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ORIGINAL ARTICLE

Recombinant adeno-associated virus vector for gene transfer to the transplanted rat heart

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Summary

Efficient durable viral vector transduction of the transplanted heart remains elusive. This study assesses the potential of recombinant adeno-associated virus (rAAV) mediated gene delivery to the transplanted rat heart. rAAV serotype 1, 2 and 5 vectors encoding the green fluorescent protein (GFP) gene (1×10^{11} viral particles/ml) were diluted in cold University of Wisconsin solution and circulated through the coronary vasculature of the donor organs for 30 min before syngeneic rat heterotopic heart transplantation was performed. Study 1: animals ($n = 5$ each serotype) were killed at 21 days post-transplant to evaluate the efficiency of GFP transduction using RT-PCR and expression by fluorescence microscopy. Study 2: using rAAV-1, animals ($n = 5$ each group) were killed at 7, 21 and 84 days to evaluate the durability of GFP expression. The maximum cardiac GFP expression at 21 days was observed in rAAV-1. GFP expression by rAAV-1 was detectable at 7 days, improved at 21 days, and was still evident at 84 days. This study demonstrates cardiac rAAV gene transduction with a cold perfusion preservation system of the donor heart. These data show that AAV-1 is superior to AAV-2 and AAV-5 for this purpose and that durable expression is achievable.

Introduction

Cardiac allograft vasculopathy (CAV) is the greatest obstacle to long-term patient survival after cardiac transplantation. Gene therapy has emerged as a potential approach to normalize coronary arterial vasoreactivity and reduce CAV after the transplantation. Major challenges to successful clinical gene therapy include the need for optimal vectors and delivery systems to transfer genetic material to tissues *in vivo*. Organ transplantation may be an ideal setting for gene therapy as the donor organ is uniquely available for genetic modification between the times of procurement and implantation when the transplant organ is not required to function.

As delivery of genes to tissues *in vivo* is inefficient, a number of viral vector systems have been used to enhance efficiency. Adenoviral vectors have been used extensively for cardiovascular gene transfer due to the quiescent nature of cells to be targeted. These vectors can be easily manipulated and grown to high titres. Adenoviral vectors, however, have been shown to suffer a number of safety and biosafety concerns including induction of an inflammatory response and a limited duration of transgene expression. A viral vector system with potential advantages over adenoviral vectors is the recombinant adeno-associated virus (rAAV) vector. This vector system has shown particular promise in skeletal muscle [1,2], hepatic [3] and cerebral [4] gene transfer. rAAV vectors have been shown to result in prolonged transgene expression without inflammation in these organ systems. However, there is less experience in cardiac gene transfer with rAAV vectors, especially in the setting of transplantation.

Multiple distinct AAV serotypes have been recently identified, each one differing from the others with respect to receptor usage and biodistribution of transduced cells. Among the various serotypes of AAV, rAAV vectors based on AAV serotype 2 have been most extensively investigated as gene delivery vectors. However, the transduction efficiency of AAV-2 mediated gene transfer to the heart *in vivo* remains unsatisfactory. Finding the optimal AAV serotypes for efficient and tissue-specific transduction has become imperative successful gene therapy.

Use of an effective and clinically applicable gene delivery system is crucial for sufficient and targeted transgene expression. Several methods of gene delivery to the heart have been studied, including direct injection into the myocardium [5–7], bolus injection into the coronary arteries [7–9] and coronary perfusion systems [8–10]. In previous studies by our group, most efficient gene transfer to the transplanted heart was achieved by perfusion of the vector through the donor organ using a peristaltic pump [11].

This series of experiments was designed to investigate the efficiency of rAAV mediated gene transfer after *ex vivo* circulation of the viral vector carrying the green fluorescent protein (GFP) reporter gene through the coronary vasculature of the donor organ in a heterotopic rat heart transplant model. The present study examined whether specific AAV serotypes would differ in their ability to mediate gene transduction in transplanted rat heart. In addition, we sought to evaluate the time-course of transgene expression following gene delivery.

