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ORIGINAL ARTICLE

Ex vivo intracoronary gene transfer of adeno-associated virus 2 leads to superior transduction over serotypes 8 and 9 in rat heart transplants

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Summary

Heart transplant gene therapy requires vectors with long-lasting gene expression, high cardiotropism, and minimal pathological effects. Here, we examined transduction properties of *ex vivo* intracoronary delivery of adeno-associated virus (AAV) serotype 2, 8, and 9 in rat syngenic and allogenic heart transplants. Adult Dark Agouti (DA) rat hearts were intracoronarily perfused *ex vivo* with AAV2, AAV8, or AAV9 encoding firefly luciferase and transplanted heterotopically into the abdomen of syngenic DA or allogenic Wistar–Furth (WF) recipients. Serial *in vivo* bioluminescent imaging of syngraft and allograft recipients was performed for 6 months and 4 weeks, respectively. Grafts were removed for PCR-, RT-PCR, and luminometer analysis. *In vivo* bioluminescent imaging of recipients showed that AAV9 induced a prominent and stable luciferase activity in the abdomen, when compared with AAV2 and AAV8. However, *ex vivo* analyses revealed that intracoronary perfusion with AAV2 resulted in the highest heart transplant transduction levels in syngrafts and allografts. *Ex vivo* intracoronary delivery of AAV2 resulted in efficient transgene expression in heart transplants, whereas intracoronary AAV9 escapes into adjacent tissues. In terms of cardiac transduction, these results suggest AAV2 as a potential vector for gene therapy in preclinical heart transplants studies, and highlight the importance of delivery route in gene transfer studies.

Introduction

Heart transplantation is a standard therapy for many end-stage heart diseases. Nowadays, acute rejection of cardiac allografts is effectively inhibited by modern immunosuppressive drugs [1]. However, these drugs fail to prevent ischemia-reperfusion injury and also have many adverse side effects, such as increased incidence of comorbidities, infections, and malignancies. Moreover, the long-term survival of cardiac allografts is challenged by the development of heart failure due to cardiac allograft vasculopathy (CAV) and cardiac fibrosis [2].

Experimental studies have highlighted the role of gene therapy as a potential therapeutic tool in the treatment of cardiac allografts. Local expression of a therapeutic gene in the heart transplant would have many advantages over systemic drugs: it could exclusively target the transplant, eliminate systemic adverse effects, and remove the risk of

low drug compliance. Possible therapeutic genes could target inflammatory pathways involved in ischemia-reperfusion injury, acute rejection, and fibroproliferative pathways critical in the development of primary or late graft failure [3]. Preclinical studies with gene transfer of cytoprotective heme oxygenase-1, immunomodulatory IL-4 and IL-10, and T-cell costimulatory activation inhibitors CTLA4-immunoglobulin (CTLA4Ig) and CD40Ig to cardiac allografts have been shown to prolong graft survival [4–8]. However, progress toward clinical trials has been slow, and the first applications of gene therapy to clinical transplantation are still pending.

Although perfusion of an organ transplant with a viral vector is technically feasible, the choice of the viral vector is of vital importance. It has to be free of any pathogenicity and be able to provide a long-lasting and stable transgene expression specifically in the target tissue. Adeno-associated virus (AAV) has been subject to a wide array of research due to its relatively low pathogenicity, ability for long-lasting and stable transgene expression, and broad tissue tropism. The first clinical trials of AAV gene therapy on heart failure patients have shown encouraging results regarding vector safety and improvement in patient condition [9].

Adeno-associated virus is a *Dependovirus*, thus belonging to the *Parvoviridae* family. Its genetic structure includes a single strand of DNA with two open reading frames (ORFs) flanked by inverted terminal repeats (ITRs) [1,10]. The ORFs are called rep and cap. Rep encodes four REP proteins involved in replication of the viral genome, while cap encodes the three structural proteins VP1, VP-2, and VP-3. These proteins are produced via alternative splicing and construct the viral capsid. ITRs are elements necessary for integration, replication, and packaging of the viral genome into the capsid shell [2,11]. There are over 100 naturally occurring serotypes of AAV, of which AAV1–9 have been most widely studied in terms of kinetics, tissue tropism, and pathogenicity. These serotypes differ mainly in the properties of their viral capsids; AAV2 has receptors for heparan sulfate proteoglycan, allowing it to bind faster and more readily to the extracellular matrix than AAV8 and 9 [3,12,13]. Serotypes 6, 8, and 9 have the highest cardiotropism of the investigated serotypes 1–9 [9,14–17]. However, there is a direct relation between virus titer and cardiac tissue transduction, with higher doses resulting in higher transduction [16]. Moreover, mice cardiac tissue transduction is not dependent on animal age, as opposed to the aorta, liver, and kidney [18].

As different AAV serotypes have not been directly compared in *ex vivo* intracoronary gene transfer in the heart transplants, we investigated the transduction properties of AAV2, AAV8 and AAV9 in syngenic and fully MHC-mismatched allogenic heart transplants in adult rats.

Materials and methods

Experimental design

The long-term kinetics of the vectors was compared in syngenic heart transplants. For this purpose, we used recombinant AAV serotypes 2 (AAV2-Luc; $n = 6$), 8 (AAV8-Luc; $n = 6$ in syngenic), and 9 (AAV9-Luc; $n = 7$) encoding firefly luciferase (titer = 2.68 vg/μl) and fully major histocompatibility-matched, specific pathogen-free inbred adult male rats of the Dark Agouti strain (DA, Scanbur, Stockholm, Sweden) weighing 225–275 g. The recipients received no immunosuppression. The effects of an alloimmune response on vector activity were examined using fully MHC-mismatched allogenic heart transplant Wistar–Furth (WF) rat recipients. For this purpose, we used AAV serotypes 2 (AAV2-Luc; $n = 4$, titer = 1.09×10^9) and 9 (AAV9-Luc, $n = 6$, titer = 7.82×10^8). To inhibit acute allograft rejection, and to allow moderate chronic inflammation, recipients were postoperatively immunosuppressed with s.c. Cyclosporine A (Novartis, Basle, Switzerland), at a dose of 2 mg/kg/day for the first 7 days, tapering it down to 1.5 mg/kg/day from day 14 onward. A control group with no virus was included in all experiments. The *in vivo* kinetics and distribution of the serotypes were characterized with bioluminescent imaging of the recipient rats up to 6 months in the syngenic model, and 30 days in the allogenic model. Vector activity and reporter gene expression were measured *ex vivo* with quantitative PCR, real-time RT-PCR, and luminometer analyses of the heart transplants. Graft inflammation, CAV, and cardiac fibrosis were measured by immunohistochemical and histological analyses.

