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Improved cardiac gene transfer by transcriptional and transductional targeting of adeno-associated viral vectors

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Abstract

Objective: Vectors based on recombinant adeno-associated virus 2 (AAV-2) are a promising tool for cardiac gene transfer. However, potential therapeutic applications need to consider the predominant transduction of the liver once AAV-2 vectors enter the systemic circulation. We therefore aimed to increase efficiency and specificity of cardiac vector delivery by combining transcriptional and cell surface targeting.
Methods: For analysis of transcriptional targeting, recombinant AAV vectors were generated harboring a luciferase reporter gene under control of the cytomegalovirus (CMV) promoter or the 1.5-kb cardiac myosin light chain promoter fused to the CMV immediate-early enhancer (CMV_{enh}/MLC1.5). Luciferase activities were determined in representative organs three weeks after intravenous injection of the vector into adult mice. Transductional targeting was studied using luciferase-reporter constructs crosspackaged into capsids of AAV serotypes 1 to 6 and modified AAV-2 capsids devoid of binding their primary receptor heparan sulfate proteoglycan.
Results: Intravenous injections of AAV-2 vectors harboring the CMV_{enh}/MLC1.5 promoter enabled a specific and 50-fold higher reporter gene expression in left ventricular myocardium of adult mice compared to vectors containing the CMV promoter. Comparison of AAV-2 vector genomes crosspackaged into capsids of AAV-1 to -6 showed that AAV-1, -4, -5, and -6 capsids increased cardiac transduction efficiency by about 10-fold. However, transduction of other organs such as the liver was also increased after systemic administration. In contrast, AAV-2-based vectors with ablated binding to their primary receptor heparan sulfate proteoglycan enabled a significantly increased efficiency of cardiac gene transfer and reduced transduction of the liver.
Conclusions: Combining transcriptional targeting by the CMV_{enh}/MLC1.5 promoter and AAV vectors devoid of binding the AAV-2 primary receptor results in an efficient cardiac gene transfer with a significantly reduced hepatic transduction.

Keywords: Gene therapy; Gene transfer; Gene expression; Heart; Mouse; Rat; In vivo

This article is referred to in the Editorial by A. Gödecke (pages 6–8) in this issue.

1. Introduction

Adeno-associated virus (AAV) vectors are a promising vector system for gene transfer into myocardium since they enable a long-term transduction [1]. AAV-2 mediated gene transfer of the heme oxygenase gene has shown a prophylactic effect on development of an ischemia/reperfusion injury in a rat model [2] and expression of δ-sarcoglycan or a pseudophosphorylated mutant of phospho-

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lamban remarkably improved hemodynamic parameters in hamsters with hereditary cardiomyopathy [3,4].

However, transduction of and/or gene expression in extracardiac tissue may result in potential undesired side effects in clinical approaches. A strategy to restrict expression of a transferred gene to the target tissue is transcriptional targeting by use of tissue-specific promoters. Several cardiac-specific promoters have been used so far to target gene expression from adenoviral [5–8] and AAV vectors [9,10] into the myocardium. Although activity of a 0.26 and 2.1 kb myosin light chain (MLC)-2v promoter fragment was highly restricted to ventricular myocardium [11], expression is downregulated in adult myocardium [12]. However, fusion with the cytomegalovirus immediate-early (CMV) enhancer [13] resulted in high levels of transcriptional activity also in adult myocardium [14,15].

Biodistribution studies showed that AAV-2 vector genomes were predominantly found in liver and spleen, but not in heart or lung after tail vein injections in mice [16]. Efforts have been made to identify receptor molecules on the cellular surface responsible for transduction of a certain cell type. For adeno-associated virus type 2 (AAV-2), heparan sulfate proteoglycan has been shown to act as a primary receptor [17]. After binding to the cell surface, AAV-2 is thought to engage a secondary receptor like αVβ5 integrins [18] or human fibroblast growth factor receptor-1 [19] which mediates cell entry. Systematic deletions of potential heparin-binding motifs on AAV-2 capsids indicated that heparin binding is a prerequisite for efficient transduction of liver, but not kidney, lung or heart tissue [20]. Vectors packaged in capsids detargeted from binding to heparan sulfate therefore show decreased hepatic transduction and may be more suitable for transfer into non-hepatic tissue [20].

A further approach of making AAV vectors more efficient for specific tissues is based on crosspackaging recombinant AAV-2 vector genomes into capsids of other AAV serotypes. It has been shown that those so-called pseudotyped AAV vectors have different cell-type specificities [21,22]. In vivo analyses elucidated that pseudotyped vectors with capsids from AAV-1, -3, -5, -7, and -8 revealed an increased transduction efficiency of skeletal muscle [23,24].

Aim of our study was to develop strategies to increase the efficiency and specificity of cardiac gene transfer with respect to a potential therapeutic gene transfer. We elucidated the role of (i) a promoter with strong cardiac activity, (ii) cross-packaging of vector genomes into capsids of AAV serotypes 1 to 6, and (iii) finally AAV-2 capsids with ablated liver tropism. We found that combination of transcriptional and transductional targeting enabled an efficient cardiac gene transfer with a significantly reduced hepatic transduction.

