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***INHERITED DISEASE*** ***RESEARCH ARTICLE***

# *Early adenovirus-mediated gene transfer effectively prevents muscular dystrophy in alpha-sarcoglycan-deficient mice*

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*Limb-girdle muscular dystrophy type 2D (LGMD 2D) is the most common cause of LGMD with a sarcoglycan defect. We recently engineered a murine model for this progressive disease and we investigated the possibility of preventing the development of muscular dystrophy in these animals by adenovirus-mediated gene transfer of human α-sarcoglycan. Here we report that a single intramuscular injection of a first generation adenovirus into the skeletal muscle of neonate mice led to sustained expression of α-sarcoglycan at the sarcolemma of transduced myofibers for at least 7 months. The morphology of transduced muscles was consequently preserved. In addition, we have used contrast agent-enhanced magnetic resonance imaging (MRI) to investigate sarcolemmal integrity in adenovirus-injected animals and have thereby demonstrated maintenance of sarcolemmal function. In conclusion, we provide evidence that early virus-mediated gene transfer of a sarcoglycan protein constitutes a promising therapeutic strategy for LGMDs and that the benefits of this approach can easily and effectively be monitored by noninvasive methodologies such as MRI.* Gene Therapy (2000) **7,** 1385–1391.

**Keywords:** *limb girdle muscular dystrophy; gene transfer; adenovirus; sarcoglycan; murine model*

## *Introduction*

The sarcoglycans are single-pass transmembrane proteins localized at the sarcolemma of muscle fibers which, along with sarcospan (SSPN), a tetraspan-like protein, constitute the sarcoglycan-sarcospan (SG-SSPN) complex within the larger dystrophin-glycoprotein complex (DGC).[1–8] Mutations in four genes encoding sarcoglycan proteins (α-, β-, γ- and δ-SG) are responsible for various forms of autosomal recessive limb-girdle muscular dystrophy (LGMD 2D, 2E, 2C and 2F, respectively).[9–16] This heterogeneous group of diseases nevertheless shares a common feature in that a primary deficiency of any of the sarcoglycan proteins leads to the reduction or the absence of all other members of the SG-SSPN complex.[17] In non-consanguineous populations, α-sarcoglycan deficiency is the most frequent cause of autosomal recessive LGMD with a sarcoglycan defect (sarcoglycanopathy).[18–20] Thus, the development of therapeutic approaches in animal models for these diseases is a crucial step toward providing therapies for human patients. Because of their small size, virus-mediated delivery of the sarcoglycan genes can easily be achieved using either recombinant adenoviruses (Ad) or adenovirus-associated viruses (AAV). Both approaches have proven successful in delivering δ-SG to the skeletal muscle of a naturally occurring model for LGMD 2F, the BIO 14.6 cardiomyopathic hamster.[21–23] In addition, we recently reported preliminary data on adenovirus-mediated delivery of α-SG to the skeletal muscle of mice lacking this protein and displaying a progressive muscular dystrophy closely mimicking the human LGMD 2D pathology.[24] Just as with δ-SG, α-SG gene transfer restores the expression of the other sarcoglycan proteins at the sarcolemma of transduced fibers.[21–24]

In this study, we present extensive data supporting the feasibility of an α-SG gene transfer approach to prevent the dystrophic process in *Sgca*-null mice. Direct intramuscular injection of a first-generation adenoviral vector (Ad5RSV-SGCA) into the skeletal muscle of *Sgca*-null mice resulted not only in a high efficiency of gene transfer, but also in a robust level of expression of α-SG at the sarcolemma that was sustained for at least 31 weeks. More importantly, sarcolemmal function was maintained as assessed *in vivo* by Evans Blue dye assay,[25] as well as contrast agent-enhanced magnetic resonance imaging (MRI) of adenovirus-injected proximal hind-limb muscles of *Sgca*-null mice. Our data demonstrate efficient prevention of the muscle pathology by adenovirus-mediated gene transfer of α-sarcoglycan when delivered before the onset of the disease. Moreover, we have been able to monitor the consequences of gene transfer by MRI, a commonly used noninvasive approach. This procedure is

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thus expected to be useful and important when considering the potential application of virus-mediated sarcoglycan gene transfer to human patients.

## Results

### *Sustained expression of α-SG and restoration of the SG–SSPN complex*

Newborn *Sgca*-null mice (3- to 4-day-old) were injected directly in the quadriceps femoris muscle with $10^7$ p.f.u. of a first-generation adenovirus containing the human α-SG cDNA under the control of the RSV promoter.[24] Immunofluorescence analysis of muscle cryosections was performed between 15 ($n = 6$) and 31 ($n = 3$) weeks after injection (the latest time-point analyzed). Figure 1a demonstrates that α-SG expression was sustained for at least 7 months and that the protein was indeed correctly localized at the sarcolemma of transduced fibers. However, only the vastus lateralis muscle was transduced with an efficiency of transduction of about 90% suggesting that this was the muscle that was targeted during the injection (Figure 1a). The fact that the rectus femoris muscle was not transduced at all illustrates the inability of adenoviral particles to cross the perimysium separating muscle fascicles (Figure 1a). In order to analyze single muscle groups, we also performed injections into the tibialis anterior (TA) or the extensor digitorum longus (EDL) muscle of newborn mice. Again, at least 90% of the myofibers were transduced (data not shown). In all cases, expression of all the other components of the SG–SSPN complex was restored at the sarcolemma of transduced fibers (Figure 1b). In addition, immunostaining analysis of glycoprotein preparations from muscles harvested 21 days after adenoviral gene transfer demonstrated that a single intramuscular injection of Ad5RSV-SGCA into the quadriceps femoris of neonate mice produces about 30% of the amount of α-SG detected in the control sample (Figure 2). This level reflects the partial transduction of the entire quadriceps femoris muscle, wherein only the vastus lateralis muscle was consistently transduced. This result is in accordance with previous data from gene transfer experiments of δ-SG in dystrophic hamsters.[23] Indeed, restoration of the entire SG–SSPN complex can also be detected by immunostaining of glycoproteins from injected muscle samples (data not shown).

