). Error bars represent mean ± SD.

the cardiac gene transfer efficiency of two additional serotypes, 8 and 6. Serotype 8 was chosen because it has relatively high cardiac tropism in the mouse[3–5,8] and rat,[9] and serotype 6 was chosen because it has a relatively high cardiac tropism in the pig when compared to serotypes 2 and 5.[32,33] We continued the study with the superior scAAV vector designed to express CB-EGFP at a dose of 5×10^{11} gc/kg. Although the cardiac gene transfer efficiency of

scAAV8 was similar to that of scAAV9, scAAV6 was significantly more efficient than the other vectors evaluated in this study ($P = 0.0001$) (**Figure 2a–d**). With scAAV6, high-level expression of GFP was evident throughout the LVFW and IVS in ~60% of the cardiomyocyte area (**Figure 2b** and **d**). Representative 4′-6-diamidino-2-phenylindole-counterstained sections for each vector and saline control can be found in **Supplementary Figure S1**.

Biodistribution of AAV vectors after transendocardial injection

AAV vector genomes were detected in all tissues examined (**Figure 3a**); however, the ratio of genomes detected in the heart to genomes detected in other tissues varied significantly from vector to vector (**Figure 3b**). Of the four vectors examined, scAAV6 had the highest cardiac specificity with heart-to-tissue ratios ranging from 15 in the liver to ~100 in the spleen and up to 4,000 in other tissues. In contrast, the heart-to-organ ratios for the other vectors evaluated were ~2 or less for the liver and 10 or less in the spleen (**Figure 3b**). GFP fluorescence was also minimal in liver sections from animals treated with scAAV6 compared to other sc vectors ($P = 0.002$) (**Figure 3c** and **d**).

Safety of percutaneous transendocardial delivery of AAV vectors

There was no mortality associated with our protocol. However, ventricular ectopy with secondary hypotension was noted in 14 of 16 dogs at the time of AAV injection. The dog treated with saline only also experienced ventricular ectopy. These arrhythmias were amenable to lidocaine infusion in all 14, and lidocaine was discontinued uneventfully once the injections were completed. Ventricular arrhythmias had resolved in all dogs by the time they had recovered from anesthesia. Color flow Doppler of the aortic valve, which was performed in a subset of animals ($n = 6$ dogs) before and after procedure, revealed no change in valve leakage secondary to transaortic catheterization in all dogs examined. Histological analysis of hematoxylin and eosin- and trichrome-stained cardiac sections revealed minimal amounts of fibrosis and mononuclear cell infiltration at the sites of injection (**Supplementary Figure S2**).

DISCUSSION

Cardiac gene therapy has great therapeutic potential, but its application has been hindered due to the lack of a safe and effective delivery method in a large animal model. We report here a simple, relatively noninvasive protocol in the canine which results in highly efficient, global cardiac gene transfer mediated by scAAV6 after delivery via a percutaneous injection catheter. This procedure is superior to previously reported large animal cardiac gene transfer techniques because it combines the advantages of direct injection with those of a noninvasive percutaneous delivery system.[17–38] Because it results in high-level transgene expression throughout the LVFW and IVS, this technique could be useful to treat diseases which affect the heart globally, such as cardiomyopathy. Furthermore, localized delivery is also possible, and importantly can be achieved in poorly perfused areas. This offers a tremendous advantage over vascular approaches in treating regional ischemia, which is important considering that ischemic cardiomyopathy accounts for ~40% of all heart failure cases.[39]

Under fluoroscopic guidance, the catheter is steerable and needle-tip length is adjustable so that multiple injections can be targeted throughout the left ventricle from base to apex and from endocardium to epicardium. Fluoroscopy images recorded during the procedure demonstrate how the catheter tip can be deformed to target a specific region of the heart and how contrast media is used to track injections (**Supplementary Figure S3**). Therefore, the technique lends itself to global gene delivery or targeted gene delivery for addressing a regional defect.

Moreover, our delivery method is simple, safe, and relatively noninvasive, suggesting it would be better tolerated in patients with significant heart disease than procedures requiring thoracotomy, cardiopulmonary bypass, or electromechanical mapping before injection. The catheter has been safely employed to deliver skeletal myoblasts to large animals and humans,[40–43] but to our knowledge this is the first report of its use for gene delivery. The only adverse event associated with the injection procedure was ventricular ectopy that was amenable to lidocaine infusion. Because ectopy was noted in the saline-injected animal as well, it is likely a result of the intramyocardial injections themselves and not the AAV vector. Pathology at 7–10 days revealed only small fields of fibrosis at the injection sites with minimal mononuclear infiltration, which is similar to what was observed following direct myocardial injection in a porcine model at 2 weeks.[23] Finally, our technology does not require the use of expensive and sometimes dangerous vasodilators, such as vascular endothelial growth factor, for effective delivery.