2. Materials and methods

2.1. Generation of AAV vectors and determination of titers

The vector genomes used for packaging are shown in Fig. 1. For generation of rAAV-vector pUFCMV-Luc the firefly

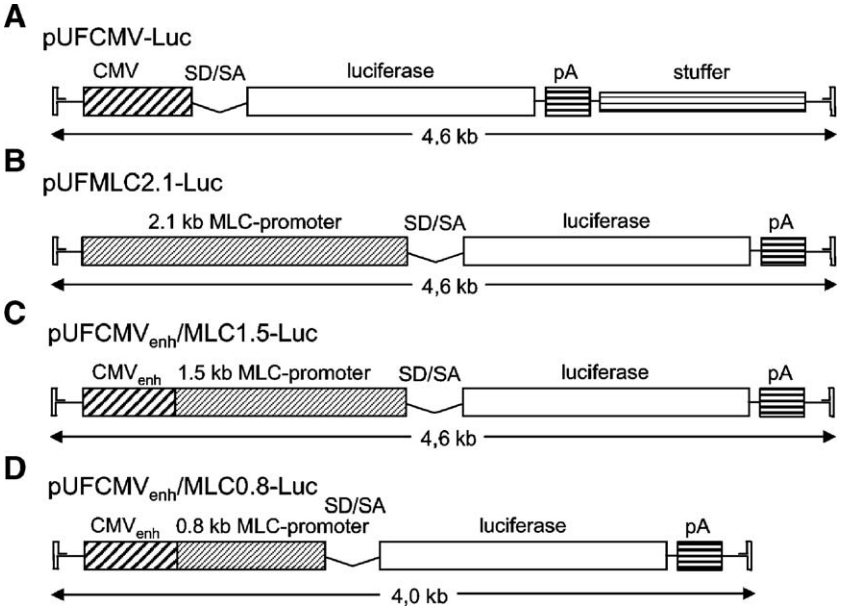

Fig. 1. Construction of recombinant AAV vector plasmids. All constructs contain a splice donor/splice acceptor signal (SD/SA) [25], the firefly luciferase sequence from pGL3 (Promega) under the control of different regulatory sequences, and the SV40 late poly(A) signal, flanked by the AAV inverted terminal repeats (ITR, shown as hairpin loops). (A) Plasmid pUFCMV-Luc contains the 619 bp CMV promoter and additional stuffer sequences from pUF3 [25] in order to achieve similar vector lengths. (B) In plasmid pUFMLC-Luc luciferase expression is driven by the 2.1 kb 5′ regulatory region (promoter) of the gene encoding rat myosin light chain-2v (MLC) [12]. (C) Plasmid pUFCMV$_{enh}$/MLC1.5-Luc carries the CMV-IE enhancer and a 1.5 kb fragment of the MLC regulatory region to control expression of the luciferase reporter. (D) Plasmid pUFCMV$_{enh}$/MLC0.8-Luc contains the proximal 0.8 kb of the MLC regulatory region fused to the CMV-IE enhancer in front of the luciferase reporter gene.

luciferase sequence from pGL3-basic (Promega, Mannheim, Germany) was inserted into the *Hind*III and blunted 3′ *Xho*I site of pUF3 [25]. To construct the rAAV vector pUFCM-V_{enh}/MLC1.5-Luc a intermediary vector was generated by cloning the *Hind*III/*Kpn*I CMV-SD/SA-fragment from pUF3 into pGL3 followed by insertion of the *Kpn*I CMV_{enh}/MLC1.5 fragment from $pCMV_{enh}$/MLC1.5-EGFP, which was derived by *Nhe*I/*Xba*I excision of 743 bp 5′ regulatory sequence from $pCMV_{enh}$/MLC-EGFP [26]. The final construct was obtained by excising the CMV promoter by *EcoR*I/*Xho*I and inserting the *Kpn*I/*BsaB*I CMV_{enh}/MLC1.5-luciferase fragment into the *Kpn*I and blunted 3′ *Sph*I site of pUF2 [25]. $pUFCMV_{enh}$/MLC0.8-Luc was constructed simultaneously after *Mro*I/*Xba*I excision of 5′ regulatory sequences from $pCMV_{enh}$/MLC-EGFP. pUFMLC-Luc was obtained by replacing the *Kpn*I/*Nhe*I CMV_{enh}/MLC1.5 fragment from $pUFCMV_{enh}$/MLC1.5-Luc by the MLC promoter excised *Kpn*I/*Nhe*I from pMLC-EGFP [26].

AAV-2 vectors with wild-type capsids were generated by cotransfection the helper plasmid pDF2 [21]. For production of AAV-2 vectors with detargeted capsids we used pDG(R484E; R585E) containing mutations of two amino-acids involved in heparin binding of AAV-2 (R484E; R585E) [20]. Crosspackaging of pseudotyped vectors was accomplished with pDP1, pDP3, pDP4r, pDP5, and pDP6 [21]. After 2 days, cells were harvested and viruses were purified using iodixanol gradients [27]. Gradient fractions were concentrated using VIVASPIN columns (Sartorius, Göttingen, Germany) thereby exchanging iodixanol against PBS. The AAV genomic, capsid, and replicative titers were determined as described previously [28]. Vector genomes were detected with a 660 bp *Eco*NI luciferase fragment from pGL3-basic.

