AAV-MEDIATED GENE TRANSFER TO MODELS OF MUSCULAR DYSTROPHY: INSIGHTS INTO ASSEMBLY OF MULTI-SUBUNIT MEMBRANE PROTEINS.

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AAV-MEDIATED GENE TRANSFER TO MODELS OF MUSCULAR DYSTROPHY: INSIGHTS INTO ASSEMBLY OF MULTI-SUBUNIT MEMBRANE PROTEINS.

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The sarcoglycanopathies are a subset of the limb girdle muscular dystrophies (LGMD) caused by mutations in the sarcoglycan genes (α, β, γ and δ). In collaborative studies, δ-sarcoglycan was delivered to deficient hamsters using a recombinant adeno-associated virus (AAV), which rescued muscle biochemically, histologically, and functionally. Murine knockouts for the other sarcoglycans permitted us to pursue AAV-mediated gene delivery. AAV-mediated gene delivery of β-sarcoglycan to deficient mice provided long-term biochemical and histological rescue. AAV-mediated gene delivery of α-sarcoglycan to deficient mice showed initial rescue of biochemical and histological defects, although expression was not persistent. Severe Combined Immune-Deficient (SCID) mouse studies indicated that α-sarcoglycan over-expression leads to cytotoxicity. The apparent cytotoxicity can be interpreted with emerging models of sarcoglycan complex assembly. These studies show that AAV-mediated delivery of even closely related proteins can lead to different outcomes, and aspects of protein biochemistry can alter efficacy of gene delivery.

Inherited muscle disorders typically have defined primary biochemical defects. However, there are likely secondary responses that mitigate gene delivery success. To dissect such variables, we studied the immunostimulatory properties of dystrophic muscle. We hypothesized that immune cell infiltrate accompanying degeneration/regeneration could be

immunostimulatory, which could elicit an immune response to delivered transgenes, hampering the success of gene delivery. To study this, we tested antibody response to and persistence of, β-galactosidase in normal and dystrophic muscle. Consistent with our hypothesis, dystrophic muscle showed increased immune surveillance and recognition of β-galactosidase, evidenced by antibody titers and clearance of transduced cells. Furthermore, biochemical rescue of the dystrophy quenched the immune response. This indicated that dystrophic muscle is more prone to immune responses and that aspects of tissue pathology influence the persistence and efficacy of gene delivery. Our results suggest that full biochemical rescue will attenuate immunostimulatory effects.

We also address a hurdle facing AAV-mediated gene therapy; namely, delivery methods. We developed an injection manifold, which was used to safely, accurately, and consistently deliver genes to 20 mm^2 regions of muscle.

Taken together, these results more clearly define barriers to gene delivery. Future research will finely tune regulation of transgenes and enable full rescue of biochemical defects.

FORWARD

I would like to dedicate my dissertation work to two amazing people in my life. The first person is my grandfather, Edward Galiskis. Although he passed away prior to the completion of my dissertation, I know that he was with me in spirit when I finished. The second person that I would like to thank and dedicate my work to is my fiancé, Heather Gordish. She was very helpful with my statistical analyses, but more importantly she has also been a major source of inspiration and support during the completion of my dissertation, and I am looking forward to spending the rest of my life with her. Heather, thank you for all your time and help with my dissertation, especially with all your emotional support throughout this process.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1.0

Introduction

1.1 Limb-girdle muscular dystrophies 2C, 2D, 2E and 2F, the sarcoglycanopathies

Four forms of limb-girdle muscular dystrophy (LGMD 2D, 2E, 2C, and 2F) are caused by mutations in any one of the four genes encoding the dystrophin-associated sarcoglycan proteins (α, β, γ, and δ). These four limb-girdle muscular dystrophies are a group of genetically heterogeneous autosomal recessive muscular dystrophies (Lim *et al.* 1995; Ozawa *et al*., 1995; Bonnemann *et al.* 1996a; Bonnemann *et al.* 1996b; Duggan and Hoffman 1996; Jung *et al.* 1996a; Jung *et al.* 1996b; Nigro *et al.* 1996; Piccolo *et al.* 1996; Sewry *et al.* 1996; Dincer *et al.* 1997; Quinlivan *et al.* 1997). Mutations in any one of the four sarcoglycan genes, termed the "primary sarcoglycanopathies," lead to a secondary biochemical deficiency in all of the other sarcoglycan proteins (Jung *et al.* 1996a; Ozawa *et al*., 1998). This secondary loss is now thought to occur post-translationally, potentially due to inaccurate assembly and stabilization of the dystrophin-associated protein complex (Chen *et al.* 2000). The disease presentation and progression of the sarcoglycanopathies are similar to that of Duchenne muscular dystrophy, and patients with primary sarcoglycanopathies are clinically indistinguishable from those with primary dystrophinopathies. Approximately 10-20% of cases with a Duchenne muscular dystrophy phenotype but normal dystrophin findings by muscle biopsy are caused by sarcoglycan gene mutations. The relative occurrence for each of the different sarcoglycanopathies in the American/European population is $\alpha > \beta > \gamma > \delta$ in about a 6:3:2:1 ratio (Duggan *et al.* 1997a).

1.2 The Sarcoglycan Proteins

Alpha, beta, gamma, and delta sarcoglycans are single-pass transmembrane glycoproteins that assemble stoichiometrically to form a tetrameric sarcoglycan complex (Yamamoto *et al.* 1993; Yoshida *et al.* 1997; Yoshida and Ozawa 1990; Yoshida *et al.* 1994). This complex is one of the components of the dystrophin-glycoprotein complex located at the myofiber plasma membrane (Figure 1) (Ervasti *et al.* 1990). The sarcoglycans are all N-glycosylated with large extra-cellular domains containing cysteine clusters and short intra-cellular domains. α-, γ- and δ-sarcoglycan are primarily expressed in striated muscle, while β-sarcoglycan is moderately expressed in other tissues as well (Bonnemann *et al.* 1995; Lim *et al.* 1995; Jung *et al.* 1996; Nigro *et al.* 1996; Roberds *et al.* 1993a; Noguchi *et al.* 1995).

Investigation of the assembly of the sarcoglycan complex revealed that the four transmembrane proteins are synthesized in the rough endoplasmic recticulum (ER) and glycosylated in both the ER and Golgi apparatus. The individual sarcoglycans are then assembled in the Golgi to form the sarcoglycan complex, which further assembles with other dystrophin associated protein complexes that are all transported to the cell surface. A hierarchical order of assembly has been established with β-sarcoglycan nucleating the complex. The tetramer is completed by subsequent addition of γ- or δ-sarcoglycan added in either order, and followed by the addition of α-sarcoglycan (Noguchi *et al.* 2000). Prior to the elucidation of the assembly of the sarcoglycan complex, Holt and Campbell (1998) used myc-tagged sarcoglycan proteins in a heterologous expression system and found that efficient and correct targeting of the sarcoglycan complex could only be completed with the expression of all four sarcoglycan proteins.

The sarcoglycans are believed to interact with both the dystroglycans and dystrophin. δ-sarcoglycan is thought to interact directly with dystroglycan (Vainzof *et al.* 1996; Sakamoto *et al.* 1997; Chan *et al.* 1998), while the complete complex interacts with the C-terminus of dystrophin (Suzuki *et al.* 1994; Ozawa *et al.* 1998; Suzuki *et al.* 1992; Ahn and Kunkel 1995; Rafael *et al.* 1996; Yang *et al.* 1995; Jung *et al.* 1995; Duclos *et al.* 1998; Araishi *et al.* 1999; Iwata *et al.* 1993; Roberds *et al.* 1993a). These interactions have provided some of the first clues to the function of the sarcoglycan complex.

Although the sarcoglycan complex may help stabilize the interactions of dystrophin and the dystroglycans, contributing to the structural integrity of the myofiber plasma membrane during contraction-induced mechanical stress (Suzuki *et al.* 1994; Ozawa *et al.* 1998; Araishi *et al.* 1999; Hack *et al.* 2000; Yoshida *et al.* 2000), however, this stabilization may not be the only function of the sarcoglycan complex. Database searches revealed homology of the sarcoglycans to a number of epidermal growth factor-like (EGF-like) domains of receptors, a nerve growth factor receptor, and a low-density lipoprotein receptor. The sarcoglycans are arranged at the membrane so that these EGF-like domains are extracellular, which would enable them to interact with a ligand (Ibraghimov-Beskrovnaya *et al.* 1992). The sarcoglycan phosphorylation sites are located cytoplasmically (Bonnemann *et al.* 1995; Lim *et al.* 1995; Noguchi *et al.* 1995; Roberds *et al.* 1993a), which may imply a role in signal transduction. Betto *et al.* (1999) has also shown that α-sarcoglycan can act as an ecto-ATPase. Therefore, deficiencies in α-sarcoglycan may compromise the ability of myofibers to protect themselves against extracellular ATP, which in turn could regulate the activity of purinergic receptors and may have a role in potential calcium dysregulation. In addition, the sarcoglycan proteins may also interact with a calcium channel. Therefore the sarcoglycan proteins could play a role in the regulation of calcium as they are well

positioned at the plasma membrane to directly or indirectly control the influx of calcium (Hack *et al.* 2000). There has also been an interaction observed between the integrins and the sarcoglycans, which could indicate a role in the bi-directional signaling and regulation of the α5β1 integrin in striated muscle (Yoshida *et al.* 1998). Thus, deficiencies in the sarcoglycans could disrupt the proper coupling between signaling and mechanical complexes in muscle (Hack *et al.* 2000).

1.2.1 α-sarcoglycan

The α-sarcoglycan gene was first cloned in rabbit (Roberds *et al.* 1993a) and was later localized in humans to chromosome 17q21 (McNally *et al.* 1994). The human genomic DNA covers over 5.4 kb and contains 10 exons (Roberds *et al.* 1994), while the human cDNA comprises 1410 base pairs (bp), which includes an open reading frame of 1161 bp encoding 387 amino acids (Roberds *et al.* 1993a). The predicted weight is 42.9 kD, although the native protein weighs about 50 kD (Yoshida and Ozawa 1990; Ervasti and Campbell 1991). Initially, the protein was called adhalin ("adhal" in Arabic means muscle) as patients suffering from severe childhood autosomal recessive muscular dystrophy (SCARMD) (prevalent in Arabic countries) were missing this protein (Roberds *et al.* 1993a). α-sarcoglycan is a Type I transmembrane protein with an amino-terminal signal sequence, two consensus sites for phosphorylation (one Ca^{2+}-calmodulin protein kinase site Ser^{377} and one casein kinase II site Thr^{336}), a single-pass transmembrane domain, two predicted N-linked glycosylation sites (Asn^{174} and Asn^{246}), a cytoplasmic carboxyl-terminus, five extracellular cysteine residues, and an ATP binding site in the extracellular domain (Betto *et al.* 1999) (Figure 2). α-sarcoglycan is primarily expressed in striated muscle, but low amounts are detected in the bladder, lung, and small intestine (Roberds *et al.* 1993a; Roberds *et al.* 1994).

1.2.2 β-sarcoglycan

The human β-sarcoglycan gene is located on 4q12 and spans about 13.5 kb of genomic DNA containing 6 exons (Bonnemann *et al.* 1996b). The human cDNA for this gene is 1225 bp with an open reading frame of 956 bp that encodes 318 amino acids (Bonnemann *et al.* 1995; Lim *et al.* 1995). The predicted weight is 34.8 kD, with the native protein weighing 43 kD. β-sarcoglycan is a type II transmembrane protein with a short N-terminal cytoplasmic domain containing a large hydrophobic region, a potential protein kinase C phosphorylation site (Ser^{21}), a single transmembrane region, a large extracellular domain (C-terminal), five extracellular cysteine residues, three potential N-linked glycosylation sites (Asn^{158}, Asn^{211} and Asn^{258}) (Figure 2). β-sarcoglycan is expressed most abundantly in striated muscle, but can also be found in brain, liver, kidney, lung, placenta, and adult pancreas (Lim *et al.* 1995; Bonnemann *et al.* 1996b).

1.2.3 γ-sarcoglycan

The human γ-sarcoglycan gene is located on 13q12 and spans over 100 kb of genomic DNA containing 8 exons (McNally *et al.* 1996). The human cDNA open reading frame consists of 873 bp that encodes 290 amino acids (Noguchi *et al.* 1995). The predicted weight is 32 kD, while the native protein weighs 35 kD (Ervasti *et al.* 1990; Yoshida and Ozawa 1990). γ-sarcoglycan is a type II transmembrane protein with a large extracellular domain (C-terminal), a potential N-linked glycosylation site (Asn^{110}), and five conserved cysteine residues, a single transmembrane domain, one potential phosphorylation site (Yoshida *et al.* 1998), and a short intracellular domain (Noguchi *et al.* 1995) (Figure 2). γ-sarcoglycan is expressed exclusively in striated muscle (Noguchi *et al.* 1995).

1.2.4 δ-sarcoglycan

The human δ-sarcoglycan gene is located on 5q33-q34, spanning over 100 kb of genomic DNA with 8 exons. The human cDNA has an open reading frame of 870 bp encoding 290 amino acids (Nigro *et al.* 1996). The predicted molecular weight is 32 kD with a native weight of 35 kD (Yoshida *et al.* 1997). δ-sarcoglycan is a type II transmembrane protein with a short cytoplasmic domain (N-terminal), a single transmembrane domain, a large extracellular domain, five cysteine residues, and three potential N-linked glycosylation sites (Asn^{61}, Asn^{109} and Asn^{285}) (Figure 2). It differs from the other sarcoglycans, as it does not appear to have any phosphorylation site. However, δ-sarcoglycan and γ-sarcoglycan have the highest amino acid homology among the sarcoglycans, with 55% amino acid identity and 70% amino acid similarity (Nigro *et al.* 1996). It is believed that one of these two sarcoglycans originated by way of a gene duplication event of the other (McNally *et al.* 1996; Nigro *et al.* 1996). δ-sarcoglycan is expressed predominantly in striated muscle, with an alternate splice variant (utilizing an alternative exon 8 which lacks the conserved cysteine residues) expressed outside of muscle (Jung *et al.* 1996).

1.3 Sarcoglycan deficient animal models

Animal models for all four of the sarcoglycanopathies are now available for study. The first model to be identified was a naturally occurring hamster model for δ-sarcoglycanopathy. However, since the identification of the gene defect in the hamster, there has been the subsequent production of knockout mouse models for each of the sarcoglycan deficiencies including δ-sarcoglycan. Two different groups have generated a knockout model for each of the sarcoglycans, except δ-sarcoglycan, which only has one mouse model. This duplication in production may lead to discrepancies between the knockout models, as different areas of each sarcoglycan gene were disrupted in the two models. These discrepancies create the opportunity for genotype/phenotype differences; however, as all knockouts generated have demonstrated no protein production from the null allele, it is likely that any discrepancy between knockout models is not due to the type of knockout generated. Each knockout strain is addressed below in greater detail.

1.3.1 α-sarcoglycan deficient mice

Two separate groups have reported the generation of α-sarcoglycan deficient mice (Duclos *et al.* 1998; Liu and Engvall 1999). Both models for this disease show a progressive muscular dystrophy. More specifically described in Duclos *et al.* (1998), these mice exhibit the muscular dystrophy hallmarks of muscle fiber necrosis, degeneration/regeneration, central nucleation, atrophy, hypertrophy, fiber splitting, and endomysial fibrosis. The two groups generated their mice slightly differently, by targeting different exons of the α-sarcoglycan gene. Duclos *et al.* (1998) knocked out exons 2 and 3 with a neomycin resistance gene. These two exons were targeted because they are the location of one third of the mutations in human patients. However, the mutation did not eliminate the N-terminal signal sequence. Liu and

Engvall (1999), on the other hand, disrupted exons 1 and 2 to delete the N-terminal signal sequence and both exons. Both groups describe the generation of mutations in the α-sarcoglycan gene that created null alleles (Duclos *et al.* 1998; Liu and Engvall 1999).

Duclos *et al.* (1998) indicate that Southern blot analysis showed homologous recombination, and northern blot analysis revealed an absence of full-length coding sequence in skeletal and heart muscle. However, RT-PCR analysis indicated a minor transcript, which is the result of using a cryptic splice site in the neomycin cassette. Sequencing of this product revealed that it encoded exon 1 and 516 bp from the inverted neomycin cassette spliced into exon 4. This splice event inserted 172 amino acids from the neomycin cassette and maintained a reading frame with exon 4 to finish the rest of the α-sarcoglycan protein. However, this transcript would result in a protein lacking 91 amino acids encoded by exons 2 and 3, including part of the signal sequence. Analysis of cardiac and skeletal muscle to identify production of the mutant protein by immunoblot or immunofluorescence with the use of a C-terminal peptide antibody against α-sarcoglycan did not find any mutant protein. α-sarcoglycan protein was absent from the membranes of both cardiac and skeletal muscle. The α-sarcoglycan deficiency leads to a dramatic reduction of sarcospan, β-, γ- and δ-sarcoglycan with a slight reduction of α-dystroglycan and patchy staining of dystrophin at the sarcolemma. In addition, there is a ~10-fold increase in serum creatine kinase (CK) activity compared with control animals (Duclos *et al.* 1998).

Liu and Engvall (1999) did not describe the analysis of the mice in as much detail as Duclos *et al.*(1998). However, Southern blot analysis verified disruption of the α-sarcoglycan gene. Immunofluorescence and immunoblot analysis showed no expression of α-sarcoglycan in homozygous knockout animals, and consistent with Duclos *et al.*(1998), there was a significant

decrease in β-, γ- and δ-sarcoglycan present at the sarcolemma, resulting in a progressive muscular dystrophy phenotype (Liu and Engvall 1999).

1.3.2 β-sarcoglycan deficient mice

Two groups have also described the generation of β-sarcoglycan knockout mice, which develop a progressive muscular dystrophy (Araishi *et al.* 1999; Durbeej *et al.* 2000). Araishi *et al.* (1999) was the first group to describe the generation of such mice. This model has targeted disruption of exon 2 of the β-sarcoglycan gene with a neomycin gene. Exon 2 contains the intracellular and membrane spanning domains of the β-sarcoglycan protein. Durbeej *et al.* (2000) targeted exons 3, 4, 5 and 6 of β-sarcoglycan, which encode part of the transmembrane domain and some of the extracellular portion of the protein. They utilized the phosphoglycerate kinase promoter and neomycin phosphotransferase cDNA for disruption of the β-sarcoglycan gene. Both groups describe the successful generation of null alleles.