Furthermore, we have identified an ideal gene transfer vector in a large animal model. While many investigators have relied on unstable vectors such as adenovirus and plasmid DNA in their large animal cardiac models, we have focused on the stable scAAV vector with the goal of achieving long-term expression with rapid onset. The scAAV vectors contain a mutation in one of the replication termination sequences that allows synthesis and packaging of a dimeric inverted repeat.[15] As a result, these vectors bypass the rate-limiting requirement for second strand synthesis and have been reported to be more efficient than the traditional ssAAV vectors in several species and tissues.[13–15] Our findings confirm these previous reports for the first time in the canine heart. We demonstrate that scAAV is several logs more potent than ssAAV in the canine heart, at least within the short time frame of these experiments (7–10 days). It may be that the ssAAV expression would approach that of the scAAV over weeks to months;[44] however, this would still limit its possible therapeutic applications as compared with scAAV vectors because many myocardial diseases, such as ischemia/infarction would demand rapid onset of expression for ideal therapeutic efficacy. Although we did not look for expression earlier than 1 week, small animal data suggest that transgene expression mediated by AAV can initiate as early as 1–4 days.[44,45] Further studies are needed in this canine model to determine a time course of expression.

We also observed that AAV6 is ~1 log more potent than AAV8 and AAV9 in the canine heart. This result was unanticipated based on a review of the literature. Although data from porcine hearts suggest that AAV6 is superior to 2 and 5,[32,33] a study in rats that excluded serotype 9 demonstrated the superiority of AAV8 over serotypes 1–7 in the heart,[9] and three reports in mice demonstrated that AAV9 is superior to 1 and 8 in the heart.[3,4,7] This led us to hypothesize that serotype 9 would be superior to 8, which would be superior to 6. However, the small animal data did not accurately predict the large animal outcome because we observed quite the opposite result. Based on our data, scAAV6 is ~1 log more potent than the other scAAV vectors evaluated in this study (**Figure 2d**). We screened a subset of our dogs for the presence of

pre-existing neutralizing antibodies against AAV9, 8, and 6 and detected extremely low-titer antibodies (<1:20 to 1:40 serum dilution) only for AAV6. Because we observed that AAV6 was the most efficient cardiac gene transfer vector, it is unlikely that differential titers of pre-existing neutralizing antibodies were responsible for the differential cardiac transduction reported in this study.

A recent study examined the mechanism of high-efficiency cardiac gene transfer by AAV6 and found that serotype 6 displays both enhanced cellular internalization and nuclear uncoating in cardiomyocytes compared with serotype 2.[16] Perhaps, the canine heart contains a higher density of the AAV6 receptor compared with other species, and this fact, combined with its high efficiency of nuclear uncoating and self-complementary DNA structure, may explain the superior performance of scAAV6 in this study. Further investigation is needed to confirm this hypothesis. It will also be important to conduct future studies to determine the time course of expression because this may vary depending on the serotype used.

In addition to being highly efficient, scAAV6 is also relatively cardiac specific compared with the other serotypes evaluated, which further increases its utility as a cardiac gene therapy vector. In terms of vector biodistribution, the heart-to-liver ratio was 15 for scAAV6 compared with 2 or less for the other serotypes. The heart-to-organ ratio of scAAV6 was also very favorable in other organs and ranged from 100 in the spleen up to 4,000 for the gonads. This preferential cardiac transduction of AAV6 has also been reported in the mouse[8] and pig.[32] Although the transduced cell types in the spleen and other organs were not investigated, another report has identified the ability of AAV to transduce monocytes in the spleens of nonhuman primates.[46]

It should be noted, however, that although our canine data suggest that AAV6 may be the vector of choice for clinical trials of cardiac gene transfer, the human heart may display a different tropism for AAV serotypes. Indeed, a study in nonhuman primates showed that AAV9 was able to provide fourfold greater expression than AAV1 in the heart.[4] Although AAV1 and AAV6 share >95% sequence homology in their capsids,[11] a recent report noted that the relative tissue tropisms of AAV1 and 6 were substantially different in several organs examined, including an almost 2 log advantage for AAV6 in the heart.[8] Therefore, despite high sequence homology, AAV1 and 6 may not necessarily perform similarly in the heart. In addition, the nonhuman primates were injected within 1 hour of birth, and because protein expression profiles change throughout development, the receptors for different AAV serotypes may be differentially expressed in the neonate compared to the adult. A comprehensive comparison of AAV serotypes in adult nonhuman primate hearts, which would be the large animal model phylogenetically closest to humans, may be necessary to identify the ideal vector for human clinical trials.