2.2. Cell culture

293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 1 mg/ml of streptomycin at 37 °C in an atmosphere of 5% CO_2. Primary neonatal rat cardiomyocytes were generated and maintained as previously described [29].

2.3. In vitro gene transfer

Cultures of primary neonatal rat cardiomyocytes grown in 24 well plates were infected with a multiplicity of infection (MOI) of 100 vector genomes ($n=3$ for each group). After 48 h, cells were washed with PBS, harvested in reporter lysis buffer (Promega), and stored at −80 °C until luciferase activities were determined.

2.4. In vivo gene transfer

All procedures involving the use and care of animals were performed according to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the German animal protection code. Female immunocompetent NMRI mice (6–8 weeks of age) and Sprague–Dawley rats (250 g) were purchased by the German branch of Charles River Laboratories (Wilmington, MA).

Mice were injected intravenously via the tail vein with concentrated vector (in PBS) using a 30-gauge needle. Gene transfer in rats was achieved with a catheter based approach described by Hajjar et al. [30]. Rats were anesthetized with inhalation of isofluran and placed on a ventilator (Hugo Sachs Elektronik KG, March, Germany). Analgesia was provided by injection of temgesic (0.03 μg/g body weight) and carprofen (5 μg/g body weight). Upon opening of the chest and the pericardium a 7–0 suture was placed at the apex of the left ventricle. The aorta and pulmonary artery were identified and a 22 G catheter was advanced from the apex of the left ventricle to the aortic root. AAV vector solution was injected while aorta and pulmonary arteries were clamped. Clamping was released after 10 s and the chest was closed.

After three weeks animals were sacrificed and representative organs (heart, skeletal muscle, lung, liver, intestine, spleen, kidney, ovary, uterus, brain) were harvested and frozen in liquid nitrogen. Unless stated specifically, skeletal muscle means the quadriceps femoris muscle.

2.5. Measurement of luciferase activities

Luciferase reporter activities were determined with the luciferase assay kit from Promega (Mannheim, Germany) in a luminometer (Lumat LB9501; Berthold, Bad Wildbad, Germany). Luciferase activities in cellular lysates were expressed as relative light units (RLU) per well. Frozen tissue samples were homogenized in reporter lysis buffer and centrifuged for 10 min at 10,000 $\times g$. Protein content in tissue homogenates was determined with the Nano-Orange Kit (Molecular Probes, Leiden, The Netherlands) and luciferase activities were expressed as RLU per mg protein. Unless otherwise stated heart means left ventricular myocardium.

2.6. Detection of AAV genomes by PCR

Genomic DNA was extracted from organs using the Qiamp Tissue Kit (Qiagen, Hilden, Germany). 800 ng genomic DNA was used for PCR-amplification (40 cycles) of a 677 bp fragment of the luciferase gene using the primers 5′-GACGCCAAAAACATAAAGAAAG-3′ and 5′-CCAAAAATAGGATCTCTGGC-3′ under standard conditions. Integrity of DNA was determined by amplifying a 492 bp region of the murine β-actin gene using the primers 5′-ATGTTTGAGACCTTCAACAC-3′ and 5′-AACGTCAC-ACTTCATGATGG3′. PCR products were analyzed by gel electrophoresis (1.5%; w/v).

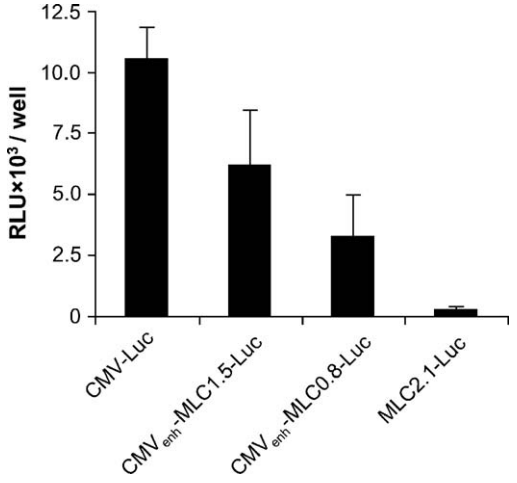

Fig. 2. Influence of the regulatory sequence on expression levels in cultivated primary neonatal rat cardiomyocytes. Luciferase reporter activities are given in relative light units (RLU) per well. Constructs without the CMV-IE enhancer revealed only marginal reporter activities at an MOI of 100, whereas constructs with the CMV-enhanced MLC promoter or the CMV itself enabled an efficient luciferase expression at the same MOI.

2.7. In vivo imaging of luciferase expression

Mice were anesthetized with isofluran using the XGI-8 gas anesthesia system (Xenogen, Alameda, USA) and were injected with an aqueous solution of luciferin (150 mg/kg body weight) intraperitoneally 10 min prior to imaging. Animals were placed under the chamber of the CCD camera system (IVIS 100 imaging system, Xenogen) and photons were counted for 5 min.

2.8. Statistical analyses

All data were expressed as mean ± standard deviation. To test for statistical significance an unpaired Student's *t*-test (level of significance < 0.05) was applied.