Permission for the animal experiments in this study was obtained from the State Provincial Office of Southern Finland. The animals received humane care in compliance with the *Guide for the Care and Use of Laboratory Animals* prepared by the National Academy of Sciences and published by the National Academy Press (ISBN 0-309-05377, revised 2011).

Recombinant vector production, cell lines, and plasmids

The detailed production and purification of viral vectors has been previously published [1]. The firefly luciferase (Luc) gene was cloned into the psub-CMV-WPRE of AAV serotypes 2, 8, and 9. A recombinant rAAV vector plasmid, the AAV packaging plasmid pAAV/Ad-rep (ACG), and the adenovirus helper plasmid pBS-E2A-VA-E4 were used to cotransfect 293 T-cells. The cells were collected 48 h after transfection, and rAAVs were released by three freeze–thaw cycles in liquid nitrogen. rAAV2 was purified by an iodixanol-gradient ultracentrifugation and heparin-sepharose high-performance liquid chromatography. Due to their lack of heparan sulfate receptors, rAAV8 and rAAV9 were purified

using only iodixanol-gradient ultracentrifugation. For detailed information on methods, please find the online-only Supplemental Material (Expanded Methods S1).

Statistical analyses

Data in longitudinal studies were analyzed and compared by both Kruskal–Wallis and repeated measures ANOVA. The Dunn *post hoc* test was used to compare the results of multiple groups in longitudinal studies, and both one-way ANOVA with LSD *post hoc* test and Kruskal–Wallis test for multiple groups presented in box plots. Data are presented as the mean ± standard error of the mean (SEM) for longitudinal measurements. Continuous variables were presented as box plots showing the upper extreme, upper quartile, median, lower quartile, and lower extreme. All analyses were carried out using SPSS, version 19.0 software (SPSS, Inc., Chicago, IL, USA). A P-value < 0.05 was considered statistically significant.

Results

Ex vivo intracoronary perfusion of donor syngrafts with AAV9 results in rapid and strong luciferase activity in the abdominal region of the recipients

To determine the efficiency and kinetics of transgene expression of AAV serotypes 2, 8, and 9, DA rat hearts were *ex vivo* intracoronarily perfused with AAV-Luc vector serotypes 2, 8, or 9 and heterotopically transplanted into abdominal position of syngenic recipients.

Bioluminescent imaging showed that none of the recipients of AAV-Luc-perfused syngrafts expressed any luciferase activity when imaged at 2 days after transplantation (Fig. 1a). Recipients with AAV2-Luc-perfused syngrafts showed an onset of expression at 2 weeks, strictly localized to the region of the transplanted heart in the abdominal area. At the end of the *in vivo* imaging at 6 months, all of the AAV2-Luc activity was exclusively restricted to the same area. The recipients of AAV8-Luc-perfused syngrafts also showed the onset of expression at 2 weeks. In contrast to AAV2-Luc, transient luciferase activity was detected at the region of the neck, quadriceps, the tail, and abdominal midline, but localized mainly to the abdominal region where the heart was implanted from the 3rd week onward. The recipients of AAV9-Luc-perfused transplants showed an onset of expression already at 1 week, localized mainly in the head, thorax, and both upper and lower extremities. However, the luciferase expression gradually shifted to the abdominal region, and from the 2nd week onward, the signal was mostly concentrated in the abdominal area of the recipient.

Quantitative analysis of the luciferase signals from the abdominal region of the heart recipients revealed that AAV2 luciferase expression peaked at 4 weeks, AAV8 at 16 weeks, and AAV9 at 8 weeks (Fig. 1b). The peak levels of luciferase expression in the recipients of AAV9-Luc-perfused syngrafts were fourfold and up to eightfold higher than the peak levels of the recipients with AAV8-Luc- and AAV2-Luc-perfused syngrafts, respectively ($P < 0.05$). The luciferase expression levels of all the serotypes remained stable throughout the 6 months. At 6 months, the luciferase signals of the recipients with AAV9-Luc- and AAV8-Luc-perfused syngrafts were 10× and 4× higher in the abdominal region when compared with the signals of recipients with AAV2-Luc-perfused syngrafts, respectively.

Ex vivo intracoronary perfusion of syngrafts with AAV2-Luc results in the highest transduction efficiency and luciferase activity in the heart transplant

We performed *ex vivo* quantitative PCR and luminometer analyses of the syngraft apex to determine the transduction efficiency, luciferase mRNA levels, and luciferase enzyme protein activity in the transplanted heart.

The results revealed that syngrafts perfused with AAV2 contained the greatest amount of viral DNA, followed by syngrafts perfused with AAV8-Luc and AAV9-Luc ($P < 0.05$; Fig. 2a). These results were mirrored in luciferase mRNA expression ($P < 0.05$; Fig. 2b). *Ex vivo* luminometer analysis of the syngraft apex showed the highest luciferase activity in syngrafts perfused with AAV2 followed by syngrafts perfused with AAV8-Luc and AAV9-Luc ($P < 0.05$; Fig. 2c). Also, very low levels of vector particles were detected in recipient livers (Fig. 2d).

Reperfusion of the transplant results in the escape of AAV9 after *ex vivo* syngraft intracoronary perfusion

To clarify the conflicting results of *ex vivo* and *in vivo* bioluminescent imaging, we performed midline laparotomy and subsequent bioluminescent imaging to an anesthetized recipient of an AAV9 perfused syngraft 6 months after transplantation. The images revealed a strong bioluminescent signal in the abdominal muscle tissue of the recipient (Fig. 2e). Furthermore, *ex vivo* imaging of explanted tissues and organs of the same AAV9-perfused syngraft recipient revealed only weak luciferase activity in the syngraft but strong activity in the abdominal muscle, while none in the abdominal fat (Fig. 2f).

