Dose-Escalation of Systemically Delivered Adeno-Associated Virus–Mediated α-Sarcoglycan in a Mouse Model With Limb-Girdle Muscular Dystrophy Type 2D

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BACKGROUND

- Limb-girdle muscular dystrophy type 2D (LGMD2D) is an autosomal recessive disorder caused by loss-of-function mutations in the α-sarcoglycan (α-SG) gene, leading to deterioration of skeletal and respiratory muscles and loss of ambulatory and respiratory function[1,2]
- There are no disease-modifying therapies for LGMD2D
- LGMD2D is an ideal candidate for gene transfer therapy since its pathology is of monogenic origin

OBJECTIVE

- To test the efficacy and safety of systemically delivered scAAVrh74.tMCK.hSGCA (MYO-102) for skeletal muscle deficits in *sgca-/-* mice

METHODS

Animals

- All procedures were conducted in accordance with approval by The Research Institute at the Nationwide Children's Hospital Institutional Animal Care and Use Committee
- C57BL/6-TgN (*sgca-/-*) homozygous knockout mice were gifted by Kevin Campbell. C57/BL6 were obtained from Jackson Labs. All animals were maintained under standardized conditions on a 12:12 hour light:dark cycle with food and water provided ad libitum
- Non-human primates (NHP): Rhesus macaques were socially housed under standardized conditions. NHPs were fed a Harlan Teklad diet supplemented with fresh vegetables, fruits and grains

scAAVrh74.tMCK.hSGCA (MYO-102) Construct

- Self-complementary adeno-associated virus vector, AAVrh74, containing a codon-optimized human α-SG (hSGCA) transgene driven by a muscle-specific promoter, shortened muscle creatine kinase (tMCK) was designed as previously described and utilized in these studies[3]
- FLAG-tagged modified construct, scAAVrh74.tMCK.hSGCA.FLAG, was developed to assess expression and localization of delivery of an exogenous transgene and protein product in NHP
- Constructs were diluted in lactated Ringer's solution and were delivered intravenously (IV) or using an isolated limb procedure (ILP), as previously described at doses noted in figure legends

Biological endpoints

- hSGCA expression was evaluated by immunofluorescence and western blot, as previously described[4]
- For histological evaluation, 12-µm thick cryosections of muscle were processed using 2 staining methods:
  - Hematoxylin & eosin staining for morphometric analysis, as previously described[5]
  - Picrosirius red staining (Polysciences Inc., Mount Arlington, NJ, Catalog #24901) was used to assess collagen content, as a marker of fibrosis
  - Images were captured using a Zeiss (Germany) AxioCam MRC5 camera and processed using National Institutes of Health's ImageJ software or Zeiss Axiovision LE4 software
- Serum analysis
  - Liver enzymes and blood glucose were assessed to evaluate toxicity
  - Creatine kinase was measured using the Creatine Kinase SL Assay (Sekisui Diagnostics; Charlottetown, PE, Canada; Catalog #326-10)
- Taqman quantitative PCR on various tissues was performed to quantify the number of vector genome copies to assess biodistribution, as previously described[4,6,7]

Functional endpoints

- Locomotor activity (ambulation and rearing) was assessed using laser monitoring of an open-field activity chamber using a previously described protocol[8,9]
- Tetanic contraction was assessed in intact mice in the tibialis anterior and *ex vivo* in the diaphragm, as previously described[8,10-12]