3. Results

3.1. Comparison of expression levels driven by different promoter elements in vitro and in vivo

Different promoter-reporter constructs were generated to elucidate whether it is possible to transcriptionally target gene expression into the heart (Fig. 1). Promoter-reporter constructs were packaged into AAV-2 capsids. The activity of these vectors was assessed in vitro on primary neonatal rat cardiomyocytes. Highest expression levels were obtained by transduction with AAV-2-CMV-Luc, followed by vectors with CMV_{enh}/MLC1.5 and CMV_{enh}/MLC0.8 promoter sequences (Fig. 2). Luciferase expression was almost absent with the 2.1 kb

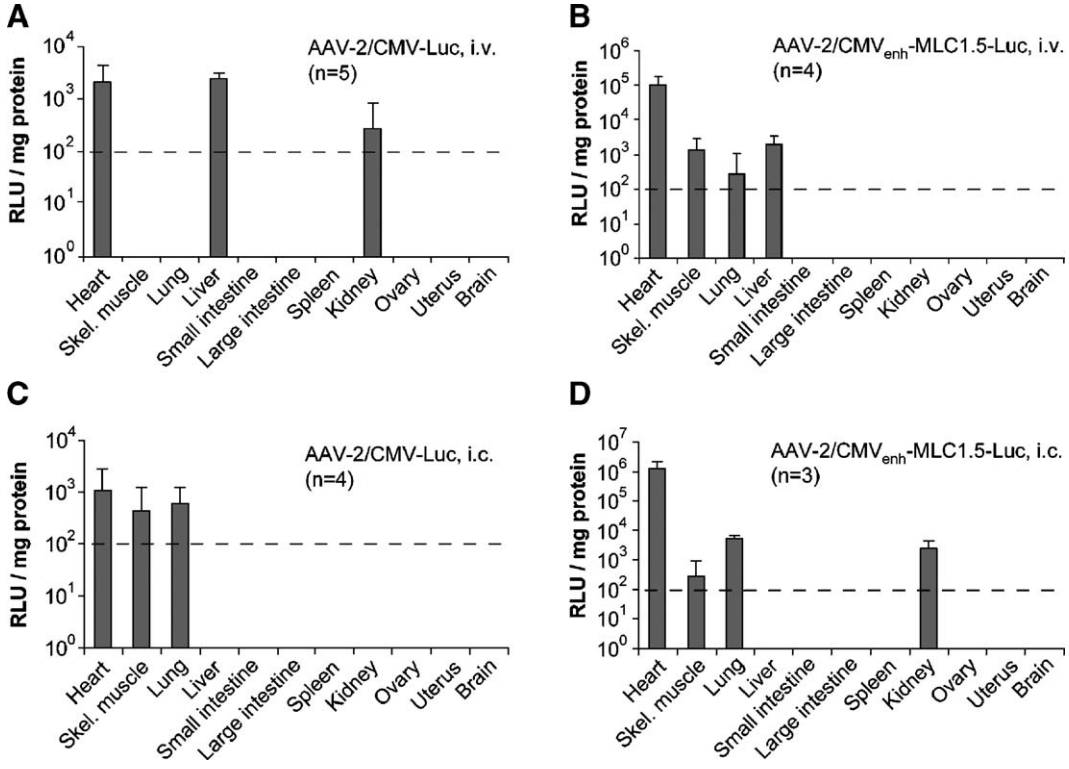

Fig. 3. Influence of the regulatory sequence and route of administration on reporter gene expression. Luciferase activities (in relative light units (RLU) per mg protein) were determined in representative organs three weeks after administration of 10^{11} vector genomes. (A) Intravenous injection (i.v.) of AAV-2-CMV-Luc into adult mice; (B) intravenous injection of AAV-2-CMV_{enh}-MLC1.5-Luc into adult mice; (C) intracoronary perfusion (i.c.) of AAV2-CMV-Luc in adult rats; (D) intracoronary perfusion of AAV2-CMV_{enh}-MLC1.5-Luc in adult rats. The dashed line shows the detection limit of luciferase expression.

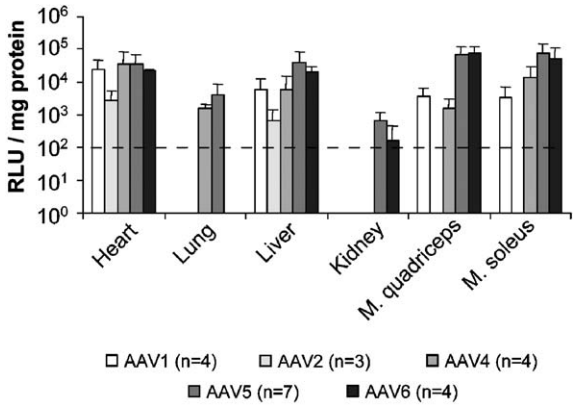

Fig. 4. Expression profile of pseudotyped AAV vectors expressing luciferase under control of the CMV promoter. Luciferase activities (in relative light units (RLU) per mg protein) were determined in representative organs three weeks after intravenous injection of 2×10^{10} vector genomes into adult mice. The dashed line shows the detection limit of luciferase expression.

MLC2v promoter/control region without the CMV-enhancer element.