Materials and methods

Animals

Inbred male Lewis rats (Harlan, Madison, WI, USA), weighing 250–300 g, were used as donors and recipients for syngeneic heart transplantation. All animals were housed and received humane care in accordance with the standards established by the Institutional Animal Care and Use Committee of the Mayo Clinic and Foundation and as described in the Guide for the Care and Use of Laboratory Animals prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (NIH publication No. 86–23, revised 1985).

Recombinant adeno-associated virus vector

rAAV production

The rAAV stocks were produced in a lepidopteran cell line, *Sf*9, derived from *Spodoptera frugiperda* as previously described [12]. Briefly, a set of three recombinant *Autographa californica* nuclear polyhidrosis viruses, or baculovirus expression vectors (BEVs), were used to infect *Sf*9 cells: two differentBEVs express the structural and nonstructural AAV proteins while a third provides the rAAV genome. One baculovirus expresses AAV2 Rep 52 and Rep 78, which is the only virus encoded proteins required for vector DNA replication. A second BEV is used to express the three AAV capsid proteins at stoichiometric levels. A different BEV for each capsid serotype was used. The third BEV provides the rAAV genome which is 'rescued' in the presence of Rep proteins and replicates to high copy number. The linear, single-stranded DNA vector genomes are then packaged into the AAV capsids. The details of producing different rAAV serotypes in *Sf*9 cells were described previously. The rAAV concentrations were determined by quantitative (q) PCR using plasmid DNA standards and biological activities were determined by hrGFP transduction assays in HEK293 cells. Western blot analyses using anti-capsid serum were used to determine capsid protein composition.

Processing rAAV

Small scale insect cell cultures (≤0.4 l) were processed as follows: the entire culture was subjected to three freezing/thawing cycles and then the supernatant was clarified by centrifugation at $2000 \times g$ for 10 min at RT. To further purify the vector and to separate empty capsids from vector genome (vg)-containing capsids, the pellet is resuspended in CsCl solution (density = 1.42 g/cm^3, with reflective index = 1.372) and centrifuged in a swinging bucket rotor (Beckman SW40, Beckman Coulter Inc., Fullerton, CA, USA) for 66–72 h at 38,000 rpm at 20 °C. The gradients were fractionated by puncturing the tube and collecting approximately 10×1 ml fractions. The densities of the fractions were determined by measuring the refractive index and samples were either stored in CsCl or dialyzed against PBS plus 2 mM $MgCl_2$. The fractions corresponding to vg-containing particles are virtually devoid of empty capsids which have a lower buoyant density than vg-containing capsids.

Heart transplantation and vector delivery

Donor procedure

After anesthesia with intraperitoneal pentobarbital (70 mg/kg), the donor rat was intubated and mechanically ventilated with a Harvard Rodent Ventilator Model 683 (Harvard Apparatus Inc., South Natick, MA, USA). The heart was exposed via a median sternotomy. After heparinization with 200 units of aqueous heparin injected into the inferior vena cava, the innominate artery was cannulated with a 24-gauge cannula, and the venae cavae and the pulmonary veins were ligated *en bloc* with 4/0 silk. The aorta was tied distal to the cannula, and the heart was arrested by infusion of cold University of

Wisconsin (UW) solution into the aortic root through the indwelling cannula for 2 min.

Vector delivery

The donor heart was harvested and a total volume of 5 ml UW solution containing 5×10^{11} viral particles rAAV-GFP (1×10^{11} viral particles/ml) was circulated through the coronary vasculature of the donor organ for 30 min by means of a peristaltic pump (Rainin, Emeryville, CA, USA). The viral vector solution was infused into the donor organ through the cannula inserted into the aorta and was collected in a vial placed under the heart ejecting the solution to be recirculated through the pulmonary artery. A flow rate of 2.4 ml/min was chosen to generate a perfusion pressure in the aortic arch of 70–80 mmHg as determined in previous experiments [11]. During the perfusion period, the vial containing the viral vector solution was kept on ice not to exceed 4 °C. In control animals, virus-free UW solution was administered. At the end of perfusion, the heart was removed from the perfusion apparatus and stored in UW solution at 4 °C during the time needed for the following recipient preparation (30–40 min).