To reveal the possible escape of intracoronary AAVs into the abdominal tissue after reperfusion, we perfused cardiac syngrafts intracoronarily *ex vivo* with small-molecular fluorescence marker indocyanine green (ICG) and imaged the surgical region immediately after reperfusion before closing the laparotomy.

In vivo imaging revealed the strongest ICG signal in the area of the transplant, but a significant signal also in the

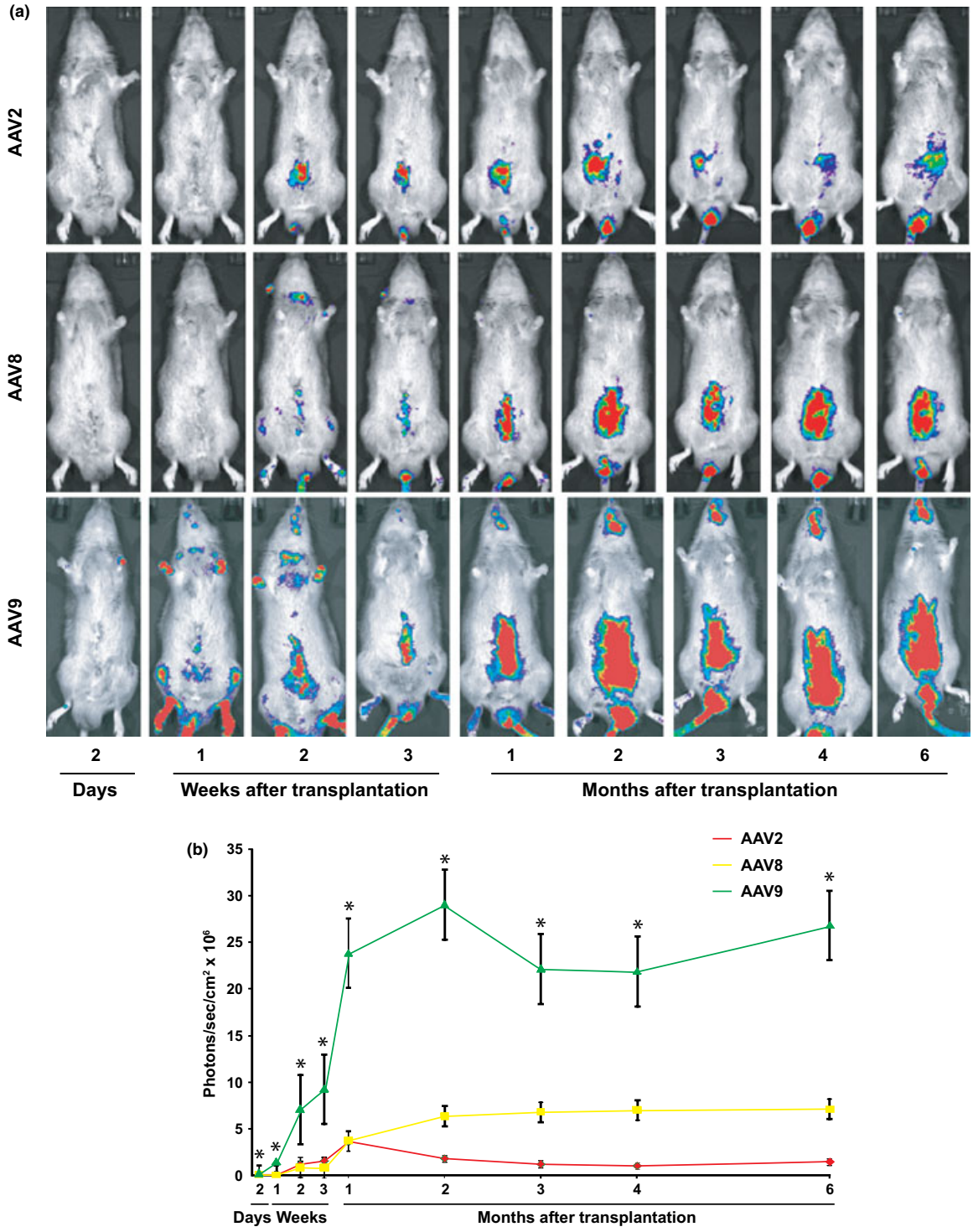

Figure 1 *In vivo* imaging and quantification of luciferase activity in the recipients of cardiac syngrafts after intracoronary delivery of adeno-associated virus (AAV) serotypes 2, 8, and 9. (a) Luciferase substrate luciferin was injected into the penile vein of the recipients of AAV vector-perfused DA syngrafts, and the recipients were subjected to bioluminescent imaging with an IVIS Xenogen bioluminescence camera. The rats were imaged at specific time points for a period of 6 months after transplantation. (b) The bioluminescence signal was quantified to create a graph of luciferase expression signal versus time. The IVIS software measured luciferase activity as photons emitted per unit time per unit area. *$P < 0.05$ versus AAV2 and AAV8 at all points. Both repeated and one-way ANOVA with LSD *post hoc* test were used to compare data between groups. Data are presented as mean ± SEM.