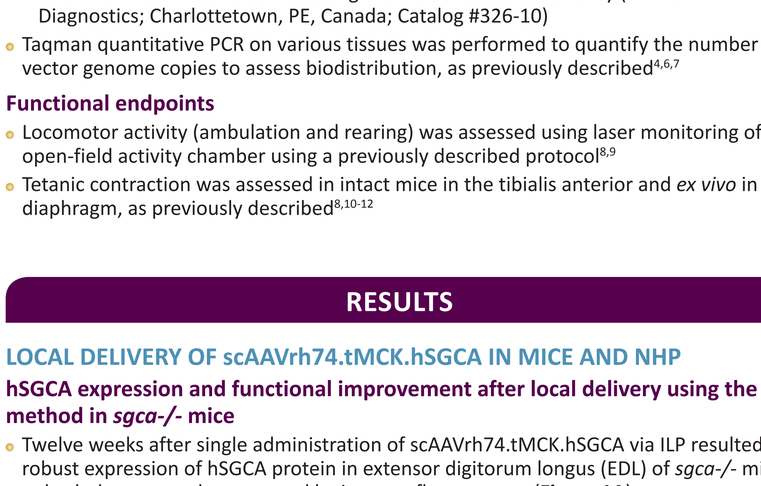
RESULTS

LOCAL DELIVERY OF scAAVrh74.tMCK.hSGCA IN MICE AND NHP

hSGCA expression and functional improvement after local delivery using the ILP method in *sgca-/-* mice

- Twelve weeks after single administration of scAAVrh74.tMCK.hSGCA via ILP resulted in robust expression of hSGCA protein in extensor digitorum longus (EDL) of *sgca-/-* mice at both doses tested as assessed by immunofluorescence (**Figure 1A**)
- scAAVrh74.tMCK.hSGCA delivered by ILP dose-dependently increased maximum specific force generation in *sgca-/-* mice and protected against contraction-induced injury (**Figure 1B-C**)

Figure 1. Local delivery of scAAVrh74.tMCK.hSGCA using ILP promotes hSGCA protein expression and improves muscle function



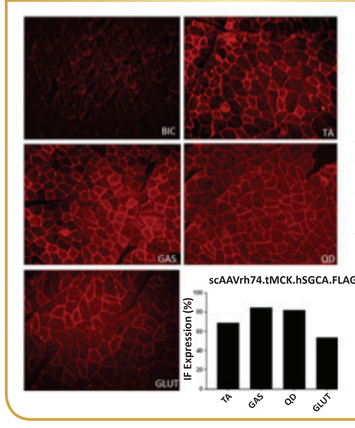
Sgca-/- mice received scAAVrh74.tMCK.hSGCA either at $2x10^{12}$ vg/kg or $6x10^{11}$ vg/kg via the isolated limb perfusion method. Representative images of hSGCA protein expression in the extensor digitorum longus (EDL) muscles 12 weeks post-treatment, as assessed by immunofluorescence (**A**). Maximum specific force generation (**B**) and contraction-induced injury as measured as the specific force generated relative to initial force over eccentric contraction cycles (**C**) in the EDL of *sgca-/-* mice. Data represents n=12 per treatment group. *p<0.05 and **p<0.01 vs. untreated *sgca-/-* mice as determined by One-way ANOVA followed by Tukey's post hoc analysis.

RESULTS continued

Treatment with scAAVrh74.tMCK.hSGCA.FLAG by ILP in NHP confirms transgene delivery and expression

- Immunofluorescent imaging of hSGCA protein expression using anti-FLAG antibody in NHP dosed with $6x10^{12}$ vg/kg a FLAG-tagged construct (scAAVrh74.tMCK.hSGCA.FLAG) demonstrates robust expression at the sarcolemma membrane
  - On average, 70% of muscle fibers from lower limb positively expressed hSGCA.FLAG compared to the control (**Figure 2**)

Figure 2. hSGCA protein expression following delivery of scAAVrh74.tMCK.hSGCA.FLAG using ILP in NHP



