Prevention of cardiomyopathy in δ-sarcoglycan knockout mice after systemic transfer of targeted adeno-associated viral vectors

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Aims δ-Sarcoglycan is a member of the dystrophin-associated glycoprotein complex linking the cytoskeleton to the extracellular matrix. Similar to patients with defects in the gene encoding δ-sarcoglycan (Sgcd), knockout mice develop cardiomyopathy and muscular dystrophy. The aim of our study was to develop an approach for preventing cardiomyopathy in Sgcd-deficient mice by cardiac expression of the intact cDNA upon systemic delivery of adeno-associated viral (AAV) vectors.

**Methods and results** We packaged the Sgcd cDNA under transcriptional control of a myosin light chain promoter fused with a cytomegalovirus enhancer into AAV-9 capsids. Vectors carrying either the Sgcd cDNA or an enhanced green fluorescent protein (EGFP) reporter gene were intravenously injected into adult Sgcd knockout mice. After 6 months, immunohistochemistry revealed almost complete reconstitution of the sarcoglycan subcomplex in heart but not skeletal muscle of mice with the Sgcd vector. Furthermore, Sgcd gene transfer resulted in prevention of cardiac fibrosis and significantly increased running distance measured by voluntary wheel running. Left ventricular function remained stable in mice expressing Sgcd while it deteriorated in EGFP controls within 6 months, paralleled by increased expression of brain natriuretic peptide, a molecular marker of heart failure.

**Conclusion** Our study establishes an approach to specifically treat hereditary cardiomyopathies by targeting gene expression into the myocardium upon systemic application of AAV vectors.

1. Introduction
Gene therapy approaches in cardiology have focused on treatment of acquired disorders, such as ischaemic heart disease, in which a therapeutic effect is expected already with a transient and localized expression of the therapeutic gene. In contrast, gene replacement approaches for inherited diseases of the myocardium such as hereditary cardiomyopathies require a sustained and homogenous transfer of the intact cDNA throughout the heart. Adeno-associated virus (AAV) vectors enable a long-term gene transfer into heart in animal models and skeletal muscle in humans. A further advantage of AAV vectors is the ability of different serotypes to enable an efficient systemic gene transfer into hearts of mice and hamsters. Among those serotypes, AAV-9 vectors revealed the highest tropism for murine heart. Specificity of AAV-mediated gene transfer could be additionally increased by transcriptional targeting using a cardiac-specific promoter such as the CMV-enhanced myosin light chain (MLC) 2v-promoter. To elucidate whether systemic transfer of transcriptionally targeted AAV-9 vectors is suitable for specifically treating cardiomyopathy, we investigated the effect of cardiac gene transfer of δ-sarcoglycan (Sgcd) in Sgcd knockout mice.

δ-Sarcoglycan is a transmembrane glycoprotein and forms together with α-, β-, γ-, ε-, and ζ-sarcoglycan the sarcoglycan complex, which is a member of the dystrophin-associated glycoprotein complex (DGC). The DGC plays a central role in maintaining integrity of the cell membrane by forming a structural link between the extracellular matrix and the cytoskeleton, thus protecting muscle fibres from contraction-induced damage and necrosis. Patients with mutations in δ-sarcoglycan may present with isolated dilated cardiomyopathy or limb girdle muscular dystrophy 2F with frequent cardiac involvement. Corresponding to the clinical picture, mice devoid of δ-sarcoglycan develop cardiomyopathy and muscular dystrophy with signs of progressive disease such as necrosis, muscular regeneration, inflammation,
and fibrosis within their first 3 months of life.\textsuperscript{19,20} So far, cardiomyopathy of \(\delta\)-sarcoglycan-deficient mice has been successfully treated using genetic approaches including mating with myostatin- and calcineurin- deficient mice or crossbreeding with a transgenic line expressing \(\delta\)-sarcoglycan under the control of a cardiac-specific promoter.\textsuperscript{21–23} Although several therapeutic approaches addressed gene transfer to treat cardiomyopathy in a hamster model of \(\delta\)-sarcoglycan deficiency due to a spontaneous mutation,\textsuperscript{11,24,25} a systemic transfer of AAV vectors transcriptionally targeted to the heart has not been studied so far.

