Adeno-Associated Virus Mediated β-Sarcoglycan Gene Replacement Therapy for the Treatment of Limb Girdle Muscular Dystrophy Type 2E

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Eric Richard Pozsgai

Biomedical Sciences Graduate Program

The Ohio State University

2016

Dissertation Committee:

Louise R. Rodino-Klapac, PhD, Advisor
Jerry R. Mendell, MD
Scott Harper, PhD
Paul Janssen, PhD
Copyright by

Eric Richard Pozsgai

2016
Abstract

One major class of muscular dystrophy is limb-girdle muscular dystrophy (LGMD), encompassing many different subtypes. LGMD2E is one of the most severe forms of LGMD, resulting from recessive mutations in the β-sarcoglycan (SGCB) gene, causing loss of functional protein. SGCB is a structural protein component of the dystrophin-associated protein complex (DAPC), which as a whole provides structural and mechanical stability to the sarcolemma. Due to the loss of protein, a devastating disorder ensues with widespread progressive muscle wasting, leading to loss of function. Disease progression is directly correlated with age, where the most severe cases have symptom onset in early childhood and many patients are rendered non-ambulant by their teens. In addition to skeletal muscle weakness, significant respiratory failure and fatal cardiomyopathy are common features in more severe LGMD2E patients. To date, no effective therapy exists to treat this debilitating disease. Thus, with an urgent need for a viable treatment option for LGMD2E patients, this work attempted to develop the first viral-mediated approach to restore wild-type (WT) SGCB.

Providing a relevant model to test therapeutic efficacy, the Sgcb-null (sgcb<sup>−/−</sup>) mouse recapitulates the clinical phenotype and shares many of the pathologic features of LGMD2E patient biopsies including myofiber necrosis, central nucleation, inflammation, and fibrosis. We have engineered a viral mediated gene replacement therapy using adeno-associated virus (AAV) carrying a codon optimized human SGCB gene (hSGCB) driven by one of two different muscle specific promoters. We first
established proof-of-principle efficacy of scAAVrh.74.tMCK.hSGCB by intramuscular (IM) injection and isolated-limb perfusion (ILP) in young and aged mice, which resulted in long-term widespread transgene expression accompanied by histological and function benefits. Notably, we saw a considerable reduction in fibrotic tissue, a significant component of the disease and a potential major obstacle of gene transfer. After switching promoters to the MHCK7 promoter which provides robust expression in cardiac tissue, we then demonstrated efficacy of systemic delivery of scAAVrh.74.MHCK7.hSGCB in treating skeletal and cardiac muscle deficits in sgcb−/− mice to provide a potential rationale for meaningful results in a clinical trial. This led to nearly 100% transgene expression in numerous muscles throughout the limbs, torso, and the heart, which was again accompanied by improvements in muscle histopathology and function, as well as increased overall activity. Importantly, a formal GLP toxicology study of AAV.hSGCB gene transfer in WT mice showed no adverse effects. In this well-defined model of LGMD2E, we have established a clinically relevant path for AAV mediated SGCB gene replacement therapy that has great promise for LGMD2E patients.

After demonstrating the importance of functional deficits in dystrophic muscle to serve as outcome measures for functional recovery from SGCB gene therapy, we applied these concepts to the investigations of several other forms of LGMD, Types 2B and 2L, involving dysferlin (DYSF) and anoctomin5 (ANO5), respectively. In these studies, we optimized and validated muscle fiber isolation with a laser induced membrane repair assay to aid in thoroughly characterizing these diseases and test therapeutic interventions like viral-mediated gene transfer. Using this technique, we are able to directly measure a functional parameter of both DYSF and ANO5 and quantify the membrane repair ability in different physiological settings. Taken together, we report
here an experimental method that can be a powerful tool for pre-clinical studies of muscular dystrophy. We also emphasize the importance of the pre-clinical efficacy study with SGCB gene replacement therapy and the impact it has in the translational pathway in providing a clinically meaningful therapy for LGMD2E patients.
Dedication

This dissertation is dedicated to my family and friends for supporting me throughout graduate school and all of the patients and families afflicted with LGMD2E.
Acknowledgments

I would like to first thank my advisor Dr. Louise Rodino-Klapac for the incredible opportunity to become a member of her lab for my graduate studies and carryout high-level impactful research on gene therapies for muscular dystrophies. I want to acknowledge my great appreciation for all of the mentoring and support Dr. Rodino-Klapac has given me throughout my time in her lab. I have gained tremendous experience in biomedical and translational research as a result of her mentoring and I could not have progressed as I did without her. I would also like to thank the other members of my committee; Dr. Jerry Mendell, Dr. Scott Harper, and Dr. Paul Janssen for their advisement throughout my studies and their help in advancing my research project. I have grown immensely as a scientific researcher and as a professional under their guidance. Additionally, I would like to acknowledge all former and current members of the Rodino-Klapac lab for the outstanding help and insight they provided to my project. I am also thankful to the viral vector core at Nationwide Children's Hospital for producing the therapeutic viruses used in these investigations. Finally, I would like to emphasize great appreciation and thanks to the GFB Italian Olnus and the OSU Muscle Group/NIH T32 Graduate Student Training Fellowship (NINDS-T32 NS077984 “Training in Neuromuscular Diseases”) as the major funding sources for all of this work.
Vita

June 2006.................................John Adams High School, South Bend, IN

2010..................................................B.S. Biochemistry, Indiana University

Bloomington

2011 to present ............................Graduate Research Associate, Biomedical

Sciences Graduate Program, College of

Medicine, The Ohio State University

Publications


Fields of Study

Major Field: Biomedical Sciences Graduate Program
Table of Contents

Abstract ............................................................................................................................... ii
Dedication ............................................................................................................................. v
Acknowledgments ................................................................................................................. vi
Vita ........................................................................................................................................ vii
Publications ........................................................................................................................... vii
Fields of Study ....................................................................................................................... viii
Table of Contents .................................................................................................................... ix
List of Tables ........................................................................................................................... xv
List of Figures ........................................................................................................................... xvi
Chapter 1: Introduction ............................................................................................................ 1
    1.1. Classification of Limb-Girdle Muscular Dystrophy .................................................... 1
    1.2. β-Sarcoglycan and LGMD2E Pathogenesis ............................................................... 2
    1.3. Clinical Manifestation of LGMD2E ......................................................................... 3
    1.4. Evolving Therapeutic Interventions for Muscular Dystrophy ............................... 5
    1.5. Gene Replacement Therapy for LGMD ..................................................................... 7
    1.6. Murine Model of LGMD2E ..................................................................................... 9
    1.7. Summary of β-sarcoglycan Gene Transfer .............................................................. 11
Chapter 2: β-Sarcoglycan Gene Transfer Decreases Fibrosis and Restores Force in LGMD2E Mice

2.1. Abstract ......................................................................................................................... 16

2.2. Introduction ..................................................................................................................... 17

2.3. Results ............................................................................................................................ 20

2.3.1. scAAVrh.74.tMCK.hSGCB construction and vector potency ................................. 20

2.3.2. Intramuscular delivery of scAAVrh.74.tMCK.hSGCB ......................................... 20

2.3.3. Functional correction in skeletal muscle following scAAVrh.74.tMCK.hSGCB gene transfer .......................................................... 22

2.3.4. Treatment of Aged Muscle with scAAVrh.74.tMCK.hSGCB ............................. 23

2.3.5. Isolated-Limb Perfusion of scAAVrh.74.tMCK.hSGCB in sgcb⁻/+ mice .......... 23

2.3.6. Long-Term Therapeutic Efficacy of β-Sarcoglycan Gene Transfer .................. 25

2.3.7. Safety and Biodistribution of rAAVrh.74.tMCK.hSGCB ................................. 27

2.3.8. Formal GLP Safety and Toxicity Study of IM and ILP Delivery of scAAVrh.74.tMCK.hSGCB .......................................................... 28

2.4. Discussion ....................................................................................................................... 30

2.5. Materials and Methods ............................................................................................... 32

2.5.1. Animal Models ........................................................................................................... 32
3.5.11. Biodistribution qPCR Analysis .......................................................... 79
3.5.12. Picrosirius Red Stain and Collagen Quantification ......................... 80
3.5.13. X-Ray Images .............................................................................. 80
3.5.14. Laser Monitoring of Open Field Cage Activity .......................... 81
3.7. Tables .................................................................................................. 83
3.6. Figures .................................................................................................. 85

Chapter 4: Establishing Outcome Measures for Translational Studies of Autosomal Recessive Limb-Girdle Muscular Dystrophies ........................................... 92
4.1. Abstract ............................................................................................... 92
4.2. Introduction ......................................................................................... 93
4.3. Results .................................................................................................. 96
  4.3.1. Restoration of membrane repair following intramuscular delivery of AAV5.DYSF ................................................................. 96
  4.3.2. Restoring membrane repair with delivery of AAVrh.74.DYSF.DV ................. 97
  4.3.3. Systemic delivery of AAVrh.74.MHCK7.DYSF.DV improves membrane repair 98
  4.3.4. AAVrh.74.DYSF.DV systemic gene transfer in aged mice ................. 99
  4.3.5. Ano5 facilitates membrane repair ..................................................... 100
4.4. Discussion ............................................................................................ 100
4.5. Materials and Methods ..................................................................... 104
  4.5.1. Dysferlin deficient mouse strains .................................................... 104

xiii
4.5.2. Anoctamin5-deficient Mouse Strain .......................................................... 104
4.5.3. Intramuscular Delivery ........................................................................... 105
4.5.4. Systemic Delivery .................................................................................. 106
4.5.5. Membrane Repair Assay ....................................................................... 106
4.5.6. Statistical analysis .................................................................................. 108
4.6. Figures........................................................................................................ 110

Chapter 5: Discussion ....................................................................................... 116
5.1. Requirement for LGMD2E Therapy ............................................................. 116
5.2. Novel Viral-Mediated Human SGCB Gene Transfer .................................... 117
5.3. Proof-of-Principle Studies .......................................................................... 118
5.4. Systemic Delivery of AAV-β-Sarcoglycan .................................................. 119
5.5. Future Work with LGMD2E Therapy .......................................................... 120
5.6. Functional Outcomes for Dysferlin and Anoctomin5 Myopathies .......... 124
5.7. Conclusions ............................................................................................... 125

References ......................................................................................................... 126
List of Tables

Table 2.1. Quantification of immune cells present in uninjected SGCB-/- mice, and scAAVrh.74.tMCK.hSGCB treated and untreated muscle.......................................................... 41

Table 2.2. Safety study: Isolated-Limb Perfusion of scAAVrh.74.tMCK.hSGCB in sgcb +/- mice........................................................................................................................................... 41

Table 2.3. Quantification of hSGCB vector genome copies present in all offsite organs and tissues ............................................................................................................................................. 42

Table 2.4. Analysis of circulating antibody titers for IM & ILP delivery of scAAVrh.74.tMCK.hSGCB............................................................................................................................................. 43

Table 3.1. Quantification of hSGCB transgene expression 6 months post-gene transfer of scAAVrh.74.MHCK7.hSGCB in SGCB-/- mice................................................................. 83

Table 3.2. Quantification of central nucleation and muscle fiber diameter 6 months post-gene transfer of scAAVrh.74.MHCK7.hSGCB in SGCB-/- mice................................. 84
List of Figures

Figure 1.1. Autosomal Recessive Limb-Girdle Muscular Dystrophies................................. 14
Figure 1.2. Dystrophin-Associated Protein Complex ............................................................. 15
Figure 2.1. AAV mediated β-sarcoglycan expression restores dystrophin associated proteins and protects membrane integrity ................................................................. 44
Figure 2.2. Histological analysis of β-SG deficient treated skeletal muscle. scAAVrh.74.hSGCB treatment normalizes histological parameters of sgcb−/− mice.... 45
Figure 2.3. scAAVrh.74.hSGCB intramuscular delivery corrects tetanic force and resistance to contraction-induced injury ................................................................. 46
Figure 2.4. Analysis of Aged Mice Treated Intramuscularly with scAAVrh.74.tMCK.hSGCB ....................................................................................................................... 47
Figure 2.5. Isolated-limb perfusion of low dose scAAVrh.74.hSGCB ........................................ 48
Figure 2.6. Vascular delivery of high dose scAAVrh.74.hSGCB. ............................................. 49
Figure 2.7. Histological Analysis of Vascular Delivery of High Dose rAAVrh.74.tMCK.hSGCB ........................................................................................................... 50
Figure 2.8. Reduction of fibrosis in ILP treated β-SG KO mice. ................................................ 51
Figure 2.9. ILP delivery of scAAVrh.74.tMCK.hSGCB leads to long-term hSGCB expression and reversal of histopathology in β-SG KO muscle ........................................... 52
Figure 2.10. Quantification of histological parameters following long-term ILP treatment with scAAVrh.74.tMCK.hSGCB ................................................................................ 53
Figure 2.11. Functional assessment of long-term scAAVrh.74.tMCK.hSGCB ILP treatment.................................................................................................................................................. 54
Figure 2.12. Long-term SGCB expression following IM delivery allows for functional recovery.................................................................................................................................................. 55
Figure 2.13. Vector biodistribution and protein expression................................................................................................................................................................................................. 56
Figure 2.14. Beta-Sarcoglycan expression in 15 tissues from mice treated with lactated ringers solution (LRS) or scAAVrh.74.tMCK.hSGCB........................................................................................................................................................................ 56
Figure 3.1. Restoration of SGCB expression following intravenous delivery of scAAVrh.74.MHCK7.hSGCB ................................................................................................................................................................................................. 85
Figure 3.2. Effect of systemic treatment with scAAVrh74.MHCK7.hSGCB on muscle pathology........................................................................................................................................................................................................ 86
Figure 3.3. Reduced collagen deposition in intravenous treated β-SG KO mice............ 87
Figure 3.4. Correction of kyphoscoliosis in thoracic spine............................................. 88
Figure 3.5. Assessment of cardiomyopathy in heart muscle........................................... 89
Figure 3.6. Diaphragm function correction and increased open-field cage activity....... 90
Figure 3.7. Biodistibution and off-target transgene expression analysis of systemic scAAVrh.74.MHCK7.hSGCB delivery. ........................................................................................................................................................................................................ 91
Figure 4.1. Membrane repair process is calcium dependent........................................ 110
Figure 4.2. AAV5.DYSF treatment restores membrane resealing.............................. 111
Figure 4.3. Dose-dependent membrane resealing activity following IM AAVrh.74.MHCK7.DYSF.DV delivery.................................................................................................................................................................................... 112
Figure 4.4. Systemic delivery of AAVrh.74.MHCK7.DYSF.DV restores membrane repair deficits in BlaJ mice.. .............................................................................................................................................. 113
Figure 4.5. Systemic AAVrh.74.MHCK7.DYSF.DV delivery in aged BlaJ mice improves membrane repair.......................................................... 114

Figure 4.6. Membrane Repair is defective in Ano5<sup>−/−</sup> mice................................................................. 115
Chapter 1: Introduction

1.1. Classification of Limb-Girdle Muscular Dystrophy

Muscular dystrophies (MDs) are a group of genetic musculoskeletal disorders involving progressive muscle wasting, and collectively are classified by nine major types: Duchenne MD, Becker MD, Congenital MD, Distal MD, Emery-Dreifuss MD, Facioscapulohumeral MD, Limb-Girdle MD, Myotonic MD, and Occulopharyngeal MD. One of the first descriptions of Limb-Girdle Muscular Dystrophy (LGMD) was in 1959 by Chung and Morton in a large heterogeneous study of MD patients, where they were able to delineate the common features of this specific form of the disease [1]. They described the LGMDs as being associated with initial pelvic and shoulder girdle weakness followed by generalized progressive muscle wasting with incidents of asymmetrical wasting, calf hypertrophy, and/or contractures [1]. The rate of progression varied, but most saw severe disability including ambulatory loss 20 to 30 years within disease onset, and death shortly thereafter up to middle age. Most importantly, they noted 59% of the LGMD cases could be ascribed to autosomal recessive inheritance [1]. This initial case study began to distinguish the two major types of LGMD, which are divided into the autosomal dominant Type 1 LGMDs and the autosomal recessive Type 2 LGMDs, collectively making up 30-35% of all cases of MD [2]. The more prominent forms of the autosomal recessive LGMDs are shown in Figure 1.1 and are named as subtypes A-M.
in the chronological order in which their corresponding mutation was discovered [3, 4]. Muscle proteins located at or near the sarcolemma are often involved; whose functions include structural or mechanical maintenance of membrane integrity [3, 4]. Four recessive LGMDs result from mutations in one of the sarcoglycan (SG) genes, accounting for ~15% of the Type 2 LGMDs [5], yet almost 70% of severe cases of limb-girdle [6]. This includes LGMD2E, representing one of the most severe LGMDs in the United States with worldwide reports of incidence of 1/200,000-1/350,000 [3].

1.2. β-Sarcoglycan and LGMD2E Pathogenesis

In 1995 Lim et. al. first showed LGMD2E to be caused by mutations in the gene encoding β-sarcoglycan (SGCB) (Figure 1.1) [7]. The resulting loss of functional protein correlated with chronic muscle fiber loss, inflammation, fat replacement and fibrosis, all leading to muscle weakness and progressive muscle wasting [8, 9]. The SGCB gene originally cloned by Lim et. al. and also later mapped by another group, Bonnemann et. al., was found to be located on chromosome 4q12 [7, 8]. Bonnemann et. al. reported that the gene spans 13.5 kb of genomic DNA, is encoded by 6 exons, and produces a 957 bp cDNA [9]. The corresponding 43 kDa protein is a 318 amino acid protein containing a 63 amino acid intracellular N-terminal region, a 27 amino acid transmembrane region, and a 228 amino acid extracellular C-terminal region [7, 9]. SGCB together with the four other SG proteins (α-, γ-, δ-, and ε-) all of similar size and structure ranging from 35-50kD, make up the SG complex localized in the sarcolemma of muscle fibers [7, 9]. The SG complex (α, β, γ, and δ-SG), along with dystrophin and other structural components make up the dystrophin-associated protein complex (DAPC) (Figure 1.2). This large multimeric protein complex creates a structural link between the
extracellular matrix and the intracellular actin cytoskeleton of muscle cells, contributing to membrane integrity and protection from mechanical stress during muscle activity [10]. Disruption of this link through the loss of SGCB leads to the concomitant loss of the other SG and DAPC proteins, resulting in muscle membrane instability and eventual myofiber damage and death [10].

1.3. Clinical Manifestation of LGMD2E

Numerous impactful reports of LGMD2E cases have been published through the years, which helped to define the LGMD2E patient population. Jackson and Strehler in 1968 reported an isolated population of patients with autosomal recessive LGMD in 5 Amish families from southern Indiana, and indicated a possible link between disease inheritance, severity, and consanguinity [11]. Several decades later in 1995, a homozygous missense mutation in the SGCB gene was identified in 2 Amish patients from that same region in southern Indiana clinically diagnosed with LGMD2E, leading to the cloning of SGCB mentioned in the previous section [7]. Furthermore, a young girl of Italian descent who exhibited generic autosomal recessive LGMD symptoms and formally diagnosed at the age of 3 was found to be compound heterozygous for a truncating SGCB mutation on both alleles, ablating most of the extracellular domain of the protein [8].

Additional cases of LGMD2E in Italy were reported as a founder effect involving a homozygous 8-bp duplication in the SGCB gene affecting this same extracellular region in the SGCB protein. These along with a few compound heterozygous mutations involving this 8-bp duplication in the extracellular domain-coding region of the SGCB gene occurred in several unrelated families, and in some cases were eventually shown
to cause severe fatal cardiomyopathy, with death in two patients at 18 and 27 years of age [12, 13]. An intriguing study again by Bonnemann et al. in 1998 reported members of a Tunisian family with β-sarcoglycanopathy that had a missense mutation in exon 3 of the SGCB gene, affecting once again the same extracellular domain of SGCB [14]. In all cases, patient muscle biopsies showed the significant or complete loss of not only SGCB but other SG complex and DAPC components, which led investigators to hypothesize that the extracellular domain of SGCB may be essential for assembly and/or maintenance of the complex, via disulfide-bond formation with another SG through a cysteine residue in the precise domain affected by these mutations [14]. Finally, Trabelsi et al. in 2008 identified biallelic SGCB gene mutations in 17.3% of a large (~70) heterogeneous patient population with a clinical diagnosis of autosomal recessive LGMD, and 7 of those 9 LGMD2E patients were determined to have novel mutations involving a hotspot region in exon 1 [15].

Taken together, while LGMD2E is most prevalent in Europe, it is evident from all of these clinical cases involving numerous mutations that LGMD2E displays a variety of clinical phenotypes. In a high percentage of cases, however, the typical age of onset of symptoms is between infancy and early childhood with diagnosis typically occurring by adolescence, loss of ambulation by mid to late teens, and loss of life in the third decade, all of which demonstrate the severity of LGMD2E [8, 16-18]. Several studies of a large cohort of Brazilian families have confirmed the severity of β-sarcoglycanopathy, with one study reporting 100% of patients identified as having LGMD2E had a severe phenotype [6, 19]. LGMD2E patients display typical LGMD symptoms including proximal shoulder and girdle muscle weakness, elevated creatine kinase (CK) levels, and difficulty rising from the floor or walking [8, 16-18]. Respiratory failure is also seen in a high
percentage, and cardiac involvement, often in the form of dilated cardiomyopathy with impaired ventricle function, heart rhythm abnormalities, and eventual myocardial remodeling, has been reported to occur in greater than 50-60% of cases [13, 18, 20-22].

1.4. **Evolving Therapeutic Interventions for Muscular Dystrophy**

The economic burden on families with a member who has muscular dystrophy is estimated to be in the tens-of-thousands per year due to the team of medical specialists, physical therapists, medical devices, and various medications required for the care of an MD patient. As debilitating as they are, no proven therapies for any of the MDs currently exist. In many forms of the disease, exercise and physical therapy may be used to maintain muscle strength and joint flexibility, however, this is used to slow disease progression rather than fully treat and cure the disease [23].

Corticosteroids, including glucocorticoids, have been shown to be beneficial in the management of symptoms and delaying disease progression [23-25]. Mendell et. al. in 1989 clearly demonstrated an improvement in muscle strength, pulmonary function, and time rising from the floor in patients on a continuous regiment of prednisone [24]. Additional studies with prednisone and other glucocorticoids reported increased ambulation, reduced inflammation, and delayed disease progression in treated patients [23, 25]. These benefits are limited however because they do not treat the primary cause of the disease and have been shown effective only in DMD. In addition, the side-effects from prolonged use include hypertension, loss of bone density, and increased fracture risk [23-25]. An investigation into vorapamil, a calcium-channel blocker approved for use in cardiac disease, saw a benefit in the treatment and prevention of cardiomyopathy in dystrophic beta- and delta-sarcoglycan deficient mice, though the
degree of improvement was variable [26], and this involves only management of a secondary aspect of the disease. One final method to indirectly circumvent a defect in \textit{dystrophin} in Becker Muscular Dystrophy (BMD), a milder form of DMD, is to target the myostatin pathway to increase muscle size. Pre-clinical studies leading to clinical trials in BMD showed efficacy of follistatin gene therapy [27, 28]. Delivery of this powerful antagonist of the muscle growth regulating myostatin pathway resulted in increased muscle mass and strength, allowing for functional improvements in a 6-minute walk test (6MWT) [29].

New potential therapies have emerged recently that are aimed at a more permanent cure for these diseases by directly targeting the genetic defects with gene manipulation. Examples include exon skipping with small molecules and gene editing with the CRISPR/Cas system. Beginning with exon skipping, this technique uses small oligomers to target pre-mRNA transcripts allowing for the omission of one or more mutation-containing exons during transcription of DNA to RNA [30]. The resulting product is a condensed RNA transcript comprising a gene sequence with the necessary regions for functionality that is restored to the open reading frame, allowing for the production of a truncated but still functional protein [30]. Two drug candidates (Drisapersen and Eteplirsen) were developed for use in exon 51 skipping in the \textit{DMD} gene for Duchenne Muscular Dystrophy and are both in advanced clinical trials [31-33], with Eteplirsen showing the most potential with its safety profile and functional benefit in patients[34].

On the other hand, gene editing with CRISPR(Clustered Regularly Interspaced Short Palindromic Repeats)/Cas(CRISPR-associated genes) uses pre-existing cellular machinery from bacteria, specifically endonucleases involved in adaptive immunity, to
make precise targeted alterations to the genome of cells [35]. Following removal by the nuclease of a short (~20-30 bp) genomic sequence containing the mutation, supplementation of this short sequence in its normal wild-type (WT) form allows for incorporation into the genome, restoration of the entire WT sequence, and production of a full-length, functional WT protein [35]. Several studies published recently have shown efficacy with CRISPR/Cas9 in correcting the exon 23 point mutation in the DMD gene of mdx mice, the murine model for Duchenne Muscular Dystrophy [36-38]. This technology however is still in early pre-clinical investigation and requires extensive further optimization before translating to human patients.