This novel protocol has great therapeutic potential because it can be safely used to transfer a vector capable of rapid onset, efficient, long-term expression to the heart via a simple, relatively noninvasive procedure. Its potential applications are numerous, and include most forms of acquired and genetic cardiomyopathy in both the veterinary and human setting. Limitations of our study include a short follow-up period after procedure and lack of experience with specific therapeutic transgenes. This period

was necessitated by the reporter transgene used in our study. GFP expression was analyzed 7–10 days after injection so that we could appreciate true transfer efficiency before a T-cell response against the cells expressing the foreign reporter protein was mounted.[21] However, injection of empty capsid (AAV6) in three dogs did not result in any clinical or myocardial change for 6 months after procedure, and no T cells reactive to AAV6 capsid were detected by enzyme-linked immunosorbent spot (**Supplementary Figure S4** and **Supplementary Materials and Methods**). This suggests that AAV6 has potential as a long-term expression vector in the heart, which is important in light of a recent report which described a cellular immune response against the capsids of AAV2 and 6 following direct injection into canine skeletal muscle.[47] In this model, immunosuppression was necessary for long-term expression.[48] It may be noted that the immune response to AAV varies from tissue to tissue, and long-term expression in the heart may be possible without immune modulation. It is also possible that differences in vector preparation could alter the immune response. Further studies with nonimmunogenic transgenes are necessary in our model to confirm this hypothesis.

Our protocol could be optimized in several ways to meet specific clinical needs. The DNA packaging capacity of scAAV is limited to half that of the traditional ssAAV vectors due to the fact that a dimeric repeat is encapsidated.[15] If lower expression levels and/or delayed expression could be tolerated, ssAAV6 could be used to deliver a much larger therapeutic gene at the cost of less efficient and/or delayed expression. For safety, in order to restrict expression outside of the heart, transcriptional and/or transductional targeting could be used.[49] In patients with heart disease, considering the frequency of ventricular ectopy in our healthy dogs, a lidocaine-constant rate infusion should probably be initiated at the time of anesthetic induction. Finally, increased expression efficiency may be achieved by increasing the number and/or volume of injections, and delivery could easily be directed to the right ventricle following a femoral or jugular vein catheterization. Investigation is underway to evaluate the feasibility of these approaches.

MATERIALS AND METHODS

Vector design and production. Each vector was designed to express the EGFP reporter gene under control of the constitutive chicken β-actin promoter with cytomegalovirus enhancer (CB promoter). Vectors were produced according to the previously described pseudotyping protocol by the Vector Core of the University of Pennsylvania.[11] Briefly, recombinant AAV genomes containing AAV2 inverted terminal repeats were packaged by triple transfection of 293 cells with a *cis*-plasmid containing the EGFP transgene, an adenovirus helper plasmid, and a chimeric *trans*-plasmid containing the AAV2 rep gene fused to the capsid gene of the AAV serotype of interest. Self-complementary vectors contained a mutation in the termination sequence of the 5′-inverted terminal repeat to allow synthesis and encapsidation of a dimeric inverted repeat of the transgene cassette.[15]

Animal use and vector delivery protocol. All animals were handled in compliance with National Institutes of Health and institutional guidelines that were approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania. Twenty mongrel dogs (5–10 kg, 3–6 months old) were used in this study. Sixteen mongrel dogs were randomized to receive one of the four vectors: ssAAV9, scAAV9, scAAV8, or scAAV6 (*n* = 4 per group). An additional dog received saline-only injection to serve as a negative control for GFP fluorescence and biodistribution studies, and three dogs were treated with AAV6 empty capsid for long-term (6 months)

safety evaluation. Pre-existing, neutralizing antibody titer for AAV9, 8, and 6 was determined in a subset of canines ($n = 8$) from the colony, all of which were vaccinated against parvovirus. Titer was determined by incubating Huh7 cells with serial dilutions of canine serum and AAV-CMV-EGFP of the serotype in question and observing the dilution at which the number of GFP-positive cells was reduced by 50% compared to control wells.

Procedures were performed under general anesthesia, and dogs were placed in left lateral recumbency. Heart rate, respiratory rate, systolic blood pressure, electrocardiogram, and oxygen saturation were monitored throughout the anesthetic period. In a subset of dogs ($n = 6$), transesophageal echocardiography was also performed throughout the procedure to monitor patency of the aortic valve.