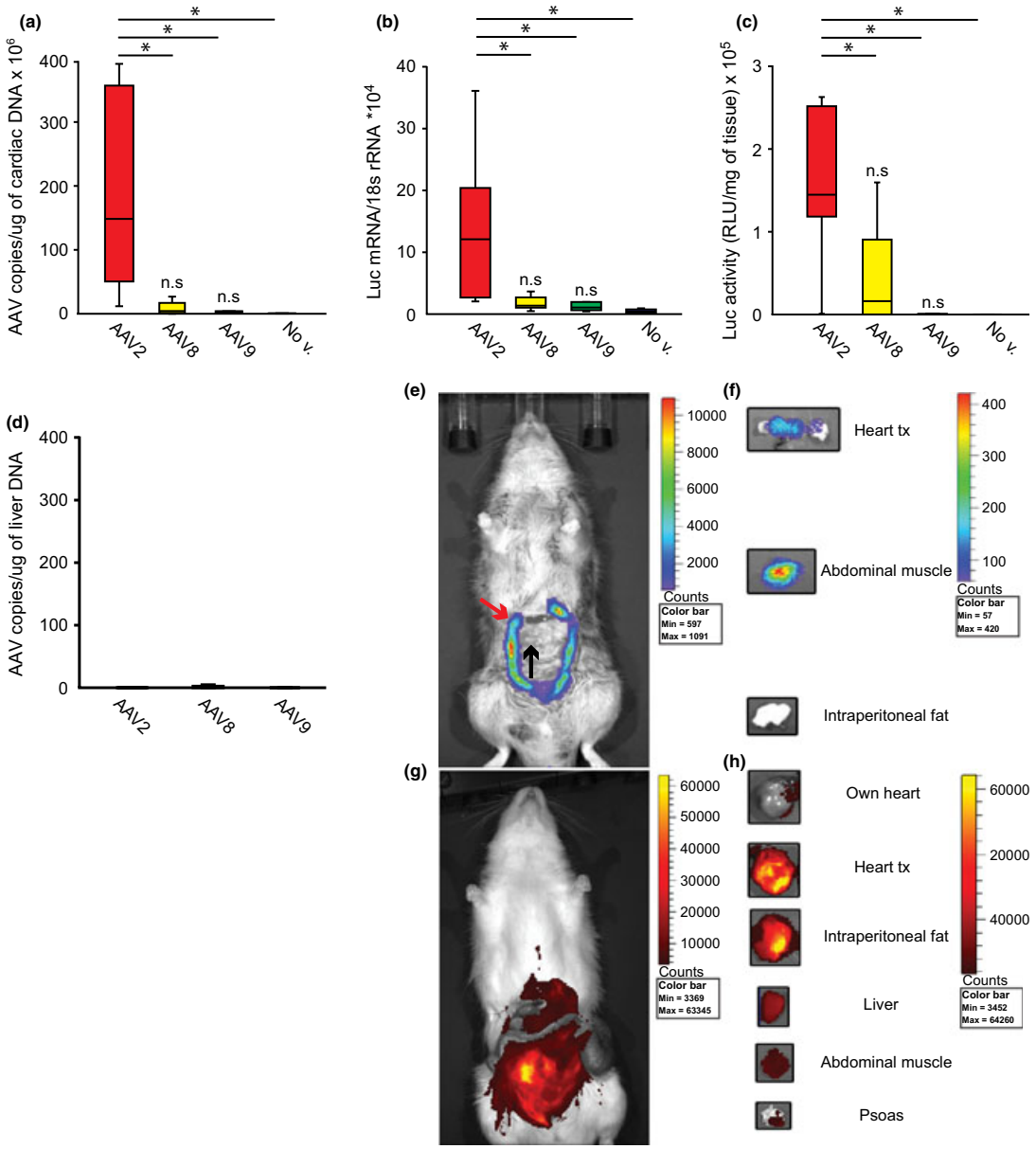

Figure 2 *In vivo* and *ex vivo* localization of vector activity in the recipients of adeno-associated virus (AAV) vector-perfused DA syngrafts using bioluminescence imaging, RT-PCR- and luminometer analysis at 6 months after transplantation. (a) Tissue samples from heart transplants were analyzed with real-time PCR to determine the amount of intragraft viral vector DNA particles 6 months after heart transplantation. 10 μg of DNA per tissue sample was used as template material. $n = 6$ in AAV2 and AAV8 group, $n = 7$ in AAV9 group. (b) Samples were analyzed with real-time RT-PCR for luciferase mRNA. The results were normalized to 18S rRNA and are given as a ratio to mRNA expressed in hearts immediately after syngraft removal. $n = 6$ in AAV2 and AAV8 group, $n = 7$ in AAV9 group. (c) The levels of luciferase activity [Real light units (RLU)/mg total protein of the tissue] were measured 6 months after intracoronary perfusion of syngrafts with AAV vectors. (d) Samples from the livers of syngraft recipients were removed at 6 months and analyzed with real-time PCR for viral DNA to determine the tendency of each serotype to enter systemic circulation. 10 μg of DNA per tissue sample were used as template material. $n = 6$ in AAV2 and -8 group, $n = 7$ in AAV9 group. (e) We applied *in vivo* bioluminescent imaging to a laparotomized, AAV9 perfused syngraft recipient at 6 months after transplantation (f) Tissue samples from the transplanted heart, abdominal muscle, and intraperitoneal fat from the same animal were removed and imaged *ex vivo* for luciferase activity. (g) To test the possible leakage of nonextracellular binding AAV9 vector from the syngraft though disconnected lymphatics after heart transplantation, we used an ICG marker to visualize the behavior of albumin binding extravasated material after intracoronary perfusion of syngrafts. The recipient of a syngraft was imaged immediately after reperfusion. (h) The transplanted heart and the recipient's own heart, as well as samples from the intraperitoneal fat, liver, abdominal muscle and psoas muscle were removed and analyzed *ex vivo* with bioluminescent imaging. *$P < 0.05$ versus all other groups, NS marks nonsignificant results. Data were analyzed by Kruskal Wallis and One-way ANOVA with LSD *post hoc* test and is presented by box plots.

abdominal cavity of the recipient (Fig. 2g). Furthermore, *ex vivo* imaging of explanted tissues confirmed that a strong ICG signal was present in the muscles of the abdominal wall, abdominal fat, liver, and psoas muscle (Fig. 2h).