Recipient procedure

Heterotopic abdominal heart transplantation was performed using standard microsurgical techniques [13]. The recipient rat was anesthetized by administration of intraperitoneal pentobarbital (70 mg/kg) followed by continuous inhalative anesthesia with isoflurane (2–4%). In brief, the heart was transplanted into the recipient by anastomoses of the donor aorta to the recipient abdominal aorta and the donor pulmonary artery to the recipient inferior vena cava in an end-to-side fashion using 10/0 monofilament sutures. During surgery, the heart was wrapped in gauze and kept cold by use of topical ice-cold saline solution. Animals received analgesia with buprenorphine 0.1 mg/kg i.m. postoperatively and recovered with oxygen in a warm environment. Function of the graft was checked daily by palpation of the beating transplanted heart.

Assessment of transgene expression

Study 1: transplanted hearts transduced by rAAV-1, 2, and 5 were removed at 21 days ($n = 5$ each group), respectively, to determine the kinetics of GFP transgene expression. Study 2: using rAAV-1, animals ($n = 5$ each group) were killed at 7, 21, and 84 days to evaluate GFP expression. At each time period, control animals ($n = 5$ each group) were also killed. At the termination of each experiment, animals were killed by an intraperitoneal injection of pentobarbital (70 mg/kg). The transplanted hearts were removed and flushed with saline. Midventricular cross-sections were cut and embedded in OCT compound (Tissue-Tek; Sakura Finetek Inc., Torrance, CA, USA) and snap-frozen in a liquid nitrogen-cooled isopentane bath. Five 5-μm-thick cryostat sections were then cut at 25-μm intervals for each portion and immediately analyzed by fluorescence microscopy. For determination of DNA for GFP, the remaining apical ventricular segments were snap-frozen in liquid nitrogen and stored at −70 °C.

Real Time (RT)-PCR for GFP DNA

Genomic DNA was extracted from 20–25 mg of frozen myocardial tissue using QIAmp DNA kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions with RNase treatment. Concentrations of DNA in each sample were measured with a Beckman DU640 spectrophotometer at 260 nm absorbance. RT-PCR was performed in accordance with guidelines from Applied Biosystems (Foster City, CA, USA). A forward primer AGAACGGCATCAAGGTGAAC; a reverse primer TGCTCAGGTAGTGGTTGTC and a dual-labeled probe 5′-FAM-TCGCCGACCACTACCAGCAGAACAC-BHQ_1–3′ were synthesized by Integrated DNA Technologies (Coralville, IA, USA). Reaction volumes of 50 μl contained 50 ng of DNA, 300 nmol/l forward primer, 300 nmol/l reverse primer, 150 nmol/l target probe, and 2X TaqMan Universal PCR Master Mix. DNA samples were amplified in triplicate using an ABI 7700 Sequence Detection System (Applied Biosystems). RT-PCR was performed at 50 °C for 2 min and 95 °C for 10 min and was then run for 40 cycles at 95 °C for 15 s and at 60 °C for 1 min. The threshold cycle number (C_T), at which the initial amplification becomes detectable by fluorescence, was determined. Standard curves were generated using a pEGFP-N2 plasmid (Clontech, Palto, CA, USA) and converting the concentration (μg/ml) to copy number of gene/μl. The standard curve represents the C_T number (*y*-axis) versus the copy number of reference standard (*x*-axis). Primers and probe for rat GAPDH were designed to normalize the GFP DNA quantitation. A forward primer CAGTCAAGGCTGAGAATGGGA, a reverse primer GGGATCTCGCTCCTGGAAG and a probe 5′FAM-CTGGTCATCAACGGGAAACCCATCA-BHQ_1–3′ amplified a 71-bp product. Standard curves were obtained using a page purified oligonucleotide. Quantification of DNA for GFP was expressed as the ratio of GFP copies to GAPDH copies per 50 ng of DNA.

Statistical analysis

Data are presented as arithmetic mean ± SE. Rank sum test was used to assess differences between groups. *P*-values of 0.05 or less were considered statistically significant.

Results

At the time of organ harvest for transplantation, all hearts stopped rapidly when perfusion with cold UW solution was started. Similarly, all hearts showed early spontaneous recovery of sinus rhythm at the time of reperfusion after transplantation. Function of the heart was checked daily by palpation. All transplanted hearts showed good contractility at the time of harvest for assessment of GFP expression.