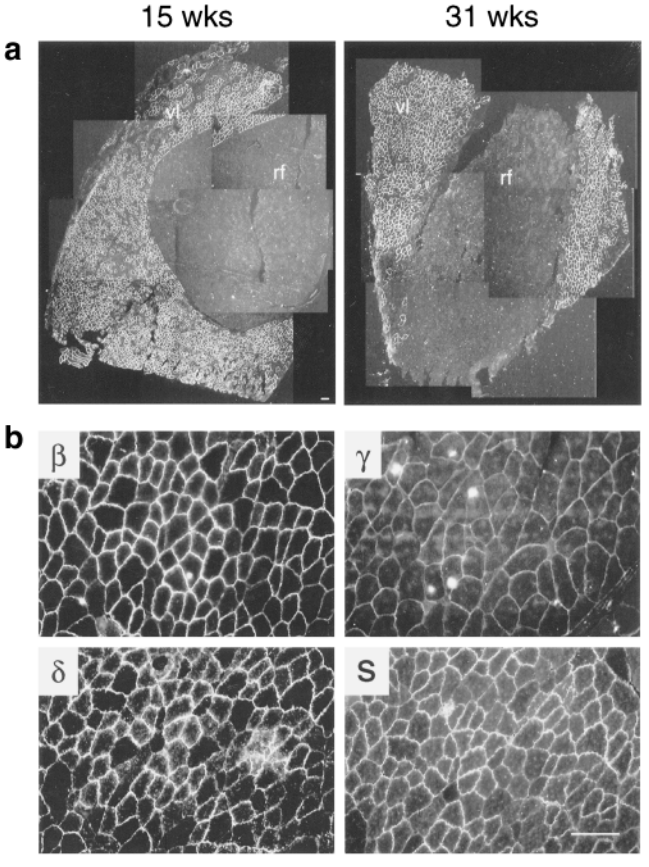


***Figure 1*** *Sustained expression of α-SG and restoration of the SG-SSPN complex. (a) Intramuscular injection of Ad5RSV-SGCA leads to high efficiency of transduction of myofibers and correct localization of α-SG. Mice were killed 15 and 31 weeks following injection and quadriceps femoris muscles were harvested and analyzed by immunofluorescence using a rabbit polyclonal antibody against α-SG. Composites represent images taken at a magnification of 5 ×. vl, vastus lateralis; rf, rectus femoris. (b) Detection of β-, γ- and δ-SG and sarcospan (S) at the sarcolemma of transduced muscle fibers. The bars represent 100 μm.*

### *Long-term expression of α-SG is mediated by the persistence of viral DNA in muscle*

To confirm that α-SG expression was indeed due to sustained translation of the human cDNA carried by the adenoviral vector, we investigated the persistence of viral DNA in injected muscles. We used oligonucleotides from the E3 region of the adenovirus serotype 5 genome (which constitutes the backbone of our vector) to perform PCR amplification on total DNA isolated from muscle cryosections. At all time-points investigated, PCR products from injected muscles yielded the expected 1.5 kb fragment (Figure 3, lanes 3, 5, 7 and 8) whereas DNA from contralateral noninjected muscles failed to produce a PCR fragment (Figure 3, lanes 2, 4 and 6). It is worth

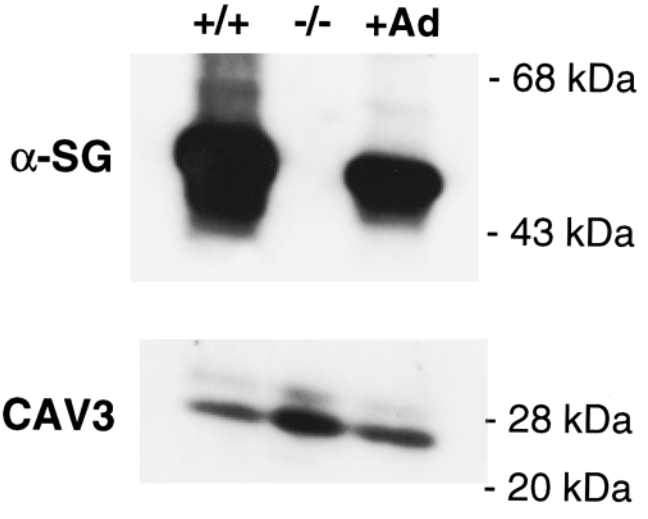


***Figure 2*** *Detection of the α-sarcoglycan in purified glycoprotein preparations from injected quadriceps femoris muscles from wild-type control mice (+/+); Sgca-null mice (–/–) and Ad5RSV-SGCA-injected mice (+Ad). Nitrocellulose membranes were first incubated with the monoclonal antibody Ad1/20A6 recognizing α-sarcoglycan (α-SG) and then with a monoclonal antibody against caveolin 3 (CAV3) to confirm equal loading. Molecular weights in kDa are indicated on the right.*