Ex vivo intracoronary perfusion with AAV vectors results in chronic inflammation

The numbers of intragraft $OX62^+$ dendritic cells, MPO^+ neutrophils, $ED1^+$ macrophages, and $CD4^+$ cells were similar in the viral vector-perfused syngrafts and in the nonperfused syngrafts at 6 months (Fig. 3a–d,f). However, there was a significant increase in the number of intragraft $CD8^+$ T-cells in AAV8-Luc and AAV9-Luc-perfused syngrafts when compared with nonperfused control syngrafts ($P < 0.05$; Fig. 3e and f arrows). No differences in the development of CAV and cardiac fibrosis were observed between the groups (data not shown).

Ex vivo intracoronary injection of AAV2 into cardiac allografts results in higher amounts of intragraft vector particles than AAV9

Cardiac allografts were removed at 4 weeks and analyzed *ex vivo* for the amount of vector particles, reporter gene mRNA levels, and luciferase enzyme activity.

PCR detection of intragraft vector DNA revealed a 3× higher quantity of vector particles in the AAV2 group ($P < 0.05$; Fig. 4a). However, there was no significant difference in the amount of allograft luciferase protein activity between the AAV2 and AAV9 groups (Fig. 4b).

We observed a significant amount of AAV9 vector particles in the liver of recipients when compared with control group livers ($P < 0.05$, Fig. 4c). No significant amounts of AAV2 vector particles were measured from the liver of recipients (Fig. 4c).

AAV9 induces chronic allograft inflammation at 1 month after *ex vivo* intracoronary injection into heart transplants

The numbers of intragraft MPO^+ neutrophils, $OX62^+$ dendritic cells, $CD8^+$- and $CD4^+$ T-cells were similar in vector syngrafts and in the control group syngrafts at 1 month (Fig. 5a–f). However, there was a significant rise in intragraft $ED1^+$ macrophages in AAV9-Luc-perfused allografts when compared with nonperfused control allografts ($P < 0.05$; Fig. 5b and f, arrows).

Discussion

Gene therapy in clinical heart transplantation could be an attractive tool for providing local therapy to allografts, without systemic adverse effects. In addition, the administration of the gene vector would be technically feasible *ex vivo* during allograft preservation. However, the choice of the gene vector is extremely important. AAV vectors could be ideal tools for gene therapy of cardiac allografts as they possess only small pathogenic qualities. Also, there has been a lack of direct comparison between different AAV serotypes in a heart transplantation model.

Previous experiments on AAV vector transduction properties have mapped the *in vivo* and *ex vivo* tissue distribution of vectors after intravenous injection. In each study, vector serotypes 6, 8, and especially 9 have been the most cardiotropic vectors, and AAV2 has been a more hepatotropic one [14–16,19]. To clarify that the vectors we used in our transplant models behaved according to results from previous studies, we injected AAV2, AAV8, and AAV9 systemically into rats. Our results indicated that AAV9 indeed was the most cardiotropic one and AAV2 the least (Fig. S2). However, in clinical heart transplantation, intravenous delivery is not suitable to transduct donor hearts. This is due to the long clearance time of many vectors, the relatively small amount of vector particles that eventually transduct the cardiac tissue, and the prolonged time the graft should remain in the donor after brain death. Moreover, intravenous injection of viral vectors into recipients would result in unspecific transduction of other tissues and organs. Although therapeutic genes would be used for the treatment of cardiac allografts, those same genes and their products could have deleterious effects in healthy tissues and organs of the recipient. However, in our experiment with both intracoronary and intravenous injection of AAV vectors, we did not note any specific side effects in the animals.

Here, we compared the transduction properties of AAV2, AAV8, and AAV9 serotypes after *ex vivo* intracoronary perfusion of cardiac syngrafts and those of AAV2 and AAV9 in cardiac allografts. Successful tissue transduction would first require crossing the vascular endothelial barrier. Then, the vector needs to tether to the target cell and initiate receptor-mediated endocytosis [12,20–23]. Next, the virus is transported to the nucleus [20,22,24–26]. In the nucleus, the viral capsid is uncoated, and the single-stranded DNA is transformed into double-stranded DNA [20–22,24,27,28]. We discovered that *ex vivo* intracoronary perfusion of cardiac syngrafts with AAV2-Luc resulted in superior cardiac transduction levels than perfusion with AAV8-Luc or AAV9-Luc, as measured by the highest amount of intragraft vector DNA and luciferase activity. Similar results were observed in the comparison of AAV2-Luc and AAV9-Luc in an allogenic model.

The low cardiac transduction rate of AAV9 was surprising, as noninvasive *in vivo* bioluminescent imaging showed strong abdominal luciferase expression at the site of the transplant. We detected that part of AAV9 escaped to the