In study 1, transplanted hearts were removed at 21 days, to determine the efficiency of GFP transduction using RT-PCR and transgene expression by fluorescence microscopy. Maximum expression of GFP was detected by in rAAV-1 postoperative day 21 (Fig. 1). RT-PCR for GFP DNA identified 130,786 ± 54,877 GFP copies/50 ng DNA in rAAV-1, 12 186 ± 3767 GFP copies/50 ng DNA in rAAV-2, and 2.47 ± 1.41 GFP copies/50 ng DNA in rAAV-5, respectively. The mean GFP/GAPDH ratio was 0.482 ± 0.256 in rAAV-1, 0.043 ± 0.014 at 21 days, and 0.0001 ± 0.0001 in rAAV-5, respectively (Fig. 2). GFP transduction at 21 days using rAAV-1 and 2 was higher than rAAV 5 (1 vs. 5: $P = 0.01$ and 2 vs. 5: $P = 0.01$).

In study 2, using rAAV-1, animals were killed at 7, 21 and 84 days to evaluate GFP transduction and expression. At each time period, control animals were also killed. Expression of GFP was already detectable at 7 days after rAAV mediated gene transfer. While a peak of expression was observed at 21 days, transgene expression was diminished but still evident at 84 days post-transplant (Fig. 3). Transgene expression was detected in all regions of the cross-sections, neither revealing differences between left and right ventricle nor between subepicardial and myocardial zones. As shown in Fig. 3, fluorescence microscopy revealed maximum GFP expression at 21 days after rAAV-1 mediated gene delivery. RT-PCR for GFP DNA