1.5. Gene Replacement Therapy for LGMD

As an alternative to the indirect management of secondary symptoms or use of less efficient genetic manipulation techniques like those discussed above, more efficient and clinically applicable therapies to either enhance muscle growth and recovery or fully correct the genetic defects are under investigation. One emerging form of therapy that has great promise as a potential treatment for these disorders is viral-mediated gene delivery to supplement proteins involved in muscle recovery or restore WT DNA and consequently WT protein in affected muscle, to ultimately restore muscle function. Adeno-associated virus (AAV) is a single-stranded DNA virus able to efficiently transduce multiple target tissues, both dividing and non-dividing (such as muscle) cell types, with long-term persistence [39-41]. Studies have shown that the majority of AAV vector DNA appears to persist as transcriptionally active concatameric episomes [42-44]. Therefore when considering potential risks for translation into patients, rAAV vectors pose little risk for genomic integration, making oncogenic mutagenesis unlikely [43].
Although some reports indicate sporadic marginal immune responses to AAV transduction [45-50], AAV is generally considered to be non-pathogenic in nature with no known human disease [51] and low immunogenicity, which helps in achieving long-term high-level gene expression with relatively low risk to patients [52-54]. The safety profile of AAV combined with the high transduction efficiency, wide tropism, and numerous available serotypes has permitted AAV to be an ideal delivery vehicle for use in gene therapy.

Various studies have investigated the use of AAV gene therapy in the different LGMDs. A series of studies in our group looking at AAV transfer of DYSF for the treatment of dysferlinopathies demonstrated efficacy using both a single vector system with AAV5 and a dual vector system with AAVrh.74 to produce full-length DYSF and restore its function in sarcolemmal patch repair [55, 56]. These studies have led to a Phase I clinical trial in LGMD2B patients [57]. A study by Dressman et. al. of AAV gene therapy for the sarcoglycanopathies reported construction of both rAAV2.SGCA and rAAV2.SGCB constructs, with both recombinant vectors showing widespread biochemical and histological rescue of dystrophic muscle, however, only AAV.SGCB showed long-term persistence of vector DNA and transgene expression [58]. The use of a cytomegalovirus (CMV) promoter rather than a muscle specific muscle creatine kinase promoter (MCK) was the likely cause of the loss of SGCA transgene expression that was observed over time, due to promoter shut down.

Our group has also shown proof-of-principle for efficacy of gene replacement for α-sarcoglycanopathy (LGMD2D), involving the highly similar SGCA, with rAAV1.tMCK.hSGCA gene transfer in α-SG KO mice [59], which resulted in a clinical trial in LGMD2D patients [47, 49]. In the pre-clinical study, we drove human SGCA gene
expression with either the CMV promoter or one of several muscle specific promoters including desmin, MCK, or a further modified MCK (tMCK) promoter [59]. Sustained SGCA transgene expression was observed up to 12 weeks post gene transfer with the greatest expression seen using the MCK based promoters [59]. Translation of the pre-clinical study to LGMD2D patients reported 4.5-5 fold increased hSGCA expression in treated muscle compared with control, the full restoration of the SG complex, and an increase in muscle fiber size. While an initial short-lived T-cell and neutralizing antibody response to AAV was seen (presumably due to pre-existing AAV immunity), no significant immune or toxicity related safety concerns in patients were detected [47, 49]. This study provided a clear model for SGCB gene therapy, increasing the chances of success for this form of treatment for LGMD2E.

1.6. Murine Model of LGMD2E

Several different mouse models of β-sarcoglycanopathy have been created for the study of LGMD2E pathogenesis and corresponding therapeutic interventions. Initially, Araishi et. al. generated a SGCB-deficient transgenic mouse by incorporating a targeting vector that disrupted exon 2 (encoding most of the intracellular and transmembrane domains of the protein), creating a null mutation resulting in complete absence of SGCB RNA and protein [60]. A second mouse model of LGMD2E (B6.129-Sgcb\textsuperscript{tm1Kcam}/1J), the one used in our pre-clinical studies, was developed in the laboratory of Dr. Kevin Campbell at the University of Iowa [10]. Here, a targeting vector containing a neomycin resistance cassette was used to replace exons 3-6 of the SGCB gene (encoding part of the transmembrane domain and the extracellular domain). Northern blot analysis of skeletal muscle with an exon 6 probe revealed no transcript in
homozygous mutant mice, and Western blotting for SGCB protein indicated complete absence of protein [10].

Analogous to the disease progression of LGMD2E, the mice develop severe muscular dystrophy and cardiomyopathy. Severe dystrophic changes including necrosis, central nucleation, dystrophic calcification, fatty infiltration, fibrosis, and muscle atrophy and hypertrophy are detected in multiple skeletal muscles and cardiac muscle as early as 4-weeks old, and accumulate with age [10]. The severity of the LGMD2E phenotype in this mouse was illustrated by significant prominence of endomysial fibrosis, where connective tissue deposition was much greater compared to other animal models of MD [61]. Additionally, the SG-sarcospan, dystroglycan, and differentially expressed smooth muscle-specific ε-sarcoglycan (SGCE) containing complexes were all disrupted in skeletal, cardiac, and smooth muscle membranes. Consequences of this, particularly in smooth muscle, were vascular irregularities in heart, diaphragm, and kidneys, which the investigators ascribe as a contributing factor to the development of cardiomyopathy in LGMD2E [10]. The authors ultimately concluded that loss of the SG complex and sarcospan in skeletal muscle alone is sufficient to cause muscular dystrophy, that SGCB is an important core protein for formation of the SG complex and with sarcospan, and that the role of the SG-sarcospan complex is in part to strengthen the DAPC connecting the extracellular basement membrane with the intracellular cytoskeleton [10, 60].

Finally, one previous study suggested a dysfunction of the calcium release channel Ryanodine Receptor type 1 (RyR1) as a contributing factor to disease pathogenesis [62]. They reported impaired muscle function in the Durbeej et. al. sgcb−/− mice where analysis of muscle physiology revealed reduced specific force compared to healthy WT controls [62]. Demonstration of the force deficits seen in LGMD2E patients
in this murine model of the disease provided a basis for correction following AAV treatment. We concluded from these reports that these mice provide an excellent model in which to determine preclinical efficacy of AAV.hSGCB gene transfer because they accurately recapitulate the disease phenotype of LGMD2E patients.

1.7. **Summary of β-sarcoglycan Gene Transfer**

As was discussed previously, the severity of the LGMD2E phenotype has a profound impact on the quality of life of patients and in many instances leads to a dramatically shortened lifespan. Due to the limited effectiveness and applicability of the treatment options outlined above, no therapy exists to treat LGMD2E and little advancement in the clinical management of symptoms beyond what was presented has occurred in some time. Thus, there is clearly an urgent need to develop a viable treatment option for patients. The overall objective of this work was to provide pre-clinical proof-of-principle for effectiveness of AAV-mediated SGCB gene transfer in sgcb<sup>-/-</sup> mice to reintroduce the WT gene and produce WT protein, all in hopes of restoring muscle function. The work outlined here provides evidence of successful completion of this pre-clinical study, which not only provides a path to the clinic for AAV-mediated SGCB gene therapy, but also gives support for viral-mediated gene replacement therapy in other diseases.

1.8. **LGMD Functional Outcomes Measures**

Two other groups of MDs, involving dysferlin (DYSF) and anoctomin5 (ANO5) separately, exhibit similar characteristics and ones that resemble those of the LGMDs
described earlier. Dysferlinopathies (including LGMD2B) caused by mutations in DYSF [63-68], and recently identified anoctamin5 myopathies (including LGMD2L), resulting from mutations in ANO5 (TMEM16E) [69-71], present with characteristic LGMD features of progressive muscular dystrophy with chronic myofiber loss, limb muscle weakness, and deteriorating muscle function [66, 69-72]. The unique aspect of these forms of the disease however is the presence of membrane defects and sarcolemmal lesions in muscle fibers from patients [69-71, 73, 74]. Strong molecular, structural, and functional evidence in mice, particularly in DYSF association with Ca\(^{2+}\) and phospholipid binding [75-77], suggests a role for DYSF in Ca\(^{2+}\)-dependent sarcolemmal membrane repair [78-81]. The similarities between ANO5 and DYSF myopathy phenotypes, along with an unknown function for ANO5 [82, 83], led to the belief that ANO5 may also be involved in membrane repair.

One point of focus in this report is the necessity for pre-clinical functional efficacy of therapies for MD, and a thorough characterization of disease states in murine models is critical to establish functional outcome measures. Under normal physiological conditions, muscle contractions cause mechanical stress and consequently membrane damage, therefore patch repair mechanisms involving intracellular membrane vesicle fusion at the injury site are required [84-86]. This combined with the roles of DYSF and ANO5 in that process and in their disease pathogeneses create an ideal target to establish functional deficits for outcome measures. The Flexor Digitorum Brevis (FDB) muscle in mice is useful for experimental analysis, due to its small size and location in the footpad, making it easily accessible for treatment and extraction [87, 88]. Individual fibers dissociated from the FDB muscle provide the model to measure sarcolemmal repair. A multi-photon laser microscope is targeted at the membrane to replicate
mechanical injury, and the rate of passive infiltration of a phospholipid-activated fluorescent dye [87] accurately quantifies membrane repair ability. This quantifiable functional measure is a predictor of pre-clinical gene replacement therapeutic efficacy, or membrane resealing dysfunction in dystrophic muscle.

1.9. Summary

This report details the pathogenesis and progression of multiple forms of autosomal recessive Limb Girdle Muscular Dystrophy. As these diseases progress, a debilitating outcome ensues which drastically reduces patients quality of life and ultimately the lifespan itself. Multiple therapeutic strategies are under investigation with intricate experimental techniques to determine the ability of these therapies to correct and reverse the disease state. We utilize the specific function of muscle proteins, DYSF and ANO5, to develop these experimental methods with in vivo microscopy as a tool to test therapeutic efficacy. Finally, using a viral-mediated gene replacement approach, we hypothesize that restoration of the wild-type cDNA of an affected gene, in this case SGCB, will result in production of a wild-type, fully functional protein and correction of the disease state. Overall, this dissertation presents in detail strong pre-clinical evidence of powerful functional outcomes for the study of certain LGMDs, and efficacy of SGCB gene replacement therapy for LGMD2E.
1.10. Figures

### Autosomal Recessive LGMD

<table>
<thead>
<tr>
<th>DISEASE</th>
<th>PROTEIN</th>
<th>GENE</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGMD2A</td>
<td>Calpain3</td>
<td>CAPN3</td>
</tr>
<tr>
<td>LGMD2B</td>
<td>Dysferlin</td>
<td>DYSF</td>
</tr>
<tr>
<td>LGMD2C</td>
<td>γ - Sarcoglycan</td>
<td>SGCG</td>
</tr>
<tr>
<td>LGMD2D</td>
<td>α - Sarcoglycan</td>
<td>SGCA</td>
</tr>
<tr>
<td>LGMD2E</td>
<td>β - Sarcoglycan</td>
<td>SGCB</td>
</tr>
<tr>
<td>LGMD2F</td>
<td>δ - Sarcoglycan</td>
<td>SGCD</td>
</tr>
<tr>
<td>LGMD2G</td>
<td>Telethonin</td>
<td>TCAP</td>
</tr>
<tr>
<td>LGMD2H</td>
<td>TRIM32</td>
<td>TRIM32</td>
</tr>
<tr>
<td>LGMD2I</td>
<td>Fukutin-related protein</td>
<td>FKRP</td>
</tr>
<tr>
<td>LGMD2J</td>
<td>Titin</td>
<td>TTN</td>
</tr>
<tr>
<td>LGMD2K</td>
<td>O-Mannosyl transferase-1</td>
<td>POMT1</td>
</tr>
<tr>
<td>LGMD2L</td>
<td>Fukutin</td>
<td>FKTN</td>
</tr>
<tr>
<td>LGMD2M</td>
<td>O-Mannose β – 1, 2-N-acetylgulosaminyl transferase</td>
<td>POMGn1</td>
</tr>
<tr>
<td>LGMD2N</td>
<td>O-Mannosyl transferases-2</td>
<td>POMT2</td>
</tr>
</tbody>
</table>

**Figure 1.1. Autosomal Recessive Limb-Girdle Muscular Dystrophies.** Major forms of the Type 2 LGMDs caused by recessive mutations in a variety of muscle related proteins. LGMD2E caused by a mutation in β-sarcoglycan is one of the more prominent and severe type 2 LGMDs.
Figure 1.2. Dystrophin-Associated Protein Complex. Schematic of the dystrophin-associated protein complex (DAPC) with β-sarcoglycan located in the transmembrane sarcoglycan complex. The DAPC is a large multimeric protein complex that links the intracellular actin cytoskeleton with the extracellular matrix, providing structural and mechanical stability to the sarcolemma.
2.1. Abstract

Limb-girdle muscular dystrophy type 2E (LGMD2E) results from mutations in the β-sarcoglycan (SGCB) gene causing loss of functional protein and concomitant loss of dystrophin-associated proteins. The disease phenotype is characterized by muscle weakness and wasting, and dystrophic features include muscle fiber necrosis, inflammation, and fibrosis. The Sgcb-null mouse recapitulates the clinical phenotype with significant endomysial fibrosis providing a relevant model to test whether gene replacement will be efficacious. We directly addressed this question using a codon optimized human SGCB gene (hSGCB) driven by a muscle specific tMCK promoter (scAAVrh74.tMCK.hSGCB). Following isolated limb delivery (5x10^{11} vg total dose), 91.2% of muscle fibers in the lower limb expressed SGCB, restoring assembly of the sarcoglycan complex and protecting the membrane from Evans blue dye leakage. Histological outcomes were significantly improved including decreased central nucleation, normalization of muscle fiber size, decreased macrophages and inflammatory mononuclear cells, and an average of a 43% reduction in collagen deposition in treated muscle compared to untreated muscle at endpoint. These measures correlated with improvement of tetanic force and resistance to eccentric contraction. In 6 month-old mice, as indicated by collagen staining,
scAAVrh74.tMCK.hSGCB treatment reduced fibrosis by 42%. Finally, we were able to demonstrate long-term therapeutic efficacy up to 1 year post-delivery. This study demonstrates the potential for gene replacement to reverse debilitating fibrosis, typical of muscular dystrophy, thereby providing compelling evidence for movement to clinical gene replacement for LGMD2E.

2.2. Introduction

Limb-girdle muscular dystrophy (LGMD) type 2E is an autosomal recessive disorder resulting from mutations in the gene encoding β-sarcoglycan (SGCB), causing loss of functional protein [8]. LGMD2E represents a relatively common and severe form of LGMD in the United States with worldwide reports of incidence of 1/200,000-1/350,000 [3]. The absence of SGCB leads to a progressive dystrophy with chronic muscle fiber loss, inflammation, fat replacement and fibrosis, all resulting in deteriorating muscle strength and function [10, 60]. As a complex, the sarcoglycans (SG) (α-, β, γ-, δ-), ranging in size between 35-50kD [9], are all transmembrane protein components of the dystrophin-associated protein complex (DAPC). This complex links the intracellular actin cytoskeleton and extracellular matrix, providing stability to the sarcolemma and offering protection from mechanical stress during muscle activity [60]. Loss of SGCB in LGMD2E usually results in varying degrees of concomitant loss of other SG proteins contributing to the fragility of the muscle membrane leading to loss of myofibers [8]. Although the range of clinical phenotype in LGMD2E varies, diagnosis typically occurs before age 10 and with loss of ambulation occurring by mid to late teens [8, 16, 17]. Patients present with elevated serum creatine kinase (CK), proximal muscle weakness,
difficulty arising from the floor, and progressive loss of ambulation. Cardiac involvement occurs 50% or more of cases [20-22].

Currently, there is no cure or treatment for LGMD2E [89], although, deflazacort has been reported to benefit two siblings with this condition [90]. Gene replacement therapy has intuitive appeal but many questions remain unanswered (e.g., delivery system, dosing, vehicle for gene transfer). A suitable model for translational studies is the murine model of LGMD2E that completely lacks SGCB (sgcb<sup>-/-</sup> mouse), and has clinical-pathological features in skeletal and cardiac muscle that replicate the human disease [10, 13]. The prominence of the endomysial fibrosis in this model is particularly attractive for testing therapeutic products since early onset fibrosis and the extent of connective tissue deposition is much greater compared to many other dystrophic animal models [61]. Considering that a major question unanswered by gene replacement therapy is potential efficacy once significant degrees of connective tissue have infiltrated dystrophic muscle, the studies described here in the β-sarcoglycan knock out mouse have particular relevance for planning future clinical trials.

For this proof of principle study we delivered the full-length SGCB cDNA under control of a muscle specific promoter (tMCK) using a self-complementary adeno-associated virus (scAAV) [91, 92]. A major rate-limiting step in the transduction efficiency of AAV is the requirement of synthesis of a complementary strand of DNA from the single-stranded AAV genome by the host-cell machinery. The self-complementary AAV vector was engineered by McCarty et. al. to bypass this synthesis step by creating a deletion of the terminal resolution site from the AAV terminal repeat sequence [91, 92]. This allows for the packaging of single-stranded dimeric inverted repeat DNA molecules [91, 92]. Use of the scAAV vector greatly reduces the time taken
to begin producing the exogenous therapeutic protein and increases efficiency of transduction with higher transgene expression at lower doses with no added difficulty in vector cloning or virus production. Our initial efforts were directed at demonstration of SGCB expression by intramuscular (IM) injection to establish vector potency. More importantly, as part of a dose escalation study beginning with an initial dose of $1 \times 10^{11}$ vg total dose we were able to deliver $5 \times 10^{11}$ vg total dose of scAAVrh.74.tMCK.hSGCB through an isolated-limb perfusion (ILP) system[93-95], achieving 90% or greater expression throughout the tibialis anterior (TA) and gastrocnemius (GAS) muscles of the lower limbs. The restoration of SGCB expression correlated with improvements in functional outcomes assessed by absolute and specific force generation and resistance to contraction induced injury. Of particular note was the reduction in endomysial fibrosis by approximately 50% following treatment. Importantly, when we extended the study to aged mice, we found hSGCB gene transfer alone was sufficient to reverse pre-existing fibrosis by 42%. We ultimately showed sustained long-term expression of hSGCB following both 6 and 12 months of treatment, which again reversed the dystrophic pathology in muscle and restored functional outputs. These findings have particular relevance to translational considerations for scAAVrh.74.tMCK.hSGCB gene therapy for LGMD2E.
2.3. Results

2.3.1. scAAVrh.74.tMCK.hSGCB construction and vector potency

We constructed a transgene cassette containing a codon-optimized full-length human SGCB cDNA (Figure 2.1a). The cassette includes a consensus Kozak sequence (CCACC), an SV40 chimeric intron, a synthetic polyadenylation site, and the muscle specific tMCK promoter [96] used to drive expression of the cassette. It was packaged into a self-complementary (sc) AAVrh.74 vector that is 93% homologous to AAV8 [97]. AAVrh.74 has been shown in mice and non-human primates to be safe and effective, particularly in crossing the vascular barrier when delivered to muscle through the circulation [93, 94, 98]. Direct intramuscular injection into the left tibialis anterior (TA) muscle of the Sgcb-null (sgcb⁻/⁻) mouse was performed to establish vector potency. Delivery of $3 \times 10^{10}$ vg transduced $70.5 \pm 2.5\%$ (±SEM) of muscle fibers and $1 \times 10^{11}$ vg transduced $89.0 \pm 4.0\%$ of muscle fibers, 3-weeks post gene transfer.

2.3.2. Intramuscular delivery of scAAVrh.74.tMCK.hSGCB

Following vector potency, studies were extended to analyze the efficacy of therapy 6 and 12 weeks post-gene transfer. As a result of the high levels of expression following the short 3-week potency study, a dose of $3 \times 10^{10}$ vg total was selected for subsequent studies in order to use the lowest effective dose. Five-week old sgcb⁻/⁻ mice were treated with $3 \times 10^{10}$ vg total dose of scAAVrh.74.tMCK.hSGCB IM to the TA and SGCB expression was demonstrated using immunofluorescence in $88.4 \pm 4.2\%$ of muscle fibers 6 weeks post-injection (n=9), and in $76.5 \pm 5.8\%$ of muscle fibers 12 weeks post-injection (n=6) (Figure 2.1b), and expression was confirmed via Western
 blotting (Figure 2.1b). β-sarcoglycan expression was accompanied by restoration of components of the DAPC (β-sarcoglycan and dystrophin) (Figure 2.1c). Using Evans blue dye (EBD) as a marker for membrane permeability [99, 100] we found all fibers expressing exogenous SGCB were protected from leakage and EBD inclusion (Figure 2.1d). Muscle from sgcb⁻/⁻ mice exhibit a severe muscular dystrophy with centrally nucleated fibers, frequent muscle fiber necrosis, fibrotic tissue, and significant fiber size variability represented by both atrophic and hypertrophic fibers [10, 60]. As seen in Figure 2.2a, hematoxylin & eosin staining shows an overall improvement in the dystrophic phenotype of diseased muscle including a reduction in central nuclei (sgcb⁻/⁻ untreated – 76.8 ± 2.3% vs. AAV.hSGCB treated – 38.86 ± 3.5%; p<0.0001) (Figure 2.2c). We also saw normalization of fiber size distribution, with an increase in the average fiber diameter following treatment (sgcb⁻/⁻ untreated – 32.6 ± 0.31 μm vs. AAV.hSGCB treated – 35.56 ± 0.22 μm; p<0.0001) (Figure 2.2d).

The histopathological hallmark of the scgb⁻/⁻ mouse is fibrosis characterized by widespread replacement of muscle tissue primarily with collagens along with other extracellular matrix components such as fibronectin, elastin, laminin, and decorin [61]. This replacement of muscle tissue by connective tissue challenges the potential value of gene replacement and may limit the degree of improvement [29]. To test this, we assayed the mice treated for 12 weeks for reduction in fibrosis. We specifically assessed the TA muscle since we established its inherent degree of fibrosis in the KO model and because it represents a potential target following vascular ILP gene delivery. Picrosirius red staining for collagen, types I and III, of TA muscles showed a significant reduction (52.74%) in the amount of collagen present within AAV.tMCK.hSGCB treated muscle compared to untreated sgcb⁻/⁻ mouse muscle (20.7 ± 0.57% vs. 43.8 ± 2.3%,
Untreated \(sgcb^{−/−}\) muscle from 5-week old mice at the age of injection had 24.05 ± 1.5% collagen deposition, indicating there was a slight (14.0%) reduction in the amount of collagen following the 12 weeks of treatment.

### 2.3.3. Functional correction in skeletal muscle following scAAVrh.74.tMCK.hSGCB gene transfer

To determine whether hSGCB gene transfer can improve muscle function, we assessed the functional properties of the TA muscle from \(sgcb^{−/−}\) mice treated with AAV.tMCK.hSCGB. Following intramuscular delivery of \(3 \times 10^{10}\) vg of scAAVrh.74.tMCK.hSCGB to the TA of 4-week old \(sgcb^{−/−}\) mice, 6 weeks post-treatment the TA muscles were subjected to in situ force measurements (n=4). Treated muscles were compared with untreated contralateral muscles and those from C57BL/6 WT mice. AAV.tMCK.hSCGB treated muscle showed significant improvement in both absolute tetanic force and normalized specific force (Figures 2.3a,b). Treated muscles had an average absolute force of 1436.9 ± 199.5 mN compared to 770.9 ± 118.3 mN for untreated \(sgcb^{−/−}\) controls (p<0.01). Similarly, treated TA muscles produced an average specific force of 254.01 ± 6.9 mN/mm\(^2\) and untreated muscles produced 124.2 ± 13.9 mN/mm\(^2\) of force (p<0.01). Finally, muscles treated with AAV.tMCK.hSCGB showed greater resistance to contraction-induced injury compared to the untreated control muscles (Figure 2.3c). Treated TA muscles lost 34.0 ± 5.1% of force from that produced after the first contraction whereas untreated diseased muscle lost 54.1 ± 3.8% (p<0.01) of force following the eccentric contraction protocol. These data show that hSGCB gene transfer does provide a functional benefit to diseased muscle deficient for SGCB.
2.3.4. Treatment of Aged Muscle with scAAVrh.74.tMCK.hSGCB

Studies of disease progression in this mouse model of LGMD2E have shown that although the most severe tissue remodeling in muscle occurs between 6-20 weeks, the histopathology of the muscle continues to worsen with age, resembling the disease progression in patients [10, 60, 61]. Consequently, to mimic a clinical setting where treatment would occur at an older age with more advanced muscle deterioration and endomysial fibrosis, we treated 6-month old sgcbr+ mice (n=5) intramuscularly in the TA with 3x10^{10} vg total dose of scAAVrh.74.tMCK.hSCGB. Following 12 weeks of treatment, at 9-months of age, 80.1 ± 4.8% of muscle fibers were transduced (Figure 2.4a). Picrosirius red stain for collagen types I and III showed a 42.2% reduction in the amount of collagen present in treated mice compared to untreated sgcbr− mouse muscle (AAV.hSGCB treated – 20.0 ± 0.80% vs. sgcbr− untreated – 34.6 ± 1.4%, p<0.0001) (Figures 2.4b,c). At the age of treatment, 6-month old sgcbr− mice have 30.8 ± 2.0% collagen deposition (n = 4, 4 male); thus these results indicate that AAV.tMCK.hSCGB treatment not only prevents, but has the potential to reverse existing fibrosis.