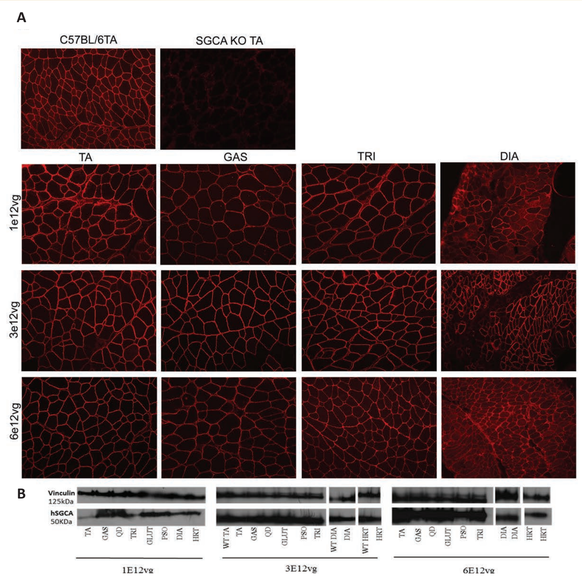
Immunofluorescence imaging of anti-FLAG expression in non-human primates, as a marker of expression of hSCGA protein delivered by scAAVrh74.tMCK.hSGCA.FLAG. Abbreviations: BIC, bicep; GAS, gastrocnemius; GLUT, gluteal; QD, quadriceps; TA, tibialis anterior.

SYSTEMIC DELIVERY OF scAAVrh74.tMCK.hSGCA IN MICE

hSGCA expression after systemic delivery by IV administration in *sgca-/-* mice

- Twelve weeks after hSGCA transgene delivery, robust hSGCA protein expression at the sarcolemma membrane was demonstrated in the tibialis anterior (TA), gastrocnemius (GAS), quadriceps (QUAD), gluteal (GLUT), psoas major (PSOAS), triceps (TRI), diaphragm (DIA), and cardiac (HRT) muscles after treatment with all 3 doses tested, as assessed by immunofluorescence (**Figure 3A**)
  - Muscle fibers expressing hSGCA protein ranged from 70%-93% compared to untreated *sgca-/-* control mice
  - hSGCA protein expression in the HRT muscle remained at 75%, independent of dose
- Western blots confirm protein expression in all muscle groups examined from scAAVrh74.tMCK.hSGCA treated *sgca-/-* mice (**Figure 3B**)

Figure 3. hSGCA protein expression after systemic treatment with scAAVrh74.tMCK.hSGCA

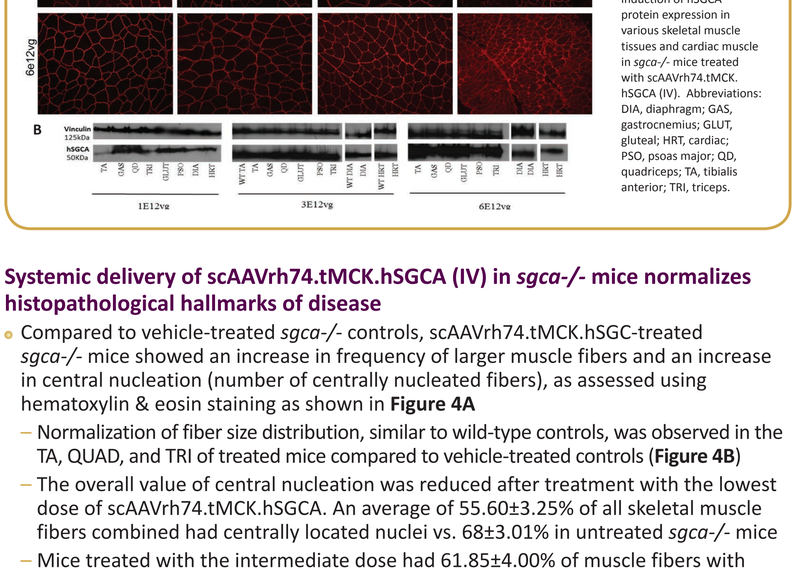


(A) Representative images of hSGCA protein expression by immunofluorescence shows membrane expression in various skeletal muscle groups and cardiac muscle from *sgca-/-* mice systemically (IV) treated with $5x10^{13}$ vg/kg, $1x10^{14}$ vg/kg, and $2x10^{14}$ vg/kg (corresponding to a total dose of $1x10^{12}$ vg, $3x10^{12}$ vg, $6x10^{12}$ vg, respectively) (n=6 per group). (B) Western blots show a dose-dependent induction of hSGCA protein expression in various skeletal muscle tissues and cardiac muscle in *sgca-/-* mice treated with scAAVrh74.tMCK.hSGCA (IV). Abbreviations: DIA, diaphragm; GAS, gastrocnemius; GLUT, gluteal; HRT, cardiac; PSO, psoas major; QD, quadriceps; TA, tibialis anterior; TRI, triceps.