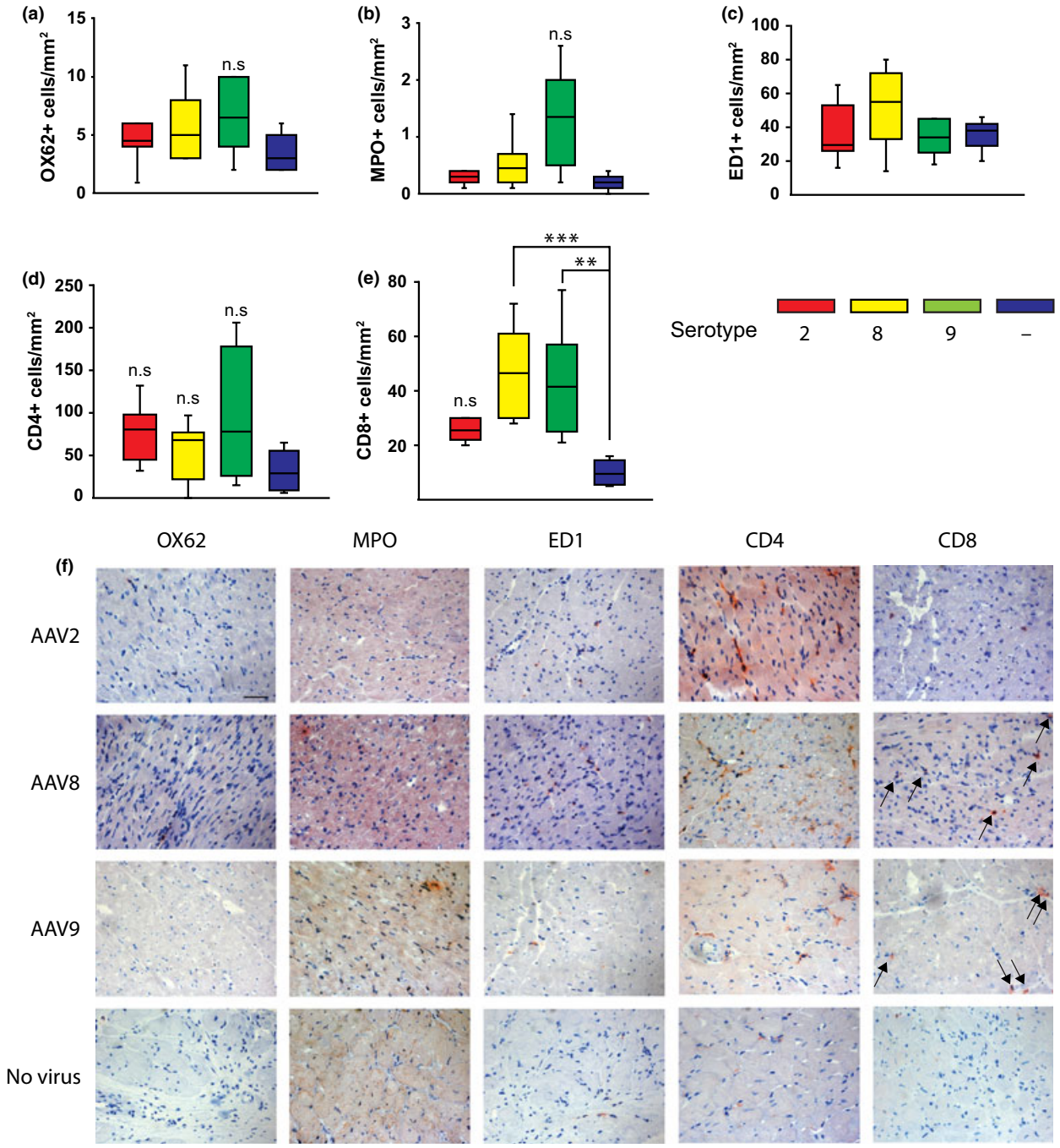

Figure 3 *Ex vivo* intracoronary perfusion with adeno-associated virus (AAV) serotypes 8 and 9 induces chronic T-cell inflammation in the syngrafts. (a–f) The number of intragraft infiltrating OX62$^+$ dendritic cells, MPO$^+$ neutrophils, ED1$^+$ macrophages, and CD4$^+$ and CD8$^+$ T-cells at 6 months were analyzed by immunohistochemical staining of AAV vector-perfused and control group syngraft samples at 6 months after transplantation. Data is presented as the number of immunoreactive cells per mm^2 of cardiac cross-section (a–e). The black bars = 5 μm. No v, no virus control. *$P < 0.05$, NS marks nonsignificant results. Arrows indicate positive inflammatory cells where relevant. Data were analyzed by Kruskal–Wallis and One-way ANOVA with LSD *post hoc* test and are presented by box plots.

adjacent tissues of the transplant, which was also confirmed with open-abdomen imaging of an AAV9 syngraft recipient, revealing the major source of the signal to be the abdominal tissues above the syngraft. We confirmed these results by intracoronary perfusion of a heart transplant with a small-molecular fluorescent probe; after graft

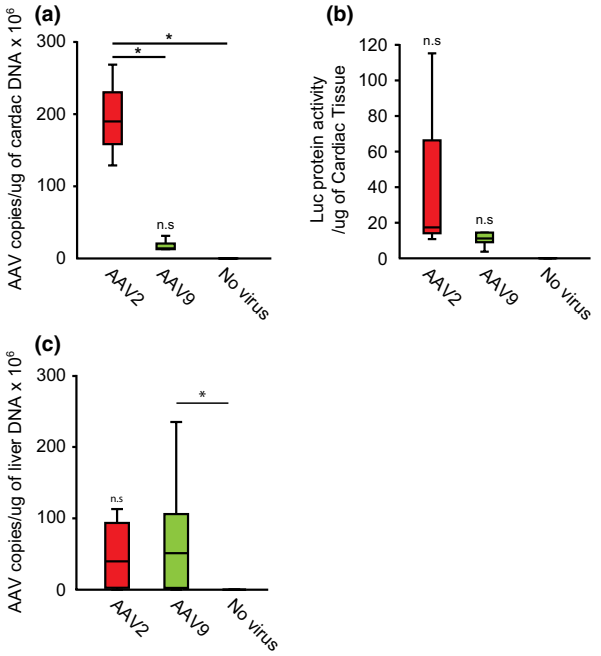

Figure 4 *Ex vivo* localization of vector activity in the recipients of adeno-associated virus (AAV) vector-perfused DA allografts using RT-PCR- and luminometer analysis. (a) Samples were analyzed with real-time PCR for viral DNA to determine the amount of vector particles in cardiac allografts at 4 weeks after transplantation. 10 μg of DNA per tissue sample were used as template material. $n = 4$ in AAV2 group, $n = 6$ in AAV9 group and no virus group. (b) The levels of luciferase activity [Real light units (RLU)/mg total protein of the tissue] were measured 4 weeks after *ex vivo* intracoronary perfusion of allografts with AAV vectors. $n = 4$ in AAV2, $n = 6$ in AAV9 group and no virus group. *$P < 0.05$ versus all other groups. NS marks nonsignificant results. (c) Samples from the livers of allograft recipients were removed at 4 weeks and analyzed with real-time PCR for viral DNA to determine the tendency of each serotype to enter systemic circulation. 10 μg of DNA per tissue sample were used as template material. $n = 4$ in AAV2 group, $n = 6$ in AAV9 group and no virus group. Data were analyzed by Kruskal–Wallis and One-way ANOVA with LSD *post hoc* test and are presented by box plots.

implantation, the abdominal distribution of the probe molecule mimicked that of the AAV9. Also, *ex vivo* analyses of cardiac transplant samples revealed that the amount of intragraft vector particles, luciferase mRNA, and protein activity were the lowest in the AAV9 group, confirming its probable escape into the abdomen.