To analyze the influence of the promoter sequence in vivo, 10^{11} AAV vector genomes were injected into the tail vein of adult mice. After three weeks, animals were sacrificed and luciferase activities were determined in representative organs. As shown in Fig. 3A, vectors with the CMV promoter enabled a low reporter gene expression in heart, liver and kidneys. In contrast, the CMV_{enh}-MLC1.5 promoter showed 50 times higher luciferase activities in the heart as compared to skeletal muscle and liver (Fig. 3B). Analysis of animals 3 months after intravenous administration of AAV-2-CMV_{enh}/MLC1.5 vectors confirmed the predominant expression in the heart (data not shown).

In order to elucidate the influence of the route of administration, the same constructs were administered at the same titer intracoronary into rat hearts according to a technique described previously [30]. Expression driven by the CMV promoter was as low as observed after intravenous injections in mice (Fig. 3C). Intracoronary perfusion of constructs carrying the CMV-MLC promoter in contrast resulted in an increase in transduction levels by one order of magnitude compared to intravenous transfer (Fig. 3D). However, a low level non-specific transduction of skeletal muscle (290 RLU/mg protein), lung (5460 RLU/mg protein), and kidney (2301 RLU/mg protein) was also observed.

3.2. Effects of pseudotyping

Crosspackaging recombinant AAV-2 vector genomes into capsids of other AAV serotypes results in an altered gene transfer in vitro and in vivo [21,22,24,33]. In a first attempt we analyzed reporter gene expression of pseudotyped AAV vectors under the control of the CMV promoter in representative organs of adult mice three weeks after intravenous injections. Expression levels in heart, liver, and skeletal muscle were elevated by injection of AAV-1, AAV-4, AAV-5, and AAV-6 pseudotyped vectors compared to AAV-2 (Fig. 4). AAV-4 and -5 vectors also enabled a low level reporter gene expression in pulmonary tissue and AAV-5 and -6 a low level of expression in the kidney. No detectable expression was observed after systemic transfer of AAV-3 pseudotyped vectors (not shown).

In order to analyze whether promoter targeting with the CMV_{enh}/MLC1.5 luciferase cassette might further increase transduction efficiency of AAV-6 pseudotyped vectors, organ distribution of reporter gene expression was determined after tail vein injection in adult mice (Fig. 5). Although high cardiac reporter activities could be detected, there was still a significant reporter gene expression in other organs such as skeletal muscle, lung, and liver despite using the CMV_{enh}/MLC1.5 promoter.

3.3. Combination of transcriptional targeting and AAV-2 capsid mutants devoid of binding to heparan sulfate

We previously showed that two distinct mutations in the AAV-2 capsid (R484E; R585E) are sufficient to completely ablate binding to Hela cells and to heparin as surrogate for binding to heparan sulfate proteogylcan which is thought to act as a primary receptor for AAV-2 cell-attachment [20]. Since binding to heparan sulfate is required for transduction of hepatic, but not cardiac tissue we packaged the CMV_{enh}-MLC1.5-Luc expression cassette into mutated AAV-2 capsids devoid of heparin binding (R484E; R585E). Luciferase activities were measured in representative organs after tail vein injection of the mutant vectors and compared to those obtained with vectors carrying wild-type capsids. Mutant vectors enabled a significantly increased reporter gene expression in the left ventricle, while reporter activities were reduced close to background levels in the liver (Fig. 6A). A significant luciferase expression was also observed in right ventricular myocardium as well as in quadriceps and

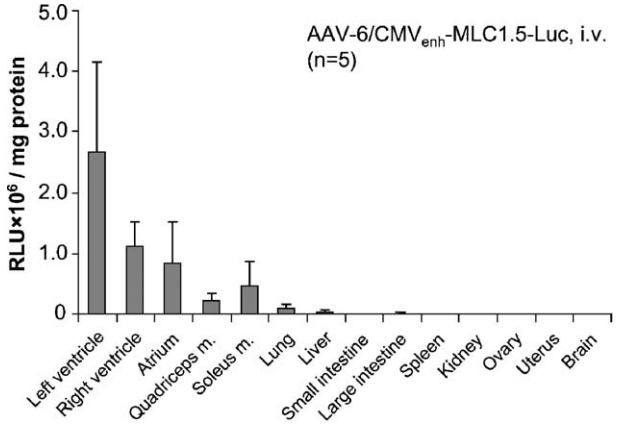

Fig. 5. Reporter gene expression of AAV-6 pseudotyped vectors carrying the CMV_{enh}-MLC1.5-Luc transgene in different organs of adult mice. Luciferase activities (in relative light units (RLU) per mg protein) were determined in representative organs three weeks after intravenous injection of 2×10^{10} vector genomes into adult mice.