Therefore, we have investigated the efficiency and specificity of transcriptionally targeted AAV-9 vectors to deliver \(\delta\)-sarcoglycan into the heart of \(\delta\)-sarcoglycan-deficient mice. We found an almost complete reconstitution of \(\delta\)-sarcoglycan expression throughout the heart, but not in skeletal muscle 6 months after systemic transfer of \(2 \times 10^7\) genomic vector particles. In addition, gene transfer prevented progression into heart failure as indicated by contractile and biochemical parameters.

2. Methods

2.1 Production of adeno-associated virus vectors

The open reading frame of Sgcd was amplified with the Expand PCR kit (Roche, Mannheim, Germany) using oligo-dT-primed heart cDNA of mouse strain C57BL/6 as template and primers XbaSgcdF (GCTCTAGAGCAGTGAGAGCGGGAAATGCTG) and XbaSgcdR (GCTCTAGAGCTTTCAAGGACGACATTGTGTG). The polymerase chain reaction (PCR) product of \(~1.1\) kb was first cloned in vector pMOS (Amer sham, Freiburg, Germany) and sequenced. The cDNA insert was then cut out with XbaI and recloned into the XbaI-digested AAV vector pHUCMV\textsubscript{enh/MLC1.5-luc},\textsuperscript{4} resulting in pHUCMV\textsubscript{enh/MLC1.5-Sgcd}.

For production of AAV9-Sgcd pseudotyped vectors, this plasmid was used for cotransfection of 293T cells together with p5E18-VD2-9 (kindly provided by Drs Guangping Gao and Jim Wilson),\textsuperscript{26} encoding the AAV-9 cap sequence, and pDGdelpVR,\textsuperscript{27} containing the AAV-2 rep gene as well as adenoviral helper sequences. For generation of the control vector AAV9-EGFP, a vector genome with enhanced green fluorescent protein (EGFP) under the control of the CMV-enhanced MLC promoter was used. AAV vectors were produced, purified, and titrated using standard procedures.\textsuperscript{28,29}

2.2 Animals and in vivo vector delivery

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. \(\delta\)-Sarcoglycan-deficient mice (B6.129-Sgcd\textsuperscript{tm1Mcn/J}) were obtained from Jackson Laboratory (Bar Harbor, ME, USA) and bred as previously described.\textsuperscript{20} Nontransgenic littermates were used as controls. Vector was intravenously injected into the tail vein of adult (2 months old) mice as 150 \(\mu\)L bolus using a sterile syringe and 29-gauge needle. After 6 months, animals were euthanized by cervical dislocation. Organs were dissected and rapidly frozen in liquid nitrogen. For histological analyses, tissue was embedded individually in tissue freezing medium (Jung, Nussloch, Germany) and frozen in liquid nitrogen.

2.3 Immunohistochemistry

For immunofluorescence staining, unfixed cryosections (7 \(\mu\)m thickness) were immediately blocked in phosphate-buffered saline (PBS) containing 10% goat serum, 1% bovine serum albumin (BSA), and 0.1% Triton X-100 for 45 min at room temperature. Sections were rinsed twice with 0.1% BSA-PBS and subsequently incubated overnight at 4°C with primary antibodies against \(\alpha\)- and \(\delta\)-sarcoglycan. Both the monoclonal mouse \(\alpha\)-sarcoglycan (Novoceastra, Newcastle, UK) and the polyclonal rabbit \(\delta\)-sarcoglycan antibody (Santa Cruz H55, Heidelberg, Germany) were diluted 1:50 in PBS. After incubation with biotinylated anti-mouse or anti-rabbit secondary antibodies diluted 1:100 in PBS, samples were overlaid with Fluorescein-Streptavidin diluted 1:100 and kept in the dark for 15 min. Between every step, sections were washed twice in PBS containing 0.1% BSA. Immunostained sections were mounted with VECTASHIELD Hard Set Mounting Medium (Vector Laboratories, Burlington, CA, USA) and analysed by fluorescence microscopy (Nikon Eclipse 90i upright automated microscope, Nikon, Düsseldorf, Germany) using a D1QM camera (Nikon). Representative microphotographs of those sections were used to determine the number of \(\delta\)-sarcoglycan positive cardiomyocytes by counting fluorescent cells.