We hypothesize that the escape is due to vector- and tissue-related factors governing the transduction properties of AAVs. AAV2 has a short blood clearance time after systemic administration [29], it preferentially enters the tissue via vascular beds with fenestrated endothelium [30] and efficiently binds to extracellular matrix through its primary receptor heparan sulfate proteoglycan [12,13]. On the other hand, the targets of AAV8 and AAV9 receptors are not fully uncovered and particularly the slow blood clearance time of AAV9 after systemic delivery indicates that it needs a long time to cross the endothelial layer for cardiac transduction [29]. Therefore, the slow transendothelial access of AAV9 to the target tissue may have been the most important determinant for our results after intracoronary administration of AAV9-Luc with 1-h graft ischemia time.

Increasing the graft ischemia time or endothelial permeability could enhance cardiac AAV9 transduction. However, this may not be clinically feasible due to the predisposition of ischemia-reperfusion injury and harmful long-term effects. In contrast, intramyocardial delivery of AAV9 could bypass the endothelial barrier and lead to improved transduction without harmful effects to the transplant. However, this method has the disadvantage of producing cardiac transduction only in the vicinity of the injection site [31]. Also, recently an experimental continuous cardiac perfusion model has been found to be effective in distributing vector particles into cardiac tissue without the graft suffering excess injury [32]. In addition, further studies with genetically modified vectors may yield AAV variants that have ideal receptor-binding and endothelial-crossing properties for heart transplant transduction [33]. Also, self-complementary AAV vectors (dsAAV) could provide a faster and more effective transgene expression in tissues [34].

In vivo detection of reporter gene activity revealed that efficient transgene activity was observed at 4 weeks after transplantation with all AAV serotypes in both the syngenic and allogenic transplant model. The expression remained stable throughout the follow-up time. Moreover, the luciferase protein activity increased throughout the 4 weeks in the allogenic model, despite moderate alloimmune inflammation (Fig. S1). This expression profile is well suited with treatment of chronic allograft rejection, the main factor challenging long-term graft survival [2]. Potential therapeutic targets could include both anti-inflammatory and antiproliferative factors [35–37]. Although AAV2 and AAV9 show some transgene activity at already 2 days postoperatively, the expression levels would most likely not be enough for the treatment of ischemia reperfusion injury and acute allograft rejection; early factors that accelerate later development of chronic allograft rejection. These results indicate that in potential AAV gene therapy in clinical heart transplantation, the recipient would need effective traditional immunosuppression for the first postoperative weeks or months. Thereafter, immunosuppressive medication could be gradually reduced, as the potential AAV-mediated therapeutic protein levels would start to reach effective levels of expression. This would minimize the long-term side effects of immunosuppressive medication, such as opportunistic infections, nephrotoxicity, and malignancies.