A right carotid arterotomy was performed, and a 7-French introducer was placed in the vessel, followed by insertion of the injection catheter. This catheter was a steerable injection catheter with an adjustable length core needle (MyoCath; Bioheart, Sunrise, FL),[42] which has previously been used to deliver skeletal myoblasts to large animals and humans.[40–43] The catheter was flushed with heparinized blood before vector infusion to prevent inactivation of the virus.[50] Next, under fluoroscopic guidance, the catheter was advanced into the left ventricular cavity, and by steering the needle-tip and adjusting the needle length, ~40 transendocardial injections of 250 µl each were performed to target the LVFW and IVS from base to apex and from endocardium to epicardium with AAV vector. Contrast media was added to the vector solution so that injection sites could be visualized. This allowed us to differentiate between injected and uninjected regions of the heart and helped to ensure that the vector solution was distributed globally throughout the myocardium. Still fluoroscopy images recorded during the procedure demonstrate how the catheter tip can be deformed to target a specific region of the heart (**Supplementary Figure S3a**) and how contrast media was used to track single (**Supplementary Figure S3a**) and multiple (**Supplementary Figure S3b**) injections during the same procedure.

A dose of 2×10^{13} gc/kg was used for ssAAV9; however, a lower dose (5×10^{11} gc/kg) was used for the self-complementary vector injections. For each procedure, vector was mixed with 2 cc of sterile contrast solution (Omnipaque) and diluted with sterile saline to produce 10 cc for injection. Lidocaine (2 mg/kg as a bolus followed by a constant rate infusion at 50 µg/kg/min) was initiated if ventricular tachycardia developed during the procedure (14 of 16 dogs). After recovery, dogs were treated with carprofen for 2 days and amoxicillin–clavulanic acid for 5 days.

Histological analysis of transgene expression and pathology. Dogs were killed 7–10 days following treatment and tissues were harvested for determination of reporter transgene expression and vector biodistribution. For analysis of GFP fluorescence, the heart was divided into four sections along the short axis from the apex to base and fixed in 4% paraformaldehyde overnight at 4 °C. The tissue was then washed 3 × 10 minutes in phosphate-buffered saline and dehydrated in 20% sucrose overnight at 4 °C. Next, each section was frozen in optimal cutting temperature embedding compound (Tissue-Tek; Sakura Finetek, Torrance, CA), and 10-µm cryosections were prepared. Slides were mounted with Vectashield 4′-6-diamidino-2-phenylindole media (Vector Laboratories, Burlingame, CA) and examined for GFP fluorescence using a Leitz DMRBE fluorescent microscope (Leica, Bannockburn, IL) equipped with a Micro MAX digital camera (Princeton Instruments, Trenton, NJ) interfaced with Image Pro Plus software (Media Cybernetics, Bethesda, MD). For each animal, four representative photographs were recorded under constant exposure conditions using the ×10 objective from the IVS and LVFW from the endocardium to the epicardium in each of the four heart sections, and the area positive for GFP fluorescence was quantified using Open Lab software (Improvision, Waltham, MA). The threshold for detection was set above background levels measured in the negative control, saline-treated dog. Immunofluorescent staining for cardiac troponin T (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) was also performed, and the GFP-positive area was normalized to the troponin-positive area to report the percent cardiomyocyte area positive for GFP. A sample of liver from each animal was also fixed, sectioned, and analyzed as described above to quantify GFP-positive hepatocytes. For pathological analysis, slides were stained independently with hematoxylin and eosin and trichrome (Sigma, St. Louis, MO) to identify mononuclear infiltrate and fibrosis, respectively.

Biodistribution analysis. For biodistribution analysis, samples were snap-frozen in liquid nitrogen. Following DNA extraction, genome copy titers were quantified by TaqMan PCR (Applied Biosystems, Foster City, CA) using primers and probes designed against the EGFP transgene. One microgram of template DNA was used for each reaction, and several controls were performed to confirm the specificity and accuracy of the PCR. A spike control was performed in which the test sample was spiked with exogenous test assay target to rule out PCR inhibition. The assay was also performed on samples from the negative control, saline-injected animal to determine background signal of the assay, which was negligible (<1 gc per 5,000 cells, or >1 log lower than the minimum gc detected in experimental samples). Finally, a control endogenous gene (glyceraldehyde 3-phosphate dehydrogenase) was run as a loading control and displayed minimal sample to sample variation (<0.25 cycles) with 1-µg template DNA.

Statistical analysis. Mean values from each experimental group were compared using the two-tailed Student's *t*-test or one-way ANOVA with Student–Newman–Keuls *post hoc* analysis.

SUPPLEMENTARY MATERIAL
Figure S1. Representative cardiac sections from each vector and saline control demonstrating GFP fluorescence with DAPI counterstain.
Figure S2. Representative cardiac sections from an AAV-injected canine and uninjected control stained with H&E and trichrome.
Figure S3. Delivery of AAV via deformable injection catheter under fluoroscopic guidance.
Figure S4. Representative ELISpot data from a canine treated with AAV6 empty capsid.
Materials and Methods.

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