Araishi *et al.*(1999) first confirmed disruption of exon 2 with a single copy of the neomycin gene by Southern blot analysis. The creation of a null allele was verified by northern blot analysis and western blot analysis. The results showed the absence of β-sarcoglycan mRNA in the homozygous mutant mice. The western blot revealed that there was no β-sarcoglycan protein detected. Immunofluorescence analysis of these animals showed deficiencies in all of the other sarcoglycan proteins and sarcospan. These mice also exhibited a number of pathologic hallmarks in muscle associated with the muscular dystrophies, such as infiltration of mononuclear cells, degenerating/regenerating fibers, centrally located nuclei, increased connective tissue proliferation, and necrotic fibers with phagocytosis. Serum CK levels were increased up to ~100 times normal levels. All of these observations confirmed the hypothesis that a progressive muscular dystrophy phenotype is present in this β-sarcoglycan deficient

mouse. This group went on to show that using wheat germ agglutinin (WGA) affinity chromatography there is a decrease in the amount of the dystrophin-dystroglycan complex isolated from the β-sarcoglycan deficient mice when compared with controls. This indicates that deficiencies in the sarcoglycan-sarcospan complex may cause instability in the dystrophin-dystroglycan complex (Araishi *et al.* 1999).

Using Southern blot analysis, Durbeej *et al.* (2000) confirmed the disruption of exons 3-6 in their model with the single homologous recombination of the phosphoglycerate kinase promoter and neomycin phosphotransferase cDNA. Northern blot analysis revealed no β-sarcoglycan transcripts were present in skeletal muscle of the β-sarcoglycan deficient animals. Using a specific probe for exon 2, small amounts of a shorter transcript, of the 4.0 kb β-sarcoglycan transcript were present. However, upon analysis of microsome preparations, from skeletal muscle, cardiac muscle, and lung tissue, with antibodies specific to amino acids in exons 1 and 2, this shorter product was not detected. Additionally, the absence of β-sarcoglycan in skeletal muscle, cardiac muscle and smooth muscle was confirmed by immunofluorescence. These β-sarcoglycan deficient mice demonstrated elevated serum CK levels and a progressive muscular dystrophy with the findings in muscle of large areas of focal necrosis, central nuclei, fiber splitting, hypertrophy, extensive dystrophic calcification, endomysial fibrosis, and fatty infiltration. This group found that these muscles were much more severely affected than the α-sarcoglycan deficient animals they had generated previously (Duclos *et al.* 1998). Immunofluorescence confirmed that the other sarcoglycans and sarcospan were concomitantly reduced, as was seen in the β-sarcoglycan deficient mice generated by Araishi *et al.* (1999). Durbeej *et al.* (2000) went on to show that the sarcoglycan-sarcospan complex is reduced in

cardiac muscle of their β-sarcoglycan deficient mice. In addition to the progressive muscular dystrophy, these mice also developed a marked cardiomyopathy (Durbeej *et al.* 2000).

1.3.3 γ-sarcoglycan deficient mice

There has only been one group to publish the generation of a γ-sarcoglycan deficient mouse (Hack *et al.* 1998), although a second group has successfully generated another γ-sarcoglycan deficient mouse and are currently working on the manuscript (personal communication, T. Sasaoka). Hack *et al.* (1998) describe the γ-sarcoglycan deficient mouse as suffering from a progressive muscular dystrophy. They targeted exon 2, which contains the initiator methionine, the cytoplasmic tail, and the transmembrane domain of the γ-sarcoglycan gene, using the phosphoglycerate kinase promoter and neomycin phosphotransferase cDNA. They confirmed single copy homologous recombination by Southern blot analysis. Immunoblot analysis was used to show that there was no production of γ-sarcoglycan, verifying the generation of a null allele. In addition to the primary deficiency of γ-sarcoglycan, there is also a secondary deficiency seen by immunofluorescence in these mice of both the β- and δ-sarcoglycan mice with only partial retention of α-sarcoglycan. Hack *et al.* (1998) also show that the γ-sarcoglycan deficient mice suffered from a cardiomyopathy, which results in death of 50% of these mice by 5 months of age. In addition, these mice have elevated CK levels and pathologic changes in muscle, characteristic of progressive muscular dystrophy, comprising fiber size variation, including fiber hypertrophy, degeneration/regeneration, central nuclei, inflammatory infiltrate, abnormal calcification, and increased fatty and fibrous connective tissue (Hack *et al.* 1998).

1.3.4 δ-sarcoglycan deficient animals

The Bio 14.6 hamster has been studied since the 1960's when it was first identified as an animal model for cardiomyopathy (Homburger *et al.* 1962; Homburger *et al.* 1963). However, the cause of both its cardiomyopathy and muscular dystrophy was unknown for over three decades. Phenotypically, these hamsters present with muscle necrosis and undergo subsequent rounds of muscle fiber degeneration/regeneration, leading to central nucleation (Homburger *et al.* 1962; Homburger *et al.* 1963). A deficiency in the Bio 14.6 hamsters of α-sarcoglycan was found to be present before the onset of myocytolysis (Roberds *et al.* 1993b; Mizuno *et al.* 1995). However, it was not until 1997 that Nigro *et al.* (1997) discovered that both the cardiomyopathy and muscular dystrophy were caused by a deficiency in the δ-sarcoglycan gene. Nigro *et al.* (1997) established that the progressive muscular dystrophy seen in the Bio 14.6 hamster was due to a deletion of the promoter region and exon 1 of the δ-sarcoglycan gene. mRNA expression of δ-sarcoglycan is slight (<3-5% of normal levels) in heart and negligible in skeletal muscle. The Southern blot analysis revealed a deletion in the first exon of δ-sarcoglycan. Linkage analysis was used to verify that the deletion in the δ-sarcoglycan gene caused both the cardiomyopathy and muscular dystrophy (Nigro *et al.* 1997).

A recently described δ-sarcoglycan deficient mouse was generated by replacing exon 2 of the δ-sarcoglycan gene, which contains 63 amino acids of the cytoplasmic domain and the entire transmembrane domain, with a neomycin resistance gene (Coral-Vazquez *et al.* 1999). Homologous recombination was confirmed by Southern blot analysis, and northern blot analysis revealed that there were no transcripts containing exon 2 of δ-sarcoglycan in skeletal muscle. However, RT-PCR analysis showed a product, which was an alternative splicing of exon 1 to exon 3, maintaining the open reading frame from exon 3 to exon 8. Translation of this smaller

transcript was not detected by western blot analysis in skeletal or cardiac muscle. Immunofluorescence revealed a secondary loss of the entire sarcospan-sarcoglycan complex. These mice also displayed a 15-20 fold increase in CK levels and some of the pathologic hallmarks of muscular dystrophies, such as muscle fiber necrosis, degeneration/regeneration, centrally placed nuclei, endosomal fibrosis, fiber splitting, hypertrophy, dystrophic calcification, and fatty infiltration. A cardiomyopathy observed is the δ-sarcoglycan deficient mice was similar to that found in the Bio 14.6 hamster (Coral-Vazquez *et al.* 1999).

1.4 The Adeno-associated Virus

The adeno-associated virus (AAV) is a member of the parvovirus family (Lukashov and Goudsmit 2001). The single-stranded DNA genome of AAV is comprised of ~ 5,000 bases, and consists of two genes, *rep* and *cap*, driven by three promoters, and flanked by two inverted terminal repeat sequences (Berns 1990). The inverted terminal repeats (ITRs) are 145 bases long. The first 125 bases of each repeat is palindromic sequence that is expected to self-base pair to form a T- or Y- shaped secondary structure, depending on the hybridization of two internal palindromes, flanked by a more extensive palindrome. The folded configuration contains only seven unpaired bases, six that enable the internal palindromes to fold and the seventh separates the two internal palindromes. The inverted terminal repeats are necessary for encapsidation of AAV's genome and are also critical for integration into the host genome (Wang *et al.* 1997).

Replication of AAV involves a series of important features. First, the inverted terminal repeats act as primers during AAV DNA replication. Second, AAV is thought to have a single-strand displacement mechanism for DNA synthesis and therefore there is no lagging strand synthesis. Finally, current replication models predict site-specific cleavage of replicative intermediates; however, the putative DNAses involved in this process have not been identified, and are presumed to be of cellular origin (Berns 1990).

The AAV virion has three coat proteins, encoded by the *cap* genes. The three proteins are VP-1 at 87 kilodaltons, VP-2 at 73 kilodaltons, and VP-3 at 62 kilodaltons (Wistuba *et al.* 1995). Comparative tryptic digestions of all three proteins give very similar patterns, indicating that all three proteins were determined by a common DNA sequence (Berns 1990); this has been verified by DNA and RNA studies of the virus (Wistuba *et al.* 1995). In addition, the coat

proteins also appear to be coded by overlapping DNA sequences. There is no evidence that any of the coat proteins are glycosylated (Berns 1990).

Of all the parvoviruses, AAV is the most dependent upon the intracellular milieu for replication. The other parvoviruses require host cells to go through S phase in order to replicate. However, AAV requires adeno- or herpesvirus co-infection of host cells in order for it to undergo replication and a productive infection. The adenovirus genes that are required for AAV to undergo an active infection include E1a, E1b, E2a, and E4 (Janik *et al.* 1981). E1a is required due to its activation of other adenovirus early genes. E1b has also been identified as necessary for AAV DNA replication to occur, but it does not appear that there is a correlation between AAV transcript accumulation and the introduction of E1b. E2a encodes a DNA binding protein that is required for adenovirus DNA synthesis. However, it is not necessary for AAV DNA synthesis, although AAV's DNA synthesis is severely hampered by mutations in the E2a gene. E4 appears to be involved in encoding a helper function protein required for DNA replication of AAV (Berns 1990). No adenovirus late genes appear to be required for AAV particle production.

The *rep* gene encodes proteins that are required for both transcription and DNA replication of AAV. It has been demonstrated that *rep* performs a trans-activation role by initiation of transcription (Tratschin *et al.* 1986; Hermonat *et al.* 1998). It also appears that AAV can use its *rep* gene to regulate its own gene expression as well as the helper virus. In the absence of a permissive intracellular environment, there is a low level of *rep* expression, which inhibits normal levels of AAV gene expression. Transcription is suppressed as *rep* gene transcripts inhibit a number of heterologous promoters, including at least one, if not all, of the *rep* and *cap* promoters. These diminished levels of transcription can be overcome by co-

infection with a helper virus. Therefore, AAV gene expression appears to be autoregulated both in a positive and a negative fashion (Berns 1990).

AAV requires other helper viruses, such as adenovirus, to undergo its reproductive cycle. Much of the machinery needed for a productive infection is derived from the helper virus and the host cell. It is thought that because AAV takes away the replication machinery from the helper virus, AAV may be able to attenuate replication of the helper virus (Alexander *et al.* 1997; Barresi *et al.* 1997). Indeed, some reports have suggested that AAV may protect against the development of some virally-induced diseases such as cervical cancer (Sprecher-Goldberger *et al.* 1971; Mayor *et al.* 1976; Georg-Fries *et al.* 1984; Coker *et al.* 2001) and polymyositis (Tezak *et al.* 2000). In addition, most human populations appear to be infected with AAV, with 80-90% testing seropositive for the virion in the first two years of life (Berns and Linden 1995) with no association with disease. Therefore, AAV is not associated with disease and may even be protective against disease.

The potentially beneficial nature of AAV, its relatively wide tropism, and its apparent non-pathogenic nature has made it a prime candidate as a gene delivery vehicle (Gertz 1973). AAV also appears able to transduce cells and maintain transgene expression with little or no cytotoxicity (Berns and Bohenzky 1987; Gaschen *et al.* 1992; Flotte *et al.* 1993; Clark *et al.* 1995; Afione *et al.* 1996; Dong *et al.* 1996; Chen *et al.* 1997; Clark *et al.* 1997). Another potential advantage of AAV is its ability to integrate stably into host chromosomes during its latent life cycle, via inverted terminal repeats. Integration is permissive for long-term expression of transgenes introduced into the virus backbone.

It appears that the wild-type genome integrates preferentially near or into a specific muscle gene (troponin T, TNNT1) on chromosome 19 (Dutheil *et al.* 2000). It was reported that

muscle tissue appears to be a site of infection and latency of wild-type AAV (Tezak *et al.* 2000). Both reports suggested that muscle tissue would be an excellent target for AAV-mediated gene delivery. While some early observations indicated that AAV was unable to transduce non-dividing cells (Berns and Linden 1995; Fisher-Adams *et al.* 1996), there has been some convincing *in vivo* evidence showing that it does transduce non-dividing cells. Transduction of non-dividing cells was shown in a variety of tissues, including mouse and rat muscle (Xiao *et al.* 1996; Greelish *et al.* 1999; Li *et al.* 1999), rat brain (Chen *et al.* 1997), and pig heart (Clark *et al.* 1997). We and others have also been able to show that AAV is able to infect both mature and immature muscle (Kessler *et al.* 1996; Fisher *et al.* 1997; Snyder *et al.* 1997; Li *et al.* 1999; Pruchnic *et al.* 2000). AAV's ability to infect muscle easily is possibly due to muscle's high expression of heparin sulfate proteoglycan (HSP) (Pruchnic *et al.* 2000; Cao *et al.* 2001), which is an attachment receptor for AAV (Summerford and Samulski 1998) and the expression of integrin $\alpha V\beta 5$ in muscle (Pruchnic *et al.* 2000), which is a second co-receptor for AAV (Summerford *et al.* 1999). AAV is also able to bypass the basal lamina that surrounds muscle fibers, while adenovirus and herpes virus cannot (Feero *et al.* 1997). The basal lamina has pores that are 40 nm (Yurchenko 1990), and because the AAV particle is only 20 nm in diameter, it is easily able to pass through these pores, while adenovirus at 70-100 nm and herpes at 120-300 nm cannot.

AAV's small size is also a major disadvantage for gene delivery purposes, as recombinant AAV has only a 5 kb DNA carrying capacity. This limitation makes it impossible to encode the full-length dystrophin cDNA, which would be ideally used to treat Duchenne muscular dystrophy. However, all of the sarcoglycans have coding sequences that are less than 2

kb, making the sarcoglycanopathies an excellent system for testing the efficacy of AAV-mediated gene therapy.

Advances in AAV production technology have facilitated the availability of a three plasmid transfection method for producing recombinant AAV, without helper virus contamination (Xiao *et al.* 1998) (Figure 3). This method utilizes one plasmid for the helper functions normally supplied by the adenovirus or other helper virus, one that contains the essential AAV genes necessary for viral capsid production, and a final plasmid with the gene of interest flanked by the ITRs. The 145 basepair *cis*-acting sequences of the ITRs are the only viral sequences necessary for integration and packaging of DNA into AAV virions. As the ITRs are the only viral sequence remaining in the AAV vector, AAV is replication deficient and has little or no chance of regenerating wild-type AAV or adenovirus during the production of the virus (During *et al.* 1998). The triple plasmid transfection method provides AAV titers of up to 10^{10} or 10^{11} transducing units (t.u.)/mL, not dissimilar to those obtained with wild-type AAV propagation (Figure 3).

1.5 AAV gene delivery as a therapeutic potential for other diseases

AAV's ability to transduce non-dividing cells with no overt pathological consequences has made it an attractive vector for a number of other non-muscle related diseases. Some of the earliest work with the AAV vector was done with cystic fibrosis, including the first *in vivo* gene transfer using this vector in 1993 and the first phase I clinical trial using AAV in 1996. As of 2001, there are two ongoing phase I clinical trials using AAV to treat CF, both of which have shown no untoward inflammatory responses to date. Use of AAV in CF patients has not yet shown clinical efficacy, due to the inefficiency of the route of delivery, the difficulty in transducing lung stem cells, and the lack of DNA capacity to package an optimal promoter (Flotte and Laube 2001).

All other AAV work reported to date has involved animal models of a variety of human diseases. Two genes, tyrosine hydroxylase (TH) and aromatic L-amino acid decarboxylase (AADC), have been investigated as means of experimental therapeutics for Parkinson's disease in the (6-OHDA)-lesioned parkinsonian rat model. When these genes are co-delivered by intra-cranial injection, they appear able to exhibit a behavioral recovery in the rat model (Ozawa *et al.* 2000). In addition, recent data has suggested that protection of dopaminergic neurons in the substantia nigra is possible by using AAV-mediated gene delivery of a glial cell line-derived neurotrophic factor (GDNF) gene to neurons (Bjorklund *et al.* 2000). AAV has also been applied to animal models of cardiovascular disease. A recent report described AAV delivery of antisense (AS) DNA of the angiotensinogen, angiotensin type I receptor (AT_1-R) and angiotensin converting enzyme genes, which are all associated with vasoconstrictive properties. A single dose of AAV-AS-AT_1-R resulted in significantly prolonged reduction of blood pressure (Phillips *et al.* 1997; Phillips *et al.* 2000).

Perhaps the most studied application of AAV to human disease has been with regards to clotting abnormalities, specifically Hemophilia B (deficiency of Factor IX). Factor IX's cDNA, being only 1.4 kb, easily fits into the AAV vector. This is in contrast to the more common Hemophilia A, where the causative Factor VIII cDNA is too large to be packaged by AAV. Factor IX is a secreted protein and readily equilibrates between extravascular and intravascular space, such that there is no prerequisite for Factor IX being delivered directly into the circulation. This has permitted the application of intramuscular administration of AAV for delivery of the gene and protein (Chuah *et al.* 2001). Work with Factor IX has led to a phase I clinical trial, and a recent report suggests that there is no toxicity or germline transduction and no formation of inhibitory antibodies against Factor IX; each of these are important aspects of proving vector safety. In addition, after treatment patients had low circulating levels of Factor IX and some Factor IX protein infusion (Fabb and Dickson 2000; Kay *et al.* 2000). There are many other diseases for which use of AAV-mediated gene delivery has led to phase I clinical trials, including muscular dystrophy (Wahl and Hesterlee 1999; Stedman *et al.* 2000), cancer (Ruan *et al.* 2001; Braun-Falco and Hallek 2001; Kanazawa *et al.* 2001), Gaucher disease (Wei *et al.* 1994), and arthritis (Robbins and Ghivizzani 1998; Cottard *et al.* 2000; Zhang *et al.* 2000). AAV has proven itself a premiere vector for gene delivery, although there are some drawbacks to its use. AAV production is labor intensive, as it requires the purification of three separate plasmids for production. As mentioned previously, the main limitation of AAV is its small packaging size; however, a number of different groups are working around this problem by using truncated proteins or smaller promoters (Chao *et al.* 1999, Gnatenko *et al.* 2000, Wang *et al*., 2000). Other groups are taking advantage of recombinant AAV's ability to form large concatamers *in vivo* by delivering two viruses. Each virus contains half of a gene and when co-

delivered form concatamers that are able to produce the full-length gene (Yang *et al.* 1999; Duan *et al.* 2000; Yan *et al.* 2000).