2.4 Histological analyses

For haematoxylin and eosin (H&E) staining, 7 \(\mu\)m cryosections were incubated for 10 min in haematoxylin (Sigma-Aldrich, Steinheim, Germany). After three wash steps in tap water, samples were stained with eosin, dehydrated in ethanol and xylol, and finally mounted with RotiHistokitt II (Roth GmbH, Karlsruhe, Germany).

2.5 Vital staining with Evans blue dye

Evans blue dye was dissolved in PBS (10 mg/mL) and sterile filtered (0.2 \(\mu\)m). Mice were injected intraperitoneally with 1 mg/g body weight and euthanized by cervical dislocation 16 h later. Heart and femoral quadriceps muscle of these animals were cryosectioned (7 \(\mu\)m thickness) and Evans blue-positive fibres were identified by fluorescence excitation at 633 nm.

2.6 Transthoracic echocardiography

Echocardiography was performed using a Sonos 5500 with a 512 transducer (12 MHz). The echocardiographer was blinded with respect to the treatment group. Mice were shaved and left ventricular parasternal short-axis views were obtained in M-mode imaging at the papillary muscle level. Three consecutive beats were used for measurements of left ventricular end-diastolic internal diameter (LVEDD) and left ventricular end-systolic internal diameter (LVESD). Fractional shortening (FS) was calculated as 
\[FS = \frac{\text{LVEDD} - \text{LVESD}}{\text{LVEDD}} \times 100\]

2.7 Voluntary wheel running

At the beginning of the study, AAV9-EGFP control-injected and the AAV9-Sgcd-injected mice were placed individually in cages containing small metal running wheels (Fahrolz, Hamburg). All mice were maintained under a standard 12:12 h light–dark cycle and had free access to the wheel.
At the end of the study, the individual daily running distance was measured with digital magnetic counters (BC 500, Sigma Elektro GmbH, Neustadt, Germany) connected to the running wheels.

2.8 Real-time polymerase chain reaction

Expression of brain natriuretic peptide (BNP) was quantified at the mRNA level using real-time PCR with following primers: forward_BNP: 5’ GCT GCT TTG GGC ACA AGA TAG 3’; reverse_BNP: 5’ GGT CTT CCT ACA ACA ACT TCA G 3’. RNA was extracted from frozen cardiac samples using TRIzol (Invitrogen, Carlsbad, CA, USA) and used for cDNA synthesis with SuperScript™ III-Kit RNase H Reverse Transcriptase (Invitrogen). For real-time PCR, quantification of GAPDH was used as housekeeping gene (forward_GAPDH: 5’ CAA CTT TGG CAT TGT GGA AGG 3’; reverse_GAPDH: 5’ ACA CAT TGG GGG TAG GAA CAG 3’). The PCR was run in an ABI Prism™ 7000 Sequence Detection System (Applied Biosystems, Foster City, CA, USA), and a 7000 System SDS Software was used for data analysis.

2.9 Statistical analyses

All data were expressed as mean ± standard error. To test for statistical significance, an unpaired two-sided Student’s t-test was applied.

3. Results

3.1 Long-term reconstitution of the sarcoglycan complex in the heart of δ-sarcoglycan knockout mice after intravenous injection of a transcriptionally targeted AAV-9 vector

We generated AAV-9 vectors containing the Sgcd cDNA under transcriptional control of the CMV-MLC promoter (AAV9-Sgcd). A total 2 x 10^11 genomic particles, a dose which was previously shown to transduce the majority of cardiomyocytes,8 were intravenously injected into the tail vein of adult δ-sarcoglycan-deficient mice. Six months after Sgcd gene transfer mice were sacrificed. Immunostaining of cardiac sections with a δ-sarcoglycan antibody localized a highly efficient δ-sarcoglycan expression within the sarcolemma of heart, but not skeletal muscle (Figure 1A). Patterns of δ-sarcoglycan expression were indistinguishable from heart sections of age-matched wild-type animals (Figure 1A). Counting cells positive for δ-sarcoglycan in representative sections revealed a transduction efficiency of 92 ± 5%. No δ-sarcoglycan signal could be detected in Sgcd-deficient mice control-injected with AAV9-EGFP.