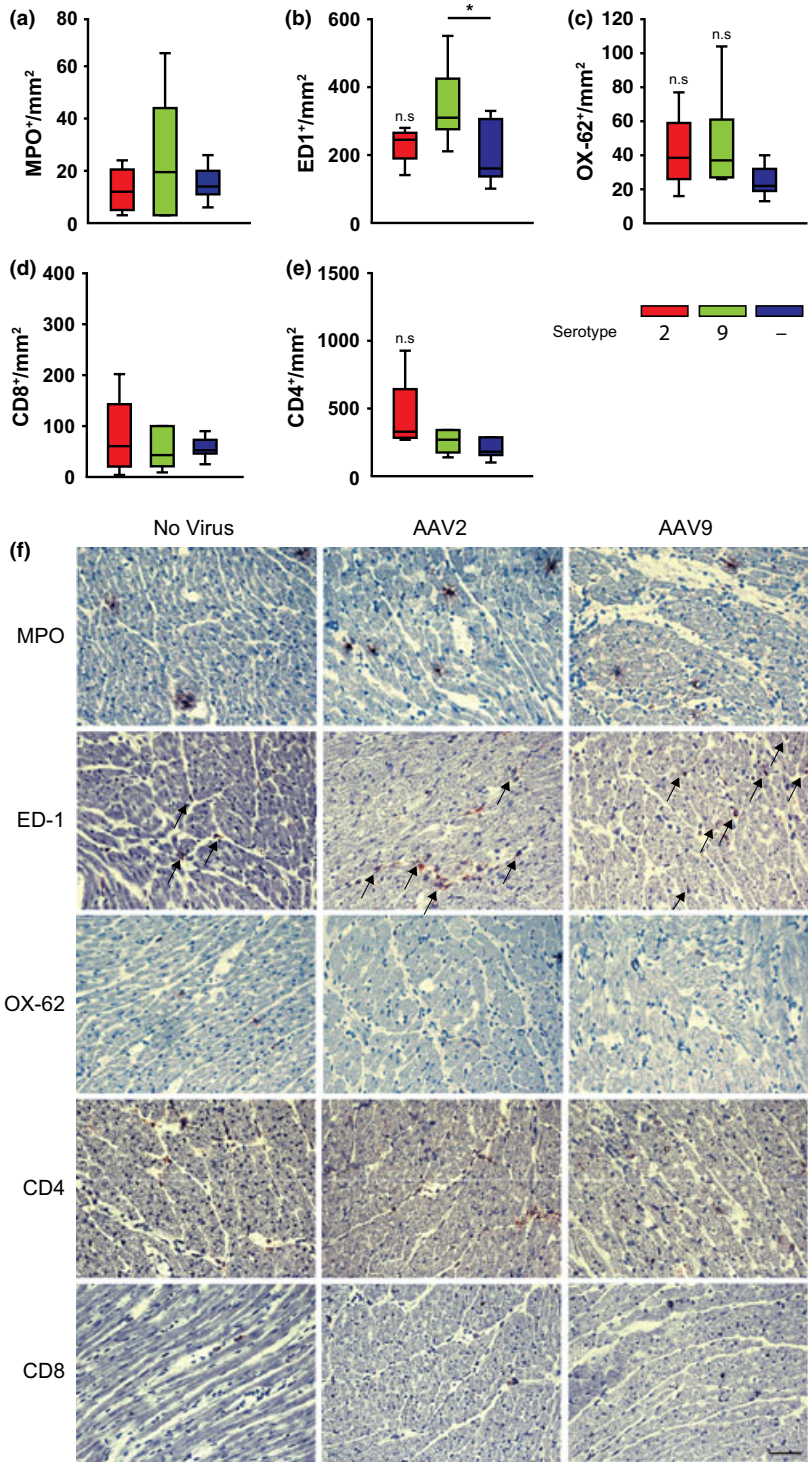

Figure 5 *Ex vivo* intracoronary perfusion with adeno-associated virus (AAV) serotype 9, but not 2, induces chronic inflammation in allografts of immunosuppressed recipients. (a–f) AAV vector-perfused and control group allografts were removed and analyzed by immunohistochemical staining for the amount of intragraft $OX62^+$ dendritic cells, MPO^+ neutrophils, $ED1^+$ macrophages, and $CD4^+$ and $CD8^+$ T-cells at 4 weeks. Data are presented as the number of immunoreactive cells per mm^2 of cardiac cross-section (a–e). (f) Representative images of immunohistochemically stained samples. The black bars = 5 μm. *$P < 0.05$, NS marks nonsignificant results. Arrows indicate positive inflammatory cells where relevant. Data were analyzed by Kruskal Wallis and One-way ANOVA with LSD *post hoc* test and are presented by box plots.

We observed small amounts of vector particles in the hepatic tissue of both allograft and syngraft recipients. However, the quantities were nearly nonexistent in the syngraft model. In the allograft model, on the other hand, we found significant amounts of AAV9 vector particles. These differences may be explained by the fact that livers were analyzed at 1 month postoperatively in the allograft model, as opposed to 6 months in the syngraft model. Hepatic tissue is highly regenerative, as opposed to cardiac tissue, and therefore, any hepatic transduction that had occurred in the syngraft model would have disappeared by 6 months [38]. Moreover, none of the animals showed any signs of hepatic damage or side effects during the follow-up time. When sacrificing the animals, no signs of hepatic damage or ascites were noted *in vivo*. These results suggest that although some hepatic transduction may occur, in the long run, it would not pose a threat to recipients in our experimental model.

It has previously been shown that AAV vectors may cause both host humoral and cellular immune reactions, in the form of viral capsid protein-specific neutralizing antibodies and $CD8^+$ cytotoxic T-cells targeted against virus-infected cells, respectively [39–43]. Moreover, previous studies have indicated that many humans are infected with latent AAV, and a large part of the human population has existing neutralizing antibodies against the viral capsid proteins [44–46]. These factors are one of the main barriers limiting recipient transgene expression in clinical trials [40,47–49]. Recent experimental studies have attempted to use immunosuppressants to extend AAV-mediated transgene expression, some successfully and some not [50–53]. As lifelong immunosuppression is standard therapy for organ transplant recipients, they would be suitable candidates for gene therapy with viral vectors also in that respect [1].

Interestingly, we observed a significant influx of $CD8^+$ T-cells in syngrafts perfused with AAV8 and AAV9, as compared to no virus controls at 6 months. Thus, the low levels of intragraft AAV9 and AAV8 vector particles and luciferase protein activity could also be explained by CD8 T-cell-mediated inflammation. The recipients of AAV-perfused allograft recipients, on the other hand, were immunosuppressed with daily doses of cyclosporine. This may have blunted the T-cell response observed in the syngenic model.

Our bioluminescent imaging results of the different AAV serotypes not only highlight the benefits of high sensitivity and noninvasiveness of the method but also underline the weaknesses of bioluminescent imaging that have to be taken into consideration when interpreting the data. Although 3D bioluminescent systems have been developed, the usual setup measures only topographical 2D bioluminescent signal and thus does not explicitly reveal the tissue source of measured bioluminescence. Concomitant evaluation of the signal-emitting tissue is thus of utmost importance.

In conclusion, our results suggest that AAV2 would be the best choice of the AAV vectors tested for the *ex vivo* intracoronary transduction of cardiac allografts in our experimental model. The implications for this are of high importance for gene therapy of cardiac transplants, as they give us new insight on how different AAV serotypes may behave. Furthermore, this gives us an indication on what vector serotype to use in our future experiments of delivering therapeutic genes to cardiac allografts. Further experiments are still required to assess the possible effects of AAV vector administration on acute cardiac allograft inflammatory responses and later development of chronic allograft vasculopathy. Also, questions on recipient immune reactions against AAV-mediated transgene expression set the stage for further studies within this field.

Authorship

JJJ: codesigned the research, performed the operations, participated in the collection and analysis of the data, and advised on the writing of the manuscript. SOS: participated in the collection of data and advised on the writing of the manuscript. RT: assisted with the operations and advised on the writing of the manuscript. RK: designed and tested the primers used in PCR and advised on the writing of the manuscript. MAIK: participated in pre-experimental preparations and advised on the writing of the manuscript. RA: assisted with laboratory procedures and methods. ER: participated in the collection of data. AA: provided the means for acquiring the viral vectors and participated in the experimental design. JS: participated in the design of viral vectors. KP: assisted with the design of viral vectors. KA: contributed the viral vectors and the means to design them. AIN: codesigned the study, participated in the operations, assisted with the collection and analysis of the data, and advised on the writing of the manuscript. KL: codesigned the study, supervised the research, and advised on the writing of the manuscript.

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Supporting information

Additional Supporting Information may be found in the online version of this article:

Expanded Methods S1. Supplemental results.

Figure S1. *In vivo* imaging and quantification of luciferase activity after *ex vivo* intracoronary delivery of adeno-associated virus (AAV) serotypes 2 and 9 in the recipients of cardiac allografts.

Figure S2. *Ex vivo* measurements of luciferase activity in the heart and liver of AAV2, -8 or -9 systemically injected rats.

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