soleus skeletal muscle after transduction with AAV-2(R484E; R585E) vectors. We also measured low reporter activities in the lung, which were less than 0.5% of the cardiac expression. Comparison of efficiency of gene transfer directed into the heart (left ventricle) versus the liver by AAV-2, AAV-6, and AAV-2(R484E; R585E) capsids pseudotyped with the transcriptionally targeted AAV-2 vector genomes (CMV_{enh}-MLC1.5-Luc) showed highest efficiency with AAV-6 capsids followed by the mutant capsids and AAV-2 (Fig. 6B). In contrast to AAV-6, which showed an increase in hepatic transduction in parallel to the higher cardiac expression levels, hepatic reporter gene activities were significantly reduced in mutant AAV-2 vectors compared to the transfer with AAV-2 wild-type vectors, resulting in an increased ratio of heart to liver expression (Fig. 6C). To prove that the reduced hepatic reporter activities observed with the heparin-binding deficient vector were due to decreased transduction of the liver, we PCR-amplified AAV vector genomes in tissue samples. Corresponding with the decreased reporter activities, less vector particles could be detected in the liver three weeks after systemic injection of 10^{11} genomic particles with ablated heparin binding, whereas no differences in vector particles could be observed in heart (Fig. 7), lung, and kidney (not shown). In order to visualize reporter expression, we performed in vivo imaging of luciferase expression using a CCD camera 3 weeks and 12 months after transfer showing stable gene transfer in the heart and skeletal muscle of the thigh over one year (Fig. 8).

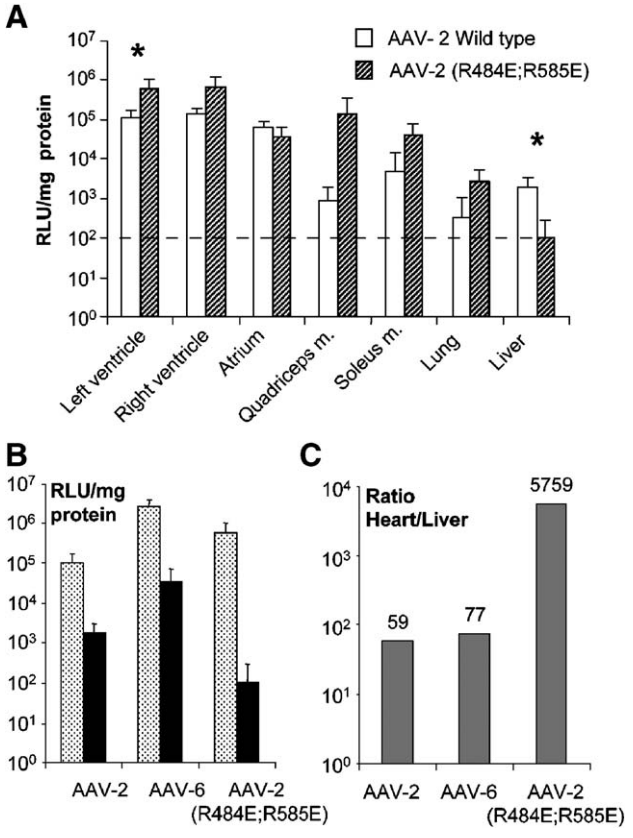

Fig. 6. (A) Transduction efficiencies of wild-type (white bars) and heparin-binding deficient (hatched bars) AAV-2 vectors carrying CMV_{enh}-MLC1.5-Luc genomes. Luciferase activities (in relative light units (RLU) per mg protein) were determined in representative organs three weeks after intravenous injection of 10^{11} vector genomes into adult mice. No activities were detected in the intestine, spleen, kidney, ovary, uterus, and brain. (*): $P<0.05$. The dashed line shows the detection limit of luciferase expression. (B) Comparison of cardiac (grey bars) and hepatic (black bars) reporter gene activities after systemic transfer of transcriptionally targeted vectors packaged into AAV-2, AAV-6, and heparin-binding deficient AAV-2(R484E; R585E) capsids. (C) Ratio of cardiac to hepatic transduction in AAV-2, AAV-6, and heparin-binding deficient AAV-2(R484E; R585E) vectors.

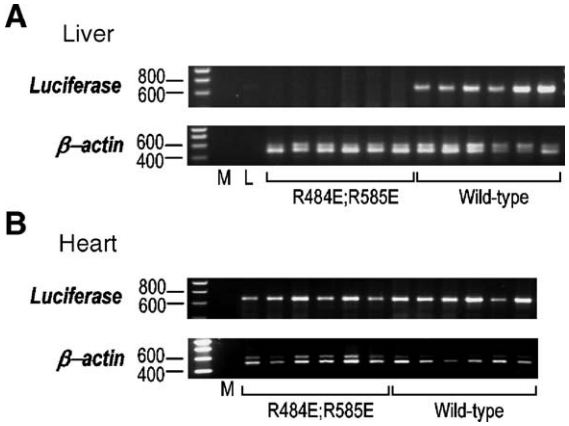

Fig. 7. Detection of vector genomes by PCR in liver (A) and heart muscle tissue (B) three weeks after intravenous injection of 10^{11} genomic particles of AAV-2(R484E; R585E)/CMV_{enh}-MLC1.5-Luc and AAV-2/CMV_{enh}-MLC1.5-Luc using luciferase and β-actin primers. The 677 bp luciferase band is faint or absent in liver tissue of mice injected with AAV carrying heparin-binding deficient AAV-2(R484E; R585E) capsids, but clearly visible in mice injected with vectors packaged into wild-type capsids. M indicates the lanes with negative controls (master mix without template); L indicates the lane with 0.1 pg pUFCMV-Luc which was used for determining the lower detection limit.