2.3.5. Isolated-Limb Perfusion of scAAVrh.74.tMCK.hSGCB in sgcbr− mice

The ability to target multiple muscles in one limb with little frequency allows for a more clinically relevant delivery method for translation to LGMD2E patients. As proof of principle, we treated 4-6 week old sgcbr− mice (n=7, 4 male, 3 female) by isolated-limb perfusion through the femoral artery beginning with 1x10^{11} vg total dose of scAAVrh.74.tMCK.hSGCB. Three months later the animals were sacrificed and treated muscles were analyzed for transgene expression as well as functional improvement. Treated muscles in the lower hindlimb showed only modest expression of hSGCB (30-
50%) but did not show functional improvement in specific force output or resistance to contraction induced injury (Figures 2.5a,b). The insufficient results from ILP treatment with a low dose of 1x10^{11} vg total dose led us to pursue the delivery of a higher dose of vector (5x10^{11} vg total dose). Delivery of 5x10^{11} vg total dose of scAAVrh.74.tMCK.hSGCB by ILP in 4-6 week old sgcb^{−/−} mice (n=9, 7 male, 2 female) was analyzed 2 months post gene transfer. β-sarcoglycan expression reached 91.8 ± 4.7% of fibers in the gastrocnemius (GAS) muscle and 90.6 ± 2.8% in TA (Figure 2.6a). ILP delivery of high dose AAV.tMCK.hSGCB resulted in significant protection from eccentric contraction induced injury (p<0.05) that was no different than WT, compared to untreated contralateral muscles (Figure 2.6c). Vascular delivery at this dose also restored muscle histopathological parameters (Figure 2.6b). Central nuclei were decreased in the TA (sgcb^{−/−} untreated – 76.9 ± 2.8% vs. AAV.hSGCB treated – 23.2 ± 5.7%, p<0.001) and GAS (sgcb^{−/−} untreated – 78.2 ± 2.4% vs. AAV.hSGCB treated – 16.8 ± 6.6%, p<0.001) (Figure 2.7a). Gene transfer also led to an increase in the average fiber size in the TA (sgcb^{−/−} untreated – 30.53 ± 0.52μm vs. AAV.hSGCB treated – 41.9 ± 0.46μm; p<0.0001) and GAS (sgcb^{−/−} untreated – 38.9 ± 0.37μm vs. AAV.hSGCB treated – 33.3 ± 0.44μm; p<0.0001), with normalization of fiber diameter distribution (Figure 2.7b). It is also important to note that through the use of immunohistochemical staining for immune cells, we found a substantial decrease (~60%) in the number of CD3 cells, CD4 cells, and macrophages (Table 2.1).

Picrosirius red staining of TA and GAS muscles also showed a significant reduction in the amount of collagen compared to untreated sgcb^{−/−} muscle following high dose vascular delivery (Figure 2.8a). Collagen levels in the TA were reduced to 21.6 ± 1.3% in treated muscle compared to 40.2 ± 1.5% in untreated sgcb^{−/−} mice at the age of
endpoint (p<0.0001) (Figure 2.8b). As indicated previously, sgcb<sup>−/−</sup> mice at the age of injection presented with 24.1 ± 1.5% collagen in TA muscle, indicating again a slight reduction (10.0%) in collagen deposition following eight weeks of treatment. Similarly, staining of the GAS muscle showed that treated mice had 22.9 ± 0.99% collagen compared to 37.9 ± 1.3% in untreated sgcb<sup>−/−</sup> mice at the endpoint (p<0.0001) (Figure 2.8b). Qualitative PCR was performed to detect collagen transcript levels in muscle, which correlate with the results of the Sirius red staining (Figure 2.8c). Taken together, these data show that AAV mediated delivery of hSGCB reduces muscle fibrosis, improves muscle function, and reverses dystrophic pathology of sgcb<sup>−/−</sup> diseased muscle.

2.3.6. Long-Term Therapeutic Efficacy of β-Sarcoglycan Gene Transfer

We have reported here the histological and functional benefits of both IM and ILP delivery of AAV.tMCK.hSGCB to diseased sgcb<sup>−/−</sup> muscle 6, 8 and 12 weeks post-injection. To better demonstrate clinical relevancy and further establish the ability of our gene therapy to be translated to LGMD2E patients, we analyzed sgcb<sup>−/−</sup> muscle treated for 6 months by ILP delivery and 12 months via IM injection. Six sgcb<sup>−/−</sup> mice were injected in the left hindlimb by ILP at 4-5 weeks of age again with 5×10<sup>11</sup> vg total dose of vector. We extended our treatment endpoint to 6 months post-injection and at this point muscles were analyzed for transgene expression. Immunofluorescence staining for hSGCB demonstrated 88.18 ± 6.44% hSGCB expression in the TA muscle and 89.58 ± 2.44% expression in GAS muscle (Figure 2.9a), indicating a remarkable ability to achieve sustained high levels of expression with no signs of any loss of expression over time.
Accompanying these high levels of transgene expression was a significant reversal of the dystrophic histopathology that develops in the muscle as the disease progresses. Demonstrated by H&E staining (Figure 2.9b), the average number of centrally nucleated fibers in \( sgcb^{-/-} \) TA muscle dropped from 70.45 ± 3.04% to 46.38 ± 4.26% (\( p<0.001 \)) following 6 months of treatment, and decreased in GAS muscle from 67.26 ± 1.81% to 36.75 ± 2.66% (\( p<0.0001 \)) in vector dosed muscle (Figures 2.10a,b). Furthermore, after treatment for 6 months general fiber size distribution was normalized similar to WT muscle in both the TA and GAS (Figures 2.10a,b). Fibers in both muscles were increased in size following gene transfer with the average fiber diameter in treated TA increasing from 27.97 ± 0.29 μm to 41.00 ± 0.21 μm (\( p<0.0001 \)) and in treated GAS increasing from 28.37 ± 0.23 μm to 35.75 ± 0.19 μm (\( p<0.0001 \)) (Figures 2.10a,b).

Picrosirius red staining of both the TA and the GAS in Figures 2.9b illustrates again the ability of our therapy to reduce collagen deposition even 6 months post-injection, indicating lower levels of fibrotic tissue following long-term treatment. Finally, we have noted the importance of providing a functional benefit to diseased \( sgcb^{-/-} \) muscle treated with AAV.tMCK.hSGCB. Supporting the sustained hSGCB transgene expression, using our \textit{in situ} TA physiology protocol we saw higher average absolute force outputs in treated \( sgcb^{-/-} \) muscle (1510.00 ± 109.8mN) compared to untreated \( sgcb^{-/-} \) muscle (408.1 ± 71.32mN) (\( p<0.0001 \)) as well as higher normalized specific force outputs in treated muscle (213.10 ± 16.65mN/mm$^2$) compared to untreated muscle (49.50 ± 7.65mN/mm$^2$) (\( p<0.0001 \)) (Figures 2.11a,b). Resistance to contraction-induced injury was also improved with treated muscles only losing 15.3 ± 2.94% of force following repetitive eccentric contractions whereas untreated \( sgcb^{-/-} \) muscle losing 24.10 ± 4.25% of force (Figure 2.11c).
As a final assessment of the long-term sustainability of hSGCB gene therapy, we extended our treatment endpoint to 12 months post-gene transfer. Six sgcb<sup>-/-</sup> mice were treated in the left TA by IM injection at 4-5 weeks of age with the standard dose used in all IM studies, 3x10<sup>10</sup> vg total dose of scAAVrh.74.tMCK.hSGCB. Impressively, we still detect 84.29 ± 6.72% hSGCB transgene expression in treated TA muscles 12 months post-injection (Figure 2.12a). Most significantly, this sustained expression of hSGCB following such a long-term treatment still provided functional recovery to the muscle. Absolute force outputs in treated TA muscle were restored to levels above those of WT muscle and significantly higher than untreated sgcb<sup>-/-</sup> (AAV.hSGCB treated – 1746.0 ± 97.99mN; sgcb<sup>-/-</sup> untreated – 923.8 ± 76.34mN, p<0.0001) (Figure 2.12b). Similar results were obtained when assessing specific force with treated muscles exhibiting 267.0 ± 12.51mN/mm<sup>2</sup> of force compared to 124.3 ± 11.30mN/mm<sup>2</sup> of force in untreated muscle (Figure 2.12b). Finally, while untreated sgcb<sup>-/-</sup> TA muscles at the age of 13-months lost 42.8 ± 7.34% of force following repetitive eccentric contractions, treated TA muscles only lost 21.30 ± 4.50% of force (Figure 2.12c). Taken together, the data presented here show the AAV vector cassette remains intact and transcriptionally active in transduced muscle cells and the therapeutic hSGCB WT protein is continuously expressed as far as 1 year post-injection. Moreover, all of the histopathological and functional benefits we see from this therapy are maintained with long-term treatment.

2.3.7. Safety and Biodistribution of rAAVrh.74.tMCK.hSGCB

Initially, normal WT mice injected with 3x10<sup>10</sup> vg total dose of scAAVrh.74.tMCK.hSGCB intramuscularly into the TA showed no signs of toxicity by H&E stain indicating no adverse effects due to the virus (data not shown). Following the
ILP vascular delivery of $5 \times 10^{11} \text{ vg}$ total dose of scAAVrh.74.tMCK.hSGCB as described previously, we next assessed the safety in a small group of mice in this cohort (n=4). We first analyzed histologically the targeted muscles with significant gene expression, as well as off target organs including heart, lung, liver, kidney, spleen, gonads, and diaphragm. An independent veterinary pathologist formally reviewed paraffin sections of treated tissues and reported no evidence of toxicity in any organ (Table 2.2). Protein expression and vector biodistribution were also assessed in all of the above tissues and organs with Western blotting and qPCR, respectively. Vector genome copies were detected in all organs tested; however we saw no protein expression in any sample other than treated muscle (Figure 2.13, Table 2.3). Finally, an analysis of wet weights of treated and untreated muscle show no significant difference or trend when comparing the average weights from either cohort (data not shown). These data provide evidence that the muscle specific tMCK promoter restricted expression to skeletal muscle and the vector is non-toxic.

### 2.3.8. Formal GLP Safety and Toxicity Study of IM and ILP Delivery of scAAVrh.74.tMCK.hSGCB

In order to translate AAV.tMCK.hSGCB therapy to the clinical, we performed an FDA guided GLP toxicology study to assess the safety of scAAVrh74.tMCK.hSGCB treatment in healthy WT mice. Vector was delivered to different cohorts of male and female C57BL/6 mice by IM injection to the TA at $2 \times 10^{11}$ vg total dose or by ILP delivery to the hindlimb at either $4 \times 10^{10}$ vg total dose or $4 \times 10^{11}$ vg total dose. Control animals were injected with Lactated Ringer’s Solution (LRS). Animals were assigned to one of five treatment groups with scheduled endpoints for terminal necropsy and tissue
collection at 6- and 12-weeks post-injection. In-life parameter and endpoint parameter analysis including body weights, clinical observations, clinical pathology (hematology and serum chemistry), anatomical histopathology, and whole blood quantitative real-time PCR (qPCR) were performed by an independent clinical research organization (CRO).

In summary, while scAAVrh74.tMCK.hSGCB transcript was not detected via qPCR in any tissues or blood from control LRS injected IM animals, vector was detected in all collected tissues and in blood from vector dosed mice as expected, however the highest levels were seen in TA and GAS muscles from the left (injected) side, and in liver and inguinal lymph nodes. Ultimately, no test-article related clinical observations, anatomical histopathology, or changes in the hematology and serum chemistry parameters were noted by the CRO throughout the study or at the 6- or 12-week endpoints.

My involvement in this study involved in-life and endpoint parameter analyses of AAVrh.74 and hSGCB transgene binding antibody titers via enzyme-linked immuno-sorbant assay (ELISA) and hSGCB transgene protein expression in muscle and non-muscle tissue via Western blotting. Circulating antibody titers to AAVrh74 capsid and hSGCB were determined in serum samples at 4 and/or 10 weeks post-ILP, and at 6 and/or 12 weeks post IM treatment (Table 2.4). Mice treated at all three vector doses had circulating antibodies to AAVrh.74 capsid as expected; titers were elevated at 4 or 6 weeks post vector administration and remained elevated at 10 or 12 weeks post administration, regardless of route or dose (Table 2.4). In contrast, no animals had circulating antibodies to hSGCB, based on negative antibody titers at 4 and 6 weeks post vector administration (Table 2.4).

TA muscles from vector dosed IM animals were positive for hSGCB expression while LRS control animals were negative (Figure 2.14a). Contralateral TA, GAS, heart
and diaphragm were not observed to have significant expression above endogenous background expression (Figures 2.14a,b). Liver, kidney, lung, spleen, gonads, pancreas, stomach, spinal cord and brain samples that tested positive by qPCR in the IM vector dosed group had no significant hSGCB expression above endogenous levels (Figure 2.14b). Together, these results indicate there was no significant off-target expression of hSGCB at the 6-week timepoint following IM delivery. Overall, the results from this study provide a No Observed Adverse Effect Level (NOAEL) of a single IM or ILP injection of scAAVrh.74.tMCK.hSGCB at multiple doses taken out to a maximum of 12 weeks post-treatment.

2.4. Discussion

An emerging form of therapy for LGMD2E is viral-mediated gene-delivery to restore WT protein to affected muscle resulting in restoration of muscle function. Considering that a subset of patients can develop cardiomyopathy [13, 20-22], this would have to be considered in the long-term care of these patients. In previous reports the sgcb⁻/⁻ mouse was well characterized. In 1999, Araishi et. al. developed the SGCB-deficient mouse with accompanying loss of all of the SGs as well as sarcospan, with at least minor preservation of merosin, the dystroglycans and dystrophin, reproducing the clinical picture seen in LGMD2E [60]. The sgcb⁻/⁻ mouse model used in our study was developed by Durbeej et. al. who reported similar characteristics to the previous model and ones that again accurately portrayed the disease phenotype of LGMD2E [10]. The histological changes in this animal model were also a prototype for the clinical counterpart, including the prominence of skeletal muscle fibrosis [61].
In a later publication in 2002, Dressman et. al. injected the TA muscle using rAAV2.CMV.SGCB. Expression persisted for 21 months and muscle fibers were protected from recurrent necrosis [58]. This was a singular study showing the potential for gene therapy in LGMD2E. This important study provided the foundation for moving forward with additional serotypes, like AAVrh.74, that are less likely to induce an immune response based on prior environmental exposure. Other additions to the gene therapy repertoire since the original report include use of self-complementary AAV to enhance transgene expression [92], a muscle specific promoter to better target skeletal muscle[59, 96], and the optimization of a human β-sarcoglycan gene (hSGCB) as an advantage for clinical trial.

In the current study, delivery of scAAVrh74.tmCK.hSGCB provided the tools to address multiple issues and whether gene replacement alone could reverse muscle fiber fibrosis. The AAVrh.74 serotype was chosen to enhance clinical efficacy through vascular delivery using ILP [93-95]. After the initial demonstration of biopotency by IM gene delivery, we showed that comparable or even better restoration of hSGCB could be achieved with vascular delivery by ILP. The efficacy was also striking with the reversal of dystrophic features including fewer degenerating fibers, reduced inflammation, and improved functional recovery by protection against eccentric contraction with increased force generation. From a clinical perspective, the most important novel finding in this study was the reversal of fibrosis from gene replacement alone. At the time of clinical gene transfer fibrosis could be a major obstacle to success. We previously reported that the degree of fibrosis can block efficacy following follistatin gene therapy and correlated with lack of improvement in the distance walked in the 6MWT [29]. Successful gene transfer to Sgcb-null mouse resulting in correction of the disease state with reduction in
fibrosis provides a pathway to the clinic for AAV-mediated hSGCB transfer for LGMD2E patients.

2.5. Materials and Methods

2.5.1. Animal Models

All procedures were approved by The Research Institute at Nationwide Children's Hospital Institutional Animal Care and Use Committee (protocol AR12-00040). B6.129-Sgcb<sup>tm1Kcam/1J</sup> heterozygous mice were purchased from the Jackson Laboratory (Strain # 006832). Sgcb<sup>-/-</sup> mice were generated by breeding heterozygous mice. KO mice were bred and maintained as homozygous animals in standardized conditions in the Animal Resources Core at the Research Institute at Nationwide Children's Hospital. Mice were maintained on Teklad Global Rodent Diet (3.8% fiber, 18.8% protein, 5% fat chow) with a 12:12 hr dark:light cycle. Identification of SGCB<sup>-/-</sup> mice was performed by genotyping using PCR. All animals were housed in standard mouse cages with food and water ad libitum.

2.5.2. Beta-sarcoglycan Gene Construction

The full-length human beta-sarcoglycan cDNA (GenBank Accession No. NM_0034994.3) was codon optimized and synthesized by GenScript Inc, Piscataway, NJ. Codon optimization through GenScript uses an algorithm that takes into account parameters that include transcription, mRNA processing and stability, translation, and protein folding to design a cDNA sequence that result in maximum expression in muscle tissue(www.genscript.com). The cDNA was then cloned into a plasmid containing AAV2
ITRs and the cassette included a consensus Kozak sequence (CCACC), an SV40 chimeric intron, and a synthetic polyadenylation site (53 bp). The recombinant tMCK promoter was a gift from Dr. Xiao Xiao (University of North Carolina). It is a modification of the previously described CK6 promoter[101] and includes a modification in the enhancer upstream of the promoter region containing transcription factor binding sites. The enhancer is composed of 2 E-boxes (right and left). The tMCK promoter modification includes a mutation converting the left E-box to a right E-box (2R modification) and a 6 bp insertion (S5 modification). The pAAV.tMCK.hSGCB vector was constructed by ligation of 1040 bp KpnI/XbaI fragment from pUC57-BSG (Genscript Inc., Piscataway, NJ) into the KpnI/XbaI sites of pAAV.tMCK.hSGCA[59].

2.5.3. rAAV Production

A modified cross-packaging approach which we have previously reported [95] was used to produce the rAAV vector. Here, a triple transfection method with CaPO₄ precipitation in HEK293 cells allows for AAV2 ITRs to be packaged into a different AAV capsid serotype [102, 103]. The production plasmids were: (i) pAAV.tMCK.hSGCB, (ii) rep2-caprh.74 modified AAV helper plasmids encoding cap serotype 8-like isolate rh.74, and (iii) an adenovirus type 5 helper plasmid (pAdhelper) expressing adenovirus E2A, E4 ORF6, and VA I/II RNA genes. Vectors were purified and encapsidated vector genome (vg) titer (utilizing a Prism 7500 Taqman detector system (PE Applied Biosystems, Carlsbad, CA)) was determined as previously described[104]. The primer and fluorescent probe targeted the tMCK promoter and were as follows: tMCK forward primer, 5-ACC CGA GAT GCC TGG TTA TAA TT-3; tMCK reverse primer, 5-TCC ATG
GTG TAC AGA GCC TAA GAC-3; and tMCK probe, 5'- FAM-CTG CTG CCT GAG CCT GAG CGG TTA C-TAMRA-3.

2.5.4. Gene Delivery

For IM injection, mice were anesthetized and maintained under 1-4% Isoflurane (in O$_2$). The anterior compartment of the lower left limb of 4-6 week old $sgcb^{-/-}$ mice was cleaned with 95% EtOH then the tibialis anterior (TA) was injected with $3 \times 10^{11}$ vg total dose of scAAVrh.74.tMCK.hSGCB diluted in saline in a 30μL volume using a 30 gauge ultra-fine insulin syringe. The contralateral muscle was left untreated to serve as a control. TA muscle from both limbs was removed at either 6 (n=9, 4 male, 5 female) or 12 (n=6, 4 male, 2 female) weeks post-injection to assess gene transfer efficiency. In experiments involving 6-month old mice (n=5, 5 male), treatment consisted of IM injection into the LTA with $3 \times 10^{11}$ vg total dose scAAVrh.74.tMCK.hSGCB. For ILP experiments, $sgcb^{-/-}$ mice were perfused at four (n=5, 5 male) and five (n=4, 2 male, 2 female) weeks of age with $5 \times 10^{11}$ vg of scAAVrh.74.tMCK.hSGCB by injection into the femoral artery as previously described[95]. Animals were euthanized and muscles were analyzed 8 weeks post gene transfer.

2.5.6. EDL Force generation and protection from eccentric contractions

A physiological analysis of the EDL muscles from mice treated by ILP was performed. The EDL muscle from both lower hind limbs of treated mice were dissected at the tendons and subjected to a physiology protocol to assess function that was previously described by our lab and others[95, 105] with some adaptations. During the
eccentric contraction protocol, a 5% stretch-re-lengthening procedure executed between 500 and 700 ms (5% stretch over 100 ms, followed by return to optimal length in 100 ms). Following the tetanus and eccentric contraction protocol, the muscle was removed, wet-weighed, mounted on chuck using gum tragacanth, and then frozen in methyl-butane cooled in liquid nitrogen.

2.5.7. TA Force generation and protection from eccentric contractions

A protocol to assess functional outcomes in the TA muscle was performed on muscles extracted from mice treated by IM injection. This TA procedure is outlined in several previous studies [106, 107]. After the eccentric contractions, the mice were then euthanized and the TA muscle was dissected out, weighed and frozen for analysis. Analysis of the data was performed blindly but not randomly.

2.5.8. Immunofluorescence

Cryostat sections (12µm) were incubated with a monoclonal hSGCB primary antibody (Leica Biosystems, New Castle, UK; Cat. No. NCL-L-b-SARC) at a dilution of 1:50 in a block buffer (1x TBS, 10% Goat Serum, 0.1% Tween) for 1 hour at room temperature in a wet chamber. Sections were then washed with TBS three times, each for 20 mins and re-blocked for 30 mins. AlexaFluor 594 conjugated goat anti-mouse secondary IgG1 antibody (Life Technologies, Grand Island, NY; Cat. No. A21125) was applied at a 1:250 dilution for 45 mins. Sections were washed in TBS 3 times for 20 mins and mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA). Four random 20X images covering the four different quadrants of the muscle
section were taken using a Zeiss AxioCam MRC5 camera. Percentage of fibers positive for hSGCB staining (>50% of muscle membrane staining intensity) was determined for each image and averaged for each muscle.

2.5.9. Western Blot Analysis

Tissue sections from the left treated TA muscle and the right contralateral TA muscle (20-20 micron thick) were collected into a micro-centrifuge and homogenized with 100μl homogenization buffer (125mM Tris-HCl, 4% SDS, 4M urea) in the presence of 1 protease inhibitor cocktail tablet (Roche, Indianapolis, IN). After homogenization, the samples were centrifuged at 10,000 rpm for 10 mins at 4°C. Protein was quantified on NanoDrop (Thermo Scientific, Waltham, MA). Protein samples (20μg) were electrophoresed on a 3-8% polyacrylamide Tris-acetate gel (NuPage, Invitrogen, Carlsbad, CA) for 1hr 5 min at 150 V and then transferred to a PVDF membrane (Amersham Biosciences, Piscataway, NJ) for 1hr 15min at 35 V. The membrane was blocked in 5% nonfat dry milk in TBST for 1hr, and then incubated in a 1:100 dilution of a polyclonal hSGCB antibody (Novus Biologicals, Littleton,CO; Cat. No. NBP-1-90300) and a 1:5000 of a monoclonal mouse gamma-tubulin antibody (Sigma Aldrich, St. Louis, MO; Cat. No. T6557). Anti-mouse (Millipore, Billerica, MA Cat. No. AP308P) and anti-rabbit (Life Technologies, Grand Island, NY; Cat. No. 656120) secondary- HRP antibodies were used for ECL immunodetection.
2.5.10. Evans Blue Dye Assay

A dose of 3x10^{10} vg total dose of scAAVrh.74.tMCK.hSGCB was delivered to 4-week old sgcb^-/- mice to the left TA through an IM injection. Four weeks post injection mice were injected in the intraperitoneal cavity on the right side at 5µl/g body weight of a filter sterilized 10 mg/ml Evans Blue Dye in 1x phosphate buffer solution. Mice were then sacrificed 24 hrs post injection and tissues were harvested and sectioned. Sections were fixed in cold acetone for 10 mins and then the immunofluorescence protocol was used to stain for hSGCB.