1.6 AAV gene delivery to the δ-sarcoglycan deficient hamster

The characterization of a sporadically occurring hamster model for δ-sarcoglycan deficiency serves to elevate the sarcoglycanopathies as a prime target for early work with gene delivery for muscular dystrophy. The Bio 14.6 hamster is a well-characterized spontaneous loss-of-function knockout for the δ-sarcoglycan gene (Nigro *et al.* 1997). Adeno-associated virus (AAV) was the vector of choice for the treatment of this model, as all of the sarcoglycan genes, including δ-sarcoglycan, have relatively small cDNA's (~2.0 kb each), and muscle has been observed as an easily transduced tissue using *LacZ* markers (Xiao *et al.* 1996; Greelish *et al.* 1999; Li *et al.* 1999). There are several reports of successful genetic, biochemical, histological and functional rescue of relatively large regions of the dystrophic muscle in this hamster model, both by us and others (Holt *et al.* 1998; Greelish *et al.* 1999; Li *et al.* 1999; Xiao *et al.* 2000).

We described the first successful use of recombinant adeno-associated virus for the biochemical complementation of the hamster model (Li *et al.* 1999). In this collaborative study, an AAV vector containing the complete human δ-sarcoglycan cDNA driven by a CMV promoter (AAV-CMV-δ-SG) was constructed and delivered to Bio 14.6 δ-sarcoglycan deficient hamster muscle by intramuscular injection. Western blot analysis of muscle that was injected with the AAV-CMV-δ-SG and showed that there was a high level of δ-sarcoglycan production (Figure 4). Immunofluorescence analysis also showed extensive and efficient genetic and biochemical complementation of the primary defect *in vivo* (Figures 5, 6).

The injected Bio 14.6 hamster muscle was analyzed by immunofluorescence to confirm that the secondary biochemical defects were corrected by the rescue of the primary deficiency. Our results revealed that the secondary loss of the sarcoglycan complex was correctly restored to

the membrane of the muscle tissue expressing the δ-sarcoglycan gene (Figures 5, 6). Interestingly, we also showed a range of expression levels of the δ-sarcoglycan gene in different myofibers. Some produced enough to have proper localization to only the membranes of the myofibers, while other fibers were over-expressing the δ-sarcoglycan gene so that there was inappropriate cytoplasmic staining (Figure 5). However, the over-expression of the δ-sarcoglycan did not lead to formation of the complete sarcoglycan complex in the cytoplasm, as the other sarcoglycans (α-, β-, γ-) were only localized to the plasma membrane (Figure 6). Along with the restoration of the sarcoglycan complex, there was also a substantial improvement in the histology of treated muscles seen with hematoxylin and eosin (H&E) staining (Figure 7). In addition, there was no cytotoxic effect seen due to the expression or over-expression of the transgene, as evidenced by a lack of T-cell infiltration. The transgene long-term expression and persistence of expression was followed and persisted for 4 months.

We then went on to test whether the rescue of the primary biochemical defect (δ-sarcoglycan) led to functional improvement of the muscle (Xiao *et al.* 2000). The functional testing of muscle was performed in Dr. Jon Watchko's laboratory. It is important to note that there is an increased mass of muscle (hypertrophy) in the dystrophic Bio 14.6 hamster model when compared with the normal F1B hamster age-matched control. This hypertrophic response is shared with most rodent models for the muscular dystrophies. Specific tetanic force generation and specific twitch force is decreased in the Bio 14.6 relative to controls (Xiao *et al.* 2000) (Figure 8).

To determine if muscle functional measures were corrected by delivery of δ-sarcoglycan, *in vitro* contractile force measurements were performed on the tibialis anterior muscle (TA) of the Bio 14.6 hamster 4 months after treatment with a single injection of the AAV-CMV-δ-SG,

untreated Bio 14.6 hamster, and normal F1B hamster. The TA muscle was carefully removed from the legs of either the controls or the treated animals and subjected to *in vitro* electrophysiological stimulation and contractile measurement on a force transducer. The treated muscle showed significant improvement in muscle mass, specific twitch, and specific tetanic force measurements, with a recovery of muscle strength of more than 97% in both specific twitch and tetanic force (Figure 8). These results showed that treatment of dystrophic muscle using an AAV vector not only complemented the primary genetic and biochemical defects by restoration of the secondary biochemical defects and improvement in muscle histology, but it also restored muscle strength and size.

1.6 Large scale gene delivery

One of the major hurdles facing gene delivery as a therapeutic approach for muscle disease is the need for large scale delivery and widespread rescue of muscle. Muscle comprises ~30% of body mass in humans, and introducing a delivery vehicle to all of the muscle groups in the body represents a daunting task. One possible solution to this problem is taking advantage of muscle being a highly vascularized tissue, and therefore attempting systemic delivery of genes using the vascular system. Some researchers have attempted this delivery method using AAV, but it appears that AAV vectors have substantial liver-tropism *in vivo*, with the majority of intravenously injected AAV vectors accumulating in liver and resulted in little to no expression in muscle (Ponnazhagan *et al.* 1997). Many groups are working on bypassing the liver-tropism by discovery of receptor molecules that show greater tropism for muscle (Feero *et al.* 1997; Bouri *et al.* 1999). However, this is a long-term approach that will require extensive characterization of potential ligands, and coupling to infectious virions. Another alternative approach was recently published, where the authors showed that administration of AAV vector through the arterial supply of an isolated limb via an extra-corporeal membrane oxygenation (ECMO) circuit appears to yield widespread expression of the transgene in hamsters and in rats (Greelish *et al.* 1999). Unfortunately, the exact nature of the ECMO procedure and its consequences has not been adequately studied, including the potential risks inherent in using histamine to induce endothelial permeabilization. Indeed, our attempts to reproduce this difficult procedure led to lethality from histamine. One final approach would be to perform multiple widespread intra-muscular injections. However, this will require multiple accurate and consistent injections, and also raises another problem of the potentially inaccessible location of some muscle groups, such as the diaphragm.

1.7 Conclusion of introduction

The sarcoglycanopathies represent a subgroup of the limb-girdle muscular dystrophies. Research on these diseases has characterized the genes and proteins of the four sarcoglycan (α, β, γ, and δ) whose deficiencies are responsible for these particular muscular dystrophies. Animal models of all sarcoglycanopathies have been made facilitating further characterization of the interaction of the sarcoglycan proteins and also permitting experimental therapeutics.

One of the treatment strategies that has been applied to the sarcoglycanopathies is gene delivery utilizing an adeno-associated virus (AAV). AAV has been shown by several groups to be safe and effective for a wide variety of diseases, and more specifically has been shown to be a premiere vector for gene delivery to muscle. AAV-mediated gene delivery of δ-sarcoglycan has been extensively studied by several groups. The work presented in this dissertation serves to extend the understanding of gene delivery to the remaining sarcoglycans, and potentially provide additional insight into the interaction between the four sarcoglycan proteins. In addition, we will gain a better understanding of some of the complicating factors involved with gene delivery to dystrophic muscle.

One complicating factor of gene delivery to disease muscle is the delivery to all of the muscle. Other research groups have attempted to overcome this problem, with some limited success. This dissertation presents a method for the transduction of large areas of muscle.

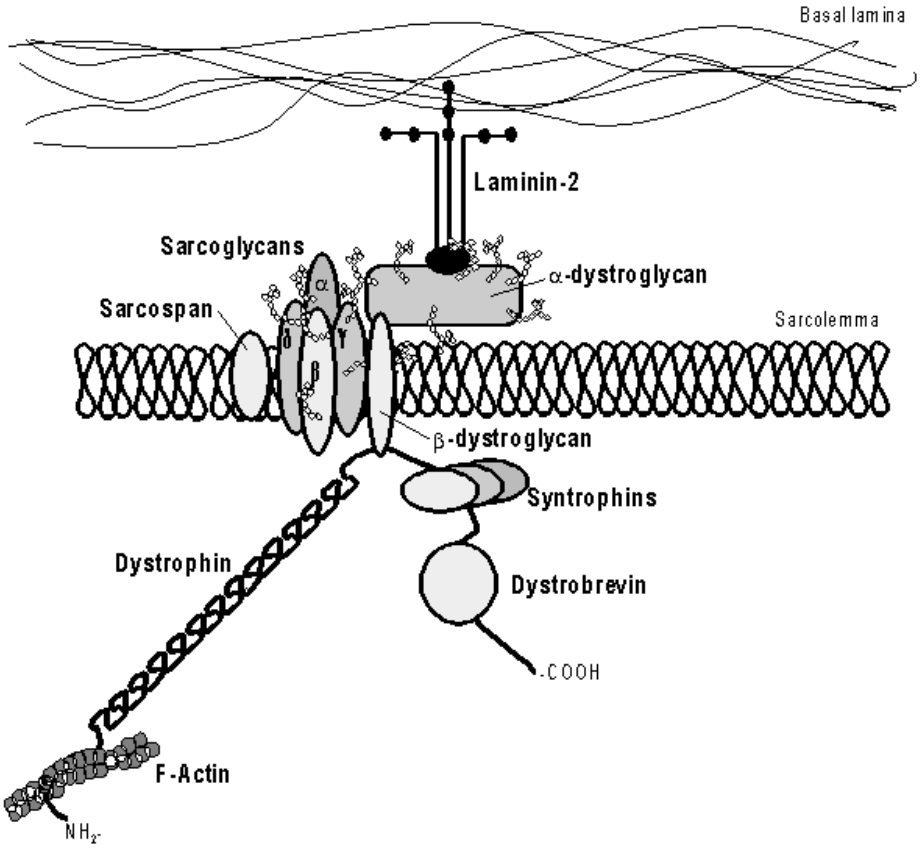


Figure 1. Schematic diagram of the dystrophin associated protein complex.

This diagram represents the location and interaction of proteins at the myofiber membrane, including the sarcoglycan complex (Leiden Muscular Dystrophy web pages).

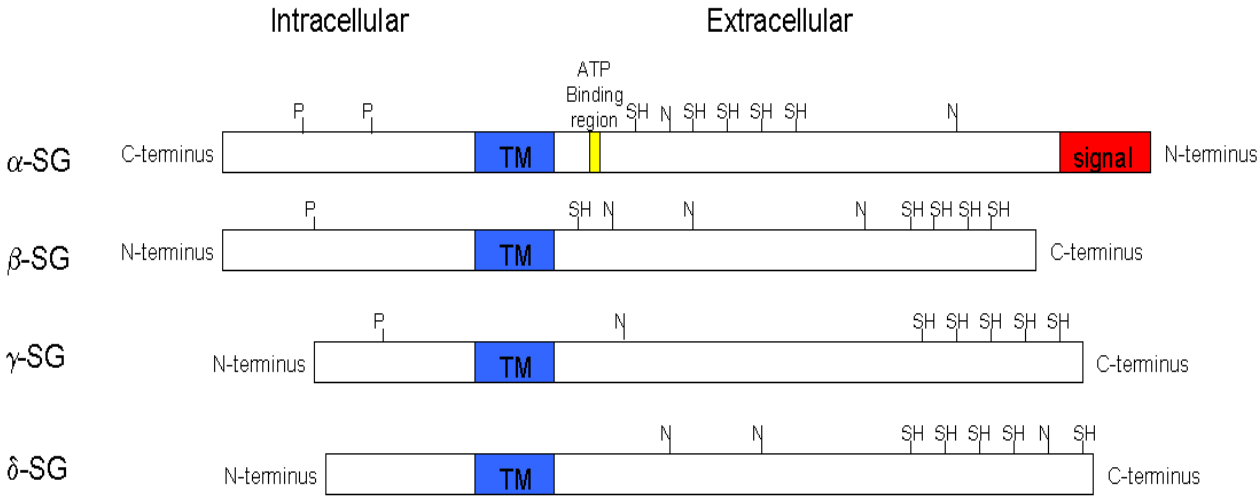
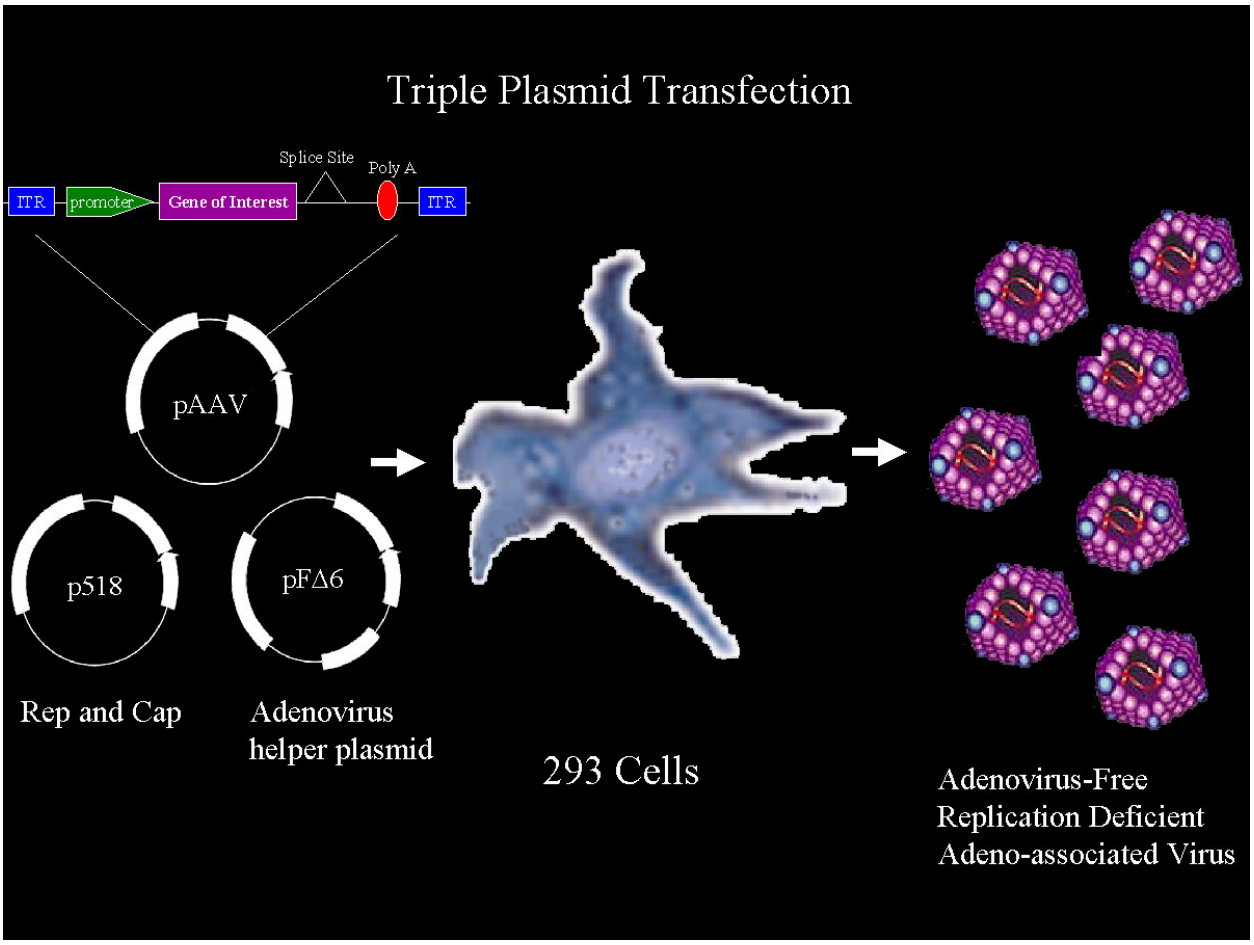


Figure 2. Comparison of human sarcoglycan proteins.

A representative diagram of the human sarcoglycan proteins showing transmembrane domains (*TM*), phosphorylation sites (*P*), cysteine residues (*SH*), potential N-linked glycosylation sites (*N*), the N-terminal signal sequence (*signal*), and the ATP binding consensus sequence (*ATP binding region*).

Figure 3. Diagram of triple plasmid transfection method for production of AAV.

AAV is currently produced in an adenovirus-free system using a triple plasmid transfection method. The cis plasmid is the pAAV plasmid, which contains a promoter, the gene of interest, a splice site, and a polyadenylation signal, all flanked by two inverted terminal repeats (ITRs) that are the signaling sequences for packaging of AAV. The trans AAV plasmid, p518, contains both the *rep* and *cap* genes from AAV to allow for replication and capsid protein production, but lacks any ITRs so that this sequence is not packaged and no wild-type AAV is produced. The final plasmid is the adenovirus helper plasmid, pFΔ6, which contains all of the necessary genes from the adenovirus genome to allow for AAV replication. These three plasmids are co-transfected into 293 cells, a human embryonic kidney cell line stably transduced with the 5' end of the adenovirus genome. The cells are subsequently harvested, and adenovirus-free replication deficient AAV containing the gene of interest can be purified.


Triple Plasmid Transfection
Splice Site
Poly A
ITR
promoter
Gene of Interest
ITR
pAAV
p518
pFΔ6
Rep and Cap
Adenovirus
helper plasmid
293 Cells
Adenovirus-Free
Replication Deficient
Adeno-associated Virus

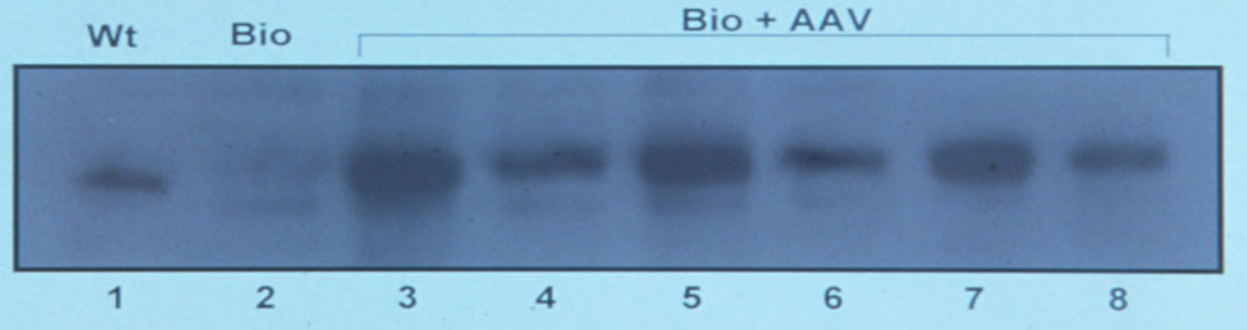


Figure 4. Western Blot analysis of *in vivo* δ-sarcoglycan production.