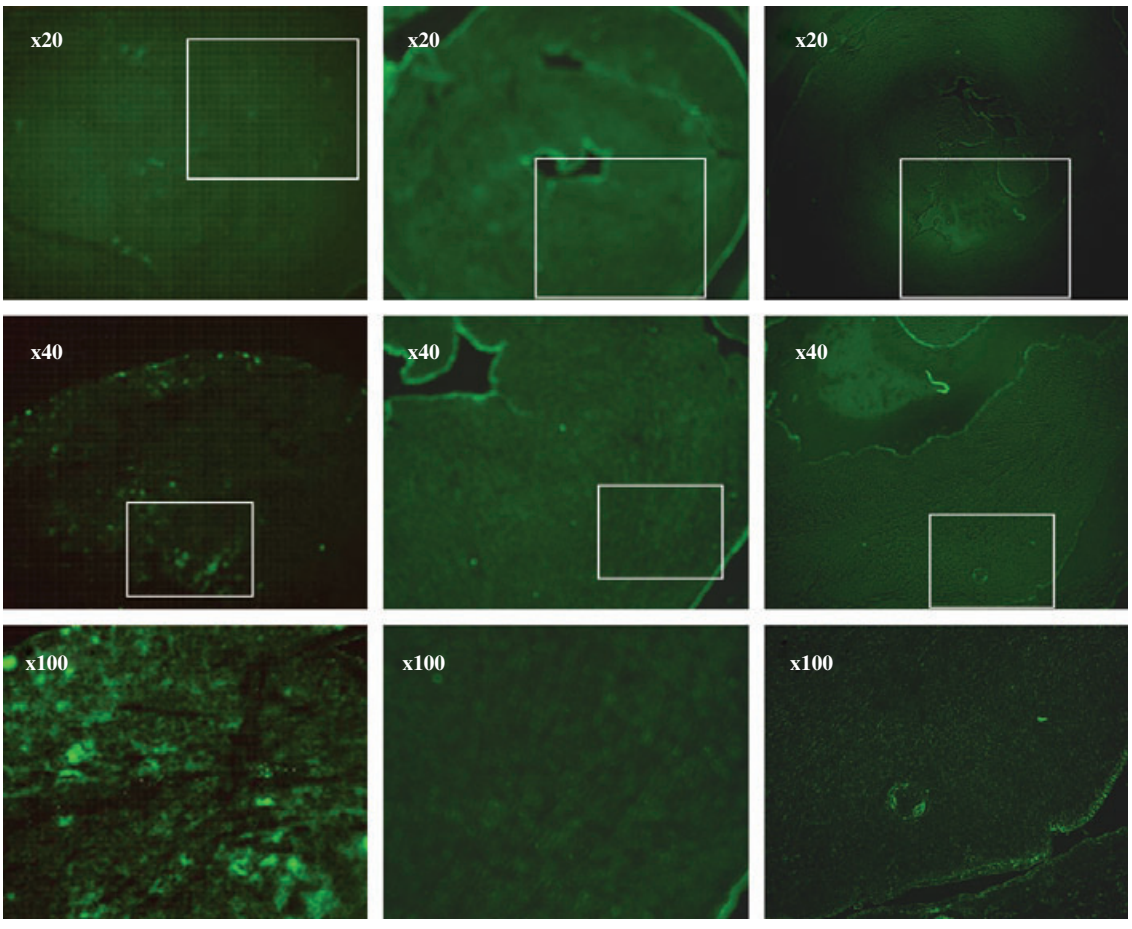

Figure 1 Images of green fluorescent protein (GFP) expression at day 21: maximum expression of GFP was detected by fluorescence microscopy in recombinant adeno-associated virus-1 postoperative day 21. The area magnified is noted by the square.

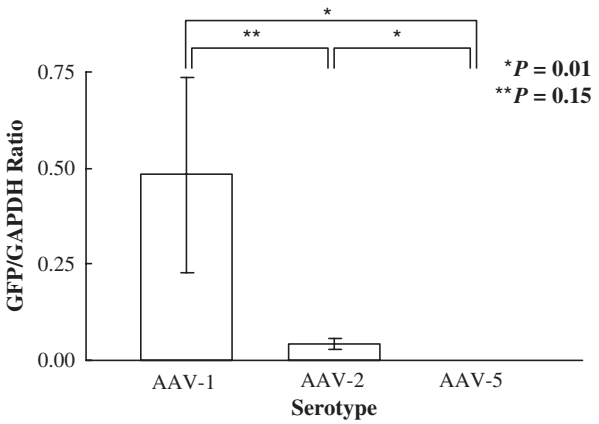

Figure 2 GFP/GAPDH ratio in AAV-green fluorescent protein transduced rats heart (50 ng of DNA): transduction of GFP at 21 days using recombinant adeno-associated virus (rAAV)-1 and 2 were higher than rAAV-5.

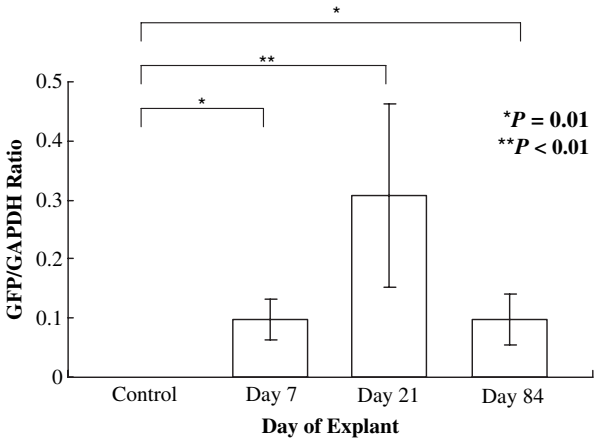

Figure 4 GFP/GAPDH ratio in AAV-1-GFP transduced rats heart (50 ng of DNA): transduction of green fluorescent protein (GFP) was already detectable at 7 days after recombinant adeno-associated virus mediated gene transfer. A peak of transduction was observed at 21 days, and was still evident at 84 days post-transplant. At all time period, GFP copies of the transplanted heart which was transduced by AAV-1 were significant higher than control.

identified 59,400 ± 49,737 GFP copies/50 ng DNA, 84,460 ± 74,775 GFP copies/50 ng DNA, and 30,248 ± 29,568 GFP copies/50 ng DNA at 7, 21, and 84 days, respectively. The mean GFP/GAPDH ratio was 0.098 ± 0.077 at 7 days, 0.307 ± 0.347 at 21 days, and 0.096 ± 0.097 at 84 days after heart transplantation (Fig. 4). At all time periods, GFP copies of the transplanted heart which was transduced by AAV-1 were significant higher than control.