2.5.11. Morphometric Analysis

Muscle fiber diameters and percentage of myofibers with centrally located nuclei were determined from TA and GAS muscles stained with hematoxylin and eosin (H&E). Four random 20X images per section per animal were taken with a Zeiss AxioCam MRc5 camera. Centrally nucleated fibers were quantified using the NIH ImageJ software. Fiber diameters were measured as the shortest diameter through the muscle fiber using Zeiss Axiovision LE4 software.

2.5.12. Biodistribution qPCR Analysis

Taqman quantitative PCR was performed to quantify the number of vector genome copies present in targeted and untargeted contralateral muscle as well as nontargeted organs as previously described [94, 104]. A vector-specific primer probe set was used to amplify a sequence of the intronic region directly downstream from the tMCK promoter that is unique and located within the scAAVrh.74.tMCK.hSGCB
transgene cassette. The following primers and probe were used in this study: tMCK intron Forward Primer 5’-GTG AGG CAC TGG GCA GGT AA -3’; tMCK intron Reverse Primer 5’- ACC TGT GGA GAG AAA GGC AAA G -3’; and tMCK intron Probe 5’-6FAM-ATC AAG GTT ACA AGA CAG-GTT TAA GGA GAC CAA TAG AAA-tamra-3’ (IDT). Copy number is reported as vector genomes per microgram of genomic DNA.

2.5.13. qRT-PCR Collagen 1A Transcript Quantification

Qualitative RT-PCR (qRT-PCR) was performed to analyze the levels of collagen 1A (col 1A) transcript in BL6 WT TA muscle, uninjected contralateral RTA sgcb⁻/⁻ muscle, and ILP vector dosed LTA sgcb⁻/⁻ muscle (n=3 per group). Total RNA was extracted from each muscle using the Trizol RNA extraction kit (Invitrogen). Expression of col 1A mRNA was determined by the mRNA-specific Taqman microRNA assay kit (Life Technologies) using the ABI PRISM 7500 Detection System with GAPDH serving as a standard for normalization.

2.5.14. Immunohistochemistry for Immune Cell Staining

Immunohistochemistry was used to identify immune cells. Frozen tissue sections on Fisherbrand Superfrost charged microscope slides were incubated with rat anti-mouse monoclonal antibodies using an anti-rat Ig HRP Detection kit (BD Pharmagen Cat:551013): CD3 (Cat:555273), CD4 (Cat:550280), CD8 (Cat:550281), and Mac-3 for macrophages (Cat:550292). All primary antibodies were diluted at 1:20 with phosphate buffered saline. Positive immune staining was visualized using DAB chromagen diluted in DAB buffer with Streptavidin-HRP peroxidase extastain ABC Peroxidase. Ten
random 40X images were taken for each muscle and each corresponding stain. The number of mononuclear cells were counted and expressed as total number per mm$^2$.

2.5.15. Picrosirius Red Stain and Collagen Quantification

Frozen sections placed onto Fisherbrand Superfrost charged microscope slides were fixed in 10% Neutral Buffered Formalin for 5 min, then rinsed in distilled water. Slides were then incubated in Solution A (Phosphomolydbic acid) from the Picrosirius Red Stain Kit (Polysciences Inc. Catalog # 24901) for 2 min. After a thorough rinse in distilled water, the slides were placed in Solution B (Direct Red 80/2 4 6-Trinitrophenol) for 15 min, followed by an additional rinse in distilled water and then incubation in Solution C (0.1N Hydrochloride Acid) for 2 min. Slides were counterstained for 2.5 min with 1% Fast Green in 1% Glacial Acetic Acid from Poly Scientific (Catalog #S2114) using a 1:10 dilution in DI water. Finally, the slides were rinsed again in distilled water, dehydrated in graded ethanol, cleared in xylene and mounted with coverslips using Cytoseal 60 media from Thermo-Scientific (Cat#8310). Images were taken using the AxioVision 4.9.1 software. For analysis of Sirius red staining and % collagen quantification, the contrast between the red and the green colors were enhanced using Adobe Photoshop. The color deconvolution plugin in the ImageJ software program was selected and the RGB color deconvolution option was used. The Red image includes all connective tissue from the Sirius Red stain. The Green image includes all muscle from the Fast Green counterstain. Only the Red image and the original image were used. A threshold was then applied to the images to obtain black and white images with areas positive for collagen in black and negative areas in white. Using the measure function, the area of collagen was calculated. The total tissue area was then determined by
converting the originally image to “8-bit” and adjusting the threshold to 254, which will be one unit below completely saturating the image. The total tissue area was then measured as done previously and total area was recorded. The percentage of collagen was then calculated by dividing the area of collagen by the total tissue area. The mean percentage for each individual was calculated and reported.
2.7. Tables

<table>
<thead>
<tr>
<th>Cell type</th>
<th>Treated LTA cells/mm²</th>
<th>Untreated RTA cells/mm²</th>
<th>Uninjected SGCB-/- TA cells/mm²</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD3</td>
<td>15.6 ± 3.2</td>
<td>37.85 ± 6.2</td>
<td>29.8 ± 1.7</td>
</tr>
<tr>
<td>CD4</td>
<td>20.9 ± 4.7</td>
<td>58.1 ± 2.9</td>
<td>49.0 ± 0.8</td>
</tr>
<tr>
<td>CD8</td>
<td>8.2 ± 1.8</td>
<td>12.7 ± 2.4</td>
<td>15.5 ± 5.8</td>
</tr>
<tr>
<td>Macrophage</td>
<td>28.2 ± 5.0</td>
<td>75.2 ± 5.6</td>
<td>100.2 ± 5.9</td>
</tr>
</tbody>
</table>

Table 2.1. Quantification of immune cells present in uninjected SGCB-/- mice, and scAAVrh.74.tMCK.hSGCB treated and untreated muscle. Data shown is following ILP delivery of virus and represents the mean number of cells/mm² ± SEM, n=8 per group. A ONE-WAY ANOVA was used to compare values from the three different cohorts. Levels of immune cells were decreased with a statistically significant difference (p<0.01) between the treated LTA and untreated RTA and/or the treated LTA and uninjected TA in all stains except for CD8.

<table>
<thead>
<tr>
<th>Test Article (vector)</th>
<th>Animal ID (eartag #s)</th>
<th>Group Endpoint</th>
<th>Dose</th>
<th>Full Necropsy</th>
<th>Formal Histopath*</th>
</tr>
</thead>
<tbody>
<tr>
<td>scAAVrh.74.tMCK.hSGCB</td>
<td>811</td>
<td>2 months</td>
<td>5 x 10¹¹ vg</td>
<td>Y</td>
<td>NP</td>
</tr>
<tr>
<td></td>
<td>812</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>813</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>814</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Table 2.2. Safety study: Isolated-Limb Perfusion of scAAVrh.74.tMCK.hSGCB in sgcb +/- mice. NP= no pathology, *Histopathologic evaluation completed by Certified Veterinary Pathologist. 4 animals analyzed.
Table 2.3. Quantification of hSGCB vector genome copies present in all offsite organs and tissues. Tissues were harvested at necropsy to assess biodistribution of vector genome (vg) copies using Taqman qPCR. Vector specific primer probe sets were utilized. Shown is the average copy number in each organ ± SEM.
Table 2.4. Analysis of circulating antibody titers for IM & ILP delivery of scAAVrh.74.tMCK.hSGCB. ELISAs were performed on serum samples from control lactated ringer's solution (LRS) or AAV.tMCK.hSGCB dosed mice at either 6- or 12-weeks post-IM delivery and either 4- or 10-weeks post-ILP delivery. Titers listed are the positive endpoint serum dilutions yielding a sample to background ratio of absorbance readings of greater than or equal to 2. Samples testing <1:50 are considered negative for circulating antibodies.

<table>
<thead>
<tr>
<th>Binding Antibody ELISA</th>
<th>Test-Article</th>
<th>Delivery Route</th>
<th>Dose Level (vg/animal)</th>
<th>Time Point</th>
<th>Median Titer</th>
<th>Titer Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAVrh74</td>
<td>LRS</td>
<td>IM</td>
<td>0</td>
<td>6 weeks</td>
<td>&lt;1:50</td>
<td>&lt;1:50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12 weeks</td>
<td>&lt;1:50</td>
<td>&lt;1:50</td>
</tr>
<tr>
<td>AAVrh74</td>
<td>AAV.hSGCB</td>
<td>IM</td>
<td>2x10^{11}</td>
<td>6 weeks</td>
<td>1:3200</td>
<td>1:1600-1:12800</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>12 weeks</td>
<td>1:3200-1:6400</td>
<td>1:1600-1:25600</td>
</tr>
<tr>
<td>AAVrh74</td>
<td>LRS</td>
<td>ILP</td>
<td>0</td>
<td>4 weeks</td>
<td>&lt;1:50</td>
<td>&lt;1:50</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 weeks</td>
<td>&lt;1:50</td>
<td>&lt;1:50</td>
</tr>
<tr>
<td>AAVrh74</td>
<td>AAV.hSGCB</td>
<td>ILP</td>
<td>4x10^{10}</td>
<td>4 weeks</td>
<td>1:1600</td>
<td>1:400-1:1600</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 weeks</td>
<td>1:1600</td>
<td>1:400-1:1600</td>
</tr>
<tr>
<td>AAVrh74</td>
<td>AAV.hSGCB</td>
<td>ILP</td>
<td>4x10^{11}</td>
<td>4 weeks</td>
<td>1:6400</td>
<td>1:800-1:25600</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>10 weeks</td>
<td>1:6400</td>
<td>1:1600-1:25600</td>
</tr>
<tr>
<td>SGCB</td>
<td>LRS</td>
<td>IM</td>
<td>0</td>
<td>6 weeks</td>
<td>&lt;1:25</td>
<td>&lt;1:25</td>
</tr>
<tr>
<td>SGCB</td>
<td>AAV.hSGCB</td>
<td>IM</td>
<td>2x10^{11}</td>
<td>6 weeks</td>
<td>&lt;1:25</td>
<td>&lt;1:25</td>
</tr>
<tr>
<td>SGCB</td>
<td>LRS</td>
<td>ILP</td>
<td>0</td>
<td>4 weeks</td>
<td>&lt;1:25</td>
<td>&lt;1:25</td>
</tr>
<tr>
<td>SGCB</td>
<td>AAV.hSGCB</td>
<td>ILP</td>
<td>4x10^{11}</td>
<td>4 weeks</td>
<td>&lt;1:25</td>
<td>&lt;1:25</td>
</tr>
</tbody>
</table>
2.6. Figures

Figure 2.1. AAV mediated β-sarcoglycan expression restores dystrophin associated proteins and protects membrane integrity. (a) Self-complementary AAV vector containing the codon-optimized human β-sarcoglycan gene (hSGCB) driven by the muscle specific tMCK promoter. The cassette also contains a chimeric intron to augment processing and polyadenylation signal for stability. (b) Immunofluorescence staining with anti-β-SG antibody shows high levels of sarcolemmal staining of the SGCB transgene in five week old mice both 6 and 12 weeks post-injection. 20X images shown. Percentage of fibers expressing beta-sarcoglycan per TA muscle averaged 88.4 ± 4.2% after 6 weeks (n=9, 4 male, 5 female) and 76.5 ± 5.8% after 12 weeks (n=6, 4 male, 2 female). Protein expression confirmed in the Western blot with gamma-tubulin blot shown for a loading control. (c) AAV delivery of β-sarcoglycan leads to restoration of other members of the sarcoglycan complex; α-sarcoglycan, dystrophin. 20X images. (d) scAAVrh.74.hSGCB protects sgcb<sup>−/−</sup> membranes from damage. Image showing a large area of Evans blue positive fibers (red) juxtaposed to a cluster of β-sarcoglycan positive fibers that have been protected from Evans blue dye incorporation. 40X image is shown.
Figure 2.2. Histological analysis of β-SG deficient treated skeletal muscle. scAAVrh.74.hSGCB treatment normalizes histological parameters of sgcb<sup>−/−</sup> mice. Hematoxylin & Eosin staining and Picrosirius Red staining was performed on TA muscle from sgcb<sup>−/−</sup> mice along with normal control C57/BL6 mice and scAAVrh.74.hSGCB treated mice followed by quantification of histological parameters and % collagen staining. (a) H&E staining shows presence of centrally nucleated fibers, inflammatory cells, and large fiber diameter distribution in β-SG deficient muscle and an improvement in histopathology following gene transfer. (b) Picrosirius Red staining shows a decrease in red collagen staining in treated muscle. (c) Quantification of centrally nucleated fibers showing decrease following treatment (p<0.0001, ONE-WAY ANOVA) and (d) representation of fiber size distribution and increase in average fiber size of TA muscle from C57/BL6 controls and sgcb<sup>−/−</sup> mice compared to treated mice (p<0.0001, ONE-WAY ANOVA). (e) Quantification of % collagen in TA muscle from C57/BL6 controls and sgcb<sup>−/−</sup> mice compared to sgcb<sup>−/−</sup> treated mice (p<0.0001, ONE-WAY ANOVA). 100μm scale bar shown for 20X images.
Figure 2.3. scAAVrh.74.hSGCB intramuscular delivery corrects tetanic force and resistance to contraction-induced injury. The TA muscle of sgcb⁻/⁻ mice treated with 3x10¹⁰ vg of scAAVrh.74.hSGCB via an IM injection was harvested 6 weeks post gene transfer, and subjected to a protocol to assess tetanic force and an eccentric contraction protocol to assess resistance to contraction-induced injury. (a) AAVrh.74.hSGCB treated TA’s demonstrated significant improvement in both absolute tetanic force (p< 0.01, paired t-test) and (b) normalized specific force (p<0.05, paired t-test) which was not different from wild-type force (C57/BL6). (c) AAVrh.74.hSGCB treated TA’s exhibited significant improvement in resistance to contraction-induced injury compared to untreated sgcb⁻/⁻ controls (p<0.01, TWO-WAY ANOVA). Force retention following ten contractions is shown.
Figure 2.4. Analysis of Aged Mice Treated Intramuscularly with scAAVrh.74.tMCK.hSGCB.
(a) Immunofluorescence staining of TA muscle from 6 month old treated sgcb<sup>−/−</sup> mice 12 weeks post-injection (n=5, 5 male) shows sarcolemmal expression of the SGCB transgene at levels averaging 80% in injected mice compared to untreated (n = 4, 4 male).  (b) Picrosirius red staining of the treated and untreated TA muscle.  (c) Quantitation of collagen present in the Picrosirius red stained tissue shows a significant reduction in the amount of collagen following treatment with rAAVrh.74.tMCK.hSGCB (p<0.0001, ONE-WAY ANOVA).  100μm scale bar shown for 20X images.
Figure 2.5. Isolated-limb perfusion of low dose scAAVrh.74.hSGCB. Four week old β-SG deficient mice were treated with vector via the femoral artery to deliver the vector to the lower limb muscles at a dose of 1e11 vg. (a) At this low dose, β-SG expression ranged from 30-50% of muscle fibers among different mice that resulted in (b) no significant improvement in specific force compared to untreated animals or (c) protection from contraction induced injury. The left two panels show β-SG protein expression in two different mice. Muscle from a β-SG KO untreated mouse is shown for comparison. 40X images are shown.
Figure 2.6. Vascular delivery of high dose scAAVrh.74.hSGCB. Four (n=5, 5 male) and five (n=4, 2 male, 2 female) weeks β-SG deficient mice were treated with vector via the femoral artery to deliver the vector to the lower limb muscles. At a dose of 5x10^{11} vg, β-SG expression was 90.6 ± 2.8% in the TA and 91.8 ± 4.7% in the GAS of treated mice accompanied by improvements in histopathology that resulted in significant improvement in specific force compared to untreated animals even following an injury paradigm. (a) β-SG protein expression from three representative mice. Muscle from a β-SG KO untreated mouse is shown for comparison in the inset (lower right). 20X Images are shown. Expression in treated muscles confirmed via Western blot and gamma-tubulin is shown as a loading control. (b) Histopathology is significantly improved following high dose treatment. Upper panels- treated TA and gastrocnemius muscles. Bottom panels- untreated β-SG deficient control muscle. 100μm scale bar shown for 20X images. (c) Percentage of specific force retained in EDL muscle following 10 cycles of eccentric contraction induced injury. Treatment with 5x10^{11} vg of AAVrh.74.hSGCB led to significant improvement in force that was equivalent to WT (normal) control muscle (p<0.001, ONE-WAY ANOVA).
Histological Analysis of Vascular Delivery of High Dose rAAVrh.74.tMCK.hSGCB. Histopathology of TA and GAS muscle from sgcb−/− mice was assessed following ILP delivery of 5x10^{11} vg rAAVrh.74.tMCK.hSGCB using hematoxylin & eosin staining (Figure 2.6b) followed by quantification of histological parameters. (a) Quantification of central nuclei showed significant decrease in treated muscles compared to untreated controls (p<0.001) and (b) representation of fiber size distribution of TA and GAS muscles from SGCB−/− mice compared to C57/BL6 controls and treated mice.

![Graphs showing histological analysis](image)
Figure 2.8. Reduction of fibrosis in ILP treated β-SG KO mice. (a) Picrosirius red staining shows reduced fibrosis in treated mice indicated by a decrease in collagen deposition compared to untreated sgcb<sup>−/−</sup> mice. (b) Quantification of collagen levels in the TA and GAS muscles from BL6 WT, untreated sgcb<sup>−/−</sup> mice, and treated mice confirm reduction in collagen levels in treated mice (p<0.0001, ONE-WAY ANOVA). 100μm scale bar shown for 20X images. (c) Qualitative RT-PCR was used to detect and quantify the number of collagen 1A transcripts present in BL6 WT TA muscle, uninjected sgcb<sup>−/−</sup> TA muscle, ILP treated LTA muscle, and contralateral RTA muscle (n=3 per group). Levels are represented relative to the internal standard GAPDH. A ONE-WAY ANOVA was performed indicating a significant difference between uninjected sgcb<sup>−/−</sup> muscle and AAV.tMCK.hSGCB treated muscle (p<0.05).
Figure 2.9. ILP delivery of scAAVrh.74.tMCK.hSGCB leads to long-term hSGCB expression and reversal of histopathology in β-SG KO muscle. Six sgcβ<sup>−/−</sup> mice were treated by ILP at 4-5-weeks of age with 5x10<sup>11</sup> vg total dose of AAV.tMCK.hSGCB and analyzed 6-months post-injection. (a) Representative immunofluorescence images of LTA and LGAS from vector dosed mice demonstrating 88.18 ± 6.44% and 89.58 ± 2.44% hSGCB expression, respectively. BL6 WT and untreated β-SG KO TA muscles included for strain specific controls. 20X images are shown. (b) Representative H&E and picrosirius red images of BL6 WT, untreated β-SG KO, and vector dosed β-SG KO TA and GAS muscle. Histological disruption of tissue in untreated β-SG KO mice (middle panel) and reversal of pathology in treated β-SG KO mice (right panel). 20X images are shown.
Figure 2.10. Quantification of histological parameters following long-term ILP treatment with scAAVrh.74.tMCK.hSGCB. Assessment of histopathology of TA and GAS muscle from sgcb−/− mice 6-months post-ILP delivery of 5x10^{11} vg total dose AAV.tMCK.hSGCB (n=6) using hematoxylin & eosin staining (Figure 2.9b). (a) Quantification of central nuclei showed significant decrease in treated TA muscles compared to untreated controls (p<0.001). Fiber size distribution in the TA was normalized and average fiber size was increased following treatment (p<0.001) (ONE-WAY ANOVA). (b) Central nuclei in GAS muscle was significantly decreased from untreated KO following treatment (p<0.0001). GAS fiber size distribution also normalized and treated muscles increased in size compared to untreated KO (p<0.0001) (ONE-WAY ANOVA).
Six-months after ILP delivery of 5x10^{11} vg total dose to 4-5 week old sgcb⁻/⁻ mice (n=6), the TA muscle was harvested and tetanic force outcomes and resistance to contraction-induced injury were assessed to demonstrate functional benefits from gene-transfer. A significant improvement from untreated sgcb⁻/⁻ muscle in both (a) absolute tetanic force (p< 0.0001, ONE-WAY ANOVA) and (b) normalized specific force (p<0.0001, ONE-WAY ANOVA) was seen in AAV.tMCK.hSGCB treated TA muscles. (c) Measurement of force retention following ten eccentric contractions demonstrates an improvement in resistance to injury in TA muscles treated with AAV.tMCK.hSGCB compared to untreated sgcb⁻/⁻ controls.
Long-term SGCB expression following IM delivery allows for functional recovery. Six sgcb<sup>−/−</sup> mice were treated at 4-5 weeks of age by IM injection of 3x10<sup>10</sup> vg total dose with scAAVrh.74.tMCK.hSGCB and analyzed 12-months post-injection. (a) Immunofluorescence staining showing high levels of hSGCB transgene expression at 84.29 ± 6.72% in treated TA muscles. Representative 20X images are shown along with control BL6 WT and β-SG KO TA muscles. The TA muscles of BL6 WT, untreated sgcb<sup>−/−</sup>, and 12-month AAV dosed sgcb<sup>−/−</sup> mice were harvested at necropsy at 13-months old and subjected to the tetanic force and eccentric contraction protocol to assess functional parameters. (b) AAV.tMCK.hSGCB treated TA’s demonstrated significant improvement in both absolute tetanic force (p<0.0001, ONE-WAY ANOVA) and normalized specific force (p<0.0001, ONE-WAY ANOVA), which were both not different from WT muscle. (c) AAV.tMCK.hSGCB treated TA’s were significantly more resistance to contraction-induced injury compared to untreated sgcb<sup>−/−</sup> controls (p<0.001, TWO-WAY ANOVA).
Figure 2.13. **Vector biodistribution and protein expression.** (a) Histogram of average distribution of vector in harvested tissues from ILP treated mice given in copies of transcript per microgram of DNA added to qPCR reaction. Left limb was treated. (b) No protein expression via western blot seen in off target organs.

Figure 2.14. **Beta-Sarcoglycan expression in 15 tissues from mice treated with lactated ringers solution (LRS) or scAAVrh.74.tMCK.hSGCB.** All bands are ~43 kDa, indicative of full length SGCB expression. (a) TA muscle lysates from 2 (LRS), or 3 (AAV.hSGCB) animals sacrificed 6-weeks post-treatment. (b) Tissue lysates from representative animals sacrificed 6-weeks post-treatment. 2 LRS controls were selected randomly, while 3 vector dose treated were selected on the basis of high viral genome qPCR titers.
Chapter 3: Systemic β-Sarcoglycan Gene Therapy for Treatment of Cardiac and Skeletal Muscle Deficits in LGMD2E

3.1. Abstract

Limb-girdle muscular dystrophy type 2E (LGMD2E), resulting from mutations in the β-sarcoglycan (SGCB) gene leads to a progressive dystrophy with deteriorating muscle function as well as respiratory failure and cardiomyopathy in 50% or more of LGMD2E patients. SGCB knockout (sgcb⁻/⁻) mice share many of the phenotypic deficiencies of LGMD2E patients. The purpose of this pre-clinical study report was to demonstrate efficacy of systemic delivery of AAV.MHCK7.hSGCB in treating skeletal and cardiac muscle deficits in sgcb⁻/⁻ mice. We initially designed a self-complementary AAVrh.74 vector containing a codon optimized human SGCB transgene driven by a muscle specific promoter (tMCK), and then established proof-of-principle efficacy of vector delivery by intramuscular (IM) injection and isolated-limb perfusion (ILP). Here, altered the vector cassette replacing the tMCK promoter with the MCHK7 promoter. We next delivered AAV.MHCK7.hSGCB through the tail vein of sgcb⁻/⁻ mice to provide a potential rationale for gene delivery in clinical trial that would lead to clinically meaningful results. This resulted in nearly 100% transgene expression in numerous muscles throughout the hindlimbs, forelimbs, torso, and the heart, which was accompanied by improvements in dystrophic muscle histopathology. Kyphoscoliosis of the spine was also partially reversed, and total ambulation increased in AAV.hSGCB treated mice by
~22% while hindlimb vertical rearing increased by ~77% in treated mice compared to KO. We also saw complete restoration of diaphragm function indicated by specific force output following treatment. Importantly, no hSGCB transgene expression was detected in non-muscle tissue and no adverse effects were seen in muscle of C57 BL/6 WT mice injected systemically with AAV.MHCK7.hSGCB. In this well-defined mode of LGMD2E, we have demonstrated that systemic delivery of AAV.MHCK7.hSGCB normalizes histologic and functional outcome measures in limb, diaphragm, and heart, and is nontoxic in the mice. These findings have established a path for AAV mediated gene therapy for LGMD2E that we are currently pursuing.