Lane 1 is the wild-type positive control from the F1B hamster (Wt). Lane 2 is the untreated Bio14.6 hamster (Bio) showing an absence of δ-sarcoglycan. Lanes 3-8 are Bio 14.6 hamster gastrocnemius muscle after injection with AAV-δ-sarcoglycan (Bio + AAV) (Xiao *et al.* 2000).

Figure 5. Over-expression of δ-sarcoglycan in the cytoplasm does not disrupt the correct localization of the sarcoglycan complex.

(Panel A) AAV-δ-SG treated Bio 14.6 hamster muscle stained with antibody against the δ-sarcoglycan protein. (Panel B) Parallel section of the same treated Bio 14.6 hamster muscle stained with antibody against the α-sarcoglycan protein. It is important to note that over-expression of the δ-sarcoglycan led to cytoplasmic staining of some myofibers, but did not affect correct membrane localization of α-sarcoglycan (Xiao *et al.* 2000).

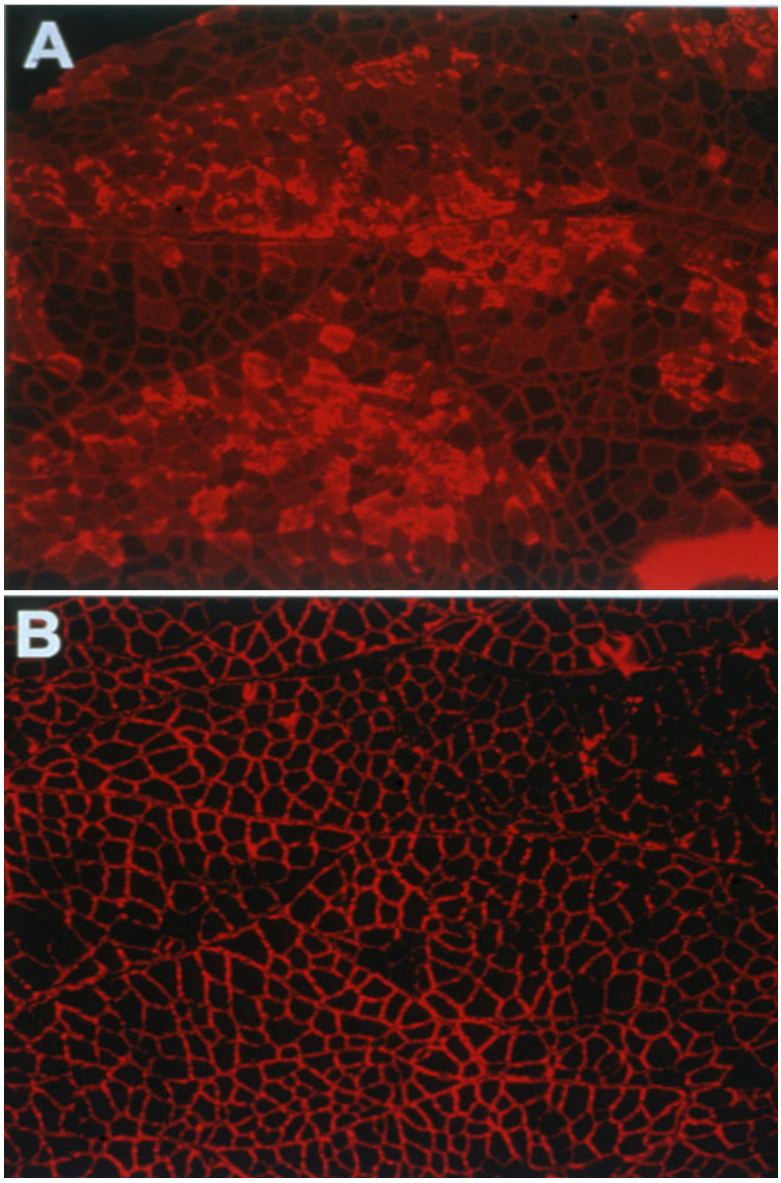

A
B

Figure 6. Complete restoration of the sarcoglycan complex in treated Bio 14.6 hamsters.

Top row consists of serial sections from a normal F1B hamster showing normal staining for the sarcoglycan complex. Note that the antibody used against human δ-sarcoglycan does not cross react with hamster. The middle row shows no staining of the sarcoglycans in the δ-sarcoglycan deficient hamster. The bottom row shows restoration of all the sarcoglycans in AAV-δ-SG treated Bio 14.6 animals (Xiao *et al.* 2000).

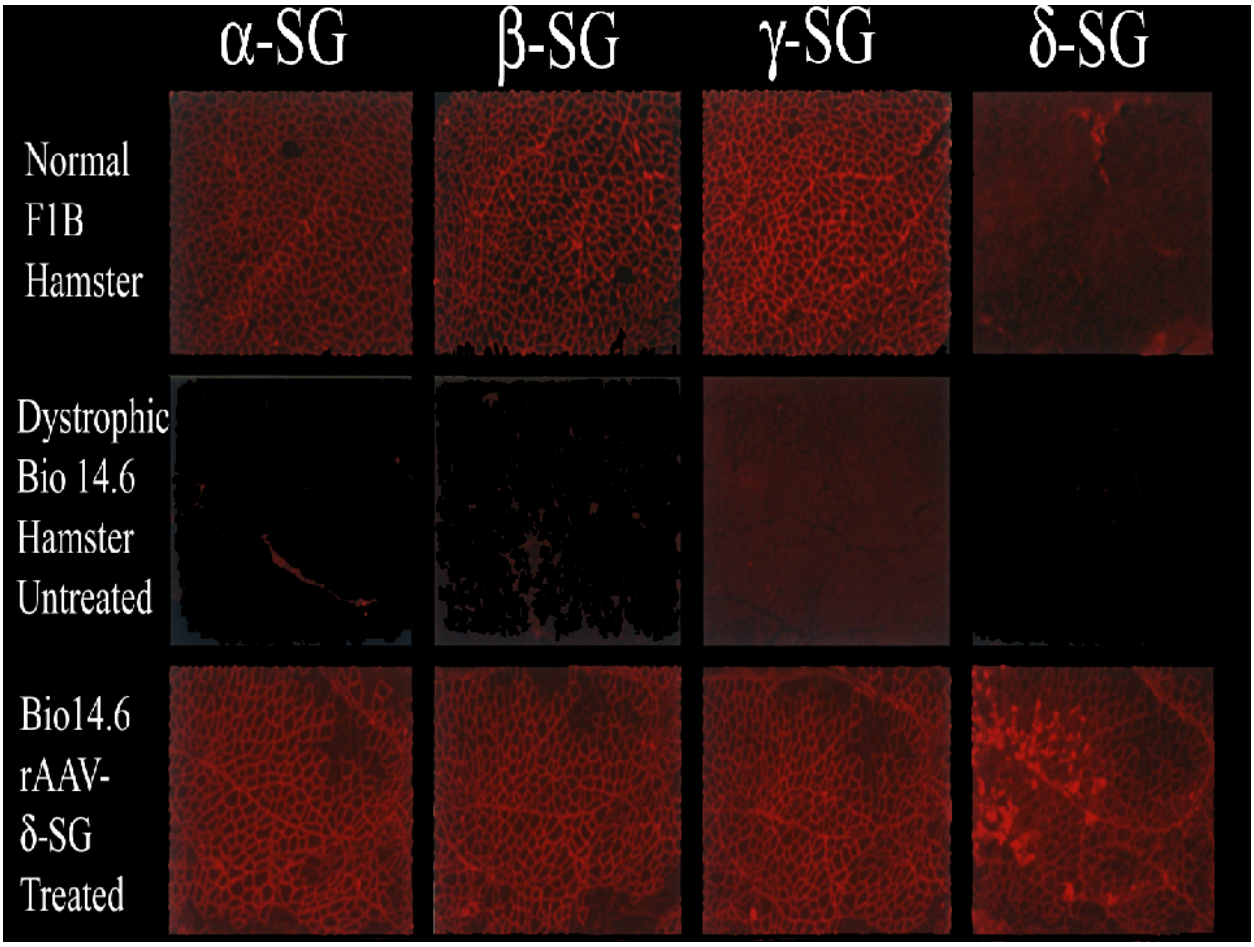

α-SG
β-SG
γ-SG
δ-SG
Normal F1B Hamster
Dystrophic Bio 14.6 Hamster Untreated
Bio14.6 rAAV-δ-SG Treated

Figure 7. Histological examination of normal vs. rescued vs. dystrophic muscle.

Panel A shows wild-type (WT) F1B hamster muscle with normal histology. Note normal myofiber size with little variation in size, peripheral nuclei, and no fibrosis. Panel B shows AAV δ-sarcoglycan rescued Bio 14.6 (Bio AAV) hamster muscle. Panel C shows the untreated Bio 14.6 (Bio) hamster muscle. Note that the treated muscle (panel B) has restored myofiber size. It also does not have the fibrosis seen in panel C. However, they both have central nuclei, indicative of degeneration/regeneration, but we believe the reason for seeing it in the treated muscle is due to treatment after degeneration/regeneration has already occurred (Xiao *et al.* 2000).

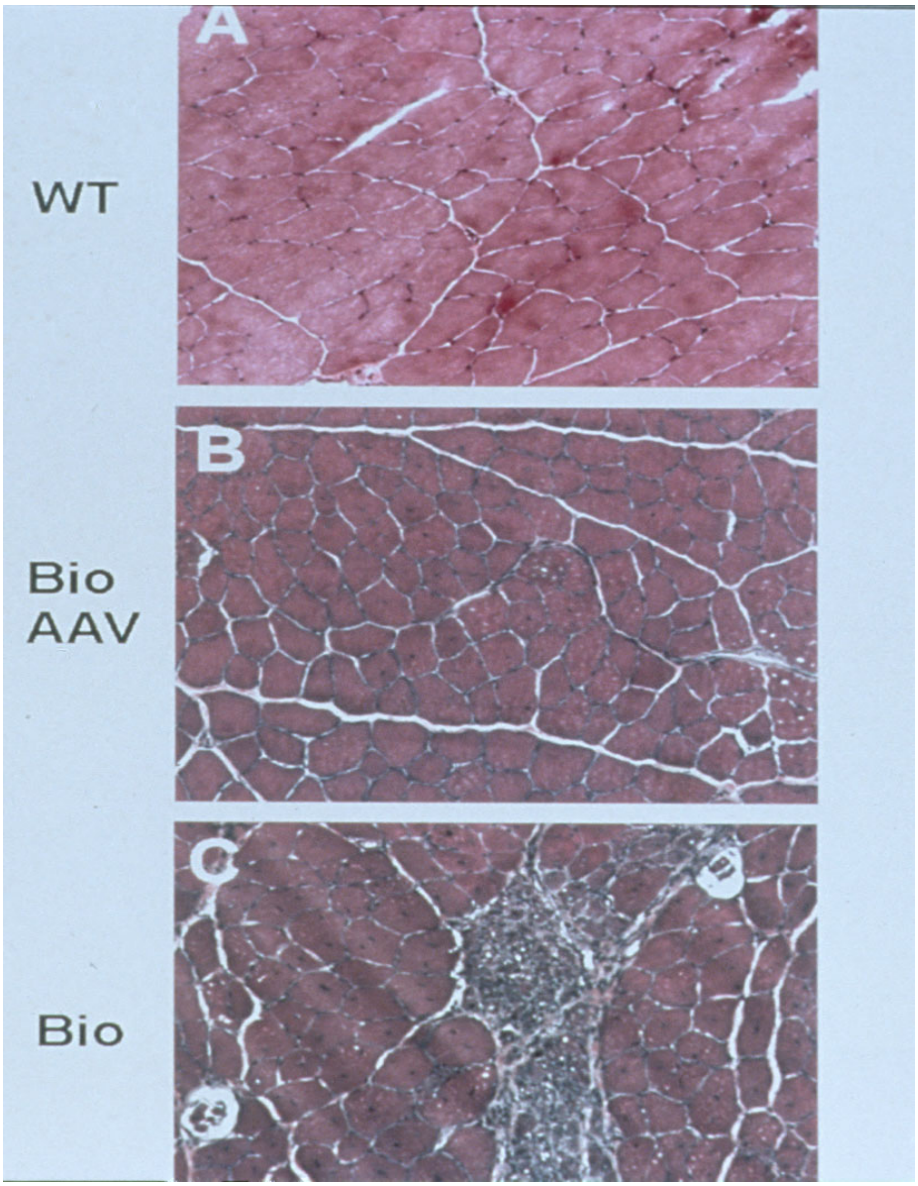
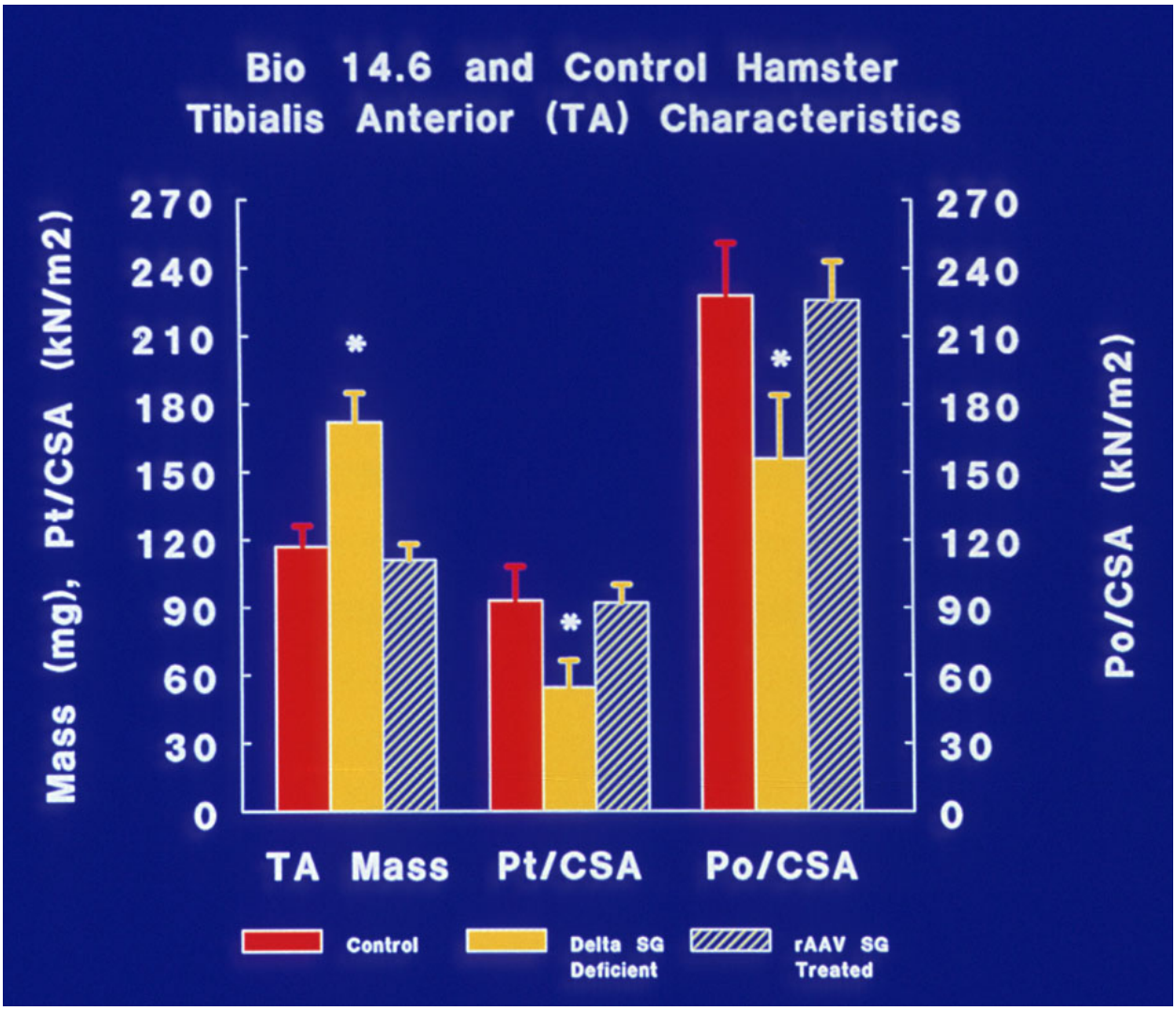

A
WT
B
Bio
AAV
C
Bio

Figure 8. Functional recovery of Bio 14.6 hamster TA muscle with a single treatment.

Bio 14.6 hamsters were injected at 40 days of age with an AAV δ-sarcoglycan containing vector and were studied at 4 months post-injection. The Bio 14.6 hamster showed functional rescue of muscle following treatment with an AAV δ-sarcoglycan containing vector as evidenced by measurements of muscle mass, specific twitch force, and specific tetanic force which show restoration to 97% of normal muscle values. Pt = twitch force, Po = tetanic force, N= newtons, CSA= cross sectional, *indicates a $p<0.05$ (ANOVA). (F1B n = 8, Bio 14.6 untreated n = 9, Bio 14.6 treated n = 4).


Bio 14.6 and Control Hamster
Tibialis Anterior (TA) Characteristics
Mass (mg), Pt/CSA (kN/m2)
Po/CSA (kN/m2)
270
240
210
180
150
120
90
60
30
0
*
*
*
TA Mass
Pt/CSA
Po/CSA
Control
Delta SG Deficient
rAAV SG Treated

2.0

Statement of the Problem

The biology of both normal and abnormal tissues is exceedingly complex, making controlled gene delivery with predictable outcomes a daunting task. However, one gene delivery vector, recombinant adeno-associated virus (AAV), is particularly promising, especially in muscle where only a few attempts at gene delivery have led to a correction of biochemical, histological, and physiological symptoms of muscular dystrophy (Holt *et al*., 1998; Greelish *et al*., 1999; Li *et al*., 1999; Xiao *et al*., 2000).