4. Discussion

Our study evaluated strategies to improve specificity and efficiency of cardiac gene transfer with AAV vectors in vivo. In particular, our experiments addressed the following questions: How can gene expression be restricted to the heart? Are AAV serotypes other than AAV-2 more suitable for cardiac gene transfer? Is there a synergistic effect of transcriptional targeting and transduction using AAV-2 vectors with ablated liver tropism? And finally with respect to potential therapeutic applications: Would expression levels by targeted vectors after systemic transfer compare to those achieved by direct cardiac administration of non-targeted vectors?

Our results demonstrate that a heterologous promoter consisting of the 1.5 kb MLC2v promoter fused to a CMV enhancer enables a predominantly cardiac reporter gene expression in adult mice after systemic administration of AAV-2 vectors. Previously, intracardiac injections of ade-

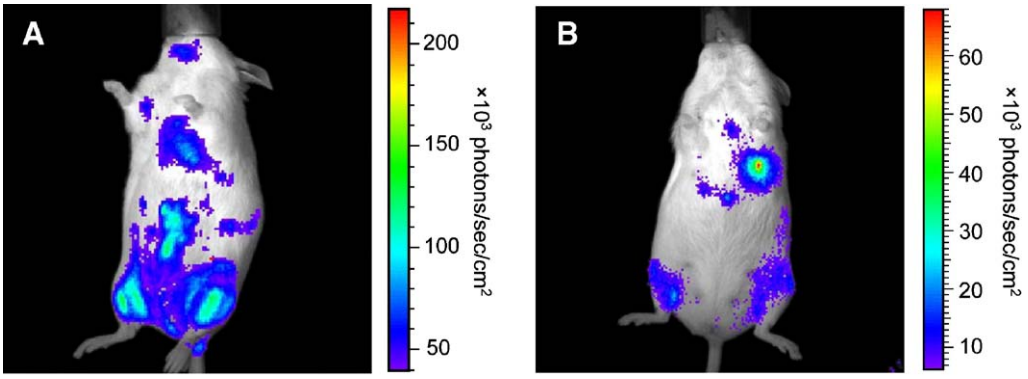

Fig. 8. In vivo imaging of long-term luciferase expression using AAV-2(R484E;R585E) vectors in cardiac and skeletal muscle especially of the thigh 3 weeks (A) and 12 months (B) after intravenous injection.

noviral vectors harboring a 0.8 kb fragment of the MLC2v promoter in newborn rats [6] and 0.26 or 2.1 kb fragments in adult rats [8] resulted in cardiac-specific gene expression. AAV-2 vectors harboring a 1.7 kb MLC2v-gfp transgene enabled also a cardiac-specific reporter gene expression after intracardiac injections in neonatal rats [10]. However, reporter gene expression could only be detected by RT-PCR after systemic administration of this vector in adult mice. The low expression driven by the MLC2v promoter in adult mice can be explained by developmental downregulation as previously shown in transgenic mice [12]. Therefore, we fused the MLC2v regulatory sequence to the CMV enhancer [13]. This fusion construct resulted in high levels of transcriptional activity in adult myocardium in transgenic rats [14,15]. The augmentation of gene expression driven by the CMV-enhanced MLC2v promoter was also observed in our in vitro transduction studies with primary cardiomyocytes, in which the 2.1 kb MLC2v promoter showed only low levels of reporter activity.

Upon tail vein injection, transcriptionally targeted AAV-2-CMV_{enh}/MLC1.5-Luc vectors enabled a 50-fold higher transduction efficiency than vectors harboring the CMV promoter. Such low activity of the CMV promoter after gene transfer in vivo was previously observed by other groups and explained by transcriptional silencing by methylation or cytokine response [31,32]. Besides the efficient cardiac gene expression driven by the CMV_{enh}/MLC1.5 promoter, only low expression levels could be detected in liver, lung, and kidney tissue which can be attributed to a low background activity of the MLC promoter in these tissues. It should be noted that the enhancer-less 1.7 kb MLC2v promoter also enabled a low hepatic gene expression in a previous study [10]. Crosspackaging CMV_{enh}/MLC1.5 promoter constructs into AAV-6 capsids further increased the overall transduction efficiency, but resulted in detection of significant reporter gene activities also in skeletal muscle and other organs ranging between 7.5% (skeletal muscle) and 0.03% (kidney) of those in left ventricular myocardium. The relative high skeletal activity is not surprising when considering the expression pattern of endogenous myosin light chain-2v, which is expressed both in cardiac and fast twitch skeletal muscle fibers [12]. A similar skeletal activity was also observed in transgenic mice harboring a CMV_{enh}/MLC2.1 luciferase transgene (own unpublished observations). The low activity in the kidneys (0.2% of cardiac activity) and lungs (0.4%) after intracoronary perfusion of CMV_{enh}/MLC1.5 promoter constructs in rats may reflect a low systemic spillover. A low pulmonary gene transfer was also reported in the original description of gene transfer by crossclamping aorta and pulmonary artery, which may be explained by a reflux over the mitral valve during cross-clamping [30]. Lacking hepatic transduction after intracoronary administration in rats shows that the systemic spillover into the liver is below the detection limit.