Similar to patients with a primary deficiency30 absence of δ-sarcoglycan results in a loss of the other sarcoglycans (α-, β-, and γ-sarcoglycan) due to impaired assembly in the endoplasmic reticulum and trafficking to the sarcolemmal membrane in an in vitro model.31 Thus, we examined...
whether expression of Sgcd also restored the secondary deficiency of α-sarcoglycan. Corresponding to the δ-sarcoglycan protein, we could also detect α-sarcoglycan in the plasma membrane of cardiomyocytes after systemic delivery of AAV9-Sgcd in Sgcd knockout mice (Figure 1B). These results suggest that transcriptionally targeted AAV-9 vectors are able to long-term reconstitute the sarcoglycan complex in heart, but not skeletal muscle.

3.2 Prevention of pathological changes in the heart upon systemic delivery of AAV9-Sgcd

We further investigated whether long-term Sgcd expression could result in a therapeutic effect on pathological changes in the myocardium. H&E staining of cardiac sections of untreated and AAV9-EGFP control-treated Sgcd knockout mice showed signs of cardiomyopathy such as pronounced fibrosis, cell degeneration, and necrosis, as described before (Figure 2).19,20 In contrast, AAV9-mediated Sgcd gene transfer prevented pathological muscle fibre degeneration and fibrosis in all mice (n = 9) (Figure 2). Furthermore, heart muscle morphology of AAV9-Sgcd-transduced Sgcd-deficient mice was indistinguishable from that of age-matched wild-type mice. Finally, no lymphocyte infiltration was observed in cardiac sections of AAV9-Sgcd and control-transduced mice, supporting the notion that targeted cardiac gene transfer using AAV-9 vectors does not elicit an immune response.

Corresponding to the lack of sarcoglycan expression in skeletal muscle after systemic delivery of AAV9-Sgcd, histological analyses of skeletal muscle still showed severe morphological alterations with extensive fibrosis and muscle cell degeneration in control-treated mice (n = 7) (Figure 2). The absence of the sarcoglycan complex results in altered membrane permeability which allows Evans blue to accumulate in damaged cardiac and skeletal muscle fibres.21 In order to analyse the effect of systemic delivery of AAV9-Sgcd on membrane permeability of heart and skeletal muscle cells in our study, Evans blue dye was intraperitoneally injected in two mice of each group 16 h before dissection.

Evans blue autofluorescence could be detected in groups of skeletal and cardiac muscle fibres of control-treated Sgcd-deficient mice (Figure 3). Dye-positive fibres also showed characteristic features of degeneration and necrosis in H&E stains. However, corresponding to cardiac reconstitution of δ-sarcoglycan and preservation of morphology, no Evans blue accumulation was observed in hearts of knockout mice upon systemic delivery of AAV9-Sgcd (Figure 3). These data demonstrate that the integrity of the sarcolemma has been restored in cardiac myofibres expressing Sgcd.

3.3 Cardiac δ-sarcoglycan expression results in preserved left ventricular systolic function and increased running distance in voluntary wheel running

To address the issue whether transfer of Sgcd into the hearts of δ-sarcoglycan-deficient mice has a beneficial effect on physiological parameters, we assessed left ventricular function by transthoracic echocardiography. Echocardiographic measurements showed that differences between the decline of FS over the period of 6 months were highly significant between the two groups (Figure 4). FS in AAV9-Sgcd-treated mice remained stable (67.6 at the beginning vs. 65.0% at the end of the study) compared with a significant decline in EGFP controls (from 69.9 to 58.1%, P < 0.05). To provide further evidence of preserved left ventricular function after long-term gene transfer of Sgcd in Sgcd knockout mice, we determined the levels of BNP, a molecular marker of heart failure.22 In parallel to the preserved left ventricular function, gene transfer of Sgcd resulted in a significantly lower cardiac BNP expression than in mice transduced with AAV9-EGFP (P < 0.05) (Figure 5).

Furthermore, we studied the consequences of AAV9-mediated Sgcd gene transfer using voluntary wheel running which can be used as an indicator of cardiac and skeletal muscle performance.34,35 We measured the daily running distances of the two groups in our voluntary wheel running setup during the last 4 weeks of the study (Figure 6). AAV9-Sgcd-treated mice revealed a significantly increased running distance compared with EGFP-controls (92.3 ± 5.7 vs. 64.3 ± 11.0 km, P < 0.05). Since skeletal muscle pathology is not improved by the transfer of Sgcd using transcriptionally targeted AAV-9 vectors, the increased running distance can most probably be attributed to the preserved left ventricular function.