We hypothesized that biochemical complementation of animal models of muscular dystrophy could be accomplished by delivery of appropriate genes to muscle by AAV. We predicted that different models and their corresponding genes would show differing levels of "success" (expression, persistence, and biochemical rescue), due to the intrinsic biochemistry of the muscle fiber and to variability in the interactions with the host immune system. We felt that the study of closely related dystrophies and complementing genes would provide valuable insight into biochemical interactions within muscle.

We employed murine and hamster models of muscular dystrophy to test the above hypotheses. However, we also postulated that dystrophic muscle pathology itself has immunostimulatory properties that may elicit an immune response to delivered transgenes. Dystrophic muscle has many secondary consequences, occurring downstream as a result of the primary defect, that are shared between most muscular dystrophies; these downstream effects could act as immunostimulatory properties. Some of these downstream effects, such as the degeneration/regeneration process and higher than normal amounts of macrophages, CD4, and CD8 T-cells (Arahata and Engel 1984; Arahata and Engel 1988), are referred to as hallmarks of muscular dystrophy. Therefore, I tested the hypothesis that a transgene delivered to diseased

muscle that does not correct the primary defect will result in generating an immune response to that transgene.

Each of the sarcoglycan disorders provides an excellent model to isolate specific biochemical and pathological factors, which may mitigate the efficacy of gene delivery. There is emerging biochemical and genetic data on each of the four sarcoglycan genes that allows careful comparison of the interaction of gene delivery with the biology of the system. Gene mutations in any single sarcoglycan gene lead to a biochemical deficiency of all four sarcoglycan proteins. Thus, there is an intimate and mutually dependent (and stoichiometrically equal) interaction between these four protein products. Considerable new data have shown a likely hierarchy in the assembly of the four sarcoglycan proteins during their transport through the golgi (Holt and Campbell 1998, Noguchi *et al.* 2000). Also, the sarcoglycans have differing orientations through the membrane: three of the sarcoglycans have the carboxyl-terminus of the protein outside the plasma membrane, while one has the opposite orientation (Roberds *et al.* 1993a; Roberds *et al.* 1994; Lim *et al.* 1995; Noguchi *et al.* 1995; Bonnemann *et al.*1996; Jung *et al.* 1996; McNally *et al.* 1996; Nigro *et al.* 1996). In addition, murine models for each sarcoglycan disease display similar muscular dystrophy phenotypes, including many of the hallmarks associated with the human disease (Duclos *et al.* 1998; Hack *et al.* 1998; Araishi *et al.* 1999; Coral-Vazquez *et al.* 1999; Liu and Engvall 1999; Durbeej *et al.* 2000).

Another hurdle facing gene therapy for the treatment of muscle disease is the safe and efficient treatment of all muscles in a human patient. This is a daunting task, as muscle consists of ~30% of the body's mass, with many muscle groups not easily accessible by physical means. New methods of gene delivery must be developed before treatment of human muscle disease with gene therapy becomes a practical option.

Over-all hypothesis: There are a series of complex variables, some disease specific, and some more generalized, which affect the efficacy of gene delivery.

Hypothesis 1: There are aspects of *protein biochemistry* that alter efficacy of gene therapy, even in closely related biochemical defects. Altering biochemical interactions during protein assembly by over-expression of a transgene leads to cytotoxicity in some models, but not in others. α-sarcoglycan gene delivery results in cytotoxicity of myofibers via improper assembly in the golgi, while delivery of β- or δ-sarcoglycan does not.

Hypothesis 2: There are aspects of *tissue pathology* that alter efficacy of gene therapy. Muscle, which is functionally rescued shows <u>little or no</u> immunological reaction to AAV delivery of foreign protein, while dystrophic (non-rescued) muscle <u>does</u> show an immunological response against transgenes.

Observation: Development of a 10-needle injection manifold will allow for an accurate, reproducible, and safe method of gene delivery to large areas of muscle.

3.0

α- and β-sarcoglycan gene delivery by AAV: efficient rescue of muscle, but differential persistence of gene expression.

3.1 Summary

The sarcoglycanopathies are a group of four autosomal recessive limb-girdle muscular dystrophies (LGMD 2D, 2E, 2C, and 2F), caused by mutations of the α, β, γ, or δ sarcoglycan genes. The δ-sarcoglycan deficient hamster has been the most utilized model for gene delivery to muscle by recombinant AAV vectors, although human patients with δ-sarcoglycan deficiency are exceedingly rare with only two patients described in the United States (Duggan *et al*., 1997b). Here I report construction and use of AAV vectors expressing α- or β-sarcoglycan, the genes responsible for the most common forms of the human sarcoglycanopathies. Both vectors showed successful short-term genetic, biochemical, and histological rescue of both α- and β-sarcoglycan deficient mouse muscle. However, comparison of persistence of expression in 50 injected mice showed substantial differences between AAV α-SG and β-SG vectors. AAV β-SG showed long-term expression with no evidence of decrease 389 days after injection, while AAV α-SG showed a dramatic loss of positive fibers between 28 days and 41 days post-injection ($p=0.006$). Loss of immuno-positive myofibers was correlated with significant inflammatory cell infiltrate, primarily macrophages. To determine if the loss of α-sarcoglycan positive fibers was due to an immune response or to cytotoxic effects of α-sarcoglycan over-expression, Severe Compromised Immune-Deficient (SCID) mouse muscle was assayed for cytotoxicity after injection with AAV α-SG, AAV β-SG, or PBS. The results were consistent with over-expression of α-sarcoglycan causing significant cytotoxicity. The cytotoxicity of α-sarcoglycan, and not β- or δ-sarcoglycan over-expression, was consistent with biochemical studies of the hierarchical order of assembly of the sarcoglycan complex. Our data suggests that even closely

related proteins require different levels of expression to avoid toxicity and achieve long-term tissue rescue.

3.2 Introduction

Mutations in the genes encoding the dystrophin-associated sarcoglycan proteins (α, β, γ, and δ) are responsible for a subset of the genetically heterogeneous autosomal recessive muscular dystrophies (LGMD 2D, 2E, 2C, and 2F respectively) (Ozawa *et al*., 1998; Hoffman 1999). Patients with primary sarcoglycanopathies can be clinically similar to patients with primary dystrophin abnormalities (Duchenne and Becker muscular dystrophies) (Duggan *et al*., 1997a; Ozawa *et al*., 1998). Similarly, corresponding mouse models show similar phenotypes. Loss of function mutations in any one of the sarcoglycan genes (primary deficiency) leads to secondary deficiencies of the other sarcoglycan proteins (Jung *et al*., 1996a; Ozawa *et al*., 1998). The secondary loss of the sarcoglycans in both sarcoglycanopathies and dystrophinopathies occurs post-translationally (Chen *et al*., 2000), presumably due to inaccurate assembly and stabilization of the dystrophin/sarcoglycan/dystroglycan complex (Suzuki *et al.* 1992; Iwata *et al.* 1993; Roberds *et al.* 1993a; Suzuki *et al.* 1994; Ahn and Kunkel 1995; Jung *et al.* 1995; Yang *et al.* 1995; Rafael *et al.* 1996; Vainzof *et al.* 1996; Sakamoto *et al.* 1997; Chan *et al.* 1998; Duclos *et al.* 1998; Ozawa *et al.* 1998; Araishi *et al.* 1999; Hack *et al.* 2000; Yoshida *et al.* 2000). Thus, there appears to be relatively stringent requirements for the macromolecular assembly of the sarcoglycans. The primary sarcoglycanopathies are estimated to account for about 10-20% of cases with a Duchenne muscular dystrophy phenotype but normal dystrophin findings by muscle biopsy (Duggan *et al*., 1997a). Here, “muscular dystrophy” refers to patients with primarily proximal weakness, persistent high serum creatine kinase levels, and no other associated symptoms. Work completed in the Hoffman laboratory has shown the relative

incidences of the different sarcoglycanopathies in an American/European population to be $\alpha > \beta > \gamma > \delta$ in about a 6:3:2:1 ratio (Duggan *et al*., 1997a).

The sarcoglycanopathies have been a prime target for early work with gene therapy of muscle disease due to the small size of their genes (~1.5 kb) and the existence of the Bio 14.6 hamster, a well characterized spontaneous loss-of-function knockout for the δ-sarcoglycan gene (Nigro *et al*., 1997). This model, coupled with the emergence of recombinant AAV as a promising delivery vector for muscle, has led to a series of reports of genetic, biochemical, histological and functional rescue of relatively large regions of muscle in this hamster model from us and others (Holt *et al*., 1998; Greelish *et al*., 1999; Li *et al*., 1999; Xiao *et al*., 2000).

AAV shows many advantages relative to other gene delivery systems for muscle. The Hoffman laboratory has recently reported that muscle tissue appears to be the preferential site of infection and latency of wild-type AAV (Tezak *et al*., 2000). In addition, the wild-type genome integrates preferentially into or close to troponin T gene (*TNNT1*) a highly muscle-specific gene on chromosome 19 (Dutheil *et al*., 2000). Both in muscle and other tissues, AAV appears to be nonpathogenic with no known association with any disease (Gertz 1973), and AAV efficiently transduces cells with no reports of induction of a cellular immune response or cytotoxicity (Berns and Bohenzhy 1987; Flotte *et al*., 1993; Clark *et al*., 1997). In contrast to all other (recombinant) viruses, AAV is also able to infect both mature and immature muscle (Li *et al*., 1999). The ability of AAV to infect muscle readily may be due to the high expression of AAV attachment receptors, heparin sulfate proteoglycan (HSP) in muscle (Summerford and Samulski 1998; Pruchnic *et al.* 2000; Cao *et al.* 2001). Muscle also expresses an AAV co-receptor, integrin αVβ5 (Summerford *et al.* 1999; Pruchnic *et al.* 2000). In addition, AAV is able to penetrate the basal lamina of muscle, while other viral vectors are not; this is possibly due to the

AAV virion being only 20 nm in diameter, while adenovirus is 70-100 nm, and herpes is 120-300 nm (Cao *et al*., 2001). However, AAV's small size also limits the packaging capacity of the recombinant vectors to inserts of 3-5 kb. This limitation makes it impossible to deliver the full-length dystrophin gene, which could be used to treat the most common muscle disease, Duchenne muscular dystrophy. Truncated dystrophin constructs encoding only the most critical regions of the dystrophin protein have recently shown functional rescue in mouse models (Wang *et al*., 2000).

Two different groups have reported functional recovery in the δ-sarcoglycan deficient hamsters using AAV-mediated delivery of the normal human gene (Greelish *et al*., 1999; Xiao *et al*., 2000). However, δ-sarcoglycan deficiency in humans is probably the most rare human muscle disease characterized to date, with only two unrelated patients reported in the United States by the Hoffman laboratory (Duggan *et al*., 1997b). Murine knockouts for α-, β-, and γ-sarcoglycan have recently been reported (Duclos *et al.* 1998; Hack *et al*., 1998; Araishi *et al.*, 1999; Liu and Engvall 1999; Durbeej *et al*. 2000), making new animal models available for testing gene delivery for the more common sarcoglycanopathies. AAV gene delivery was recently reported in γ-sarcoglycan deficient animals using the human γ-sarcoglycan gene driven by a truncated muscle creatine kinase (MCK) promoter (Cordier *et al.*, 2000). This group reported intramuscular injection of 22 mice, with the majority of animals showing between 20-40% biochemical rescue at 4-16 weeks post injection. Prevention of histopathological changes in dystrophic muscle was reported if treated at early ages (< 3 weeks). No immune complications or rejection of γ-sarcoglycan positive fibers in treated mice was described.

Here I report construction of both α- and β-sarcoglycan AAV constructs and the use of these vectors in the corresponding knockout mice. While both recombinant vectors showed

widespread biochemical and immediate histological rescue of dystrophic muscle, only AAV β-SG showed persistence of transgene expression. I present data suggesting that the loss of α-sarcoglycan expression was due to cytotoxicity from protein over-expression from the CMV promoter, likely as a result of incorrect assembly (dominant-negative effect) of the quaternary sarcoglycan complex. These data suggest that expression levels of the protein from the transgene may be a critical variable in gene delivery efforts and, further, that even closely related biochemical defects may show very different cytotoxicity profiles.

3.3 Materials and methods

3.3.1 AAV vector production

The complete coding sequences of both α- and β-sarcoglycan were amplified and cloned from primary human muscle biopsy cDNA. α-sarcoglycan cDNA was amplified by using the following primer set: forward 5' TCTGTCACTCACCGGGC 3' and reverse 5' GTGGACAGAGAAGGGAGGATGA 3'. β-sarcoglycan cDNA was amplified using the following primer set: forward 5' ACAGTCGGGCGGGGAGCT 3' and reverse 5' CATGTTGGTGACCTCTGGG 3'. The amplified coding sequences were then cloned utilizing a TA cloning vector, pGem EZ cloning vector (Promega). Each coding sequence was excised from the corresponding plasmid using NotI, and the fragments were gel purified and cloned into a cis-acting AAV vector (pAAV CMV α-SG or pAAV CMV β-SG). This placed the gene under the CMV promoter, as we have previously described (Li *et al.,* 1999). Both constructs were subjected to automated sequencing of the portions internal to the AAV terminal repeats to verify integrity of the constructs.

Recombinant AAV viral stocks were produced by the triple plasmid co-transfection method as previously described (Xiao *et al.,* 1998). Briefly, 1-2 hours prior to transfection, 293 cells (human embryonic kidney cell line) at 70-80% confluence were given IMDM media. Freshly made 2.5M $CaCl_2$ and HEBS buffer were combined with 12.5 μg cis-acting plasmid (pAAV CMV α-SG or pAAV CMV β-SG), 12.5 μg trans-acting plasmid (p518) (Xiao *et al.,* 1999), and 25 μg of adenovirus helper plasmid (pFΔ6) (Xiao *et al.,* 1999) and added to each 150mm plate of 293 cells. Media was replaced with DMEM the following day. Media was collected, and virus was harvested 5 days post transfection. AAV particles were purified through

two sequential CsCl density gradients. The particle number of each virus was determined using a slot-blot method. Typical yields of virus were 2-5 x 10^{15} particles (2-5 x 10^{12} per mL).

3.3.2 Mouse strains

A murine gene knockout for α-sarcoglycan was obtained from Dr. Eva Engvall and was constructed as previously described (Liu and Engvall 1999). The murine gene knockout for β-sarcoglycan was obtained from Dr. Eijiro Ozawa and was constructed as previously described (Araishi *et al.,* 1999). Breeding colonies were established, and mice of specified ages were used for intramuscular injection of AAV.

4-5 week old SCID (c3H/Smn.ClcrHsd-SCID) mice were obtained from Harlan.

3.3.3 Intramuscular injections

Mice deficient for either α- or β-sarcoglycan were injected with a recombinant AAV that would complement their deficiency (either AAV α-SG or AAV β-SG) in the tibialis anterior muscle. Mice were injected either prior to the initial phase of degeneration/regeneration (under 2 weeks of age), or during the later stable stage of histology after the initial round of degeneration/regeneration (~6 weeks of age) (Araishi *et al.,* 1999; Liu and Engvall 1999). Mice injected prior to degeneration/regeneration (< 2 weeks) received a single intramuscular injection of 10-15 μL (5 x 10^{10} viral particles). Mice injected at older ages (> 6 weeks) received a single intramuscular injection of 35-40 μl (2 x 10^{11} viral particles). Mice were sacrificed at ~ 27, 41, 133, or 388 days post injection.

Seven week old SCID mice were injected in both tibialis anterior muscles with 20 μL of either PBS, AAV α-SG, or AAV β-SG (1 x 10^{11} viral particles per injection) and were sacrificed at 28 or 41 days post injection.

A single intramuscular injection was given to each mouse, using a Hamilton 1702 syringe with a 22S gauge needle. The needle was inserted longitudinally through the tibialis anterior muscle at the knee. In mice older than one month the muscle was exposed by incision of the skin prior to injection, while younger mice were injected through the skin.

3.3.4 Histological and immunofluorescent analyses

Each tibialis anterior muscle was carefully dissected from both tendon insertion sites and directly flash frozen in liquid nitrogen-cooled isopentane. Muscles were stored in hydrated, airtight containers at –80° prior to sectioning. Cryosections were cut from the belly (center) of each muscle. Hematoxylin and eosin staining was done on 8 μm frozen sections, while all immunostaining was done with 4 μm sections.

For immunostaining, sections were thawed on Superfrost Plus slides (Fisher Scientific Inc.) then immediately incubated with 10% horse serum in PBS for 30 minutes. The sections were incubated with primary antibody at room temperature for an hour. The primary antibodies used were previously described, rabbit polyclonal anti-α-SG and anti-γ-SG antibody (Noguchi *et al.* 2000), rabbit anti-β-SG antibody (Araishi *et al.* 1999), rat anti-δ-SG antibody (Imamura *et al.* 2000), and rabbit anti-sarcospan antibody (Noguchi *et al.* 1999). Sections were washed 3 to 4 times with the 10% horse serum in PBS and incubated with a Cy3-conjugated secondary antibody diluted in 10% horse serum and PBS for an hour, and subsequently washed an additional three times. Sections were then mounted with Gel/Mount (Biomeda Corp.) and analyzed using a Nikon FXA microscope.

3.3.5 Quantitation of transduced myofibers

To determine the percentage of fibers that had been transduced by AAV, photomontages were produced from microscopic images of each entire TA cross-section immunostained with the

appropriate anti-sarcoglycan antibody. These montages were imported into NIH image v1.62, the area of the positive fibers was outlined and quantitated, and this value was divided by the total area of each TA. On average, 30 cells represented approximately 1% of the muscle fibers (~3,000 fibers/TA). Any muscles with less than 30 cells were reported as <1%.

3.3.6 Characterization of immune responses

Immune responses were characterized by both immunostaining, using antibodies directed against specific macrophage, T-cell, and B-cell markers, and by assay of circulating murine antibodies using an immunoblotting method. For immunostaining 4 μm cryosections were incubated with the following antibodies: anti-mouse CD4 (L3T4), anti-mouse CD8a (Ly-2), anti-mouse CD19 (1D3), and anti-mouse Mac-3 (all from PharMingen Inc).

Immune cell infiltrate was measured in both injected and non-injected muscles. Quantitation was performed by immunofluorescently labeling both nuclei and specific immune cell infiltrate (B-cells, macrophages, CD4 and CD8 T-cells), taking random fields and counting the number of areas which were double stained, giving a specific cell count.