3.2. Introduction

The limb girdle muscular dystrophies (LGMDs) are one major class of genetic disorders affecting the musculoskeletal system and its corresponding molecular components and processes. The form of LGMD targeted in this study is an autosomal recessive disorder, LGMD2E, representing one of the most severe LGMDs with worldwide reports of incidence of 1/200,000-1/350,000 [3]. In this disease, mutations in the β-sarcoglycan (SGCB) gene lead to loss of functional protein with concurrent loss of other structural components of the sarcolemmal-stabilizing dystrophin-associated protein complex (DAPC) [8, 9]. The result is progressive decline in muscle function due to necrotic muscle fiber loss, enhanced inflammatory infiltration, and development of fibrotic tissue [10, 60].

LGMD2E patients exhibit typical yet often more severe characteristics of LGMD, with widespread progressive muscle wasting, initial pelvic and shoulder girdle weakness, degrees of proximal muscle weakness, and evidence of Gower’s maneuvers [8, 16-18].
The spectrum of clinical presentation is heterogeneous, however, in the more severe Duchenne Muscular Dystrophy (DMD)-like cases, symptoms commonly onset in early childhood with increasing difficulty in mobility and eventual loss of ambulation in the second decade of life [8, 16-18]. Due to extensive musculoskeletal involvement, reports in dystrophic mice and LGMD2E patients also indicate joint contractures and kyphoscoliosis (curvature) of the thoracic spine, which when combined with the degenerating diaphragm lead to compromised diaphragm and lung function [18, 108-111]. Most significantly, evolving studies have reported cardiac involvement in approximately 50% or more of LGMD2E cases. Cardiac dysfunction manifests in the form of cardiomyopathy (often dilated), resulting in impaired left and right ventricle function, heart rhythm abnormalities, and eventual myocardial hypertrophic remodeling [13, 18, 20-22]. Importantly, when considering a clinically effective therapy for patients, it is critical that the significant cardiac involvement that is often a key morbidity factor in β-sarcoglycanopathy be a major target of any therapeutic intervention.

The sgcb−/− murine model of LGMD2E completely absent for SGCB protein displays a severe dystrophic phenotype that worsens with age with frequent development of cardiomyopathy [10]. We and others have noted the severity of a histopathological hallmark of the scgb−/− mouse, fibrosis, evident by increased deposition of collagens and other extracellular matrix components (Chapter 2) [10, 61, 112]. Dystrophic histopathology in the myocardium and the development of cardiomyopathy have also been reported, with aged mice showing similar cardiac deficits seen in LGMD2E patients [10, 60, 113]. The recapitulation of LGMD2E disease phenotype particularly in the heart in sgcb−/− mice provides an ideal model to study systemic gene therapy. We previously reported (Chapter 2) [112] construction of a self-complementary
adeno-associated viral vector (scAAV) [91, 92] carrying full-length SGCB cDNA driven by a muscle specific promoter (tMCK) and packaged into the AAVrh.74 virus serotype for enhanced transduction via the vasculature [93, 94, 98].

Following the completion of local and regional gene delivery with this transgene cassette (Chapter 2) [112], we chose to alter our cassette to include the MHCK7 promoter that expresses well in heart and skeletal muscle (including diaphragm) [114], and use a systemic (intravenous) delivery approach to achieve correction of the skeletal and cardiac dysfunction in this disease. We demonstrate in this report efficacy of a systemic delivery of our scAAV.rh.74.MHCK7.hSGCB vector with no adverse effects in treated mice. Along with nearly complete transduction of skeletal and cardiac muscle, we report functional restoration in diaphragm muscle, partial improvement in cardiomyopathy symptoms, and significantly increased activity in vector dosed mice. With no current cure or treatment for beta-sarcoglycanopathy [89], systemic delivery has become a primary goal allowing for a comprehensive therapy for this disease. This has allowed clinical trials to progress efficiently past expectations of only safety and provides optimism for efficacy. As a result of the high incidence of cardiomyopathy in LGMD2E, we feel strongly that treatment of the heart in addition to skeletal muscle is vital for maximum long-term benefit, and these pre-clinical studies with a vector optimized for systemic therapy provide clear evidence for translation to LGMD2E patients.
3.3. Results

3.3.1 scAAVrh.74.MHCK7.hSGCB construction and vector potency

Our new therapeutic scAAVrh.74.MHCK7.hSGCB transgene cassette was constructed by replacing the tMCK promoter from our previously investigated vector (Chapter 2) [112] with the MHCK7 promoter to achieve enhanced cardiac expression [114] (Figure 3.1a). The nature of the transgene cassette and scAAVrh.74 vector used here provides an ideal delivery vehicle for a systemic therapy. Viral potency in muscle was established by intravenous injection into the tail vein of two Sgcb-null (sgcb−/−) mice at a dose of 1x10^{12} vg total dose (5x10^{13} vg/kg) followed by immunofluorescence analysis of muscles one month post-gene transfer. The amount of hSGCB transgene expression in five different limb skeletal muscles, tibialis anterior (TA), gastrocnemius (GAS), quadriceps (QUAD), psoas major (PSOAS), and triceps (TRI), from both the left and right side of the mice, was quantified along with the diaphragm. We used a qualitative analysis of heart tissue to assess the relative level of transgene expression in cardiac muscle upon delivery. Mice deficient for SGCB were completely absent of the protein when analyzed by immunofluorescence. Our therapeutic dose of 1x10^{12} vg total dose resulted in an average of 97.96 ± 0.36% (± SEM) vector transduction across all skeletal muscles including the diaphragm, and approximately 95% or greater in cardiac muscle (data not shown).
3.3.2. Long-term systemic delivery of scAAVrh.74.MHCK7.hSGCB in SGCB-/- mice

To further test the strong results of the one-month potency assay, we investigated long-term 6-month systemic delivery of the hSGCB transgene cassette to sgcb-/- mice. Four-to-five week old sgcb-/- mice were treated with 1x10^{12} vg total dose scAAVrh.74.MHCK7.hSGCB intravenously in the tail vein (n=5). Mice were necropsied 6 months post-injection and hSGCB transgene expression was demonstrated using immunofluorescence in six skeletal muscles, both left and right, in addition to the diaphragm and heart of all treated mice. Skeletal muscles analyzed included the TA, GAS, QUAD, gluteal (GLUT), PSOAS, and TRI. Average hSGCB expression resulting from systemic delivery in treated mice was 98.13 ± 0.31% (± SEM) across all skeletal muscles including the diaphragm, with expression in the heart exceeding at least 95%, and representative images are shown in Figure 3.1b. The expression levels in each individual muscle type averaged from all treated mice are shown in Table 3.1 and western blotting in Figure 3.1c confirmed transgene expression in all muscles. Quantification of hSGCB transgene expression in hearts from treated mice via western blotting and densitometry indicated overexpression of hSGCB up to 72.0% above BL/6 WT levels of expression (Figure 3.1d), correlating to the high levels quantified in skeletal muscle.

An important characteristic of sgcb-/- muscle described in previous reports [10, 60] and illustrated by the hematoxylin & eosin (H&E) staining of the GAS and diaphragm in Figure 3.2a is the severe dystrophic pathology present with central nucleation, necrosis, inflammatory infiltration, and fibrosis. Treatment with our vector significantly improved this pathology, alleviating many of these dystrophic features and restoring
diseased muscle to a more normal WT phenotype (Figure 3.2a). Quantification of
histological parameters showed a significant reduction in central nucleation in the
various skeletal muscles analyzed above as a result of therapy (Figure 3.2b). With the
expected low levels of central nucleation in BL/6 WT mice across all muscles averaging
1.89 ± 0.39%, we note here, taking into account all muscles analyzed, an average of
66.85 ± 1.86% central nuclei in untreated sgcβ−/− mice compared to 36.30 ± 5.16% in
AAV.MHCK7.hSGCB treated sgcβ−/− muscle (p<0.0001) (Table 3.2). A more detailed
analysis of muscle histopathology revealed a normalization of fiber size distribution
accompanied by an increase in average fiber diameter in diseased mice treated with
vector compared with untreated sgcβ−/− mice in all three muscles examined (GAS: sgcβ−/−
untreated – 28.37 ± 0.23μm vs. AAV.hSGCB treated – 36.04 ± 0.17μm; p<0.0001)
(PSOAS: sgcβ−/− untreated – 24.75 ± 0.23μm vs. AAV.hSGCB treated – 38.43 ± 0.28μm;
p<0.0001) (TRI: sgcβ−/− untreated – 28 ± 0.31μm vs. AAV.hSGCB treated – 35.56 ±
0.22μm; p<0.0001) (Figures 3.2c,d; Table 3.2).

Due to the significant role fibrosis plays in the pathogenesis of this disease and
effectiveness of therapies, it was critical to demonstrate the same efficacy in reducing
fibrosis we saw with localized hSGCB gene transfer (Chapter 2) [112], now in multiple
muscles following systemic delivery of AAV.MHCK7.hSGCB. Using the Picrosirius red
stain for collagen types I and III, we analyzed the levels of collagen in the GAS and
diaphragm muscles of 7 month old BL/6 WT mice (n=4), untreated sgcβ−/− mice (n=4),
and treated sgcβ−/− mice (n=5) 6 months post-injection. Treated muscles displayed
significantly less collagen deposition compared to untreated sgcβ−/− muscles (Figure
3.3a). Vector transduced GAS muscle contained 17.55 ± 0.59% collagen compared to
43.55 ± 3.33% collagen in untreated sgcβ−/− GAS muscles (p<0.0001) (Figure 3.3b).
Furthermore, treated diaphragm muscle exhibited 21.67 ± 1.09% collagen compared to 44.05 ± 2.39% in untreated sgcb−/− muscle (p<0.0001) (Figure 3.3b) proving once more the ability of hSGCB gene transfer to mitigate the fibrotic component of the LGMD2E phenotype.

3.3.3. Intravenous gene transfer to SGCB−/− mice reduces kyphoscoliosis of thoracic spine

We noted previously the involvement of the skeletal system in LGMD2E patients [18, 110] as well as mouse models of muscular dystrophy with the development of joint contractures and kyphoscoliosis (curvature) of the thoracic spine [108, 109]. Degeneration of torso and paraspinal muscles due to the worsening histopathology discussed above can be attributed to kyphosis. With the gross anatomical appearance of kyphoscoliosis in sgcb−/− mice, full body x-ray radiography was used to determine the degree of kyphosis in 7-month old BL/6 WT mice (n=6), sgcb−/− mice (n=6), and treated sgcb−/− mice 6 months post-injection (n=6). The kyphotic index (KI) score determines a quantitative value for the level of kyphoscoliosis [108]. As depicted in the WT panel in Figure 3.4a, the KI score is a ratio of the length from forelimb to hindlimb compared to the length of the midline to the apex of the curvature in the spine. While sgcb−/− mice presented with a severely curved spine and lower KI score of 3.64 ± 0.16 (n=6), BL/6 WT mice have a significantly straighter spine resulting in a higher KI score of 6.01 ± 0.41 (n=6) (p<0.01) (Figure 3.4b). Treated sgcb−/− mice exhibited a significant reduction in the degree of kyphosis in the spine with an increase in the KI score to 5.39 ± 0.58 (n=6) (p<0.05) (Figure 3.4b). We can conclude from these data that intravenous delivery of
scAAVrh.74.MHCK7.hSGCB is beneficial for the overall structure of the spine and can potentially alleviate the kyphosis and joint contractures present in the disease.

### 3.3.4. Assessment of cardiomyopathy

We noted the importance of the large subset of LGMD2E patients that develop cardiomyopathy, an aspect also prevalent in the mouse model. The histological destruction of limb and diaphragm muscle presented above is also detected in the myocardium of 7-month old sgcb<sup>−/−</sup> mice particularly with the presence of myocardial necrosis and fibrosis as evident by H&E and picrosirius red staining, respectively (Figure 3.5a). The presentation of impaired heart function often in the form of dilated cardiomyopathy is evidence by reduced cardiac output and lower ejection fraction [13, 18, 20-22, 113]. To demonstrate functional recovery in cardiac muscle, we used cardiac magnetic resonance imaging (MRI) to evaluate several functional parameters of the heart and first establish functional deficits in the myocardium of sgcb<sup>−/−</sup> mice compared to BL/6 WT mice. Imaging of control mice at 7 months of age showed a reduction of 29.4% in stroke volume from 0.041 ± 0.0019mL in sgcb<sup>−/−</sup> hearts to 0.029 ± 0.0024mL in BL/6 WT hearts (p<0.01), a 31.7% lower cardiac output from 14.70 ± 0.74mL/min in sgcb<sup>−/−</sup> hearts to 12.72 ± 0.97mL/min in BL/6 WT hearts, and finally a 14.3% lower ejection fraction, 66.21 ± 3.83% in sgcb<sup>−/−</sup> hearts compared to 76.90 ± 1.67% in BL/6 WT hearts (p<0.05) (Figure 3.5b).

This indicated a modest decline in overall cardiac function at this age and a trend towards the development of cardiomyopathy. Restoring hSGCB expression in hearts of KO mice through systemic delivery partially ameliorated these deficits, improving stroke volume to 0.032 ± 0.0027mL, cardiac output to 14.66 ± 0.75mL/min, and ejection fraction
to 68.16 ± 2.31% (Figure 3.5b). While none of these improvements were statistically significant, they show a trend towards WT levels and protection from or reversal of cardiomyopathy symptoms. As a corollary to the histological and function disruption of cardiac tissue reported here, western blotting for cardiac troponin I (cTrpl) expression, an important indicator of cardiac damage, is reduced in diseased sgcb-/- hearts to 60.38% of the levels seen in BL6 WT mice, and restored following treatment to levels of 35.80% greater than the expression seen in WT hearts (Figures 3.5c,d).

3.3.5. Functional restoration in diaphragm muscle with increase in physical activity

Accompanied with the histological benefit discussed in Chapter 2, we also showed that AAV-mediated hSGCB gene transfer can restore function to dystrophic sgcb-/- muscle (Chapter 2) [112]. While the importance of these initial studies with AAV.tMCK.hSGCB focusing on limb skeletal muscle (specifically the functional properties of the TA) cannot be undermined, the significant involvement of diaphragm dysfunction and respiratory failure in LGMD2E make providing a functional benefit to the diaphragm absolutely necessary for clinical systemic therapy. With the use of an ex vivo experimental protocol on strips taken from diaphragm muscle [115], we assessed the functional properties to determine whether restoring hSGCB expression provides a functional benefit to this severely compromised muscle.

In accordance with the significant histopathology in 7-month old diaphragms from diseased mice, sgcb-/- diaphragms (n=4) exhibited a functional deficit first with a significant (51%) reduction in specific force output compared to BL/6 WT mice (n=5) (116.24 ± 10.49mN/mm² vs. 236.67 ±15.87mN/mm², respectively, p<0.001), as well as a
greater loss of force from that produced after the first contraction following a rigorous fatigue protocol (23 ± 1.0% loss in $sgcb^{−/−}$; 7.0 ± 3.0% loss in BL6 WT, p<0.05) (Figure 3.6a). Six months following tail vein delivery of $1 \times 10^{12}$ vg total dose of scAAVrh.74.MHCK7.hSGCB, we noted a dramatic improvement in specific force output which increased to 226.07 ± 27.12 mN/mm$^2$ (n=5) (p<0.05 compared to $sgcb^{−/−}$) and better protection of the muscle from repeated fatigue with only a 12.0 ± 4.0% loss of force (p<0.05 compared to $sgcb^{−/−}$) (Figure 3.6a). Overall, these data support our previous findings in the TA muscle and show that restoring SGCB provides functional recovery in diaphragm muscle.

Symptoms of increased fatigue and reduced overall activity are frequently reported in many neuromuscular diseases [116, 117], partially attributed to the occurrence of kyphosis as well as diaphragm and cardiac dysfunction. As a result and taking into account the phenotype of LGMD2E, we hypothesized $sgcb^{−/−}$ mice would naturally be less active compared to healthy WT mice, and moreover systemic delivery of AAV.MHCK7.hSGCB to $sgcb^{−/−}$ mice would result in more physically active mice. In order to test this hypothesis, a laser-monitoring of open-field cage activity protocol similar to that used in previous reports [118, 119] was performed on all groups of mice. The graphs in Figure 3.6b depict a significant decrease by 55.5% in KO mice compared to WT, in both total ambulation (horizontal movement in the x and y planes) and hindlimb vertical rearing. The average number of horizontal ambulatory laser beam breaks over a 1 hour period in WT mice was 7355 ± 400.8 (n=6) compared to 3271 ± 483.8 (n=6) in KO mice (p<0.0001). Furthermore, the average number of vertical rearing beam breaks recorded in WT mice was 626.7 ± 53.76 as opposed to 264.5 ± 63.36 in KO mice (p<0.01) (Figure 3.6b). In accordance with our initial hypothesis, AAV.MHCK7.hSGCB
treated mice were visibly more active compared to KO which is illustrated in the quantification of activity, where total ambulation increased by 22% to 5143 ± 293.2 beam breaks (p<0.05) and hindlimb vertical rearing increased by a dramatic 77% to 615.3 ± 95.93 beam breaks (p<0.05) in MCHK7 treated mice (n=6) (Figure 3.6b).

3.3.6. Safety and biodistribution analysis of rAAVrh.74.MHCK7.hSGCB

Here we aimed to assess any potential toxicity or safety concerns of hSGCB gene therapy in sgcb−/− mice at 6 months after systemic delivery of the test article scAAVrh.74.MHCK7.hSGCB at 1.0x10^{12} vg total dose (5x10^{13} vg/kg). Vector biodistribution and off-target transgene expression were analyzed on tissue samples (TA, TRI, diaphragm, heart, gonad, lungs, kidney, liver, and spleen) from two vector dosed sgcb−/− animals using qPCR and Western blotting, respectively. Utilizing vector specific primer probe sets, scAAVrh.74.MHCK7.hSGCB transcript was detected at varying levels in all collected tissues. As expected, the highest levels were seen in skeletal muscle and the heart, indicating that the test article was efficiently delivered to all intended muscles of vector dosed mice (Figure 3.7a).

Furthermore, western blotting to detect hSGCB protein expression confirmed the functionality of the muscle specific MHCK7 promoter and the expression of transgene restricted to muscle. SGCB protein expression was observed in varying amounts in all skeletal muscle samples as well as heart samples, and importantly was not detected in any non-muscle tissue (Figure 3.7b), supported by the fact that SGCB is known to be a muscle specific protein. Finally, H&E staining was performed on cryosections of muscle tissue and all offsite organs harvested from five sgcb−/− mice along with five C57BL6 WT mice treated systemically with our vector at the therapeutic dose used in this study. A
veterinary pathologist then formally reviewed these sections for toxicity and no adverse effects were detected in any sample from any of the mice (data not shown). Taken together, these data indicate that this test article and delivery route was well tolerated by the test subjects.

3.4. Discussion

The proposed therapy investigated in this study using intravenous gene-transfer of rAAV carrying the hSGCB transgene is a promising new therapy for LGMD2E. Our investigations with AAV-mediated hSGCB gene therapy have utilized a self-complementary AAV to enhance transgene expression [91, 92], the rh.74 serotype for improved vascular delivery [93-95], muscle specific promoters to better target skeletal or cardiac muscle [59, 96, 114], and the optimization of a human β-sarcoglycan gene (hSGCB), all as advantages for clinical efficacy. In our previous studies demonstrating therapeutic efficacy using the tMCK promoter, the successful targeting of multiple muscles by vascular delivery to restore SGCB expression along with the significant reduction of fibrosis, all leading to functional recovery, provided a foundation for translating AAV-mediated hSGCB transfer to LGMD2E patients (Chapter 2) [112]. These initial studies however with IM and ILP delivery of the tMCK driven AAV.hSGCB vector were focused on regional delivery to limb skeletal muscle.

We know from initial reports characterizing the phenotype of the sgcb<sup>−/−</sup> mouse that it accurately recapitulates the clinical-pathological features of LGMD2E [10, 60], providing an advantage for pre-clinical translational studies. Based on the histological and functional deficits in sgcb<sup>−/−</sup> diaphragms and hearts, targeting the diaphragm and cardiac muscle must be considered in the long-term treatment of this disease.
Additionally, given the incidence of cardiomyopathy in patients representing a major limitation/long-term liability for treatment, the exclusive targeting to achieve skeletal muscle correction may be insufficient and potentially harmful for the LGMD2E population. There are always concerns that if we did improve limb skeletal muscle strength by localized IM or ILP gene delivery without comparable cardiac improvement that we could potentially stress the heart even further. Consequently, this signifies the necessity of a systemic delivery and the importance of cardiac and diaphragm correction in creating an effective treatment for LGMD2E patients.

Following the modification of our therapeutic AAV vector with the addition of the MHCK7 promoter allowing for enhanced transgene expression in cardiac muscle [114], we demonstrate here that intravenous injection of scAAVrh.74.MHCK7.hSGCB leads to nearly complete transduction and restoration of hSGCB expression in limb skeletal muscles, diaphragm muscle, and importantly in cardiac muscle. With systemic gene transfer in mind, the substantial involvement of diaphragm dysfunction and respiratory failure implicated in multiple forms of muscular dystrophy including LGMD2E [18, 111, 120] and their corresponding mouse models [10, 121-123] is one potentially life-threatening component of this disease. Due to deteriorating diaphragm function in many neuromuscular diseases, reports of increased fatigue and reduced overall activity are also seen [116, 117]. We detailed here the evidence of histological disruption of sgcb−/− diaphragm tissue at just 7 months of age, which is accompanied by reduced diaphragm force outputs, parameters that are improved following gene transfer. Vector dosed sgcb−/− mice are also more physically active compared to diseased sgcb−/− mice. Additionally seen in LGMD2E patients and disease models of muscular dystrophy like the mdx/utrn−/− mouse model is the development of kyphoscoliosis of the thoracic spine due to a
weakening of the muscles that support the spinal column. Kyphosis can result in the diaphragm being pushed forward, further compromising lung capacity and diaphragm function [18, 108, 109]. As additional evidence of our therapy to potentially improve the quality of life for LGMD2E patients, we were able to demonstrate the alleviation of kyphosis in \( sgcb^{-/} \) mice following systemic delivery of our vector.

Another important life-threatening complication of LGMD2E is the development of cardiomyopathy, occurring in up to 50% or more of patients, much more than other LGMD’s [13, 18, 20-22]. This suggests that compensatory mechanisms and other cardiac proteins can protect the heart in some forms of sarcoglycanopathy, but not LGMD2E. For example, \( \varepsilon \)-sarcoglycan, a SGCA homologue protects LGMD2D patients from heart involvement [26, 124]. Specifically, deterioration of cardiac muscle in LGMD2E often leads to dilated cardiomyopathy with chamber dilation, impaired contractile function marked by reduced cardiac output and lower ventricular ejection fraction, and significant myocardial hypertrophic remodeling as the disease progresses [13, 18, 20-22, 125]. Araishi et al and Durbeej et al both reported severe histopathology and significant remodeling in \( sgcb^{-/} \) cardiac tissue [10, 60], similar to what we demonstrate here. Another group studying the role of miR-669a in dystrophic cardiomyopathy reported in aged (18 month old) \( sgcb^{-/} \) mice the functional manifestation of dilated cardiomyopathy as seen in echocardiography [113].