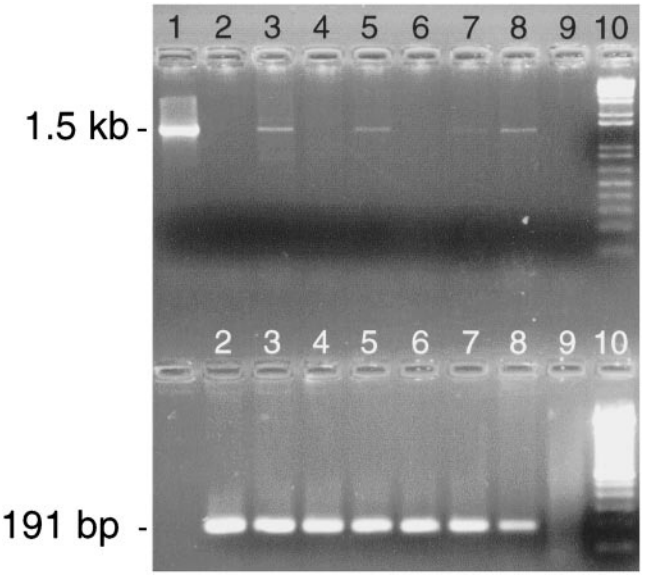


***Figure 3*** *Persistence of viral DNA in muscle mediates long-term expression of α-SG. The upper panel shows detection of viral DNA by PCR using primers specific to the E3 region on $10^9$ of UV-irradiated Ad5RSV-LacZ particles (lane 1); DNA from saline-injected quadriceps femoris muscles harvested 8 weeks (lane 2), 15 weeks (lane 4) and 31 weeks (lane 6) after injection; DNA from quadriceps femoris muscles injected with Ad5RSV-SGCA and harvested 8 weeks (lanes 3), 15 weeks (lane 5) and 31 weeks (lanes 7 and 8) after injection. Lane 9, negative control containing no DNA; lane 10, size markers. The lower panel shows the amplification of the murine adipsin gene as an internal control for DNA input. The samples are as described above.*

mentioning that some variability in the intensity of the PCR product was detected between different samples harvested at the same time-point (see Figure 3, lanes 7 and 8), likely because of injection variability.

### *Human α-SG gene transfer prevents dystrophic pathology*

In order to demonstrate functional benefit conferred by the virus-mediated expression of α-SG, we examined the morphology of injected muscles by hematoxylin and eosin (H&E) staining. Overall, within a quadriceps femoris, transduced myofibers from the vastus lateralis appeared greatly preserved when compared with the non-transduced rectus femoris muscle, with homogeneous fiber size, absence of calcifications and significantly less centrally located nuclei (Figure 4a). Moreover, transduced vastus lateralis muscles consistently displayed significantly fewer central nuclei (12% of the total number of fibers) as compared with noninjected contralateral muscles (90%, Figure 4b). To assess whether this finding truly reflected prevention of the muscle pathology, we harvested skeletal muscle from the hind-limb of 4-day-old *Sgca*-null mice. H&E staining of cryosections revealed that these muscles displayed a normal morphology as compared with age matched control muscles (Figure 5) thereby demonstrating that the Ad5RSV-SGCA injections indeed prevented the appearance of the pathology in these animals.

### *Ad5RSV-SGCA injection protects against muscle damage*

We used magnetic resonance imaging (MRI) combined with AngioMark (MS-325), a gadolinium-based blood