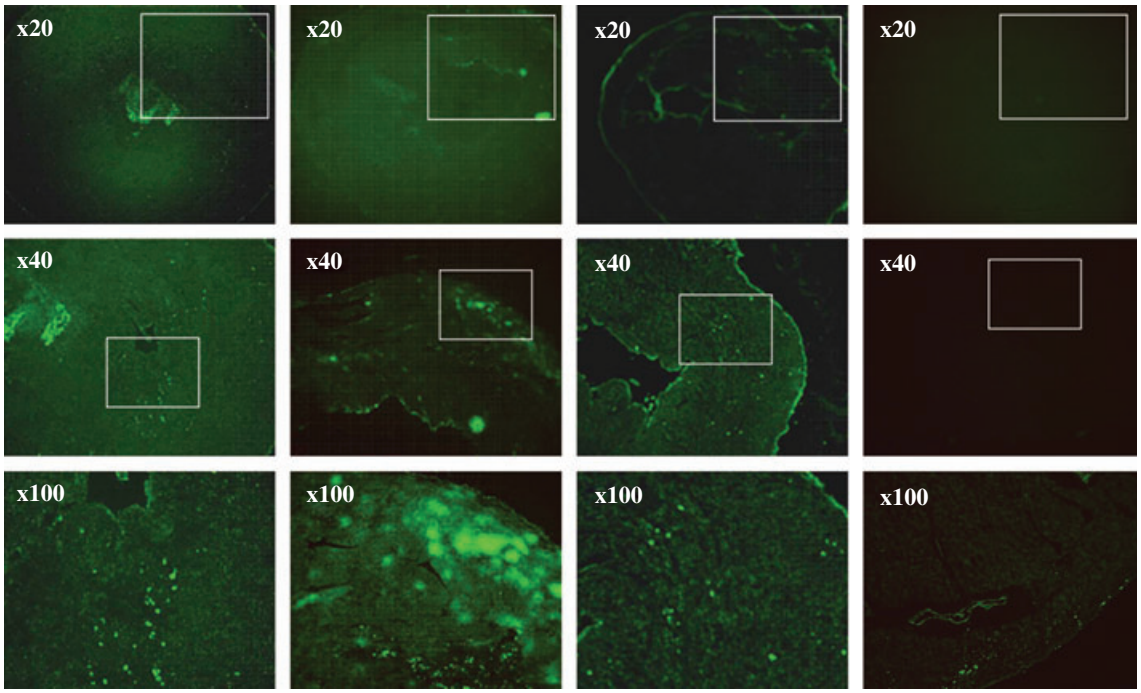

Figure 3 Images of AAV-1 green fluorescent protein (GFP) expression at day 7, 21 and 84 days: fluorescence microscopy revealed maximum GFP expression at 21 days after recombinant adeno-associated virus-1 mediated gene delivery. The area magnified is noted by the square.

Discussion

The present study demonstrates the feasibility of using rAAV vectors for gene transfer to the transplanted rat heart. This is the first demonstration of cardiac gene transduction with a cold perfusion system of the donor heart using rAAV. We compared rAAV serotype 1, 2 and 5 mediated gene transfer efficiency at 21 days after transduction and rAAV-1 mediated gene transfer at various timepoints. The maximum transgene expression was observed in AAV-1 mediated gene transfer. Peak gene expression of GFP which is transduced by AAV-1 occurred at 21 days after vector delivery and decreased thereafter. However, transgene expression was diminished but still evident at 84 days after the rAAV vector was delivered by means of a cold perfusion system.