Humoral immune responses were measured by western blotting of murine serum. Immunoblots were prepared using 100 μg total protein from human patient muscle biopsies from normal muscle, dystrophin deficiency (Duchenne muscular dystrophy), and α-sarcoglycan deficiency (LGMD 2D). The use of human biopsies for these studies was approved by the Children's National Medical Center IRB. The protein samples were resolved on a 10% SDS PAGE gel along with a lane that contained AAV. The primary antibody used was serum from injected or non-injected animals diluted 1:500. The secondary antibody was an anti-mouse IgG conjugated to HRP (Amersham Pharmacia Biotech Inc.) diluted 1:1000. An ECL (Amersham Pharmacia Biotech Inc.) western blot detection reagent was used to visualize immune complexes.

3.3.7 Statistical analysis

All statistical comparisons were done using either T-tests or analysis of variance (ANOVA). Statistical significance of multiple comparisons was defined using Scheffe's method. All analyses were completed using Minitab V.13.1.

3.4 Results

3.4.1 Biochemical rescue of β-sarcoglycan deficient mouse by AAV gene transfer.

The complete coding sequence of β-sarcoglycan was amplified and cloned from primary human muscle biopsy cDNA, placed under the control of a CMV promoter in AAV ITRs, and sequence-verified. Recombinant AAV was produced by a triple plasmid transfection method (Xiao *et al.* 1998).

After β-sarcoglycan deficient knockout mice (Araishi *et al.,* 1999) received a single intramuscular injection of AAV-CMV-β- sarcoglycan, all muscles examined showed extensive expression of human β-sarcoglycan (Figure 9). Twenty-five (25) mice were analyzed at different time points (Table 1), with an average of 15% of the TA myofibers immunostaining positive for the transgene protein. There was variability in expression, reflecting the different ages at which the mice were injected, and the difficulty in delivering precise amounts of virus by intramuscular injection. Expression of the transgene was seen at all time points, including mice sacrificed at 133 and 388 days post injection, where large areas of expression of β-sarcoglycan were still seen (Figure 10 and Table 1).

Mutations in the β-sarcoglycan gene lead to primary deficiency of β-sarcoglycan and secondary biochemical deficiencies of sarcospan, α-, γ-, and δ- sarcoglycans (Araishi *et al.,* 1999). To test if the secondary biochemical defects were restored by AAV β-SG, serial sections were immunostained with antibodies for each protein (Figure 11). All myofibers that were immuno-positive for β-sarcoglycan also demonstrated restoration of each secondary biochemical defect (Figure 11). Thus, AAV delivery of β-sarcoglycan was able to restore all primary and

secondary biochemical sarcoglycan deficiencies for long periods in β-sarcoglycan knockout muscle.

3.4.2 Histological rescue of β-sarcoglycan deficient muscle with AAV expression of β-sarcoglycan

β-sarcoglycan deficient mice do not show evidence of myofiber degeneration or regeneration until about 2-4 weeks post partum (Araishi *et al.,* 1999), similar to the other sarcoglycan deficient and dystrophin deficient mice (Hoffman and Kunkel 1989; Duclos *et al.,* 1998). Myofibers that have not undergone any degeneration/regeneration show peripheral nuclei, while fibers that have undergone one or more cycles of degeneration/regeneration show central nuclei. To determine if β-sarcoglycan delivery by AAV completely prevents myofiber degeneration, I injected mice at pre-necrotic stages (2 weeks old), then assayed for central nuclei in the post-necrotic stage. TA muscle sections were stained with H&E and assayed for dystrophic pathology and quantitation of central nucleation. β-sarcoglycan deficient mice that received AAV β-SG prior to the onset of degeneration/regeneration (2 weeks) showed complete histopathological rescue in muscle segments that expressed β-sarcoglycan**,** with all nuclei showing peripheral localization, and homogeneous fiber size in the rescued segments (Figures 9, 10). Muscle cells that receive β-sarcoglycan appear not to undergo degeneration/regeneration for over 388 days (Figure 10), which is in contrast to the β-sarcoglycan negative myofibers in the same section that show central nuclei and variable fiber size. These results demonstrate that β-sarcoglycan delivery by AAV rescues from lethality for over a year from injection.

3.4.3 Initial rescue of α-sarcoglycan deficient muscle by an AAV vector, but lack of persistence

α-sarcoglycan deficient animals were injected with an AAV α-SG and sacrificed at similar time points to those of the β-sarcoglycan deficient animals, as described in the methods

portion of this chapter. A total of 25 α-sarcoglycan deficient mice were injected and analyzed (Table 1). Mice that were sacrificed at the early (28 day) time point showed substantial biochemical rescue (Figure 12) and showed restoration of all secondary biochemical defects (Figure 11). However, histopathology was abnormal, with substantial immune cell infiltration (Figure 12). In addition, mice injected at early ages (<2 weeks) showed central nucleation (Figure 16). By 41 days post injection, the majority of muscles studied showed < 5% α-sarcoglycan positive myofibers (Table 1). Biochemical recovery did not appear to be age dependent, as all animals regardless of age initially showed biochemical rescue, but also lost expression within 40 days. Finally, all of the animals studied at 136 or 389 days post injection showed less than 1% of α-sarcoglycan positive fibers, with 3 of the 6 animals showing no positive fibers (Figure 17 and Table 1). All injected animals were grouped as <30 days post-injection, and >30 days post-injection, and the number of transduced fibers was compared for both α-sarcoglycan and β-sarcoglycan (Table 1, Figure 17, panel C). This analysis showed a highly statistically significant loss of α-sarcoglycan-positive myofibers after 30 days post-injection ($p=0.006$), while there was no loss of β-sarcoglycan-positive myofibers (Figure 17, panel C).

To characterize the immune cell infiltrate seen in muscles injected with AAV α-SG, muscles from control α- and β-sarcoglycan knockouts and muscle from AAV injected mice were stained for CD4 T-cell, CD8 T-cell, B-cell, and macrophage markers (Figure 12). Uninjected α- and β-sarcoglycan deficient muscle showed only small foci of macrophage infiltration, with less T-cell (CD4, CD8) and no B-cell positive areas (data not shown). β-sarcoglycan deficient muscle injected with AAV and studied 28 or 41 days post injection showed no inflammatory cells where biochemical rescue (α-sarcoglycan expression) was evident (H&E staining of Figure

9, 10). However, AAV α- SG injected muscle showed extensive macrophage infiltration (Figure 12), while the contralateral leg showed relatively little infiltration (data not shown). Some T-cell infiltration was also seen in the same areas that had macrophage infiltration; however, B-cells were rare (data not shown). Simon Watkins' laboratory performed quantitation of immune cell infiltrate; results indicated that AAV α- SG injected muscle has significantly more macrophages and CD4 positive T-cells than the non-injected legs (Figure 13, 14). The lack of B-cell infiltrate suggested that over-expression of human α-sarcoglycan or β-sarcoglycan did not elicit a strong humoral response, consistent with previous studies of AAV in muscle (Jooss *et al.*, 1998).

To provide further evidence that the loss of α-sarcoglycan positive fibers was not associated with a humoral immune response, serum from injected mice was used for immunoblot analysis of circulating α- or β-sarcoglycan antibodies (Figure 15). Total muscle protein from human patients with normal sarcoglycans, primary deficiency (α-sarcoglycan mutations), and secondary deficiencies (Duchenne and Becker muscular dystrophies) were stained with primary mouse sera at 1:500. Sera from both AAV α-SG injected α-sarcoglycan deficient mice and AAV β- SG injected β-sarcoglycan deficient mice showed similar levels of antibodies to both the transgene and AAV coat proteins. All AAV α- SG injected mice that showed a positive AAV response also had a positive α-sarcoglycan response, although not all animals that had an α-sarcoglycan positive response were AAV positive. All AAV β- SG injected mice that showed a positive β-sarcoglycan response had a positive AAV response, although not all β-sarcoglycan injected animals having a positive AAV response had a positive response to β-sarcoglycan (Figure 15). This data suggested that a differential humoral response was not responsible for the different levels of persistence of AAV α-SG versus AAV β- SG.

3.4.4 SCID mouse studies suggest increased cytotoxicity of α-sarcoglycan over-expression

To further address the issue of whether α-sarcoglycan over-expression caused an immune response or was cytotoxic, a cytotoxicity assay was carried out in SCID mice. As these mice are positive for the sarcoglycans, have normal muscle, and lack both humoral and cellular immune responses, immune rejection of α-sarcoglycan positive fibers should not occur. Thus, any observed loss of α-sarcoglycan positive fibers was likely due to cytotoxicity. I assayed cytotoxicity by quantitation of central nucleation, which is highly reflective of the extent of myofiber degeneration/regeneration that is caused by direct injury from the needle track and/or cytotoxicity of the transgene. Immune deficient SCID mice were injected in the TA muscles with either AAV α-SG, AAV β-SG, or PBS. Histopathology was assayed at 28 and 41 days post injection. The results showed that SCID mice injected with AAV α-SG had statistically higher number of central nuclei ($p<0.001$) than that of the PBS injected or the AAV β-SG injected animals, while numbers for the PBS injected and AAV β-SG injected animals were not statistically different (Figure 18). This data suggests that over-expression of α-sarcoglycan is cytotoxic to myofibers, while β-sarcoglycan over-expression is not.

3.5 Discussion

Inherited deficiencies in either α- or β-sarcoglycan are responsible for two of the more common limb-girdle muscular dystrophies. This report is the first to show construction and delivery of either α- or β-sarcoglycan using AAV. Two previous reports described use of an adenovirus vector to deliver α-sarcoglycan (Duclos *et al.,* 1998; Allamand *et al.* 2000). Another report showed delivery of γ-sarcoglycan using an AAV MCK-γ-sarcoglycan construct and an adenovirus MCK-γ-sarcoglycan construct (Cordier *et al.,* 2000). A final report described the use of an adenovirus to deliver β-sarcoglycan (Durbeej *et al*. 2000). All four reports showed biochemical rescue of the corresponding primary defect. Histological rescue or persistence of expression was not studied in the β-sarcoglycan adenovirus report, while the γ-sarcoglycan adenovirus and AAV vectors showed expression to four months post injection and one of the α-sarcoglycan adenovirus reports showed expression for seven months with histological correction.

Here I document biochemical complementation of both α- and β-sarcoglycan deficient murine models using the corresponding AAV construct, including complete histological protection by AAV β-SG (Figure 9, 10, 11). Our analysis of biochemistry, histology and persistence was based on 50 mice receiving a single intramuscular injection into a single large muscle group (tibialis anterior, TA), with normalization of all results to the entire cross-section of the center of each muscle. In short-term experiments, both vectors were able to biochemically and histologically rescue up to 63% of myofibers in the TA muscle after a single intramuscular injection (Figure 9). The extent of transduction was not dependent on the age of the mouse at the time of injection. This is in contrast to herpes simplex virus and adenovirus vectors, where more efficient transduction is seen in neonatal animals (Feero *et al.,* 1997). Analysis of distribution of

delivery through the TA muscle often showed extensive transduction relatively distant from the injection site, with positive fibers distributed at distances of up to 0.5 cm (Figure 9). Relatively large volumes were injected (20 μl in 100 mg of muscle, representing a 20-30% increase in total volume), which may have facilitated delivery.

Persistence of expression in injected mice was dramatically different between the AAV α-SG and β-SG vectors (Figure 17). AAV β-SG showed long-term persistence of expression with no evidence of loss of positive myofibers through 388 days after injection. In addition, β-sarcoglycan delivery protected myofibers from cell death for over a year, as evidenced by peripheral nuclei in all transduced fibers (Figure 10). To our knowledge, this is the first demonstration of complete histological rescue for over a year in any muscle disease gene therapy model. The levels and persistence of β-sarcoglycan would be expected to be therapeutic in human muscle, if sufficiently large regions of muscle could be injected. It is also pertinent to note that the CMV promoter was not inactivated in muscle; this is in contrast to liver, where loss of expression by promoter has been observed (Loser *et al.*, 1998; Chen *et al.* 1999). Finally, there was no evidence of cytotoxicity or immune response against β-sarcoglycan, despite the fact that the human protein was delivered to β-sarcoglycan knockout (null) mice.

The observed efficacy using AAV β-SG was not seen when α-sarcoglycan was delivered using an identical CMV promoter construct and AAV backbone (Figure 17). Delivery of α-sarcoglycan by AAV showed successful short-term biochemical and histological rescue of muscle (Figure 12), with up to 63% of the entire TA showing strong α-sarcoglycan expression at 28 days post injection. However, in contrast to the β-sarcoglycan results, expression of α-sarcoglycan was largely lost by 41 days post-injection (Figure 17, Table 1). Loss of transduced myofibers was associated with extensive macrophage infiltration (Figure 12), and remaining α-

sarcoglycan positive fibers often showed dystrophic histopathology (central nuclei and variable diameter), suggesting that these fibers may have undergone cycles of degeneration and regeneration. Indeed, the presence of central nuclei in α-sarcoglycan knockout mice injected at young, "pre-necrotic" ages suggests that these positive fibers may have originated from transduced myoblasts (satellite cells), as α-sarcoglycan positive fibers that contain central nuclei would have been regenerated from an α-sarcoglycan positive myoblast (Figure 12, 16). We do not believe that this loss of α-sarcoglycan-positive fibers is due to difference in levels of over-expression of AAV α-SG versus AAV β-SG, as the constructs are identical with regards to promoter, vector, and the virus was produced using identical methods.

To investigate the mechanism of the loss of α-sarcoglycan transduced myofibers, immunodeficient mice (SCID) were injected with AAV α-SG, AAV β-SG or PBS (sham control). I found evidence for cytotoxicity of the α-sarcoglycan expressing AAV (Figure 18), in that α-sarcoglycan positive fibers were lost even in the lack of an immune system. Our SCID data was consistent with the relative absences of T- and B-cell infiltration seen in the immune competent knockout mouse model data, as cytotoxicity due to the over-expression of α-sarcoglycan is not dependent on T- or B-cells, indicated by central nucleation in SCID mice and loss of positive fibers from the α-sarcoglycan deficient mice (Figure 12).

In a recent report of over-expression of γ-sarcoglycan in transgenic mice, cytotoxicity was seen only if expression levels were 150 to 200-fold (36-57 gene copies) over their normal levels; over-expression at 5 times normal levels (3-4 copies) did not cause any cytotoxicity (Zhu *et al.,* 2001). These authors concluded that expression levels of γ-sarcoglycan are important for appropriate correction of the defect, similar to the conclusions that I have drawn here with regards to α-sarcoglycan. While it is difficult to directly compare mRNA and protein levels

from studies, current studies suggest that α-sarcoglycan over-expression is more cytotoxic than γ-sarcoglycan over-expression. This is shown by comparing our results of over-expression of α-sarcoglycan by AAV-mediated gene delivery being cytotoxic with the results from Zhu *et al.* (2001) of 5 times over-expression of γ-sarcoglycan in a transgenic mouse not causing any toxicity and the results from Cordier *et al.* (2000) showing the over-expression of γ-sarcoglycan by AAV-MCK-γ-SG mediated gene delivery also not having any toxic effects.

Allamand *et al.* (2000) show that they can maintain expression of α-sarcoglycan at below normal levels, for seven months, using an adenovirus whereas I did not observe expression of α-sarcoglycan for that amount of time (Allamand *et al.* 2000). We believe that differences in persistence are due to one or a combination of differences in viral delivery methods, animal models, and/or the strength of the promoter driving the transgene. First, there is the primary difference in their use of adenovirus and our use of AAV. Second, different animal model knockouts were used. Their animal knockout has disrupted exons 2 and 3 (Duclos *et al.*, 1998), while the animal knockout that I use has disrupted exons 1 and 2 (Liu and Engvall 1999). However, both models do not produce any α-sarcoglycan protein products. Finally, the most significant difference between our two results is most likely due to the use of different promoters. They report the use of the rous sarcoma virus (RSV) promoter, which upon western blot analysis does not show over-expression. In fact, according the data presented, when a muscle is transduced at ~90% there is at most ~50% normal levels of expression of the α-sarcoglycan protein (Allamand *et al.* 2000). I used the cytomegalovirus (CMV) promoter, which in an earlier collaborative study showed vast over-expression of δ-sarcoglycan both by western blot analysis and by immunofluorescence (Li *et al.* 1999). CMV over-expression is seen in this study by cytoplasmic staining of α-sarcoglycan in muscle injected with AAV. Our results

taken with those of Allamand *et al.* results, have led us to propose some of the future studies that are discussed below.

There are a number of possible explanations for the observed cytotoxicity of α-sarcoglycan, and the lack of toxicity with the over-expression of the other three sarcoglycans (Figures 20, 21, and 22). First, recent biochemical studies of the sarcoglycan complex have shown that the four protein transmembrane complex (α, β, γ, δ) is assembled in the golgi prior to transport to the cell surface (Noguchi *et al.,* 2000). This assembly has been found to be hierarchical, where β-sarcoglycan nucleates the complex, with subsequent addition of γ- or δ-sarcoglycan added in either order. Finally the addition of α-sarcoglycan completes the tetramer (Noguchi *et al.,* 2000) (Figure 19). We hypothesize that over-expression of α-sarcoglycan results in abnormal tetramers, with too many α-sarcoglycan subunits inappropriately filling the γ- or δ- binding sites. These $\beta\alpha_2$ or $\beta\alpha_3$ complexes may initially make it to the surface, but do not fulfill their normal function and/or accumulate in the golgi, and thereby lead to cytotoxicity (Figure 21). Similarly, there is the potential that over-expression of α-sarcoglycan causes premature binding of α-sarcoglycan to β-sarcoglycan, which causes biochemical changes in the conformation of β-sarcoglycan so that it is unable to bind γ or δ-sarcoglycan. This βα complex could in turn fail to transport to the cell surface, accumulate in the golgi, and again lead to cytotoxicity (Figure 22). Indeed, I found some evidence of α-sarcoglycan-positive intracellular inclusions and cytoplasmic staining (see myofibers, top of Figure 10B), which were not seen with β-sarcoglycan. It is important to state that both of these models allow for the possibility of low levels of proper assembly of the sarcoglycan subunits, which is seen with early rescue of the sarcoglycan complex (Figures 21, 22). Therefore, the data suggests that accumulation of improperly formed complexes overtime leads to cytotoxicity.