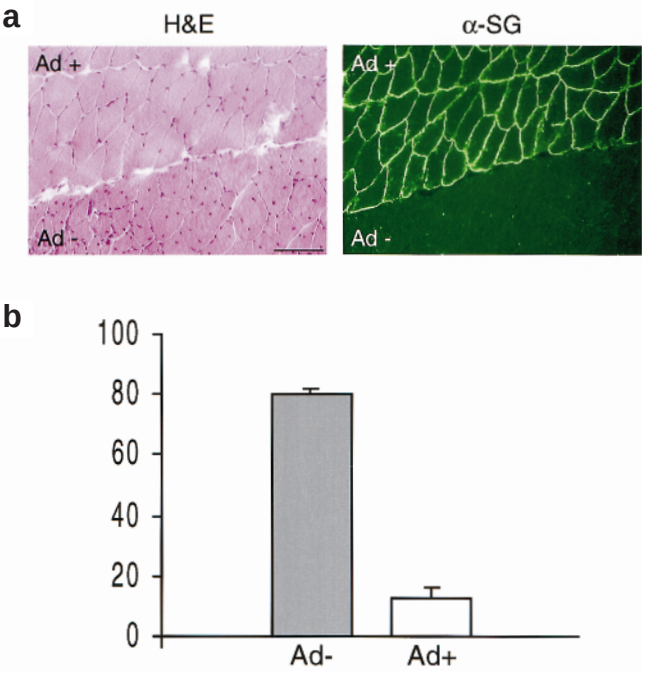


***Figure 4*** *Adenovirus-mediated expression of α-SG protects myofibers from central nucleation. (a) Left panel: Hematoxylin and eosin (H&E) staining of a 10-μm cryosection showing the adjacent vastus lateralis (Ad+) and rectus femoris (Ad–) 15 weeks after intramuscular injection of Ad5RSV-SGCA. The right panel presents a serial section stained with a polyclonal antibody against α-SG. The bar represents 100 μm. (b) Percentage of fibers containing centrally located nuclei in either non-injected (Ad–) or injected (Ad+) vastus lateralis muscles after intramuscular injection of Ad5RSV-SGCA. A total of 553 and 2112 fibers were counted from the noninjected and injected muscles, respectively.*

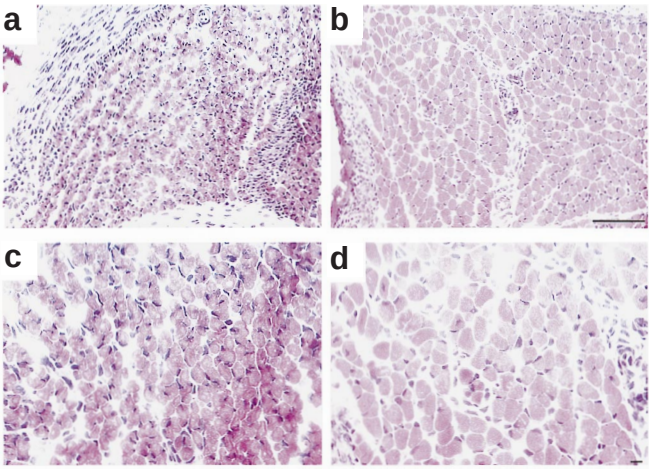


***Figure 5*** *Histological analysis of neonatal hind-limb muscles by H&E staining of 7 μm cryosections from a 3-day-old C57BL/10 control (a, c) and a 4-day-old Sgca-null pup (b, d). The bars represent 100 μm (a, b) and 10 μm (c, d).*

pool contrast agent that binds reversibly to serum albumin, to assess muscle damage in two groups of *Sgca*-null mice: injected ($n = 7$) or not ($n = 5$) with Ad5RSV-SGCA in their right quadriceps femoris and hamstring muscles 3 to 4 days after birth. The animals were imaged 8 to 10 weeks following adenoviral injection and enhancement percentage resulting from MS-325 uptake in skeletal

muscle was calculated. T1-weighted spin-echo images obtained from the proximal hind-limb muscles of noninjected *Sgca*-null mice showed that extensive areas of muscle damage could be detected by visualizing the uptake of MS-325 in skeletal muscle (Figure 6a). Interestingly, in all injected animals, there was a significant decrease in enhancement levels in the right hind-limb muscles (52.902 ± 8.192, $n = 21$ images analyzed) as compared with the left hind-limb muscles (106.818 ± 13.569, $n = 21$ images analyzed; $P = 0.0003$; Figure 6b). A few days following the MRI procedure, animals were systemically injected with Evans Blue Dye (EBD) according to Straub and colleagues[25] and killed 5 h later. Indeed, injected muscles were impermeable to EBD, whereas uninjected contralateral muscles displayed a significant uptake of the vital dye tracer (data not shown) confirming that Ad5RSV-SGCA gene transfer maintained sarcolemmal integrity in these mice.