A number of vector systems have been used for cardiac gene transfer. Several groups, including our own, have studied the use of adenoviral vectors in the context of heart transplantation. We have demonstrated that administration of an adenoviral vector to the transplanted heart results in widespread transgene expression [11,14]. However, the transient nature of transgene expression most likely due to the episomal nature of the adenoviral DNA and the expression of proteins from nondeleted regions of the viral genome eliciting immunological elimination of transduced cells via a $CD8^+$ lymphocyte-mediated cell lysis limits the application of adenoviral vectors as a gene delivery tool in chronic cardiovascular disorders [15,16]. Most studies using adenoviral mediated gene transfer have reported a peak of gene expression around day 7 followed by total loss of gene expression between 3 and 4 weeks after gene delivery [17–19]. Moreover, adenoviral-mediated gene transfer is associated with a marked inflammatory response [20].

Compared with 70 nm for adenovirus, rAAV particles are only 20 nm diameter which is likely to influence vascular extravasation and would therefore optimize its use in a setting of *ex vivo* perfusion gene transfer. In recent comparison of rAAV serotypes 1–5, the efficiency of gene transduction in mouse cardiomyocytes *ex vivo* and in mouse hearts after i.v. virus administration is highest in rAAV-1 [21]. Su *et al.* showed that AAV-1 mediates early onset of gene expression in mouse hearts compared with AAV-2 and results in better therapeutic effect. They speculated that AAV serotype selection or genetic manipulation of virion coat protein might lead to better AAV transduction [22]. Our study is consistent with this data. Recently, especially *in vivo* study, rAAV serotypes 8 and 9 were reported as promising vectors which have strong tropism to myocardium [23–25]. It will therefore be interesting to test other rAAV serotypes in gene delivery to the transplanted heart.

Recombinant adeno-associated virus is a single-stranded DNA virus that has to be converted into a transcriptionally active double stranded form. This may explain the delay in gene transduction after vector delivery in our experiments resulting in only moderate GFP expression at 7 days. On the other hand, an immune response to the reporter protein GFP may have influenced the persistence of the gene product in our study. In a clinical trial of gene therapy for hemophilia using AAV-Factor IX, the expression of factor IX was curtailed by host immune response [26]. However, the need for immunosuppression in transplantation may modify this immune response and facilitate prolonged gene expression. Previous experiments have shown significantly greater transgene expression at 8 weeks in immunosuppressed syngeneic and allogeneic animals when compared with nonimmunosuppressed animals after adenoviral gene transfer in the rat heart transplantation model [27].

Physical variables for gene transfer to the heart include both the specific method of transduction and the temperature at the time of gene transfer. Methods of transduction include direct injection into the myocardium [5–7], bolus injection into the coronary arterial tree [7–9,27], and cold or warm perfusion systems [8–11,28,29]. Direct injection of vector into the myocardium has tended to be localized to the site of injection [5,7]. Single bolus injections into the coronary arteries have been demonstrated by our group and others to result in relatively inefficient, but evenly distributed, transduction with cardiomyocytes being the predominant site of transduction [7,8,30]. Use of a cold perfusion system, as opposed to single bolus administration, has shown significantly enhanced transduction in our laboratories, with cardiomyocytes remaining the predominant cell type expressing the transgene [8,10,11]. In contrast to direct injection or *in vivo* vascular gene transfer, *ex vivo* perfusion appears to represent an ideal setting for administration of the vector via the blood vessels as the inevitable period of donor organ ischemia following harvesting allows a prolonged dwell time within the target tissue. In addition, no GFP positive cells were detected in other organs of the recipient in this study. Therefore, gene therapy may be particularly applicable in cardiac transplantation as the donor organ is uniquely available for genetic modification *ex vivo* prior to implantation into a recipient. Vessel distension increasing interendothelial cell space during bolus injection or perfusion may also play a role in increased transduction [31,32]. We chose low temperature in these transduction experiments as it reflects current clinical techniques of cardiac preservation using cold cardioplegic solutions, recognizing the need to preserve the heart during the ischemic period between removal from the donor and reperfusion after transplantation.

In conclusion, this work demonstrates for the first time the applicability of gene transfer by means of a cold perfusion system using rAAV vectors in a heterotopic rat heart transplant model. Efficient myocardial transduction is achievable with a prolonged duration of transgene expression. Studies in small and large animal models are required to further optimize the efficiency of rAAV gene transfer and evaluate the potential of rAAV mediated gene transfer to modify cardiovascular disorders after heart transplantation, such as graft rejection and CAV, using therapeutic genes [33].

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