An additional possibility is that the human α-sarcoglycan protein is not sufficiently similar to the murine orthologue to functionally replace the protein in murine muscle. The α-sarcoglycan protein shows 89% identity between mouse and human, while the β-sarcoglycan protein shows 95% identity (Appendix A, B). In addition, α-sarcoglycan is distinct from the other three sarcoglycans in that the N terminus is extracellular, while the other three have an intracellular N terminus (Figure 2). The fact that I found short-term histological rescue of α-sarcoglycan deficient muscle suggests the first mechanism, as a lack of function of human α-sarcoglycan in murine muscle, would probably not result in time-dependent loss of transduced fibers. In addition, our observation of cytotoxicity in SCID mice, which co-express murine α-sarcoglycan, suggests that the human α-sarcoglycan has a dominant cytotoxic effect on transduced muscle, likely due to over-expression (i.e. co-expression of murine α-sarcoglycan was not protective). Another potential reason for the cytotoxicity caused by over-expression of α-sarcoglycan is that α-sarcoglycan contains a particularly large cytoplasmic domain relative to the other three sarcoglycans, and this domain is presumably involved in protein-protein interactions at the cytoplasmic face of the plasma membrane. Over-expression of α-sarcoglycan could lead to mislocalization or inappropriate binding of intracellular factors, to a greater degree than the other, smaller sarcoglycans. Finally, a close relative of α-sarcoglycan, ε-sarcoglycan, is also expressed in skeletal muscle myofibers, and these two subunits are thought to be somewhat interchangeable in the sarcoglycan complex (with β, γ, and δ). However, ε-sarcoglycan expression does <u>not</u> change in α-sarcoglycan-deficient mice, suggesting that ε-sarcoglycan <u>cannot</u> functionally rescue the sarcoglycan complex in myofibers (Liu and Engvall 1999). Nevertheless, it is possible that ε-sarcoglycan has some protective role in skeletal myofibers, and

over-expression of α-sarcoglycan may exclude ε-sarcoglycan binding to the complex, thereby mediating toxicity via a dominant-negative effect on ε-sarcoglycan function.

In conclusion, I show that delivery of two closely related proteins by AAV can lead to very different outcomes, with complete, long-term rescue of muscle by β-sarcoglycan, but significant cytotoxicity and muscle damage by α-sarcoglycan. Studies are underway to determine if the use of the autologous α-sarcoglycan protein or gene promoter mitigate the cytotoxicity. While our data suggests that human clinical trials using AAV over-expression of α-sarcoglycan may be premature, delivery of β-sarcoglycan is likely to provide a long-term therapeutic benefit.

3.6 Acknowledgements

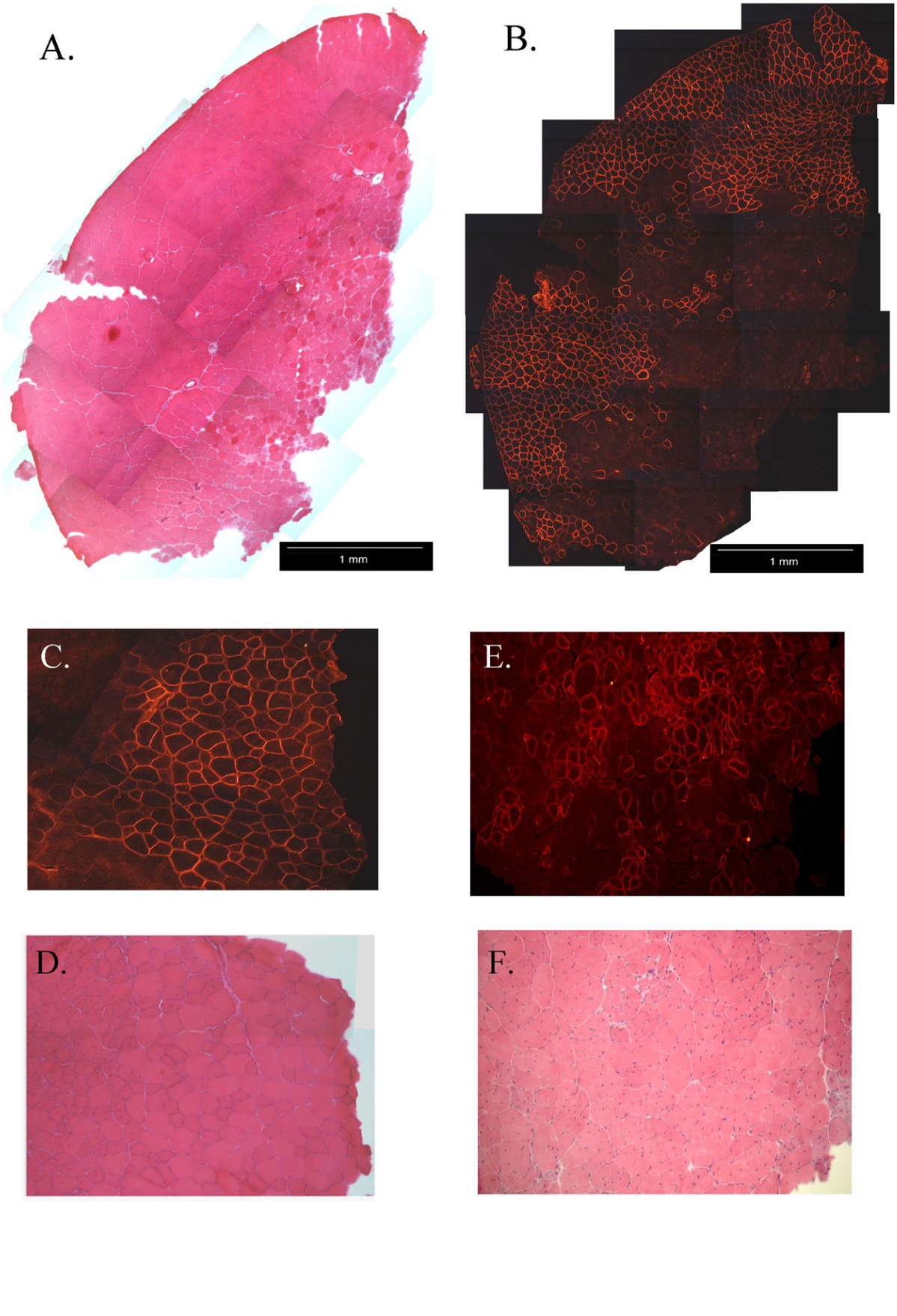
I would like to thank both Dr. Xuehai Ye and Dr. Marina Jerebtsova for their extensive help with AAV. In addition, I would like to thank Heather Gordish for her help with the statistical analysis. This work was supported by a PO1 grant from the NIH to EPH (5PO1AR45926-03), and by a pre-doctoral fellowship award from the American Heart Association to DD.

Table 1. Summary of AAV α-SG and AAV β-SG injections to correspondingly deficient mice.

Mouse #	Amount of Virus/ Viral Particles	Age Injected	Days post-Injection	Percentage of sarcoglycan positive fibers	Mouse #	Amount of Virus/ Viral Particles	Age Injected	Days post - Injection	Percentage of sarcoglycan positive fibers
Alpha sarcoglycan knockout mice					Beta sarcoglycan knockout mice				
Alpha # 1	40 μL/2x10^{11}	50	28	29%	Beta # 1	20 μL/1x10^{11}	79	14	10%
Alpha # 2	40 μL/2x10^{11}	50	28	32%	Beta # 2	20 μL/1x10^{11}	79	14	8%
Alpha # 3	40μL/2x10^{11}	50	28	16%	Beta # 3	39 μL//2x10^{11}	91	26	14%
Alpha # 4	40 μL/2x10^{11}	50	28	24%	Beta # 4	40 μL/2x10^{11}	91	26	13%
Alpha # 5	40 μL/2x10^{11}	50	28	51%	Beta # 5	80 μL/4x10^{11}	133	26	12%
Alpha # 6	40 μL/2x10^{11}	50	28	25%	Beta # 6	15μL/5x10^{10}	18	27	13%
Alpha # 7	15μL/5x10^{10}	25	28	63%	Beta # 7	15μL/5x10^{10}	8	39	33%
Alpha # 8	15μL/5x10^{10}	25	28	28%	Beta # 8	15μL/5x10^{10}	15	40	53%
Alpha # 9	15μL/5x10^{10}	10	29	<1%	Beta # 9	15μL/5x10^{10}	15	40	17%
Alpha # 10	15μL/5x10^{10}	10	29	6%	Beta # 10	15μL/5x10^{10}	15	40	1%
Alpha # 11	15μL/5x10^{10}	10	29	2%	Beta # 11	11 μL/5x10^{10}	244	41	11%
Alpha # 12	15μL/5x10^{10}	15	41	26%	Beta # 12	11 μL/5x10^{10}	244	41	9%
Alpha # 13	15μL/5x10^{10}	15	41	14%	Beta # 13	11 μL/5x10^{10}	244	41	2%
Alpha # 14	15μL/5x10^{10}	15	41	4%	Beta # 14	11 μL/5x10^{10}	244	41	18%
Alpha # 15	15μL/5x10^{10}	15	41	4%	Beta # 15	16.5 μL/5x10^{10}	244	41	11%
Alpha # 16	15μL/5x10^{10}	25	41	4%	Beta # 16	16.5 μL/5x10^{10}	244	41	8%
Alpha # 17	15μL/5x10^{10}	25	41	8%	Beta # 17	16.5 μL/5x10^{10}	244	41	8%
Alpha # 18	15μL/5x10^{10}	10	41	0%	Beta # 18	16.5 μL/5x10^{10}	244	41	7%
Alpha # 19	15μL/5x10^{10}	10	41	0%	Beta # 19	16.5 μL/5x10^{10}	244	41	4%
Alpha # 20	15μL/5x10^{10}	15	135	0%	Beta # 20	15μL/5x10^{10}	8	133	54%
Alpha # 21	15μL/5x10^{10}	15	136	0%	Beta # 21	15μL/5x10^{10}	8	133	1%
Alpha # 22	15μL/5x10^{10}	15	136	<1%	Beta # 22	15μL/5x10^{10}	8	133	35%
Alpha # 23	15μL/5x10^{10}	15	136	1%	Beta # 23	15μL/5x10^{10}	8	133	5%
Alpha # 24	15μL/5x10^{10}	15	389	<1%	Beta # 24	15μL/5x10^{10}	8	260	<1%
Alpha # 25	15μL/5x10^{10}	15	389	0%	Beta # 25	15μL/5x10^{10}	8	388	16%

Figure 9. Biochemical and histological rescue of β-sarcoglycan deficient mouse muscle after gene delivery of β-sarcoglycan by an AAV vector.

Shown is a photomontage of the entire TA of a β-sarcoglycan deficient mouse after a single intra-muscular injection of AAV-CMV-β-SG (panels A, B). Serial sections stained for histopathology (hematoxylin and eosin, panel A), and for restoration of the sarcoglycan complex (anti-α-sarcoglycan immunostaining, panel B) are shown from a TA injected at 15 days of age and sacrificed at 40 days post injection. β-sarcoglycan deficient mouse muscle receiving the AAV β-sarcoglycan vector is rescued from the primary and secondary biochemical deficiencies and from myofiber degeneration, indicated by peripheral nuclei. To compare the effect of age of the mice on delivery and transgene expression, 2 age groups (8 day old and 91 day old) were injected. Mice injected at 8 days and sacrificed 39 days post injection show the same biochemical and histological rescue (panels C and D). Mice injected at 91 days of age and studied 27 days post injection show biochemical rescue. This older group of mice exhibited central nucleation due to the degeneration/regeneration process that occurred prior to AAV delivery (panels E and F). This data shows that recovery of the biochemical defect is not affected by age at time of injection. Note that the polyclonal antibody positively stains vascular smooth muscle of blood vessels, due to cross-reaction to unknown proteins.


A.
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B.
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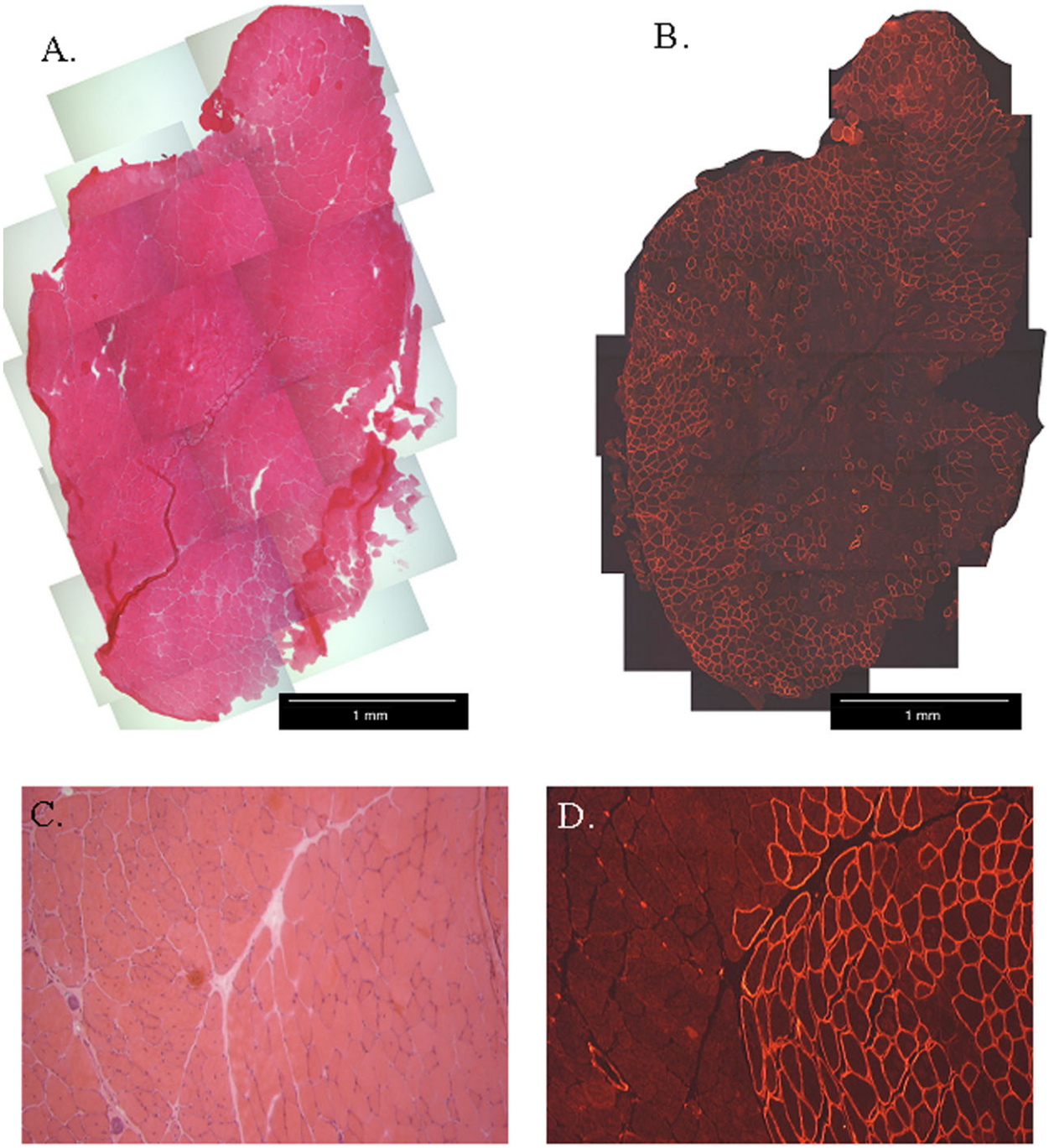
F.

Figure 10. Long-term biochemical recovery and sustained cellular rescue of β-sarcoglycan deficient muscle after a single intra-muscular injection with AAV β-sarcoglycan.