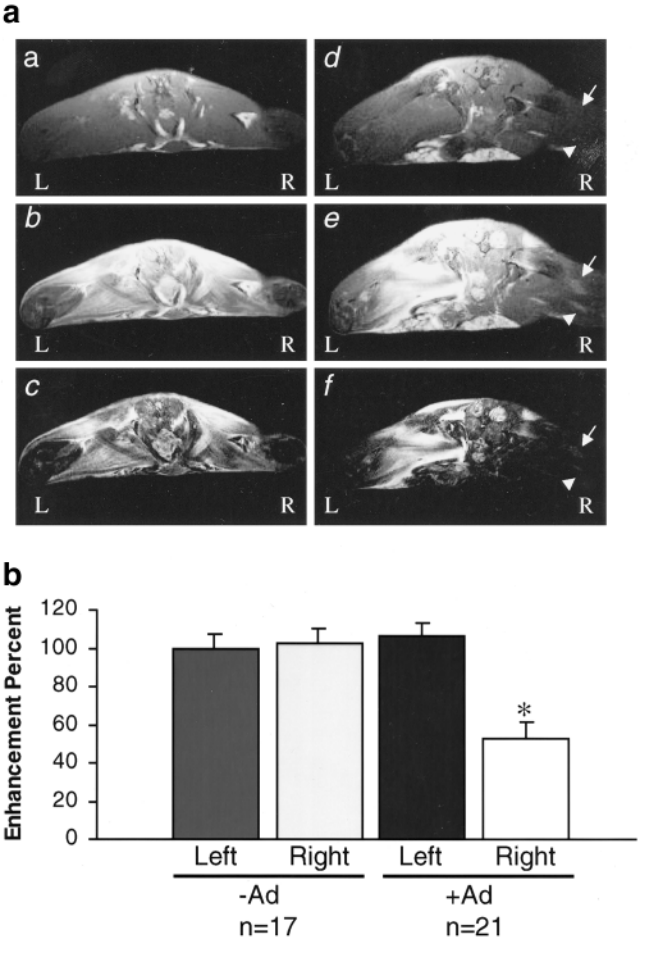


***Figure 6*** *Adenovirus-mediated gene transfer of α-SG prevents uptake of AngioMark (MS-325) in muscle groups. (a) Contrast agent-enhanced magnetic resonance imaging (MRI) of muscle groups from the pelvic girdle and proximal hind-limbs muscles of non-injected (a–c) and injected (d–f) Sgca-null mice. Neonate mice received intramuscular injections of Ad5RSV-SGCA in their right quadriceps femoris (arrows) and hamstring (arrowheads) muscles. Images were taken before (a, d) and 15 min (b, e) following systemic injection of the contrast agent. Panels c and f show the difference between post- and pre-contrast images (see Materials and methods for details). L, R: left and right hind-limb, respectively. (b) Mean enhancement percent detected in the left and right sides of noninjected Sgca-null mice (–Ad; n = 17 images taken) and Ad5RSV-SGCA injected Sgca-null mice (+Ad; n = 21 images taken). Statistical significance was examined using a paired, two-tailed t test (*P = 0.0003).*

## *Discussion*

In immunocompetent mice, adenovirus-mediated gene transfer to skeletal muscle is hampered by at least two major hurdles that result in transient expression of the therapeutic protein. First, expression of the Coxsackie adenovirus receptor (CAR) is down-regulated during muscle maturation, thus diminishing the efficiency of transduction in mature skeletal muscle.[26] Second, the strong immune response elicited both by low-level expression of adenoviral proteins[27–29] and the antigenicity of the transgene[30–33] constitutes an important obstacle, especially for the potential use of adenoviral vectors in human patients. In the course of this study, we established that adenovirus-mediated gene transfer of α-sarcoglycan elicits an immune response against the transgene in immunocompetent *Sgca*-null mice (Allamand and Campbell, personal communication).

In order to investigate long-term consequences of adenovirus-mediated gene transfer of α-SG, we therefore performed injections of a first generation adenovirus containing the human α-SG cDNA in newborn *Sgca*-null mice, an animal model for LGMD 2D.[24] It is worth noting that all the animals survived the procedure and did not show signs of inflammation or immunologic response. In particular, mice injected before maturation of their immune system did not seem to produce antibodies against α-sarcoglycan, as assessed by using the sera to stain immunoblots of DGC proteins (data not shown). Moreover, there did not appear to be a significant amount of humoral response against adenoviral proteins either, as preliminarily assessed by ELISA (data not shown). We thereby achieved high efficiency of muscle transduction (at least 90% of muscle fibers transduced) and sustained expression of α-SG (up to at least 31 weeks) following a single intramuscular injection of a first-generation adenovirus in neonates. As expected, expression of all the other sarcoglycan proteins as well as sarcospan was restored and persisted at the sarcolemma of transduced muscle fibers, reinforcing the hypothesis that each of the sarcoglycan proteins needs to be present in order for the sarcoglycan–sarcospan complex to be correctly localized at the sarcolemma.[8] Adenovirus-mediated expression of α-SG also proved fundamental for the prevention of muscle pathology since transduced myofibers were protected from the muscle phenotype whereas untransduced fibers displayed histological hallmarks of muscular dystrophy, including central nucleation, variation in fiber size, necrosis and calcification. This finding indicated that transduced myofibers had been protected from the rounds of degeneration–regeneration associated with the muscle pathology. To confirm this result, we investigated the expression of a myogenic marker, the developmental isoform of the myosin heavy chain[34] (MHCd), in injected muscles. Transduced fibers never expressed MHCd whereas groups of nontransduced fibers often displayed staining for MHCd (data not shown) again indicating that only this population of fibers was undergoing regeneration. Examination of 4-day-old *Sgca*-null muscles (the age at which adenoviral injections were performed) indicated that Ad5RSV-SGCA gene transfer indeed prevented the

development of the muscle pathology. Therefore, in LGMDs and other forms of progressive muscular dystrophies, it might be critical to intervene before the extent of muscle damage has become too great. This bears critical consequences for LGMD patients in whom *in utero* gene transfer may ultimately be necessary for treatment of these progressive diseases.

Last, we recently used magnetic resonance imaging (MRI) combined with a gadolinium-based contrast agent to assess membrane integrity in animal models for muscular dystrophy.[35] In the present study, this procedure effectively demonstrated maintenance of sarcolemmal function following α-SG gene transfer in proximal hindlimb muscles of *Sgca*-null mice.