This initial demonstration of aged mice with similar cardiac deficits to those seen in LGMD2E patients provided a basis for our study to show correction of these deficits following AAV treatment. Although histological changes in the myocardium have been indicated as early as 4 weeks of age [10, 60], findings that we have reproduced in our lab, the manifestation of severe cardiomyopathy does not correlate with the histological
deterioration of cardiac tissue. We note that cardiac MRI of sgcb<sup>−/−</sup> at 7 months of age shown here did not demonstrate severe cardiomyopathy associated with significantly reduced cardiac function, with ejection fraction in sgcb<sup>−/−</sup> mice at 66%. We believe the age of mice analyzed was the primary cause for the lack of cardiomyopathy symptoms seen here. Imaging of larger cohorts at older ages should establish a severe cardiomyopathy in sgcb<sup>−/−</sup> mice providing greater deficits for therapeutic improvement. As a corollary to the functional assessment of cardiomyopathy, we performed western blotting for cardiac troponin I (cTrpI) on heart tissue from BL/6 WT, untreated sgcb<sup>−/−</sup>, and AAV.MHCK7.hSGCB treated mice. cTrpI is an important regulator of cardiac function in cardiomyocytes and has been implicated as a biomarker for damage to the heart. One study showed a reduction in cTrpI levels in normal WT aging hearts correlated with significant diastolic dysfunction observed by P-V loop and echocardiography analysis [126]. In agreement with this, providing additional evidence for the development of cardiomyopathy and the potential cardiac benefits of our therapy, hearts from 7-month old sgcb<sup>−/−</sup> mice show about 40% lower cTrpI levels compared to healthy BL6 WT hearts, levels that are restored to above WT in treated hearts.

Finally, Sgcb<sup>−/−</sup> mice and C57BL/6 WT mice given an intravenous tail vein injection of scAAVrh.74.MHCK7.hSGCB at our proposed dose of 1x10<sup>12</sup> vg total dose (5x10<sup>13</sup> vg/kg) were fully necropsied for pathology review by an independent veterinary pathologist, who reported no adverse effects in any muscle or organ from any of the mice analyzed. The fact that we achieve such high levels of transduction in all muscles throughout the body with no adverse effects using a relatively low dose (1x10<sup>12</sup> vg total dose; 5x10<sup>13</sup> vg/kg) gives this therapy great promise for translation to LGMD2E patients. From a clinical perspective, the dose used here is much lower than the dose used for
systemic delivery of an SMN1 expressing AAV therapy delivered to babies with SMA, which is currently in clinical trial [127, 128]. Additionally, it is important to note that similar to our experience with LGMD2D (α-Sarcoglycan deficiency), while there is variability with many kinds of mutations seen, single nucleotide changes are the typical SGCB gene mutation which favors success in gene transfer serving to protect the transgene product from immunorejection[8, 9, 16-18, 47, 49]. The highly efficient restoration of SGCB expression using the MHCK7 promoter accompanied with functional benefits is very encouraging at dosing levels that could be applied clinically, and given the high incidence of heart involvement in LGMD2E patients, we feel systemic delivery provides the greatest benefit to these patients and the greatest promise for treating this debilitating disease.

3.5. Materials and Methods

3.5.1. Animal Models

All procedures were approved by The Research Institute at Nationwide Children's Hospital Institutional Animal Care and Use Committee (protocol AR12-00040). B6.129-Sgcb tm1Kcam/1J heterozygous mice were purchased from the Jackson Laboratory (Strain # 006832). Sgcb−/− mice were generated by breeding heterozygous mice. KO mice were bred and maintained as homozygous animals in standardized conditions in the Animal Resources Core at the Research Institute at Nationwide Children's Hospital. Mice were maintained on Teklad Global Rodent Diet (3.8z5 fiber, 18.8% protein, 5% fat
chow) with a 12:12 hr dark:light cycle. All animals were housed in standard mouse cages with food and water ad libitum.

3.5.2. Genotyping

DNA genotyping was used to identify \(sgcb^{+/−}\) mice. DNA from tail clippings was isolated and analyzed by OneTaq DNA Polymerase (New England Biolabs, Ipswich, MA) PCR. A series of primers were used in the PCR analysis to determine beta-sarcoglycan knockout status. The set included a common primer complementary to a region outside of the mouse beta-sarcoglycan gene in chromosome 5 (5' CAG GAC AGT GCT CAG CAA GA 3'), a mutant primer complementary to the inserted neomycin knockout cassette (5' GCC TGA AGA ACG AGA TCA GC 3'), a forward WT primer complementary to exon 4 (5' TTT TTG ACC CAA GGA CAC A 3'), and a reverse WT primer complementary to exon 5 (5' TTG CCC ATG ATG AAG ACG CC 3'). Reactions were carried out on genomic DNA for 30 cycles under the following conditions: 94°C, 30s; 57°C, 30s; 68°C, 20s.

3.5.3. Beta-sarcoglycan Gene Construction

The full-length human beta-sarcoglycan cDNA (GenBank Accession No. NM_0034994.3) was codon optimized and synthesized by GenScript Inc, Piscataway, NJ. The cDNA was then cloned into a self-complementary (sc) AAVrh.74 vector (for enhanced transduction via vascular delivery) containing AAV2 ITRs and the cassette includes a consensus Kozak sequence (CCACC), an SV40 chimeric intron, and a synthetic polyadenylation site (53 bp). The recombinant MHCK7 promoter used to drive
transgene expression is a muscle specific promoter and was a gift from Dr. Steven Huaschka (University of Washington). This is an MCK based promoter, which utilizes a 206-bp enhancer taken from ~1.2kb 5’ of the transcription start site within the endogenous muscle creatine kinase gene with a proximal promoter (enh358MCK, 584-bp) [114]. This enhancer along with a modified CK7 cassette from the MCK family of genes is ligated to a 188-bp α-MyHC (α-myosin heavy chain) enhancer 5’ of the CK portion to enhance cardiac expression [114]. The creatine kinase portion of the promoter (CK) is 96% identical between tMCK and MHCK7. Finally, the pAAV.MHCK7.hSGCB vector was constructed by ligation of the 960 bp NotI/KpnI MHCK7+Intron fragment from pAAV.MHCK7.DYSF5’DV[56] into the NotI/KpnI sites of pAAV.tMCK.hSGCB (Chapter 2) [112].

3.5.4. rAAV Production

A revised AAV cross-packaging approach previously reported [95] was used to produce the recombinant AAV vector. Here, a triple transfection method with CaPO4 precipitation in HEK293 cells allows for AAV2 ITRs to be packaged into a different AAV capsid serotype [102, 103]. The production plasmids were: (i) pAAV.MHCK7.hSGCB, (ii) rep2-caprh.74 modified AAV helper plasmids encoding cap serotype 8-like isolate rh.74, and (iii) an adenovirus type 5 helper plasmid (pAdhelper) expressing adenovirus E2A, E4 ORF6, and VA I/II RNA genes. Vectors were purified and encapsidated vector genome (vg) titer (utilizing a Prism 7500 Taqman detector system (PE Applied Biosystems, Carlsbad, CA)) was determined as previously described [104]. The primer and fluorescent probe targeted the MHCK7 promoter and were as follows: MHCK7 forward primer, 5’-CCA ACA CCT GCT GCC TCT AAA-3’; MHCK7 reverse primer, 5’-
GTC CCC CAC AGC CTT GTT C-3'; and MHCK7 probe, 5'-FAM-TGG ATC CCC-Zen-TGC ATG CGA AGA TC-3IABKFQ-3'.

3.5.5. Gene Delivery

Systemic delivery was achieved through injection of vector into the tail vein of 
\(sgcb^{-/-}\) mice. Mice were injected with \(1 \times 10^{12}\) vg of scAAVrh.74.MHCK7.hSGCB diluted in saline in a 212\(\mu\)L volume using a 30-gauge ultra-fine insulin syringe. Mice were restrained in a holding tube placing the tail back through tail slot to warm it up in order to dilate the blood vessels for ease of injection. After locating the artery down the center line of the tail the injection was performed in one of the purple/blue lateral veins that run alongside the tail artery. All treated mice were injected at 4-5 weeks of age and euthanized 6-months post-injection.

3.5.6. Diaphragm Tetanic Contraction for Functional Assessment

Mice were euthanized and the diaphragm was dissected with rib attachments and central tendon intact, and placed in K-H buffer as previously described [129-131]. A 2-4 mm wide section of diaphragm was isolated. Diaphragm strips were tied firmly with braided surgical silk (6/0; Surgical Specialties, Reading, PA) at the central tendon, and sutured through a portion of rib bone affixed to the distal end of the strip. Each muscle was transferred to a water bath filled with oxygenated K-H solution that was maintained at 37°C. The muscles were aligned horizontally and tied directly between a fixed pin and a dual-mode force transducer-servomotor (305C; Aurora Scientific, Aurora, Ontario, Canada). Two platinum plate electrodes were positioned in the organ bath so as to flank
the length of the muscle. The muscle was stretched to optimal length for measurement of twitch contractions, and then allowed to rest for 10 minutes before initiation of the tetanic protocol. Once the muscle is stabilized, the muscle is set to an optimal length of 1g and is subjected to a warm-up which consists of three 1Hz twitches every 30 seconds followed by three 150Hz twitches every minute. After a 3 min rest period, the diaphragm is stimulated at 20, 50, 80, 120, 150, 180Hz, allowing a 2 min rest period between each stimulus, each with a duration of 250ms to determine maximum tetanic force. Muscle length and weight was measured. The force was normalized for muscle weight and length.

3.5.7. Cardiac Magnetic Resonance Imaging

Cardiac function was analyzed using a 9.4T horizontal-bore magnetic resonance imaging (MRI) system and mouse volume coil (Bruker BioSpin, Billerica, MA, USA). Mice were anaesthetized with 2.5% isoflurane mixed with carbogen (1 L/min) for 3 minutes prior to placement on the imaging bed. Upon placement of mice in imaging apparatus and initiation of imaging, isoflurane/carbogen mixture was dropped to 1.5% for the remainder of the study. EKG and respiration were monitored using an MRI-compatible system (Model 1025, Small Animal Instruments, Stonybrook, NY, USA). Gated cardiac short-axis FLASH cine T1-weighted images were acquired over the entire left ventricle (LV) of the mouse (TR = 8 ms; TE = 2.8 ms; FA = 18°; matrix = 256 × 256; FOV = 3.0 × 3.0 cm; slice thickness = 1 mm, nslices=7, up to 20 frames per cardiac cycle). For image analysis, the end-diastolic and end-systolic time point of each short-axis image were identified and the endocardial and epicardial cardiac boundaries were manually traced. The papillary muscles were excluded from the endocardial boundary of the LV.
From these measured areas, end-diastolic volume (EDV), end-systolic volume (ESV), stroke volume (SV), cardiac output (CO), ejection fraction (EF), and average LV mass were calculated.

### 3.5.8. Immunofluorescence

Cryostat sections (12µm) from the tibialis anterior (TA), gastrocnemius (GAS), quadriceps (QUAD), psoas major (PSOAS), gluteal (GLUT), triceps (TRI), and diaphragm muscles along with the heart were subjected to immunofluorescence staining for the hSGCB transgene via our previously used protocol (Chapter 2) [112]. Sections were incubated with a mouse monoclonal human beta-sarcoglycan primary antibody (Leica Biosystems, New Castle, UK; Cat. No. NCL-L-b-SARC) at a dilution of 1:100. Four random 20X images covering the four different quadrants of the muscle section were taken using a Zeiss AxioCam MRC5 camera. Percentage of fibers positive for beta-sarcoglycan staining (>50% of muscle membrane staining) was determined for each image and averaged for each muscle.

### 3.5.9. Western Blot Analysis

Western blots were performed according to our previously used protocol with several modifications specific for each antibody used (Chapter 2) [112]. Samples from C57BL/6 WT mice, untreated $sgcb^{-/}$ mice, and vector dosed $sgcb^{-/}$ mice were used for each western. A 1:250 dilution of a rabbit polyclonal human beta-sarcoglycan antibody (Novus Biologicals, Littleton, CO; Cat. No. NBP-1-90300) and a 1:5000 dilution of a mouse monoclonal mouse α-actinin antibody (Sigma Aldrich, St. Louis, MO; Cat. No.
A7811) was used for hSGCB blots. A 1:500 dilution of a rabbit polyclonal mouse cardiac troponin I antibody (Abcam, Cambridge, MA; Cat. No. ab47003) and a 1:1000 dilution of a rabbit monoclonal mouse vinculin antibody (Invitrogen, Frederick, MD; Cat. No. 70062). Anti-mouse (Millipore, Billerica, MA Cat. No. AP308P) and anti-rabbit (Life Technologies, Grand Island, NY; Cat. No. 656120) secondary-HRP antibodies were used for ECL immunodetection.

### 3.5.10. Morphometric Analysis

Hematoxylin and eosin (H&E) staining was performed on 12µm thick cryosections of muscle from 7 month old C57BL6 WT mice (n=5), sgcb−/− mice (n=5), and rAAV.MHCK7.hSGCB 6 month treated sgcb−/− mice (n=5) for analysis. The percentage of myofibers with central nuclei was determined in the TA, GAS, QUAD, PSOAS, GLUT, TRI, and diaphragm muscles. Additionally, muscle fiber diameters were measured in the GAS, PSOAS, and TRI muscles. Four random 20X images per muscle per animal were taken with a Zeiss AxioCam MRC5 camera. Centrally nucleated fibers were quantified using the NIH ImageJ software and fiber diameters were measured using Zeiss Axiovision LE4 software.

### 3.5.11. Biodistribution qPCR Analysis

Taqman quantitative PCR was performed to quantify the number of vector genome copies present in targeted and untargeted contralateral muscle as well as non-targeted organs as previously described (Chapter 2) [94, 104, 112]. A vector-specific primer probe set was used to amplify a sequence of the intronic region directly
downstream from the MHCK7 promoter that is unique and located within the scAAVrh.74.MHCK7.hSGCB transgene cassette. The following primers and probe were used in this study: MHCK7 intron Forward Primer 5’-GTG AGG CAC TGG GCA GGT AA-3’; MHCK7 intron Reverse Primer 5’-ACC TGT GGA GAG AAA GGC AAA G-3’; and MHCK7 intron Probe 5’-6FAM-ATC AAG GTT ACA AGA CAG GTT TAA GGA GAC CAA TAG AAA-TAMRA-3’ (IDT). Copy number is reported as vector genomes per microgram of genomic DNA.

**3.5.12. Picrosirius Red Stain and Collagen Quantification**

A protocol to determine the levels of collagen deposition in muscle tissue was performed on 12µm cryosections from 7-month old C57BL6 WT (n=4), untreated sgcb−/− (n=4), and rAAV.MHCK7.hSGCB  6 month treated sgcb−/− (n=5) GAS and diaphragm muscles. A slightly amended protocol which we previously published was used in this study (Chapter 2) [112]. Solution A (Phosphomolydbic acid) was not used in this experimental protocol. Sections were placed straight in Solution B (Direct Red 80/2 4 6- Trinitrophenol) for 15 minutes following incubation in 10% Neutral Buffered Formalin and distilled water rinse. Here again four 20X images were taken per muscle per mouse and the mean percent collagen for each muscle was then calculated for all groups.

**3.5.13. X-Ray Images**

Whole body x-rays were performed on anesthesitized 7 month old C57BL6 WT mice (n=6), untreated sgcb−/− mice (n=6), and rAAV.MHCK7.hSGCB  6 month treated
3.5.14. Laser Monitoring of Open Field Cage Activity

An open-field activity chamber was used to determine overall activity of experimental mice. Mice at 7 months old from the C57BL6 WT (n=6) and untreated sgcb−/− (n=6) control groups along with the rAAV.MHCK7.hSGCB 6 month treated sgcb−/− mice (n=6) were subjected to analysis following a previously described protocol [118, 119] with several modifications. All mice tested were all tested at the same time of day in the early morning near then end of the night cycle when mice are most active. All mice were tested in an isolated room, under dim light and with the same handler each time. Also, as was done in the previous reports to reduce anxiety and keep behavioral variables at a minimum, which could potentially affect normal activity of the mice and consequently the results of the assay, we tested mice that were not individually housed [132]. Mice were activity monitored using the Photobeam Activity System (San Diego Instruments, San Diego, CA). This system uses a grid of invisible infrared light beams that traverse the animal chamber front to back and left to right to monitor the position and movement of the mouse within an X-Y-Z plane. Activity was recorded for 1 hour cycles at 5-minute intervals. Mice were acclimatized to the activity test room for an initial 1 hour session several days prior to beginning data acquisition. Mice were tested in individual chambers in sets of 4. Testing equipment was cleaned between each use to reduce mouse reactionary behavioral variables that could alter our results. Data collected was converted to a Microsoft Excel worksheet and all calculations were done within the Excel program. Individual beam breaks for movement in the X and Y planes
were added up for each mouse to represent total ambulation, and beam breaks in the Z plane were added up to obtain vertical activity within the 1 hour time interval.
### Table 3.1

<table>
<thead>
<tr>
<th>Muscle</th>
<th>Delivery Route</th>
<th>Dose (vg Total Dose)</th>
<th>Endpoint (Months)</th>
<th>% Fibers Expressing SGCB</th>
</tr>
</thead>
<tbody>
<tr>
<td>TA</td>
<td>IV</td>
<td>1e12</td>
<td>6</td>
<td>98.88 ± 0.55</td>
</tr>
<tr>
<td>GAS</td>
<td>IV</td>
<td>1e12</td>
<td>6</td>
<td>98.24 ± 0.82</td>
</tr>
<tr>
<td>QD</td>
<td>IV</td>
<td>1e12</td>
<td>6</td>
<td>99.32 ± 0.19</td>
</tr>
<tr>
<td>GLUT</td>
<td>IV</td>
<td>1e12</td>
<td>6</td>
<td>97.50 ± 0.39</td>
</tr>
<tr>
<td>PSOAS</td>
<td>IV</td>
<td>1e12</td>
<td>6</td>
<td>98.75 ± 0.23</td>
</tr>
<tr>
<td>TRI</td>
<td>IV</td>
<td>1e12</td>
<td>6</td>
<td>97.21 ± 1.35</td>
</tr>
<tr>
<td>Diaphragm</td>
<td>IV</td>
<td>1e12</td>
<td>6</td>
<td>97.00 ± 1.26</td>
</tr>
<tr>
<td>Heart</td>
<td>IV</td>
<td>1e12</td>
<td>6</td>
<td>≥95%</td>
</tr>
</tbody>
</table>

Table 3.1. Quantification of hSGCB transgene expression 6 months post-gene transfer of scAAVrh.74.MHCK7.hSGCB in SGCB-/− mice. Expression levels given for various muscles as the average of left and right muscles from systemically injected mice (n=5). Values indicated as AVG ± SEM.
Table 3.2. Quantification of central nucleation and muscle fiber diameter 6 months post-gene transfer of scAAVrh.74.MHCK7.hSGCB in SGCB−/− mice. Central nuclei counts and fiber diameters given for various muscles as the average (±SEM) of left and right muscles from BL6 WT, sgcb−/−, and systemically injected mice (n=5 per group).

<table>
<thead>
<tr>
<th>Animal Group</th>
<th>Dose (vg Total)</th>
<th>Muscle</th>
<th>% Central Nuclei (Avg ±SEM)</th>
<th>Combined Avg %CN (±SEM)</th>
<th>Fiber Diameter μm (Avg ±SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL6 WT</td>
<td>N/A</td>
<td>TA</td>
<td>1.76 ± 0.86</td>
<td>1.89 ± 0.39</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAS</td>
<td>0.83 ± 0.41</td>
<td>39.69 ± 0.18</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QD</td>
<td>0.98 ± 0.31</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GLUT</td>
<td>2.50 ± 0.68</td>
<td>40.96 ± 0.22</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PSOAS</td>
<td>1.26 ± 0.28</td>
<td>41.53 ± 0.24</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRI</td>
<td>2.13 ± 0.36</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIA</td>
<td>3.75 ± 1.30</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>Sgcb−/−</td>
<td>N/A</td>
<td>TA</td>
<td>70.45 ± 3.04</td>
<td>66.85 ± 1.86</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAS</td>
<td>67.26 ± 1.81</td>
<td>28.37 ± 0.23</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QD</td>
<td>63.57 ± 2.09</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GLUT</td>
<td>61.34 ± 2.05</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PSOAS</td>
<td>62.73 ± 5.20</td>
<td>24.75 ± 0.22</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRI</td>
<td>67.11 ± 2.83</td>
<td>28.74 ± 0.22</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIA</td>
<td>75.47 ± 3.79</td>
<td></td>
<td>N/A</td>
</tr>
<tr>
<td>AAV.MHCK7.hSGCB Treated</td>
<td>1.00E+12</td>
<td>TA</td>
<td>43.85 ± 3.89</td>
<td>36.30 ± 5.16</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GAS</td>
<td>38.71 ± 3.50</td>
<td>36.04 ± 0.18</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>QD</td>
<td>46.10 ± 6.26</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>GLUT</td>
<td>42.11 ± 5.48</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PSOAS</td>
<td>21.00 ± 4.69</td>
<td>38.43 ± 0.28</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TRI</td>
<td>48.39 ± 6.20</td>
<td>39.92 ± 0.27</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DIA</td>
<td>11.59 ± 2.08</td>
<td></td>
<td>N/A</td>
</tr>
</tbody>
</table>
3.6. Figures

Figure 3.1. Restoration of SGCB expression following intravenous delivery of scAAVrh.74.MHCK7.hSGCB. (a) scAAVrh.74.MHCK7.hSGCB cassette identical to our original cassette with the exception of the inclusion of the MHCK7 promoter instead of tMCK for expression in cardiac muscle. (b) Immunofluorescence imaging 6 months post-injection of skeletal muscles, diaphragm, and heart from sgcb<sup>−/−</sup> mice intravenously injected with 1e12 vg total dose scAAVrh.74.MHCK7.hSGCB. Representative images of skeletal muscles displaying an average of 98.13 ± 0.31% transduction. 20X images are shown. Representative images of heart tissue displaying high levels of hSGCB transgene expression. 10X images are shown. (c) Western blotting of all muscles from one treated sgcb<sup>−/−</sup> mouse confirming hSGCB transgene expression. (d) Western blotting for hSGCB expression in hearts of five sgcb<sup>−/−</sup> treated mice with densitometry quantification showing overexpression of hSGCB up to 72.0% of BL6 WT levels.
Figure 3.2. Effect of systemic treatment with scAAVrh74.MHCK7.hSGCB on muscle pathology. (a) H&E stain of diaphragm and QUAD muscle from C57BL/6 WT, sgcb<sup>−/−</sup>, and scAAVrh74.MHCK7.hSGCB treated mice showing normalized histopathology. (b) Quantification of reduction in centrally nucleated fibers in sgcb<sup>−/−</sup> treated muscle compared to untreated sgcb<sup>−/−</sup> muscle (TA, GAS, GLUT, diaphragm, p<0.0001) (QUAD, PSOAS, TRI, p<0.05) (c) normalization of fiber distribution in GAS, PSOAS, and TRI, and (d) increase in average fiber size in treated muscles compared to untreated sgcb<sup>−/−</sup> muscles (p<0.001) (ONE-WAY ANOVA) (n=5 per group).
Figure 3.3. Reduced collagen deposition in intravenous treated β-SG KO mice. (a) Picrosirius red staining shows reduced fibrosis in treated mice indicated by a decrease in collagen deposition compared to untreated sgcb⁻/⁻ mice in diaphragm and GAS. (b) Quantification of collagen levels in the diaphragm and GAS muscles from C57BL/6 WT mice (n=4), untreated sgcb⁻/⁻ mice (n=4), and treated sgcb⁻/⁻ mice (n=5) confirm reduction in collagen levels in both treated muscles (p<0.0001, ONE-WAY ANOVA). 100μm scale bar shown for 20X images.
Figure 3.4. Correction of kyphoscoliosis in thoracic spine. (a) Kyphoscoliosis in sgcb−/− mice as evident by X-ray radiography. (b) The Kyphotic Index (KI) score of sgcb−/− mice (3.69) is low compared to C57BL/6 WT (6.01) (p<0.01), but increases upon treatment with scAAVrh.74.MHCK7.hSGCB (5.39) (p<0.05 compared to sgcb−/−) (ONE-WAY ANOVA) (n=6 per group).
Figure 3.5. Assessment of cardiomyopathy in heart muscle. (a) H&E and picrosirius red stains of 7 month old BL6 WT, sgcb<sup>−/−</sup>, and AAV.MHCK7.hSGCB treated sgcb<sup>−/−</sup> hearts 6 months post-treatment indicating myocardial degeneration in untreated sgcb<sup>−/−</sup> muscle and improvement following treatment. (b) Cardiac MRI analysis showing reduction in sgcb<sup>−/−</sup> hearts in stroke volume (p<0.01), cardiac output, and ejection fraction (p<0.05) (ONE-WAY ANOVA) and improvements 6 months after treatment (n=6 per group). (c) Western blotting of two C57BL/6 WT hearts, two sgcb<sup>−/−</sup> hearts, and five AAV.MHCK7.hSGCB treated sgcb<sup>−/−</sup> hearts showing decreased cardiac troponin I levels in diseased mice. (d) Densitometry quantification showing reduction of cardiac troponin I (cTrpI) to 60.38% of BL6 WT levels and an overexpression of up to 135.8% of BL6 WT levels.
Figure 3.6. Diaphragm function correction and increased open-field cage activity. (a) Diaphragm muscle strips were harvested to measure force and resistance to fatigue in BL6 WT mice (n=5), sgcb<sup>-/-</sup> mice (n=4), and AAV.MHCK7.hSGCB treated sgcb<sup>-/-</sup> mice (n=5) all at 7 months of age. Six months of treatment restored force to WT levels (p<0.01 compared to sgcb<sup>-/-</sup>) and improved resistance to fatigue. (b) Overall ambulation in x and y planes is significantly decreased in sgcb<sup>-/-</sup> mice (p<0.0001) and slightly improved in MCHK7 treated mice (p<0.05). Vertical activity rearing onto hindlimbs also decreased in sgcb<sup>-/-</sup> mice (p<0.01) and significantly increased in MCHK7 treated mice (p<0.05) (ONE-WAY ANOVA) (n=6 per group).
Figure 3.7. Biodistribution and off-target transgene expression analysis of systemic scAAVrh.74.MHCK7.hSGCB delivery. (a) Distribution histogram of average vg copies of transcript per microgram DNA added to qPCR reaction in various tissues from two sgcb−/− mice after IV delivery of scAAVrh.74.MHCK7.hSGCB at 1e12 vg total dose. (b) Biodistribution westerns on muscles and organs from scAAVrh.74.MHCK7.hSGCB systemically injected sgcb−/− mice indicating no expression of hSGCB transgene in any non-muscle samples.
Chapter 4: Establishing Outcome Measures for Translational Studies of Autosomal Recessive Limb-Girdle Muscular Dystrophies

4.1. Abstract

Dysferlinopathies including LGMD2B, and Anoctomin5 myopathies including LGMD2L, comprise a family of disorders caused by recessive mutations in the dysferlin (DYSF) and anoctomin5 (ANO5) genes, respectively. These leads to progressive dystrophies characterized by chronic muscle fiber loss, inflammation, fat replacement and fibrosis; all resulting in deteriorating muscle function. The phenotypic overlap of ANO5 myopathies with DYSF-associated dystrophies leads to the hypothesis that DYSF and ANO5 may have intersecting functional characteristics. Both DYSF and ANO5 have been shown independently to be involved in repair to membrane damage in the sarcolemma, implicating a compromised ability to reseal sarcolemmal damage in these diseases. In order to thoroughly characterize these diseases and test therapeutic efficacy of viral-mediated gene replacement therapy, the development and validation of functional outcome measures specifically involving membrane repair is critical. Here we optimized a collagenase based enzymatic dissociation of the flexor digitorum brevis (FDB) muscle in mice to isolate healthy and viable muscle fibers. Utilizing a multiphoton confocal microscope to induced focal areas of membrane damage in the presence of a fluorescent dye, we were able to track and quantify the ability of muscle to reseal sarcolemmal damage by measuring the amount of dye accumulation over a period of
We first demonstrated impaired membrane repair ability in both DYSF and ANO5 deficient fibers. Following the restoration of either DYSF or ANO5 expression in the FDB through the use of adeno-associated virus (AAV), we showed membrane repair ability was improved as evident by quicker membrane resealing with less dye accumulation. Thus we report an experimental method for the study of muscular dystrophies and emerging therapeutic interventions.