Crosspackaging vector genomes into capsids of different AAV subtypes resulted in an increased transduction efficiency of skeletal muscle [23,24] and other organs [22]. Since it is not known which serotype is most suitable for a systemic gene transfer in vivo, we compared reporter gene activities after intravenous injections of AAV serotypes 1 to 6. Serotype vectors 1, 4, 5, and 6 showed a similar efficiency in transducing the heart which was on average one order of magnitude higher than that of AAV-2. High levels of transduction were also observed in liver and skeletal muscle indicating a low tissue-specificity of those serotypes, but an increased suitability for a systemic transfer. A low tissue-specificity of pseudotyped vectors with AAV-1, -4, -5, and -6 capsids after systemic administration was also observed by Grimm et al. [33] who compared transduction by these serotype vectors after systemic administration and portal vein delivery. Detection of pulmonary reporter activities with AAV-4 and -5 pseudotyped vectors is also consistent with the previously reported high abundance of AAV-4 and -5 vector genomes in the lung upon intravenous application [33]. The overall low reporter gene activities enabled by AAV-2 and AAV-3 vectors indicate that these serotypes may be less suitable for an intravenous transfer. This might reflect their common use of heparan sulfate proteoglycan as primary receptor [17,34] which may result in sequestering of these vectors on extracellular heparan sulfate proteoglycans in the vasculature or liver [35,36]. The reduced hepatic vector uptake may

result in a prolonged presence in the circulation as previously shown for peptide-modified AAV-2 vectors [37].

As mentioned above, high cardiac reporter gene activities were achieved by including the CMV_{enh}/MLC1.5 promoter into AAV-6 serotype vectors. In a recent study comparing AAV serotypes after intraperitoneal delivery into neonatal mice, AAV-1 and AAV-6 enabled higher cardiac reporter gene expression than AAV-2 [38]. Another study reported that the use of AAV serotype 1 resulted in an increased transduction of cultivated adult murine as well as human cardiomyocytes compared to AAV-2, -3, -4, and -5 pseudotyped vectors [39]. AAV-1 vectors enabled also the most efficient gene transfer upon direct intramyocardial injections in mice [39]. Since AAV-1 and AAV-6 are phylogenetically highly related, the improved cardiac transduction of AAV-6 vectors after systemic transfer in our study can be contributed to an increased transduction of cardiomyocytes. An increased transduction of cardiomyocytes with pseudotyped AAV-6 vectors was also shown by a uniform and extensive transfer of a lacZ reporter gene in mice upon systemic gene transfer [40]. Furthermore, intravenous injections of AAV-8 vectors enabled a reconstitution of δ-sarcoglycan in δ-sarcoglycan-deficient TO-2 hamsters [38], underlining the principal suitability of AAV vectors for systemic gene transfer in mice and hamsters. A potential application could be hereditary heart muscle diseases, in which a widespread gene transduction would be desirable. However, in terms of cardiac-specificity, pseudotyped AAV-6 vectors have no advantage over AAV-2 vectors since hepatic transduction is also increased (Fig. 6).

An undesired transduction of the liver in a systemic gene transfer approach to the heart can be avoided using vectors packaged into mutant AAV-2(R484E; R585E) capsids which enable a 100-fold increased ratio of cardiac to hepatic reporter activity. The AAV-2(R484E; R585E) capsids have a double mutation of the heparin-binding motif at R484 and R585 resulting in a loss of heparin binding and strongly reduced infection of mouse liver tissue in vivo without affecting transduction of the heart [20]. Therefore, transduction of cardiac tissue appears to be independent of binding to heparan sulfate proteoglycans. It is presently unknown to which receptor(s) the detargeted AAV-2 capsids bind when they infect the heart. The fact that also skeletal muscle and lung still were transduced by the vectors ablated for heparin binding indicates that further improvement of vector targeting is warranted, for example by selection of appropriate capsids from AAV-2 random peptide libraries [41].

We have used a luciferase reporter gene for detection of organ distribution of gene expression after tail vein injections since luciferase mirrors reliably promoter activity and gene expression. In contrast to other approaches using circulating gene products such as factor IX or human growth hormone [9,33], luciferase enables quantitation of gene expression in different isolated organs and thus comparison of expression profiles of different vectors [42]. Similar luciferase levels in left- and right-ventricular as well as atrial myocardium indicate a homogeneous tissue distribution which was also confirmed in recent studies reporting a systemic gene transfer with AAV-6 and -8 vectors [38,40]. Although luciferase doesn't allow analysis of tissue sections like a lacZ reporter [43] it enables an in vivo imaging using a CCD camera. Using this technique, we observed a long-term expression in the heart as well as skeletal muscle.

In summary, combining transcriptional targeting with a mutant AAV-2(R484E; R585E) vector capsid is most suitable for cardiac gene transfer upon systemic delivery. Alternative serotypes such as AAV-6 result in higher expression levels. However, a higher non-specific hepatic expression needs to be taken into account even when using a promoter appearing to be tissue-specific in the AAV-2 context. Combining transcriptional and transductional targeting of AAV vectors might become an alternative to cardiac perfusion of the vector requiring open chest surgery. Furthermore, systemic injection of targeted AAV vectors into adult mice may allow cardiac overexpression of genes as alternative to transgenic mouse models.

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