In conclusion, we herein report for the first time effective prevention of muscular dystrophy by adenovirus-mediated gene transfer of one component of the sarcoglycan complex in a murine model for LGMD. Early gene transfer protected these animals from the onset of the disease for extended periods of time (at least one-third of the life span of a mouse). However, because of the inability of adenoviral vectors to transduce multiple muscle groups, targeting each muscle would be necessary in order to achieve global prevention of the muscle pathology, a cumbersome task, even in small animal models such as mice. Unfortunately, systemic delivery of adenoviral vectors has not been achievable so far, because the endothelium constitutes an anatomical barrier that prevents the adenovirus from being delivered to muscle or any other non-liver target organs.[36] Other vectors may thus need to be used, such as adeno-associated vectors (AAV), although the transduction efficiency achieved in this study is significantly higher than that reported by using AAV.[22,23] Local gene transfer to skeletal muscle following systemic delivery of an AAV has been reported by permeabilizing the endothelium with histamine,[22] an avenue of research which may hold promise for the future but is still at a very early stage of development. The development of improved less imunogenic adenoviral vectors[37] may nevertheless provide useful tools for targeted gene transfer to specific muscle groups in animal models, as well as LGMD patients.

In addition the noninvasive MRI procedure can easily and repetitively allow monitoring of the consequences of gene transfer approaches. We believe that MRI will eventually be routinely used to follow the progression of the disease as well as to observe the outcome of various therapeutic approaches. The availability of this procedure is also expected to lessen the need for repeated muscle biopsies, a clear benefit for the patients.

## Materials and methods

### Animals

Alpha-sarcoglycan deficient[24] (*Sgca*-null) and control C57BL/10J mice were obtained from our colonies maintained in the Animal Care Unit of the University of Iowa College of Medicine, according to animal care guidelines. Alpha-sarcoglycan genotypes were confirmed as described previously.[24] The Animal Care and Use Review Committees of the University of Iowa and the Medical College of Wisconsin approved all animal procedures.

### Recombinant adenoviral vector

Adenovirus containing the human α-sarcoglycan cDNA under the control of the Rous sarcoma virus long terminal repeat (Ad5RSV-SGCA) was generated by the University of Iowa Gene Transfer Vector Core as previously described.[24]

### Adenoviral vector administration

Three- to 4-day-old *Sgca*-null pups were injected percutaneously with 1–2 × $10^9$ particles (corresponding to approximately $10^7$ p.f.u) diluted in a final volume of 10 μl of normal saline solution into the quadriceps femoris or the hamstring. Animals were housed in the Animal Care Unit of the University of Iowa College of Medicine and muscles were harvested 8 ($n$ = 4), 15 ($n$ = 6) or 31 ($n$ = 3) weeks after injection.

### Immunohistochemical analysis

At various time-points following vector injection, animals were killed by cervical dislocation. Muscle samples were harvested and snap-frozen into liquid nitrogen-cooled isopentane. Immunofluorescence analysis was performed as described previously.[24] Briefly, 7 to 10 μm-thick cryosections were obtained and blocked for 30 min in 5% BSA, 1 × PBS. Sections were incubated overnight in affinity-purified rabbit polyclonal antibodies against α-SG (rabbit 98),[9] dystrophin (rabbit 31),[38] γ-SG (rabbit 245).[39] Antibodies against β-SG (goat 026), δ-SG (rabbit 215) and sarcospan (rabbit 235) were used as previously described.[24] Excess primary antibody was removed by 3 × 5 min washes in 1% BSA, 1 × PBS, and sections were subsequently incubated for about 90 min in appropriate FITC-conjugated secondary antibodies (1:250; Jackson ImmunoResearch Laboratories, West Grove, PA, USA). Following three additional washes, sections were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) and observed under a Zeiss Axioplan microscope (Carl Zeiss, Oberkochen, Germany). Images were captured under identical conditions using the PAX-It software (Midwest Information Systems, Franklin Park, IL, USA).

### Biochemical analysis

Glycoprotein preparations were prepared from control, injected and noninjected quadriceps femoris muscles harvested 21 days after injections as previously described.[21] Twelve micrograms of the proteins were resolved under reducing conditions by 3–15% SDS-PAGE and transferred to nitrocellulose membranes (Immobilon-NC, Millipore Corporation, Bedford, MA, USA).

Detection of α-sarcoglycan was performed by using the monoclonal Ad1/20A6 antibody produced in collaboration with Dr Louise Anderson (Newcastle General Hospital, Newcastle upon Tyne, UK). HRP-conjugated secondary antibody anti mouse $IgG_1$ was used at a dilution of 1:5000 (Chemicon, Temecula, CA, USA). A monoclonal antibody against caveolin 3 (Transduction Laboratories, Lexington, KY, USA) was used as a loading control. Immunoblots were developed using enhanced chemiluminescence (SuperSignal; Pierce Chemical, Rockford, IL, USA).