4.2. Introduction

Deficits in muscle function produce muscular dystrophies (MDs) that are characterized by muscle weakness and wasting and have serious impacts on quality of life [133]. Dysferlinopathies, including limb-girdle muscular dystrophy type2B are caused by mutations in the dysferlin (DYSF) gene [63-68]. Over time, loss of DYSF leads to a progressive muscular dystrophy with chronic muscle fiber loss, inflammation, and fibrosis, all contributing to deteriorating function [66, 72]. Dysferlin, a member of the ferlin family, is composed of a C-terminal transmembrane domain and a long N-terminal cytoplasmic region with multiple C2 domains involved in calcium and phospholipid binding [75-77]. Strong evidence for dysferlin function suggests a role in Ca$^{2+}$-dependent sarcolemmal membrane repair [78, 79, 81], where previous studies reported dysf$^{-/-}$ muscle fibers fail to exclude dye entry even in the presence of Ca$^{2+}$ [80]. There is also evidence from LGMD2B patients that ultrastructural membrane defects are a present and contribute to disease pathology [73, 74]. Furthermore, a new MD with features similar to dysferlinopathies and characterized by sarcolemmal lesions has been linked to recessive mutations in ANO5 (TMEM16E) [69-71]. ANO5 mutations produce LGMD2L with a phenotype of proximal lower limb weakness, high serum creatine kinase
levels, and asymmetric muscle atrophy and weakness [69-71]. ANO5 has not been found to exhibit the typical anoctomin protein functions [82, 83], suggesting an unknown role in skeletal muscle. Given the similarities between ANO5 and DYSF myopathy phenotypes, ANO5 is hypothesized to be involved in the membrane repair process with compromised sarcolemmal resealing in ano5−/− muscle.

Due to the mechanical stress the sarcolemma experiences during muscle contraction, even healthy muscle is in constant need of repair mechanisms to patch injured membranes [84]. Sarcolemmal patch repair relies on the fusion of membrane vesicles at sites of damage [85, 86], and the attenuation of this process leads to regeneration/degeneration of fibers and is considered a putative cause of LGMD2B and potentially a major contributing factor of LGMD2L [78-81]. To better understand and characterize the disease pathogenesis and phenotype in a murine model of LGMD2L, or study potential therapeutic efficacy in a murine model of LGMD2B, the development, optimization, and validation of functional deficits to serve as outcome measures is essential.

Unfortunately, dysf−/− mouse models do not demonstrate functional deficits in widely studied muscles like the tibialis anterior and extensor digitorum longus [55, 134]. Although diaphragm force deficits occur in these models, loss of force measured by classical ex vivo techniques is difficult to correlate directly to diaphragm function in patients [55, 134]. Alternatively, with a presumed functional defect in membrane repair previously recapitulated in dysf−/− mice, a dysferlin dependent response to sarcolemmal injury can be modeled effectively for therapeutic evaluation [73, 78-81, 135].

Gene transfer (using adeno-associated virus (AAV)) to directly replace the mutated gene has proven in numerous studies to be the most promising therapy for
genetic disorders like MD [30, 47, 49, 53, 93, 105, 136], including for dysferlinopathies, to restore the critical function of muscle membrane repair [88, 137]. Treatment of the flexor digitorum brevis (FDB) muscle in diseased mice with a therapeutic AAV vector provides an ideal model to test functional efficacy. The FDB is a small, compartmentalized muscle in the footpad of mice and can be targeted reproducibly [87, 88], either intramuscularly or intravenously. To test sarcolemmal repair ability in diseased muscle, the entire FDB muscle is removed and dissociated to isolate individual fibers. Membrane damage can be induced at the sarcolemma with a multi-photon laser microscope in the presence of FM1-43 dye, a water-soluble dye that fluoresces when inserted into plasma membranes [87]. The dye is unable to penetrate membranes and therefore can only passively enter cells upon damage to the membrane. The amount of dye entry over time can be tracked and accurately quantified yielding a significant difference between diseased and healthy muscle fibers.

This quantifiable functional measure is ideal for determining gene replacement pre-clinical therapeutic efficacy, or characterizing dystrophic muscle. Following optimization of this fiber isolation and membrane repair assay in dystf<sup>-/-</sup> mice, we demonstrated a dose dependent effect on membrane repair with both single and dual AAV.MHCK7.DYSF vector systems via intramuscular and systemic treatment. We extended these studies to the ano5<sup>-/-</sup> model and report that this mouse recapitulates features of dystf<sup>-/-</sup> mice with dysfunction in sarcolemmal repair, and similar to dysferlin found an improvement in repair following ANO5 gene replacement. Thus, a laser induced injury repair model serves as a functional outcome measure for characterization and therapeutic efficacy studies, which serves as predictor for successful treatment.
4.3. Results

4.3.1. Restoration of membrane repair following intramuscular delivery of AAV5.DYSF

To begin testing the membrane repair process, we subjected normal healthy 129-WT muscle fibers to the membrane repair assay where sarcolemmal damage was induced in individual fibers using a multiphoton laser in the presence of FM 1-43 dye either containing (n=6) or absent of Ca\(^{2+}\) (n=6). The amount of fluorescence subsequently seen was indicative of membrane repair ability (higher fluorescence seen with diminished repair). The repair process was shown to be calcium-dependent, as evidenced by these preliminary experiments (Figure 4.1). The fibers damaged in the presence of Ca\(^{2+}\) showed normal repair with membrane resealing and attenuation of dye entry occurring shortly after laser damage. However, fibers damaged without Ca\(^{2+}\) showed an accumulation of fluorescence which does not stabilize or become significantly different from +Ca\(^{2+}\) at 145s post-injury, indicating the lack of Ca\(^{2+}\) affected the ability of the sarcolemma to reseal following damage (Figure 4.1).

Next, we tested the membrane repair process all in the presence of Ca\(^{2+}\) in a disease state followed by delivery of a therapeutic transgene. The results in Figure 4.2 represent changes in membrane repair capability in dysf\(^{-/-}\) muscle, and an improvement following treatment with AAV5.MHCK7.DYSF. We delivered the AAV5.MHCK7.DYSF vector at 6x10\(^{10}\) vg total dose by IM into 8-week old 129-Dysf\(^{-/-}\) (129-Dysf\(^{tm1Kcam1}\)) mice in the FDB muscle. 129-WT (129S1/SvImJ) mice (n=6) and untreated 129-Dysf\(^{-/-}\) (n=6) were sacrificed at 20-weeks of age, and AAV5.MHCK7.DYSF treated 129-Dysf\(^{-/-}\) mice (n=6) were sacrificed 12-weeks post-injection. A small area of fluorescence was
detectable in all fibers immediately following laser injury localized at the site of damage (Figure 4.2a). In WT muscle fibers, the sarcolemma repaired quickly and dye infiltration into the fiber stabilized (Figures 4.2a,b). In the fibers of dysf⁻/⁻ untreated mice, a significantly continuous increase of fluorescence was seen throughout the images over the 3-minute time course (Figures 4.2a,b), even more drastically compared to WT fibers in the absence of Ca²⁺. However, fibers from the AAV5.MHCK7.DYSF treated dysf⁻/⁻ mice showed membrane repair similar to that of WT fibers with Ca²⁺, with a significant difference from KO beginning at t=70s (Figures 4.2a,b). Corollary expression studies in the TA using immunofluorescence staining indicated 67.3±14.4% expression of the DYSF transgene (data not shown). These results show that full-length DYSF delivered directly to muscle can lead to functional improvements in membrane repair ability.

4.3.2. Restoring membrane repair with delivery of AAVrh.74.DYSF.DV

As a direct comparison to AAV5.MHCK7.DYSF, we tested the ability of the AAVrh.74.DYSF.DV vectors to effectively transduce muscle fibers, express full-length DYSF protein, and improve muscle fiber membrane repair in dysf⁻/⁻ muscle. 129-Dysf⁻/⁻ mice at 8-weeks of age were injected IM in the FDB with escalating doses of AAVrh.74.DYSF.DV; 6x10⁹ (n=6), 2x10¹⁰ (n=6), and 6x10¹⁰ (n=6) vg total dose. Untreated 129-Dysf⁻/⁻ mice (n=6) and 129-WT mice at 20-weeks of age served as strain specific normal controls. Animals were sacrificed 12 weeks after injections, FDBs were isolated, and again multiphoton laser-induced sarcolemmal damage resulted in a focal area of fluorescence near the site of damage (Figure 4.3a). Similar to previous experiments, WT fibers underwent a normal repair process and the membrane was resealed halting the influx of dye. Also repeated in dysf⁻/⁻ fibers was a compromised
repair process, hindering the ability of the membrane to reseal, and thereby allowing a continuous influx of dye (Figure 4.3a). Expression of DYSF from AAVrh.74.DYSF.DV-transduced fibers resulted in a dose-dependent improvement in resealing capacity (Figure 4.3b). For the three treatment groups, only the high dose (6x10^{10} vg total dose) restored repair to near WT levels, with significance from KO muscle occurring at t=85s post-injury. Parallel immunofluorescence expression studies show high dose fiber transduction of 87±5.6% (data not shown).

4.3.3. Systemic delivery of AAVrh.74.MHCK7.DYSF.DV improves membrane repair

To test the feasibility and efficacy of a clinically relevant systemic delivery approach, we treated a more severely affected dysf/- mouse model, Bla/J (B.6A-Dysf^+/GeneJ) mice, with either 2x10^{12} vg total dose (n=6) or 6x10^{12} vg total dose (n=6) of AAVrh.74.DYSF.DV through the tail vein. Animals were sacrificed 12-weeks post-gene transfer and FDBs were extracted to perform the membrane repair analysis. While untreated Bla/J mice replicated the defective membrane repair ability seen in the 129-Dysf^/- (Figure 4.4a), a dose-dependent response in resealing capability was observed in treated muscle. The low dose treatment (2x10^{12} vg total dose) did not improve membrane repair ability and results did not differ from untreated Bla/J (Figure 4.4a), whereas high dose (6x10^{12} vg total dose) treated fibers exhibited improved repair not significantly different than BL/10-WT (n=6), and did reach significance compared to untreated Bla/J mice at t=160s (Figure 4.4a). Quantification of total dye fluorescence intensity within muscle fibers at the final time point in the assay confirmed improved membrane resealing and reduced dye infiltration in the high dose treated cohort (Figure
4.4.  Immunofluorescence expression studies across multiple transduced skeletal muscles indicate 21.20 ± 2.94% DYSF expression in high dose treated mice (data not shown). Importantly, we have shown that clinically advantageous systemic delivery of a therapeutic DYSF transgene provides functional benefit to diseased muscle.

4.3.4.  AAVrh.74.DYSF.DV systemic gene transfer in aged mice

The above experiments testing membrane repair function in skeletal muscle were performed in younger mice with treatment occurring at the early stages of disease progression. In an attempt to determine therapeutic efficacy in aged muscle following the most severe stages of disease pathogenesis, we systemically delivered 6x10^{12} vg total dose of AAVrh.74.DYSF.DV (n=6) through the tail vein to 6-month old Bla/J mice and animals were sacrificed 3-months post-delivery. Expectedly, membrane repair in aged muscle was compromised in untreated Bla/J (n=6) mice with statistically significant differences from BL/10-WT (n=6) muscle beginning at 85s post-injury (Figure 4.5). Additionally, there was functional improvement in membrane repair upon delivery of DYSF dual vectors with statistical significance compared to KO mice seen following the 190s assay (Figure 4.5). While parallel expression studies demonstrated only an average of 15.42 ± 3.02% expression across various skeletal muscles, membrane repair analysis demonstrates AAVrh.74.DYSF.DV treatment provided a restoration of a functional parameter in severely diseased aged muscle.
4.3.5. **Ano5 facilitates membrane repair**

As an extension of our experiments in dysferlin mice, we aimed to characterize functional deficits in the less-well known ano5−/− mice. We know in a physiological state, normal exercise results in small lesions in the plasma membrane that are healed either through patch assembly of a new plasma membrane or by satellite cells that proliferate and differentiate into myocytes that fuse to repair or regenerate multinucleated muscle fibers [138]. To test the effect of loss of ANO5 expression on membrane repair, we first examined the effect of the laser-induced membrane damage on FDB muscle fibers of 4-month old ano5−/− mice. As we have reported above in dysf−/− mice, a small area of fluorescence was detected at the site of damage in all fibers immediately after laser injury (Figure 4.6a). In the disease state of ano5−/− muscle fibers (n=4), increase in fluorescence was seen at a ~2-fold faster initial rate compared to BL/6-WT fibers (n=4) (Figure 4.6b). Whereas the fluorescence intensity plateaus throughout the time course up to 190 sec in WT, the fluorescence continued to increase in ano5−/− fibers for the duration of the experiment (Figure 4.6b). To test whether restoring ANO5 expression could reverse the defect in membrane repair, we delivered AAVrh.74.ANO5.FLAG at 1x1011 vg total dose (n=4) by IM to ano5−/− FDB muscles. AAV.ANO5 treatment partially restored membrane resealing in ano5−/− muscle where a significant difference was detected at t=100s (Figure 4.6b).

4.4. **Discussion**

Development of a clinically relevant therapeutic requires preclinical efficiency with correctable functional outcome measures. The laser-induced injury assay described above is used to evaluate membrane repair ability in muscle fibers [80]. This
provides a functional outcome for characterization studies of LGMDs and testing therapeutic intervention for these disorders. The ultimate goal was to determine a correlation between repair ability and exogenous transgene expression. Although this assay tests the membrane repair ability of fibers following sarcolemmal injury, the high powered direct and acute form of laser-induced damage may not fully reflect the damage profiles seen in vivo. In some instances, the intensity of membrane damage induced by this laser was dramatic enough to result in loss or destruction of the fiber itself, thereby rendering it useless for analysis. While the damage profile from the laser may not be fully representative of in vivo mechanical damage, we believe the membrane repair profiles reported here for WT fibers, dystf−/− untreated fibers, ano5−/− untreated fibers, and all vector dosed fibers are still accurate representations of physiological sarcolemmal repair.

To begin testing the membrane repair process, we damaged WT muscle fibers in the presence or absence of calcium in the dye media. These experiments indicated a Ca²⁺-dependent repair process in muscle, where a lack of Ca²⁺ affected the ability of the sarcolemma to reseal following injury. Viral-mediated delivery of a therapeutic transgene targeting the FDB muscle is highly reproducible [56, 87, 88]. Specifically with direct IM delivery, injection directly under the footpad allows for visualization of the needle being inserted into the muscle and visual confirmation of appropriate needle location. It is also possible to detect swelling of the footpad due to infusion of the virus, which serves as a second confirmation of successful injection. Regardless of the delivery method however, it is not guaranteed that successful AAV transduction and expression of DYSF or ANO5 will have occurred in all FDB fibers included in analysis. To mitigate issues from including non-transduced fibers in the analysis, multiple fibers
per muscle were tested to increase sample size (6-8 fibers per muscle). Moreover, the muscle extraction process by nature severs muscle fibers from the *in vivo* environment and can cause damage to individual fibers. While visibly damaged fibers, appearing dead under bright field view with high intracellular fluorescent signal under multiphoton view, were not used in the assay, it is possible to unknowingly analyze fibers with underlying membrane damage. Again, variability from this issue was diminished by high sample size and screening by an experienced technician.

In addition to the experiments with and without Ca\(^{2+}\), we included studies to demonstrate compromised membrane repair capacity in *dystf*\(^{-/-}\) and *ano5*\(^{-/-}\) muscle and restored membrane repair ability following *DYSF* or *ANO5* gene replacement. The potential for *DYSF* gene replacement to improve membrane repair in the FDB has been demonstrated previously by Lostal *et al*., however improvement did not reach WT levels due to low levels of transduction (1-4%) [88]. In our studies, we utilized a single dose of a single AAV vector system (AAV5.MHCK7.DYSF) and a dual AAV vector system with multiple doses (AAVrh.74.MHCK7.DYSF.DV) to express full-length *DYSF*. Our investigations demonstrated therapeutic efficacy for AAV5.MHCK7.DYSF gene transfer that restored sarcolemmal resealing in diseased muscle.

The work here also provides proof that co-delivery of two vectors produced full-length *DYSF* through *in vivo* recombination and provided functional restoration of membrane repair in a dose-dependent manner. The infiltration of dye reflected by fluorescence intensity in damaged muscle decreased with increasing doses of virus correlating to increased levels of expression of *DYSF* when FDB muscles were treated directly. Following this work, we tested low and high systemic doses of AAVrh.74.DYSF.DV in the more severely affected Bla/J murine model, leading to ~2-6%
and ~15-27% DYSF expression, respectively. Functional outcome measures in membrane repair were restored to WT levels for the high dose, suggesting that lower levels of expression (~25%) may be sufficient to see a clinical functional benefit. In conclusion, our dual vector work is a promising therapeutic strategy for the treatment of LGMD2B patients.

Disruption of anoctamin5 (ANO5) closely phenocopies the loss of dysferlin expression in murine models [135], and dysferlin mutations cause LGMDs similar to ANO5 myopathies (LGMD2B vs LGMD2L). The observation that many membrane repair-associated proteins, including dysferlin via its C2 domains, bind phosphatidylserine (PLS), and that some ANOs can scramble phosphatidylserine [139, 140], raised the possibility that defective PLS due to ANO5 loss may explain the similar disease phenotypes. Recently, two other laboratories have reported on ANO5 knockout mice [141, 142], and in both cases, these investigators conclude that the ANO5 knockout mice do not exhibit any muscle phenotype. Here, using the single fiber isolation technique and membrane repair assay, we demonstrated an essential role for ANO5 in muscle membrane repair. We characterized an attenuation of sarcolemmal patch repair following injury in ano5<sup>_−/−</sup> fibers that is partially rescued by viral expression of ANO5. This ano5<sup>_−/−</sup> model demonstrates testable therapeutic outcomes and represents an important model for the study of ANO5-myopathy and ANO5’s role in sarcolemmal repair.

These considerations, when taken together, provide a platform to evaluate the membrane repair ability in WT, dysf<sup>_−/−</sup> and ano5<sup>_−/−</sup>, and AAV treated muscle fibers. The experiments described allow for comparison of membrane repair ability by measuring fluorescence infiltration following laser-induced sarcolemmal injury. The data presented
show a clear amelioration of membrane repair defects following muscle treatment with AAV delivery of therapeutic DYSF or ANO5 transgenes, nearly to WT levels. This work provides an alternative outcome measure to evaluate muscle function and sarcolemmal integrity, for various muscle and cellular disorders.

4.5. Materials and Methods

4.5.1. Dysferlin deficient mouse strains

Stocks of BL/10-WT (C57BL/10), 129-WT (129S1/SvImJ), 129-Dysf<sup>fl/fl</sup> (129-Dysf<sup>tm1Kcam/J</sup>) and Bla/J (B.6A-Dysf<sup>emrd</sup>/GeneJ) dysferlin deficient mice were bred and maintained as homozygous animals in standardized conditions in the Animal Resources Core at the Research Institute at Nationwide Children's Hospital. Bla/J mice were generously provided by the Jain Foundation. They were maintained on Teklad Global Rodent Diet (3.8% Fiber, 18.8% Protein, 5% fat chow) with a 12:12 h dark:light cycle. Procedures used in the experiments were approved by the Institutional Animal Care and Use Committee at Nationwide Children's Hospital (AR08-00017).

4.5.2. Anoctamin5-deficient Mouse Strain

Stocks of Ano5<sup>−/−</sup> and BL/6-WT (C57BL/6) mice were bred and maintained as homozygous animals in standardized conditions in the Animal Resources Core at the Research Institute at Nationwide Children's Hospital. The Ano5<sup>−/−</sup> mouse was created using a conditional ready, lacZ-tagged mutant allele Ano5<sup>tm1a(KOMP)Wtsi</sup> targeting vector obtained from the UC Davis KOMP Repository (PG00097_Z_1_G0) [143], in the
Embyonic Stem Cell Core at the Research Institute at Nationwide Children’s Hospital [144]. They were maintained on Teklad Global Rodent Diet (3.8% Fiber, 18.8% Protein, 5% fat chow) with a 12:12 h dark:light cycle. Procedures used in the experiments were approved by the Institutional Animal Care and Use Committee at Nationwide Children’s Hospital (AR12-00004).