Systemic delivery of scAAVrh74.tMCK.hSGCA (IV) in *sgca-/-* mice normalizes histopathological hallmarks of disease

- Compared to vehicle-treated *sgca-/-* controls, scAAVrh74.tMCK.hSGC-treated *sgca-/-* mice showed an increase in frequency of larger muscle fibers and an increase in central nucleation (number of centrally nucleated fibers), as assessed using hematoxylin & eosin staining as shown in **Figure 4A**
  - Normalization of fiber size distribution, similar to wild-type controls, was observed in the TA, QUAD, and TRI of treated mice compared to vehicle-treated controls (**Figure 4B**)
  - The overall value of central nucleation was reduced after treatment with the lowest dose of scAAVrh74.tMCK.hSGCA. An average of 55.60±3.25% of all skeletal muscle fibers combined had centrally located nuclei vs. 68±3.01% in untreated *sgca-/-* mice
  - Mice treated with the intermediate dose had 61.85±4.00% of muscle fibers with centralized nuclei, while the nucleation of muscle fibers treated with the highest dose was reduced to 37.93±12.46% (**Figure 4C**)

Figure 4. Improvement in muscle morphology by scAAVrh74.tMCK.hSGCA in *sgca-/-* mice



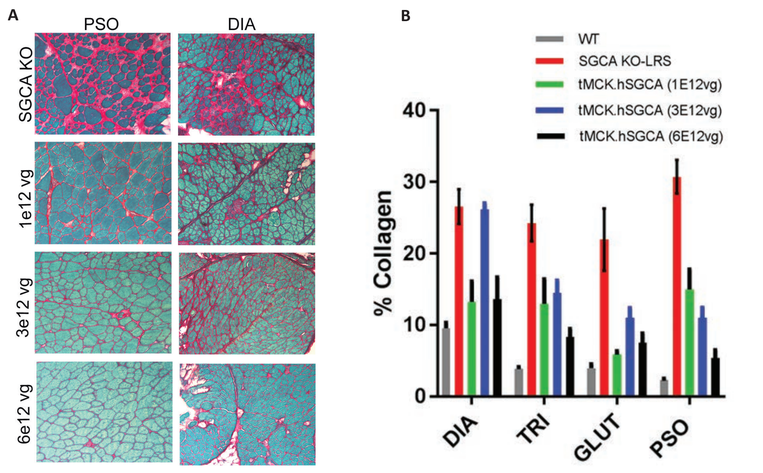
(A) Hematoxylin & eosin staining of muscles treated with $1x10^{12}$ vg, $3x10^{12}$ vg, and $6x10^{12}$ vg of scAAVrh74.tMCK.hSGCA. (B) Fiber size quantification and distribution (n=6 per group). (C) Quantification of centrally located nuclei in muscles of treated mice compared to untreated mice and wild-type controls (n=6 per group). Abbreviations: DIA, diaphragm; GAS, gastrocnemius; GLUT, gluteal; PSO, psoas major; QD, quadriceps; TA, tibialis anterior; TRI, triceps.

CONCLUSIONS

- This study showed preclinical efficacy and safety of scAAVrh74.tMCK.hSGCA (MYO-102) as well as functional and efficacy improvements relevant to disease progression
- This approach holds promise for the treatment of LGMD2D, and forms the basis for future studies

- A significant reduction in fibrosis was observed in the muscles of treated mice compared to untreated *sgca-/-* mice, as indicated by a decrease in picrosirius red staining, a marker of collagen content (**Figure 5**)

Figure 5. Reduction in fibrosis in muscle of scAAVrh74.tMCK.hSGCA-treated *sgca-/-* mice