4. Discussion

The overall aim of this study was to investigate whether systemic transfer of the δ-sarcoglycan gene using AAV-9 vectors

![Figure 2](image-url) Correction of the histological alterations in cardiac, but not skeletal muscle 6 months after AAV9-Sgcd transfer. Representative haematoxylin and eosin staining of heart and quadriceps femoris muscle sections of Sgcd knockout mice without gene transfer, after the injection of AAV9-Sgcd or AAV9-EGFP. Wild-type mice are shown for control. Bar: 50 μm.
transcriptionally targeted to the heart is suitable for preventing progression of cardiomyopathy in Sgcd knockout mice. We found reconstitution of the sarcoglycan complex and prevention of myofibre damage in cardiac, but not skeletal muscle. Moreover, cardiac expression of Sgcd resulted in a preserved fraction of shortening and increased running distance in voluntary wheel running experiments after 6 months compared with controls.

\(\delta\)-Sarcoglycan is essential for the initial stability of the sarcoglycan complex in the endoplasmic reticulum. It was proposed that it might form a core of the sarcoglycan complex with \(\beta\)-sarcoglycan. The absence of \(\delta\)-sarcoglycan results in a loss of the other sarcoglycans at the sarcoplasmic membrane due to aberrant complex assembly and trafficking. Thus, the detection of \(\alpha\)-sarcoglycan in Sgcd knockout mice with cardiac expression of \(\delta\)-sarcoglycan indicates an intact formation of the complex in the endoplasmic reticulum and transfer to the cell membrane.

The finding that Sgcd is expressed efficiently in cardiac, but not skeletal muscle may be explained by the use of the CMV-enhanced MLC 1.5 promoter which revealed a predominant cardiac activity in a previous study analysing...
transcriptional targeting of AAV vectors. Although AAV-9 mediates the most efficient cardiac gene transfer in mice upon systemic vector application, previous studies reported also a significant AAV-9-mediated transduction of skeletal muscle and other organs most probably due to the use of strong, but unspecific viral promoters in these approaches.1,8,10,36 The advantage of a tissue-specific expression has previously been shown in AAV-mediated α- and γ-sarcoglycan gene transfer using intramuscular injections which resulted in a sustained expression of the transferred genes in the corresponding knockout mouse model.37–39 In contrast, the use of unspecific viral promoters led to a dramatic drop of expression after a few weeks.37,39,40 This transient expression was explained either by a toxic effect of α-sarcoglycan overexpression or an immune response against the transferred gene product due to expression in antigen presenting cells.37,39 Since an intravenous vector application might increase the probability of transducing antigen presenting cells compared with intramuscular injections, the use of a tissue-specific promoter appears to be even more preferable and may explain the long-term expression of δ-sarcoglycan in our study.

Only few previous studies investigated a functional effect of cardiac gene transfer using systemic delivery of AAV vectors into adult animals.11,41 Zhu et al.11 used AAV-8 vectors in combination with the synthetic muscle-specific promoter C5-27, which allowed a sustained expression in both heart and skeletal muscle after systemic transfer into adult hamsters. Another study showed a beneficial effect on cardiac function by AAV-6-mediated micro-dystrophin transfer under the control of the muscle-specific CK-6 promoter into adult mdx mice.41 Besides an efficient cardiac gene transfer, both approaches revealed a high skeletal muscle expression, suggesting a potential future role for gene transfer in conditions in which skeletal muscle dystrophies are associated with cardiomyopathies, such as Duchenne muscular dystrophy or certain sarcoglycanopathies. Since cardiomyopathy frequently occurs without skeletal muscle involvement, skeletal muscle transduction may not be desirable in general. Furthermore, cardiac gene transfer approaches with vascular growth factors or modulators of the calcium homeostasis such as SERCA might even result in potential side effects in case of expression of the therapeutically sequence in extracardiac tissue. Therefore, our highly specific AAV vector might be suitable for future therapeutic approaches and could be used for validation of novel therapeutic targets in murine models.

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Conflicts of interest: none declared.

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