4.5.3. Intramuscular Delivery

For initial studies, the left flexor digitorum brevis (FDB) muscle of 8-week old 129-Dysf<sup>−/−</sup> mice was injected with 6x10<sup>10</sup> vg total dose of AAV5.MHCK7.DYSF (n=6). In subsequent studies with the dual vector system, the left FDB of 8-week old 129-Dysf<sup>−/−</sup> was injected with 6x10<sup>9</sup> vg total dose (n=6), 2x10<sup>10</sup> vg total dose (n=6), or 6x10<sup>10</sup> vg total dose (n=6) of AAVrh.74.MHCK7.DYSF.DV in normal saline buffer (1:1 ratio of AAVrh.74.MHCK7.DYSF5' and AAVrh.74.DYSF3', total volume of 30 µL). For both studies, FDB muscles were extracted 12-weeks post-injection and subjected to the membrane repair assay. FDB muscles from 20-week old129-WT mice (n=6) were used as healthy WT controls in both cases. For ANO5 studies, 4-8-week old Ano5<sup>−/−</sup> mice were injected IM in the FDB with 1x10<sup>11</sup> vg total dose of rAAVrh.74.MHCK7.huANO5.FLAG (n=4). In this case, 14-18-week old BL/6-WT mice (n=4) were used for healthy WT controls. Muscles were harvested 10 weeks post treatment and processed for membrane repair assay. In all IM experiments above, the contralateral right FDBs were used for untreated KO controls.
4.5.4. Systemic Delivery

Systemic delivery of AAVrh.74.MHCK7.DYSF.DV was achieved through tail vein injection of either $2 \times 10^{12}$ vg total dose (n=6) or $6 \times 10^{12}$ vg total dose (n=6) (1:1 ratio of AAVrh.74.MHCK7.DYSF5' and AAVrh.74.DYSF3', total volume of 150 µL) into 8-week old Bla/J mice. Mice were sacrificed 12 weeks post-injection with a full necropsy performed and the left and right FDB were harvested and subjected to the membrane repair assay. The FDBs from BL/10-WT mice (n=6) and untreated Bla/J mice (n=6) were removed at 20-weeks of age and used as WT and KO controls, respectively. Finally, for aged studies, 6-month old Bla/J mice were injected intravenously in the tail vein with $6 \times 10^{12}$ vg total dose (n=6) of AAVrh.74.MHCK7.DYSF.DV in normal saline buffer (1:1 ratio of AAVrh.74.MHCK7.DYSF5' and AAVrh.74.DYSF3', total volume of 250 µL). FDB muscles were extracted 3-months post-treatment and subjected to the membrane repair assay. Here, 9-month old FDBs from BL/10-WT mice (n=6) and untreated Bla/J mice (n=6) served as the healthy WT and diseased KO controls, respectively.

4.5.5. Membrane Repair Assay

To begin, mice were euthanized using overdose of ketamine/xylazine cocktail at 200mg/kg and 100mg/kg, respectively. Muscle extraction was initiated by cutting the skin covering the plantar surface of the foot along either side from the toe to the heel. Dissection was done without microscope magnification. While holding the heel with forceps, the skin was carefully cut from heel to toe on the dorsal side of the FDB, lifting up skin with the FDB attached as muscle is cut away. The skin was slowly peeled off the FDB and any excess fat and connective tissue was also trimmed away. The muscle
was then rinsed in 1X PBS and placed in a 35 mm dish. Individual fibers were isolated from the FDB muscle by digestion in a 2% collagenase w/v Type I solution suspended in DMEM. For this step, the muscle was incubated for 90 min at 37°C with gentle shaking at 50 rpm rotation.

Using a 1000uL pipet with the end of the tip cut off, the muscle bundle was carefully transferred to a 35mm glass-bottom dish coated with poly-D Lysine, containing 2mL DMEM + 10% FBS. The muscle was washed about 20 times in media using a 200ul pipet until the muscle was fully dissociated. All media was removed using a pipette and the muscle was centered in the glass-bottom dish. A solution comprised of 2.5 µM FM® 1-43 (Invitrogen®) in Dulbecco’s PBS (no Ca/Mg) supplemented with or without 1.5 mM Ca²⁺ was then added to the dish and the muscle bundle was covered with a glass cover slip, being careful to avoid air bubbles. The dish with muscle was placed under the microscope, and a droplet of PBS was added on top of the cover slip to bring down the objective to make contact with the PBS droplet. Using bright-field microscopy, healthy isolated muscle fibers were identified as appearing bright and shiny with striations clearly visible.

Membrane damage was induced with a FluoView® FV1000 two-photon confocal laser-scanning microscope (Olympus). Fibers were damaged with an 850 nm laser-guided circular ROI area with a diameter of 4.48µm on the edge of the sarcolemma under zoom 2X. The muscle was irradiated at 20% power for 5 s. Stimulus settings were adjusted to 40 frames spaced 5 seconds apart, where images were captured 5 s prior to injury and every 5 s after injury until 190 s post-irradiation to visualize FM1-43 dye uptake. An average of 6-8 fibers were imaged per muscle per mouse. Fluorescence intensity of dye infiltration surrounding the damage site on the membrane
was analyzed with Image J software by measuring integrated density of pixel intensity within the defined area.

A detailed procedure for analysis is as follows: On image J, measurement parameters were set under the “Set Measurements-Analyze” drop down menu and selecting “Area and Integrated density” in the subsequent pop-up window. Scale parameters were set under the “Set Scale-Analyze” drop down menu and 100 was entered for “Distance in Pixels” and “Global” was set to apply settings to all images. Using the rotate option under “Image>Transform”, the image was rotated such that the fiber was fully horizontal or vertical. For measurement, under a 2X zoom setting on ImageJ, a rectangular box measuring 7.5 μm by 10 μm (0.75pixel by 1.0pixel) region of interest (ROI) was drawn in the region of dye infiltration.

Throughout the measurement of the fluorescence intensity within the 0.75pixel rectangle in all images, the rectangle remained on the area of damage for each frame. In the analysis, measured fluorescence intensity at an individual time point was normalized to initial intensity measured at t = -5s (pre-injury) using the equation:

\[ NI = \frac{I - I_0}{I_0} \]

where \( NI \) is the normalized intensity, \( I \) is the measured intensity and \( I_0 \) is the intensity at \( t = -5s \). When fluorescence intensity was analyzed, values from all fibers from each strain were averaged together.

4.5.6. Statistical analysis

Experimental technicians were blinded to control and treatment cohorts to remove bias. A TWO-WAY ANOVA was performed to determine statistical significance between treated and untreated fibers at each time point. Data were expressed as the
mean ± SEM and analyzed using GraphPad Prism 5 (Graphpad Software, La Jolla, CA) unless otherwise specified.
4.6. Figures

Figure 4.1. Membrane repair process is calcium dependent. Individual muscle fibers isolated from the flexor digitorum brevis (FDB) muscle of 129S1/SvImJ-WT mice analyzed with and without the presence of Ca\textsuperscript{2+}. Graphical representation of fluorescence normalized to pre-injury fluorescence. *Significant difference between plus Ca\textsuperscript{2+} and no Ca\textsuperscript{2+} FM-143 dye seen starting at t=145s.
Figure 4.2. **AAV5.DYSF treatment restores membrane resealing.** Individual fibers isolated from the flexor digitorum brevis (FDB) muscle were wounded using a multiphoton laser and imaged every 5s for 195s (injury at t=0s). (a) Representative images of 129-WT, untreated 129-Dysf<sup>-/-</sup> and AAV5.DYSF fibers shown at pre-injury (t=-5s) and post-injury (t=195s). (b) Graphical analysis of fluorescence normalized to pre-injury fluorescence. *Significant difference between untreated 129-Dysf<sup>-/-</sup> and AAV5.DYSF starting at t=70s (p<0.05, TWO-WAY ANOVA).
Figure 4.3. Dose-dependent membrane resealing activity following IM AAVrh.74.MHCK7.DYSF.DV delivery. Three doses of AAV.DYSF.DV vectors were injected into the FDB of 129-Dysf<sup>−/−</sup> mice at 8 weeks of age and analyzed 12 weeks post gene transfer at 20 weeks of age (n=6 per group). Untreated 129-Dysf<sup>−/−</sup> and 129-WT mice served as strain specific normal controls. (a) Representative images pre-injury (t=-5s) (left column) and post-injury (t=190s) (right column) of 129-WT, untreated 129-Dysf<sup>−/−</sup> and treated fibers. (b) Quantification of dye infiltration over time course of imaging. AAV.DYSF.DV treatment revealed dose dependent membrane resealing. Only the high dose (6e10vg total dose) was not significantly different than WT. *Significant difference between high dose and uninjected Dysf<sup>−/−</sup> seen at t=85s (p<0.05, TWO-WAY ANOVA). Error bars represent standard error mean.
Figure 4.4. **Systemic delivery of AAVrh.74.MHCK7.DYSF.DV restores membrane repair deficits in BlaJ mice.** (a) Membrane repair assay quantification of normalized integrated density resembling fluorescence intensity of dye infiltration over time. No significant improvement at low dose, however, high dose treatment restored repair to near wild-type levels with significance compared to untreated BlaJ at t=160s (p<0.05, TWO-WAY ANOVA). Error bars represent standard error mean. (b) Quantification of total normalized integrated density of dye fluorescence intensity within muscle fiber at final analysis time point of t=190s.
Figure 4.5. Systemic AAVrh.74.MHCK7.DYSF.DV delivery in aged BlaJ mice improves membrane repair. Animals treated at 6 month of age and sacrificed at 9 months of age demonstrated improvements in membrane repair in FDB muscles systemically treated with AAV.DYSF.DV with significance at t=190s (p<0.05, TWO-WAY ANOVA).
Figure 4.6. Membrane Repair is defective in Ano5<sup>−/−</sup> mice. (a) Images of Ano5<sup>−/−</sup> and WT muscle 4 month old mice damaged by laser pulse shown 5 sec and 190 sec post-injury. Red arrows indicate the site of damage with FM1-43 dye accumulating quickly in ano5<sup>−/−</sup> muscle compared to WT and ano5<sup>−/−</sup> following delivery of 1x10<sup>11</sup> vg AAV.ANO5.FLAG vector. (b) Measurement of fluorescence intensity after laser-induced injury. ano5<sup>−/−</sup> muscle is statistically different from WT and AAV.ANO5 fibers at t=100s post-injury (p<0.001, TWO-WAY ANOVA).
Chapter 5: Discussion

5.1. Requirement for LGMD2E Therapy

From the first time LGMD and specifically LGMD2E was described clinically, it was clearly evident how debilitating this disease is [1, 7, 11]. The fact that it can affect a high percentage of patients at such an early age combined with the significant ambulatory, respiratory, and cardiac deficiencies that ensue illustrates its severity and the rapid decline in quality of life these patients experience [6-9, 11]. With no available cure, this creates great necessity for a long-term treatment option that targets and corrects the primary genetic defect in the disease. Our goal with this work was to use a gene therapy approach with AAV to reintroduce the WT SGCB gene into the affected muscles of sgcb−/− mice in order to correct the disease state. The rationale that underlies this research is that the severe disease phenotype originates from and is largely restricted to skeletal and cardiac muscle, therefore successful gene delivery to regional muscle groups would provide a functional benefit to LGMD2E patients. The research presented in this dissertation is, in our opinion, innovative, as it uses the emerging technique of viral mediated gene transfer with clinically applicable systemic delivery, a non-pathogenic virus and viral serotype with high tropism for muscle, and a muscle specific promoter to target affected muscle and restore function.
We tested this therapy in a step-wise manner with three main objectives to ultimately translate this to clinical trial: characterize the $sgcb^{-/-}$ mouse and establish functional deficits in muscle for recovery following gene transfer, demonstrate potency and efficacy of localized AAV-mediated $SGCB$ gene transfer, and establish therapeutic efficacy and safety via a clinically relevant systemic delivery approach. Success in a pre-clinical study provides the necessary support for clinical trials in LGMD2E, in order to perform the first-in-human AAV.hSGCB gene replacement studies. This document detailed the significant progress we have made along that translational pathway for therapeutic development.

5.2. Novel Viral-Mediated Human SGCB Gene Transfer

We proposed in this report the first gene transfer study of hSGCB using the recombinant scAAVrh74 serotype vector. Our previous findings with hSGCA gene therapy [47, 49, 59] provided an excellent platform for the design of the investigation here. This work with $SGCB$ gene transfer when taken together produced a novel, more safe and efficient gene replacement therapy as opposed to those previously investigated. First, we used a self-complimentary (sc) AAV vector, which decreases the time required for replication of transgene DNA and production of exogenous therapeutic SGCB protein, allowing for high transduction efficiency at lower, safer doses [91, 92]. Additionally, the rh.74 serotype transduces muscle well particularly from the vasculature [93, 94, 98]. Next, the use of systemic delivery (with the MCHK7 promoter), as opposed to IM or ILP delivery, allowed us to target all muscles throughout the body including the heart with a single intravenous injection. This delivery method is less invasive for patients than our previously studied ILP delivery [93-95]. Finally, the proposed therapy
here with $SGCB$ gene transfer, similar to our work with $SGCA$ due to the small size of the genes, involved delivery of a codon-optimized full-length WT $SGCB$ cDNA, producing entirely normal full-length $SGCB$ protein. This is advantageous for clinical application as opposed to, for example, previous studies of mini- and micro-dystrophin therapy for DMD, which resulted from the large transgene size and limited packaging capacity of AAV [105, 136]. Not only is production of a full-length protein advantageous for potentially greater functional recovery, but it also decreases the potential immunogenicity of the exogenous transgene, diminishing the chances of a significant immune response.

Mendell et. al. reported in the first IM AAV.mini-dystrophin clinical trial that AAV transduction in muscle led to a marginal immune response to the exogenous truncated mini-dystrophin that resulted in the loss of transgene expression over time, partially limiting the efficacy of the therapy [48]. Patients either with genomic deletions in a region expressed by the mini-dystrophin transgene or revertant fibers expressing dystrophin generated from a second site mutation elicited a T cell-mediated immune response to the truncated protein [48]. Moreover, as we saw in our studies with LGMD2D and $SGCA$, a wide range of mutations exist in LGMD2E patients yet single nucleotide changes are the most common $SGCB$ gene mutations, which decreases the chances for immunorejection of the transgene and increases the chances of success in clinical trials [8, 9, 16-18, 47, 49].

5.3. **Proof-of-Principle Studies**

In the first steps of our proof-of-concept investigation into pre-clinical efficacy of AAV.h$SGCB$ gene transfer, all of which are shown in Chapter 2 and a previously
publication [112], we initially demonstrated the recapitulation of the LGMD2E clinical phenotype in \( sgcb^{−/−} \) mice, particularly with significant endomysial fibrosis. IM and ILP delivery of AAV.tMCK.hSGCB to \( sgcb^{−/−} \) mice revealed highly efficient transduction and long-term transgene expression in treated muscle. Most significantly, restoring SGCB expression and consequently the DAPC and sarcolemmal integrity in diseased muscle led to a reduction in fibrosis, further contributing to therapeutic benefits including functional recovery. Another important takeaway from these initial studies when considering gene transfer in patients, who tend to show a more advanced disease state at the time of diagnosis and therefore therapeutic intervention, was the benefit we saw when treatment was given to aged mice with more severe muscle pathology. Although an ideal scenario would be to treat patients as early as possible before significant disease onset, showing therapeutic efficacy in older mice becomes an important factor supporting the versatility of our therapy. Additionally, a formal GLP toxicology study in C57BL/6 WT mice (final section of Chapter 2) revealed no observed adverse effects from a single IM injection or ILP delivery of scAAVrh.74.tMCK.hSGCB.

5.4. Systemic Delivery of AAV-\( \beta \)-Sarcoglycan

This report highlighted the evolving studies that indicate the high frequency of cardiac involvement in LGMD2E, where the number of patients with heart dysfunction can exceed 60% [13, 18, 20-22]. Importantly when considering patients for clinical trial, the cardiac involvement in LGMD2E does not correlate with age, muscle strength, or the level of dystrophic changes on muscle biopsy. In order to broaden the scope of our treatment and create a more effective therapy for LGMD2E patients, we made several changes of note to the study design (Chapter 3). We further optimized the AAV vector
by replacing the tMCK promoter with the MHCK7 promoter for enhanced transduction of cardiac tissue [114]. To support the change in promoter, we utilized an intravenous delivery approach to achieve systemic delivery of hSGCB.

Here we demonstrated in Chapter 3 substantial efficacy with systemic delivery of AAV.MHCK7.hSGCB through the tail vein of sgcb⁻/⁻ mice. This led to nearly complete transduction in numerous muscles throughout the mice, including the heart, which produced similar results to our localized delivery with AAV.tMCK.hSGCB. Histological and functional parameters were all restored to near WT levels. Furthermore, we showed benefits in treated mice only attainable through systemic gene transfer with partial reversal of kyphoscoliosis of the spine and increased ambulatory outcomes with improved overall activity. Importantly mimicking our formal GLP toxicology study, no hSGCB transgene expression was detected in non-muscle tissue and no adverse effects were seen in WT mice systemically treated with AAV.MHCK7.hSGCB. The cardiac expression in this well-defined model of LGMD2E (shown in Chapter 3) using the MHCK7 promoter is very encouraging at dosing levels that could be applied clinically. Given the high incidence of heart involvement in LGMD2E patients, we feel this has provided a rationale for the benefits of systemic gene delivery in clinical trial that would lead to clinically meaningful results for these patients.

5.5. Future Work with LGMD2E Therapy

Following the completion of the extensive pre-clinical studies outlined in this dissertation (Chapters 2 and 3), we have successfully submitted and gained FDA approval of an investigational new drug (IND) application for a Phase I/IIa, open-label, single-dose clinical trial for intravenous delivery of scAAVrh74.MHCK7.hSGCB into
LGMD2E patients. As we move forward in the translation of AAVmediate hSGCB gene therapy for LGMD2E, there are several important considerations we must take into account that can impact the outcomes of the study, because this is the first time that the scAAVrh74.MHCK7.hSGCB viral vector will be given to human patients.

The advantages of our vector delivery study design discussed earlier allow for a lower proposed viral dose in our clinical study \((5 \times 10^{13} \text{ vg/kg})\) compared to current clinical trials with AAV gene transfer, an added element of safety for LGMD2E patients. An ongoing clinical trial in our center for AAV-mediated delivery of the \(SMN\) gene to babies with Spinal Muscular Atrophy (SMA) provides strong evidence of a completely clean safety profile following systemic delivery at a much higher dose \((2 \times 10^{14} \text{ vg/kg})\) \([128]\). Along with this beneficial dosing level, preclinical and clinical studies for LGMD therapies with multiple AAV serotypes including rh.74 as well as the MHCK7 promoter indicated no treatment related adverse effects \([47, 49, 56, 59]\). It is important to note that along with the excellent results in our FDA approved GLP toxicology study with the AAV.tMCK.hSGCB vector (see Chapter 2), we can strengthen our position with a cross-reference to our GLP toxicology study using the MHCK7 promoter with the \(DYSF\) gene, which led to an FDA approved a clinical trial for LGMD2B \([57]\). While we saw the same results in our pre-clinical studies with systemic delivery of AAV.MHCK7.hSGCB, the risks of this specific therapy in humans are not known, and thus, the primary outcome of the Phase I study will be safety. For secondary outcomes, we will assess patients for transgene expression levels and functional recovery including cardiac improvement in ejection fraction and increased ambulation measured by the 6 minute walk test (6MWT).

With safety as the primary focus of this Phase I clinical trial, we will measure both binding antibodies and T cell responses to the AAVrh.74 capsid and exogenous hSGCB
transgene following gene transfer. As described, AAVrh74 is a serotype isolated from rhesus macaques, but is 93% identical to human AAV8 [97], with most humans being negative for serum antibodies to AAV8, making pre-existing immunity a concern for only a small minority of potential patients [93]. Although unlikely, in the event pre-existing immunity does occur, one option to circumvent this hurdle is the use of plasmapheresis to remove innate immune components including antibodies prior to gene delivery [98].

Cardiomyopathy in this disease is a major factor for patients in this clinical trial. Mice deficient for SGCB show myocardial dystrophic pathology as early as 4 weeks of age [10, 60], however we found no correlation between the age of development of cardiac histopathology and the age of decline in cardiac function (Chapter 3). We detect no significant functional decline in the hearts of moderately aged sgcb-/- mice, well after the initial stages of histological disruption in cardiac tissue. Eventually, hearts from sgcb-/- mice do present with cardiomyopathy symptoms with decreased ventricular stroke volume, cardiac output, and ejection fraction due to impaired cardiomyocyte contractility [113]. This delayed onset can potentially be explained by two compensatory mechanisms in the heart to extend normal function as long as possible. The Frank-Starling mechanism involves an increase in ventricular diastolic volume, which increases the stretch of myofibers leading to an increase in stroke volume [145]. Additionally, neurohormonal activation through the renin-angiotensin-aldosterone pathway contributes to increased heart rate and cardiomyocyte contractility to help buffer the drop in cardiac output [146]. Ultimately, these compensations prove to be detrimental where the progressive cardiomyocyte degeneration and increased volume overload that persist result in cardiomyopathy. When considering patients for clinical trial, these compensations may render LGMD2E patients with dilated cardiomyopathy...
asymptomatic during earlier stages of the disease. Therefore, clinical monitoring of cardiac function may be needed for an extended period of time throughout the study to ensure correction of cardiac defects.

A final point to consider is the involvement of smooth muscle dysfunction, particularly in the vasculature, in β-sarcoglycanopathy. We highlighted the findings of Durbeej et. al. that associated the loss of SGCB and the SG-sarcospan complex in vascular smooth muscle with the development and exacerbation of severe cardiomyopathy [10]. LGMD2D patients however, absent for SGCA, do not exhibit severe cardiomyopathy [4, 16, 19, 22, 26, 47, 49]. This is due to the presence of a SGCA homologue, ε-sarcoglycan (SGCE), in the SG complex in smooth muscle that rescues vascular smooth muscle function in LGMD2D, preventing the severe cardiomyopathy seen in LGMD2E [10, 124]. Durbeej et. al. reported a follow-up study in the sgcb−/− mice in which they delivered WT SGCB to diseased mice with adenovirus and saw restoration of SGCB expression in skeletal and cardiac muscle, at the neuromuscular junction, at the myotendinous junction, and in peripheral nerve, but not in smooth muscle vasculature [147].

While they saw histological and functional recovery in treated skeletal muscle, they did not detect a reversal of cardiomyopathy, which confirmed their earlier findings that smooth muscle dysfunction does not contribute significantly to disease pathogenesis in skeletal muscle. The lack of cardiac correction while not transducing smooth muscle is intriguing, and more studies are needed to determine AAVrh.74 transduction in smooth muscle. In the event LGMD2E patients do not display prevention or improvement of cardiomyopathy following systemic treatment, the specific targeting of
vascular smooth muscle potentially by altering the AAV delivery vehicle may be necessary.

5.6. Functional Outcomes for Dysferlin and Anoctomin5 Myopathies

One component in the design of a clinically relevant therapeutic for MD emphasized in this report is the requirement for correctable functional outcome measures in pre-clinical studies. To further investigate functional outcomes for MDs, we focused our attention on alternative forms of LGMD, specifically types 2B and 2L, due to the involvement of DYSF and ANO5, respectively, in the membrane resealing process and in their corresponding diseases [63-71]. We aimed to demonstrate impaired capacity to patch repair sarcolemmal lesions and determine a correlation between repair ability and exogenous transgene expression. This then provides a functional outcome for characterization studies of LGMDs and testing therapeutic intervention for these disorders.

We and others have demonstrated the reproducibility of viral-mediated delivery of a therapeutic transgene to the FDB muscle through IM injection or systemic delivery, all detailed in Chapter 4 and in previously published studies [55, 56, 87, 144]. We optimized a protocol comprising enzymatic dissociation of the FDB muscle in mice to isolate individual healthy muscle fibers [79, 87], in order to serve as an in vivo model for muscle membrane resealing. A multi-photon confocal microscope was then used to generate membrane damage and record a series of images of fluorescent dye accumulation in the fiber over time, creating a pseudo-video allowing one to visualize the membrane repair process in the muscle in real time. By measuring pixel intensity of the
fluorescent dye at the site of damage in each individual image, we quantified the rate of repair and hence the ability of muscle to reseal the sarcolemmal.

We have demonstrated impaired membrane repair ability in both DYSF and ANO5 deficient fibers, as well as restoration of repair following gene replacement in several previously published studies [55, 56, 144], also outlined here in Chapter 4. Thus, we reported an alternative experimental method as an outcome measure for the study of membrane repair-deficient LGMDs and their emerging therapeutic interventions.

5.7. Conclusions

Future clinical studies to approve AAV.hSGCB gene therapy are contingent on demonstrating safety in the Phase I trial of intravenous delivery of scAAVrh74.MHCK7.hSGCB to LGMD2E patients. Upon successful completion of a safety study in patients, a proposed Phase II study would evaluate primary long-term safety and efficacy aiming to prevent loss of muscle strength and improve muscle function. This would produce short and long-term clinical benefits with increased and sustained ambulation while preventing cardiac and respiratory complications. Overall, this dissertation presents in detail strong pre-clinical evidence of powerful functional outcomes for the study of certain LGMDs, and efficacy of SGCB gene replacement therapy for LGMD2E. We believe these results provide strong support for the ability of viral-mediated gene replacement therapy with AAV.hSGCB to translate into the clinic and finally provide a means to treat this devastating disease.
References


128. Mendell, J.R. *Phase I Gene Transfer Clinical Trial for Spinal Muscular Atrophy Type 1 Delivering AVXS-101*. 2014 2016; The purpose of this trial is to evaluate safety and efficacy of intravenous delivery of AVXS-101 as a treatment of Spinal...