### Detection of viral DNA

Muscle DNA was isolated from 50 cryosections of muscle tissue (10 μm per section) as previously described.[40]

Briefly, samples were digested overnight with 200 μg of proteinase K per milliliter in 50 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 8.0), 0.5% SDS. DNA was then purified by phenol-chloroform extraction and precipitation. Three hundred nanograms of DNA was subjected to PCR amplification using primers from the E3 region of the adenovirus genome: 5′-TGCAGCCAGGAGGAAGCT GCAATACCAGAG-3′ and 5′-GTCCAACTACAGCGA CCCACCCTAACAGAG-3′. Reactions were carried out in a final volume of 50 μl containing 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 50 mM KCl pH 8.3 and 100 ng of each primer. After 5 min at 96°C, 200 mM of each dNTP and 2.5 U Taq polymerase (Roche Molecular Biochemicals, Indianapolis, IN, USA) were added, and 35 cycles were carried out as follows: 1 min denaturation at 94°C, 1 min annealing at 57°C and 1.5 min at 72°C. PCR products were analyzed by electrophoresis on 2% Seakem agarose gel stained with ethidium bromide. As a control to the reaction, $10^9$ particles of UV-treated Ad5RSV-SGCA were subjected to the same amplification reaction. As an internal control for genome equivalent, we used primers from the mouse adipsin gene[40] to amplify a 191 bp PCR product from 300 ng of DNA in the same conditions as above.

### *Magnetic resonance imaging*

Two days before imaging, 8- to 10-week-old animals were anesthetized with ketamine (90 mg/kg, i.p.) with ace promazine (1.8 mg/kg, i.p.) and surgically instrumented with jugular vein catheters for administration of MS-325. All experiments were performed at the Medical College of Wisconsin on a 3 Tesla Bruker Medspec MR imaging system (Billerica, MA, USA). Following anesthesia, mice were placed in a 10 cm diameter, three-axis local gradient coil, together with a 2 × 4 cm quadrature transmit/receive radiofrequency surface coil. After obtaining sagittal scout images, five to seven, 2 mm axial slices were chosen to image the proximal limb muscles (eg gluteal and femoral quadriceps muscles). T1-weighted spin-echo images (TR/TE = 400 ms/19 ms, FOV = 3.2 cm, matrix = 256 × 256 giving an in-plane image resolution of 125 microns) of the proximal hind-limb muscles of anesthetized mice were acquired before and after administration of 0.1 mmole Gd/kg MS-325. A mean signal intensity was determined from all voxels in a region of interest (ROI) within the right and left leg muscles. The ROI was typically about 12 × 12 voxels, or 1.5 × 1.5 mm. Both the pre- and post-contrast mean signals were extracted from the same region of interest. Finally, the percent signal enhancement was calculated as follows: ((post-contrast signal mean – pre-contrast mean signal)/pre-contrast mean signal) × 100. A paired, two-tailed *t* test was used to determine whether the mean signal enhancement was significantly different between the right and left legs.

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## References

1 Ervasti JM, Campbell KP. Membrane organization of the dystrophin–glycoprotein complex. *Cell* 1991; **66**: 1121–1131.
2 Yoshida M *et al*. Dissociation of the complex of dystrophin and its associated proteins into several unique groups by n-octyl, β-D-glucoside. *Eur J Biochem* 1994; **222**: 1055–1061.
3 Crosbie RH *et al*. Sarcospan: the 25 kDa transmembrane component of the dystrophin–glycoprotein complex. *J Biol Chem* 1997; **272**: 31221–31224.
4 Crosbie RH *et al*. Membrane targeting and stabilization of sarcospan is mediated by the sarcoglycan complex. *J Cell Biol* 1999; **145**: 153–165.
5 Campbell KP, Kahl SD. Association of dystrophin and an integral membrane glycoprotein. *Nature* 1989; **338**: 259–262.
6 Ervasti JM *et al*. Deficiency of a glycoprotein component of the dystrophin complex in dystrophic muscle. *Nature* 1990; **345**: 315–319.
7 Yoshida M, Ozawa E Glycoprotein complex anchoring dystrophin to the sarcolemma. *J Biochem (Tokyo)* 1990; **108**: 748–752.
8 Holt KH, Campbell KP. Assembly of the sarcoglycan complex. *J Biol Chem* 1998; **273:** 34667–34670.
9 Roberds SL *et al*. Missense mutations in the Adhalin gene linked to autosomal recessive muscular dystrophy. *Cell* 1994; **78**: 625–633.
10 Bönneman CG *et al*. β-sarcoglycan mutations cause autosomal recessive muscular dystrophy with loss of the sarcolgycan complex. *Nat Genet* 1995; **11**: 266–272.
11 Lim LE *et al*. β-Sarcoglycan: characterization and role in limb-girdle muscular dystrophy linked to 4q12. *Nat Genet* 1995; **11**: 257–265.
12 Noguchi S *et al*. Mutations in the dystrophin-associated protein γ-sarcoglycan in chromosome 13 muscular dystrophy. *Science* 1995; **270**: 819–822.
13 McNally EM *et al*. Mutations that disrupt the carboxyl-terminus of γ-sarcoglycan cause muscular dystrophy. *Hum Mol Genet* 1996; **5**: 1841–1847.
14 Nigro V *et al*. Identification of a novel sarcoglycan gene at 5q33 encoding a sarcolemmal 35 kDa glycoprotein. *Hum Mol Genet* 1996; **5**: 1179–1186.
15 Nigro V *et al*. Autosomal recessive limb-girdle muscular dystrophy, LGMD2F, is caused by a mutation in the δ-sarcoglycan gene. *Nat Genet* 1996; **14**: 195–198.
16 Bushby KMD. The limb-girdle muscular dystrophies-multiple genes, multiple mechanisms. *Hum Mol Genet* 1999; **8:** 1875–1882.
17 Lim LE, Campbell KP. The sarcolgycan complex in limb-girdle muscular dystrophy. *Curr Opin Neurol* 1998; **11**: 443–452.
18 Piccolo F *et al*. Primary adhalinopathy: a common cause of autosomal recessive muscular dystrophy of variable severity. *Nat Genet* 1995; **10**: 243–245.
19 Carrié A *et al*. Mutational diversity and hot spots in the alpha-sarcoglycan gene in autosomal recessive muscular dystrophy (LGMD2D). *J Med Genet* 1997; **34**: 470–475.
20 Passos-Bueno MR, Vainzof M, Moreira ES, Zatz M. Seven autosomal recessive limb-girdle muscular dystrophies in the Brazilian population: from LGMD2A to LGMD2G. *Am J Med Genet* 1999; **82**: 392–398.
21 Holt KH *et al*. Functional rescue of the sarcoglycan complex in the BIO 14.6 hamster using δ-sarcoglycan gene transfer. *Mol Cell* 1998; **1**: 841–848.