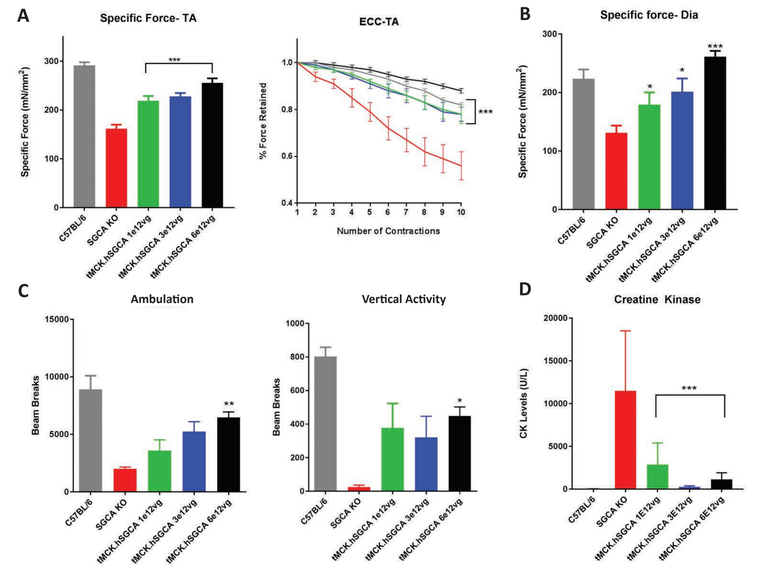


(A) Picrosirius red staining shows reduced fibrosis in treated mice indicated by a decrease in collagen deposition compared to untreated *sgca-/-* mice in various muscles. Representative 20x images shown. (B) Quantification of collagen levels in various muscles confirms reduction in collagen levels in all 3 treated groups compared to untreated *sgca-/-* mice and wild-type controls (n=6 per group). Abbreviations: DIA, diaphragm; GLUT, gluteal; PSO, psoas major; TRI, triceps.

Systemic delivery of scAAVrh74.tMCK.hSGCA (IV) in *sgca-/-* mice restores the physical activity and protects against the breakdown of muscles

- Twelve weeks after treatment, maximum specific force generation and resistance to eccentric contraction-induced damage was increased in TA muscles of all treated groups (with minimal difference between doses) compared to untreated controls (n=6 per group) (**Figure 6A**). Maximum specific force generation of the DIA muscle was increased in scAAVrh74.tMCK.hSGCA-treated *sgca-/-* mice in a dose-dependent manner compared to untreated *sgca-/-* mice (**Figure 6B**)
- Ambulation and vertical rearing activities of treated mice increased 12 weeks post-delivery of scAAVrh74.tMCK.hSGCA (**Figure 6C**)
  - Improvement ranged from 44%-69% in ambulation and 92%-94% in vertical rearing compared to untreated *sgca-/-* mice
- Serum creatine kinase levels were significantly reduced in all treated groups compared to untreated mice (**Figure 6D**)

Figure 6. Functional benefits to skeletal muscle after treatment with scAAVrh74.tMCK.hSGCA



(A) Specific force and resistance to contraction-induced damage in the tibialis anterior. (B) Specific force in the diaphragm. (C) Ambulation and rearing activity. (D) Creatine kinase levels in serum decreased in all treatment groups. Data represents n=6 per treatment group. Data were analyzed by One-way ANOVA followed by Tukey's post hoc analysis for multiple comparisons. ns=no significance, *=p<0.05, **=p<0.01, ***=p<0.001 compared to untreated *sgca-/-* mice, unless noted. Abbreviations: DIA, diaphragm; ECC, eccentric contraction cycle; TA, tibialis anterior.

Safety and biodistribution of systemic delivery of scAAVrh74.tMCK.hSGCA in *sgca-/-* mice

- Liver enzymes (ALT, AST and ALP/K) and blood glucose levels were analyzed for toxicity (n=6 per group). All chemistry values of treated *sgca-/-* mice at all doses were within the normal limits for mice
- Vector genomes were present in every tissue tested, with the highest copy number in the liver, followed by muscles, confirming successful test article delivery

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