22 Greelish JP *et al*. Stable restoration of the sarcoglycan complex in dystrophic muscle perfused with histamine and a recombinant adeno-associated viral vector. *Nature Med* 1999; **5**: 439–443.
23 Li J *et al*. rAAV vector-mediated sarcoglycan gene transfer in a hamster model for limb girdle muscular dystrophy. *Gene Therapy* 1999; **6**: 74–82.
24 Duclos F *et al*. Progressive muscular dystrophy in alpha-sarcoglycan-deficient mice. *J Cell Biol* 1998; **142**: 1461–1471.
25 Straub V, Rafael JA, Chamberlain JS, Campbell KP. Animal models for muscular dystrophy show different patterns of sarcolemmal disruption. *J Cell Biol* 1997; **139**: 375–385.
26 Nalbantoglu J, Pari G, Karpati G, Holland PC. Expression of the primary coxsackie and adenovirus receptor is downregulated during skeletal muscle maturation and limits the efficacy of adenovirus-mediated gene delivery to muscle cells. *Hum Gene Ther* 1999; **10**: 1009–1019.
27 Yang Y *et al*. Cellular immunity to viral antigens limits E1-deleted adenoviruses for gene therapy. *Proc Natl Acad Sci USA* 1994; **91**: 4407–4411.
28 Dai Y *et al*. Cellular and humoral immune responses to adenoviral vectors containing factor IX gene: tolerization of factor IX and vector antigens allows for long-term expression. *Proc Natl Acad Sci USA* 1995; **92**: 1401–1405.
29 Joos K, Ertl HCJ, Wilson JM. Cytotoxic T-lymphocyte target proteins and their major histocompatibility complex class I restriction in response to adenovirus vectors delivered to mouse liver. *J Virol* 1998; **7**: 2945–2954.
30 Tripathy SK, Black HB, Goldwasser E, Leiden MJ. Immune responses to transgene-encoded proteins limit the stability of gene expression after injection of replication-defective adenovirus vectors. *Nature Med* 1996; **2**: 545–550.
31 Yang Y, Haecker SE, Su Q, Wilson JM. Immunology of gene therapy with adenoviral vectors in mouse skeletal muscle. *Hum Mol Genet* 1996; **5:** 1703–1712.
32 Michou A *et al*. Adenovirus-mediated gene transfer: influence of transgene, mouse strain and type of immune response on persistence of transgene expression. *Gene Therapy* 1997; **4**: 473–482.
33 Joos K, Yang Y, Fisher KJ, Wilson JM. Transduction of dendritic cells by DNA viral vectors directs the immunre response to transgene products in muscle fibers. *J Virol* 1998; **72**: 4212–4223.
34 Davis CE, Harris JB, Nicholson LVB. Myosin isoform transitions and physiological properties of regenerated and re-innervated sole muscles of the rat. *Neuromusc Disord* 1991; **1**: 411–421.
35 Straub V *et al*. Contrast agent-enhanced magnetic resonance imaging of skeletal muscle damage in animal models of muscular dystrophy. *Magn Reson Med* (in press).
36 Fechner H *et al*. Expression of Coxsackie adenovirus receptor and alpha$_v$-integrin does not correlate with adenovector targeting *in vivo* indicating anatomical vector barriers. *Gene Therapy* 1999; **6**: 1520–1535.
37 Amalfitano A. Next-generation adenoviral vectors: new and improved. *Gene Therapy* 1999; **6**: 1643–1645.
38 Ohlendieck K, Campbell KP. Dystrophin-associated proteins are greatly reduced in skeletal muscle from *mdx* mice. *J Cell Biol* 1991; **115**: 1685–1694.
39 Durbeej M *et al*. Disruption of the β-sarcoglycan gene reveals pathogenetic complexity of limb-girdle muscular dystrophy type 2E. *Mol Cell* 2000; **5**: 141–151.
40 Chen HH *et al*. Persistence in muscle of an adenoviral vector that lacks all viral genes. *Proc Natl Acad Sci USA* 1997; **94**: 1645–1650.