Shown is a photomontage of the entire TA muscle of a β-sarcoglycan deficient mouse after a single intra-muscular injection at 8 days of age, and studied 133 days after gene delivery (panel A, hematoxylin and eosin; panel B anti-α-sarcoglycan immunostaining). Panel C and D showed serial sections of a β-sarcoglycan deficient mouse muscle after receiving a single intra-muscular injection at 8 days of age, and studied 388 days after gene delivery. Both positive and negative regions are shown for comparison. Protection of myofibers from degeneration/regeneration persists for over a year after delivery, as evidenced by the peripheral nuclei and homogeneous fiber size of the rescued fibers. These findings indicate that AAV-mediated gene delivery of β-sarcoglycan is able to confer long-term protection to myofibers from cellular lethality.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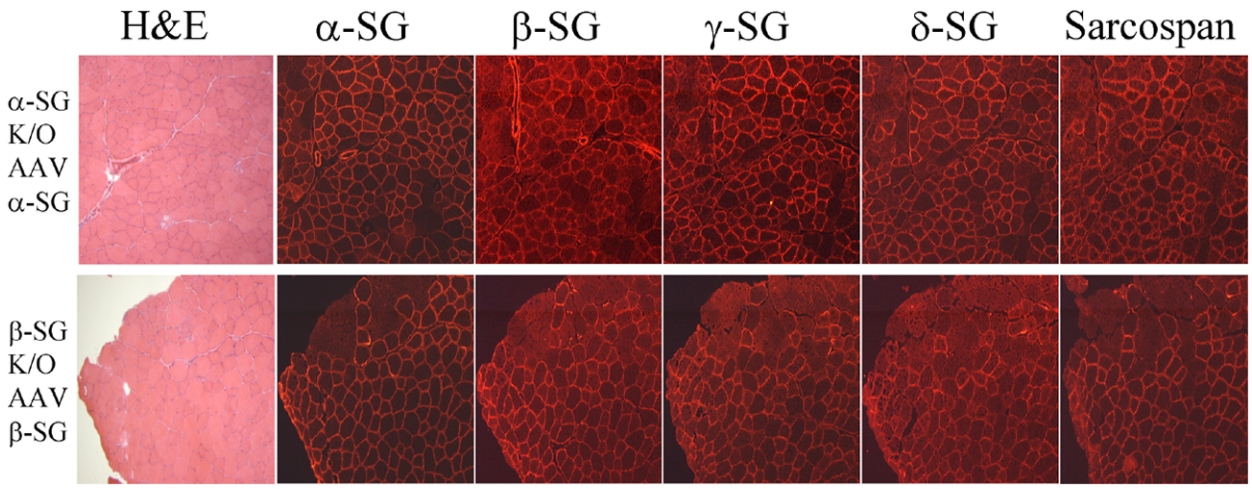
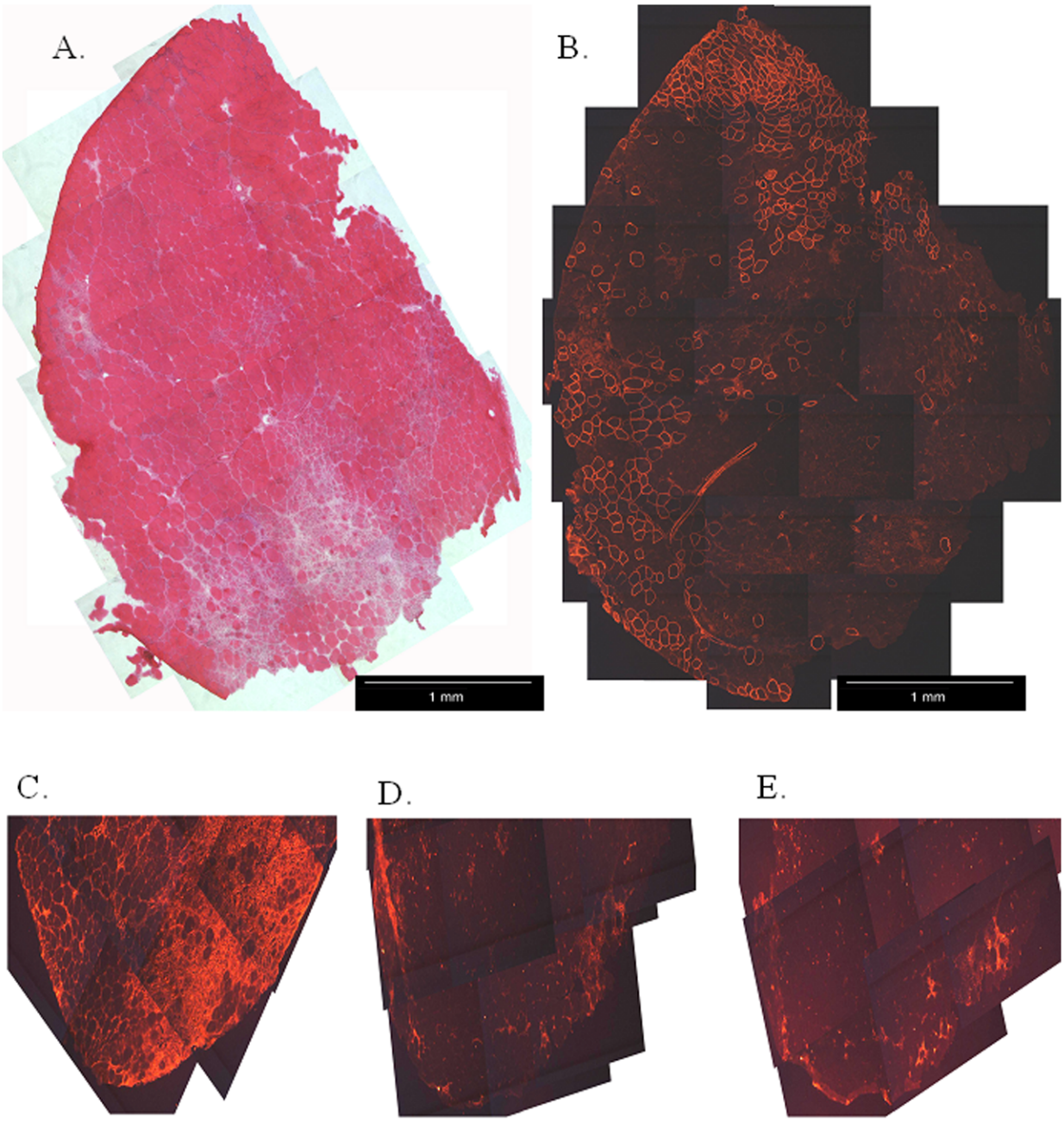


Figure 11. Restoration of all secondary biochemical defects in both α-sarcoglycan and β-sarcoglycan deficient mice following gene delivery with the corresponding AAV.

Shown are serial sections of an α-sarcoglycan deficient mouse TA that was injected at 25 days of age and studied 28 days post injection (top panels), and serial sections of a β-sarcoglycan deficient mouse TA that was injected at 15 days of age and studied at 40 days post injection (lower panels). The sections were stained with hematoxylin and eosin and immunostained with antibodies against all four components of the sarcoglycan complex (α-SG, β-SG, γ-SG and δ-SG), and sarcospan. This data shows that AAV complementing the primary defect is also able to rescue all secondary biochemical defects.

Figure 12. Delivery of an α-sarcoglycan AAV vector leads to short-term biochemical rescue of α-sarcoglycan deficient mouse muscle associated with macrophage and T-cell infiltration.

Shown are photomontages of the entire TA muscle of a mouse injected at 15 days of age with AAV α-sarcoglycan and sacrificed at 41 days post injection (panel A, hematoxylin and eosin; panel B anti-α-sarcoglycan immunostaining). The H&E staining shows considerable cellular infiltrate that was not observed in contralateral, non-injected legs (data not shown). To characterize the type of immune cell infiltrate, sections of the same treated muscle were stained for macrophages (anti-mac-3 antibodies, panel C), helper T-cells (anti-CD4, L3T4, antibodies, panel D) and cytotoxic T-cells (anti-CD8, Ly-2, antibodies, panel E). Extensive macrophage infiltration is seen with both CD4 and CD8 T-cells. B-cells were only rarely observed (not shown). These findings suggest that elimination of α-sarcoglycan positive myofibers is <u>not</u> due to an IgG mediated immune response. However an IgG immune response eliminating α-sarcoglycan positive fibers cannot be completely ruled out in these experiments and is addressed in the SCID mouse experiments (see Figure 18).


A.
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B.
1 mm
C.
D.
E.

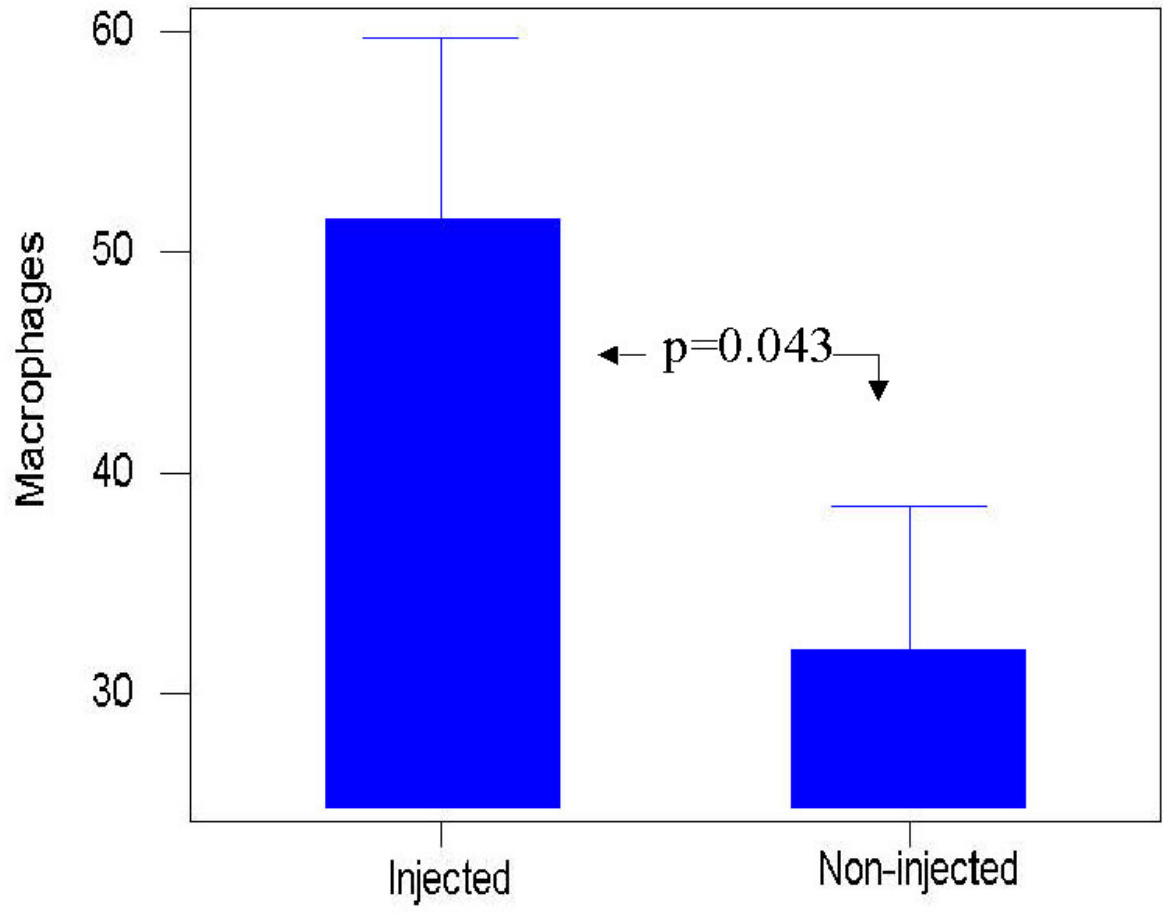


Figure 13. Quantitation of macrophage infiltration.

To determine the number of macrophages in the immune cell infiltrate of α-sarcoglycan deficient mice injected with AAV α-sarcoglycan, immunofluorescent double staining for both nuclei and macrophages was performed and quantitated. Random fields were selected for quantitation, and 8 injected muscles were compared with 4 non-injected muscles, to reveal that there are statistically more macrophages in the injected muscles than in the non-injected muscles.

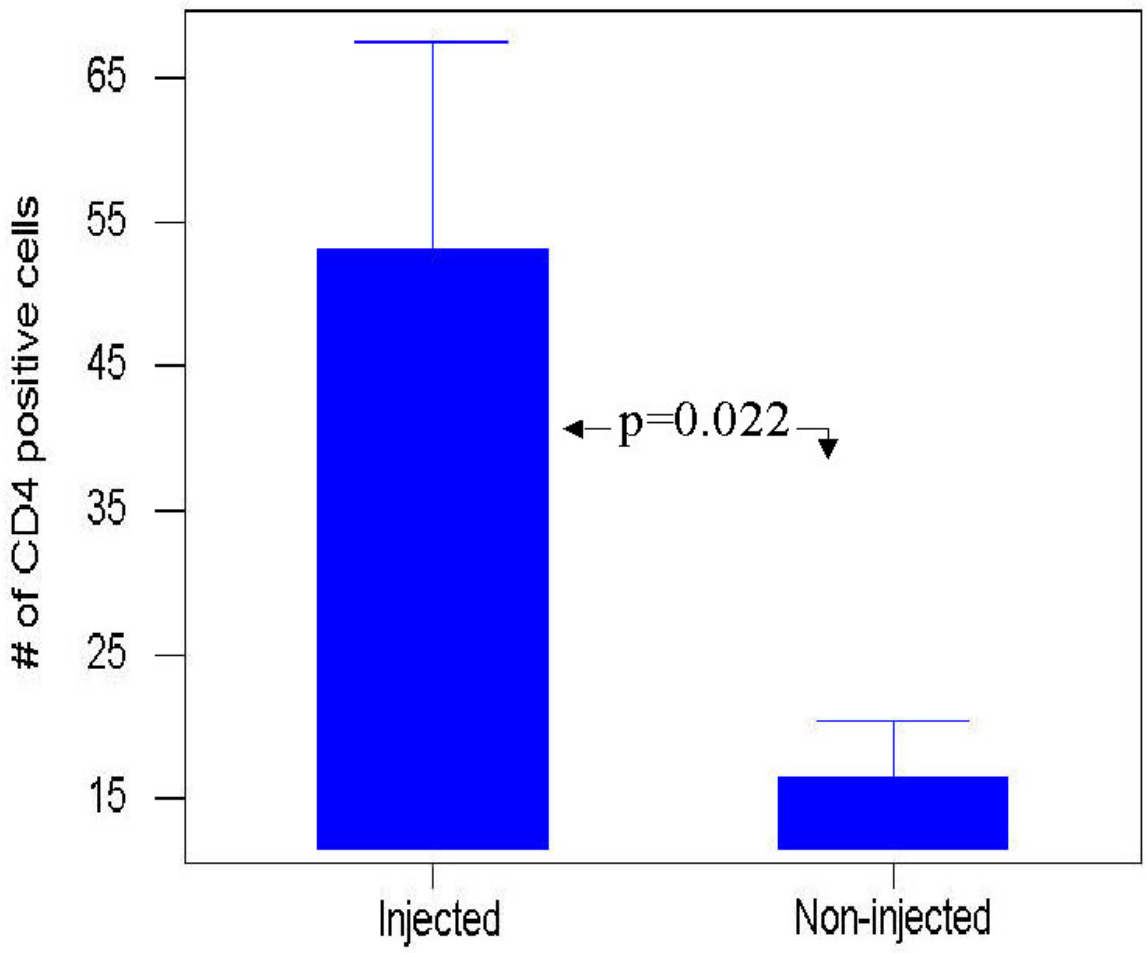
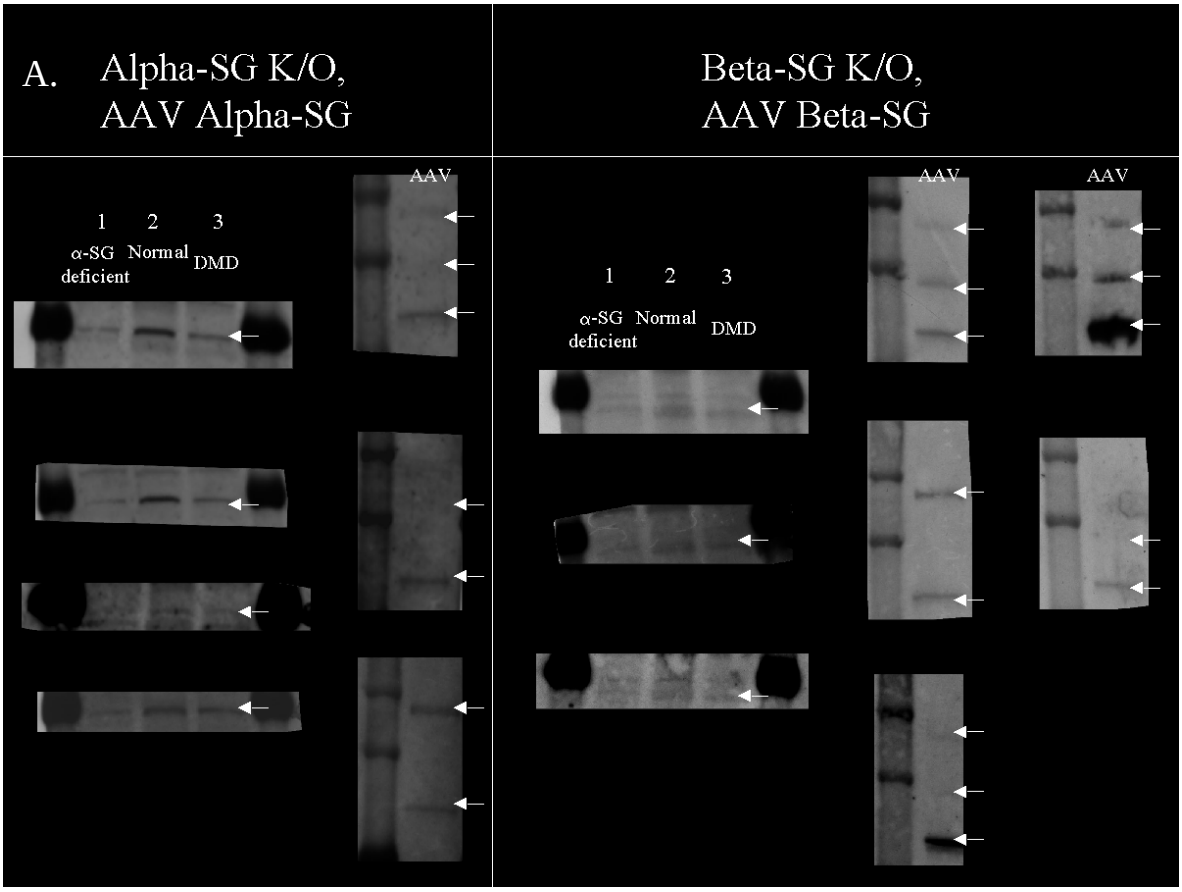


Figure 14. Quantitation of CD4 positive T-cell infiltration.

To determine the number of CD4 positive T-cells in the immune cell infiltrate of α-sarcoglycan deficient mice injected with AAV α-sarcoglycan, immunofluorescent double staining for both nuclei and CD4 positive T-cells was performed and quantitated. Random fields were selected for quantitation and 8 injected muscles were compared with 4 non-injected muscles, to reveal that there are statistically more CD4 positive T-cells in the injected muscles than in the non-injected muscles.

Figure 15. Western blot analysis of humoral immune response.

Western blots were run using protein preparations from α-sarcoglycan deficiency (α-SG deficient), normal, and dystrophin deficiency (DMD) patient muscle or AAV. The blots were probed using serum from injected or non-injected animals. The total number of animals used for each group is as follows: 6 α-SG knockout (K/O) animals injected with AAV α-SG, 6 α-SG K/O non-injected animals, 6 β-SG K/O animals injected with AAV β-SG, 6 β-SG K/O non-injected animals. Panel A shows positive bands from the western blot analysis (arrows indicate positive bands). Animals were considered to have a positive antibody response to AAV if they exhibited an antibody response to at least 2 of the 3 coat proteins of AAV. Panel B is a graphical representation of the results from all animals studied. All AAV α-SG injected animals that exhibited a positive antibody response to AAV also had a positive response to α-sarcoglycan. All AAV β-SG injected animals that had a positive antibody response to β-sarcoglycan had a positive response to AAV. The number of animals that exhibited an antibody response to their respective sarcoglycan was very similar with 4 AAV α-SG injected animal having a positive response and 3 AAV β-SG injected animals exhibiting a positive response. Therefore, loss of expression in the AAV α-sarcoglycan injected animals does not appear to be correlated with a humoral immune response.


A.
Alpha-SG K/O,
AAV Alpha-SG
Beta-SG K/O,
AAV Beta-SG
AAV
AAV
AAV
1
2
3
α-SG deficient
Normal
DMD
1
2
3
α-SG deficient
Normal
DMD

B.

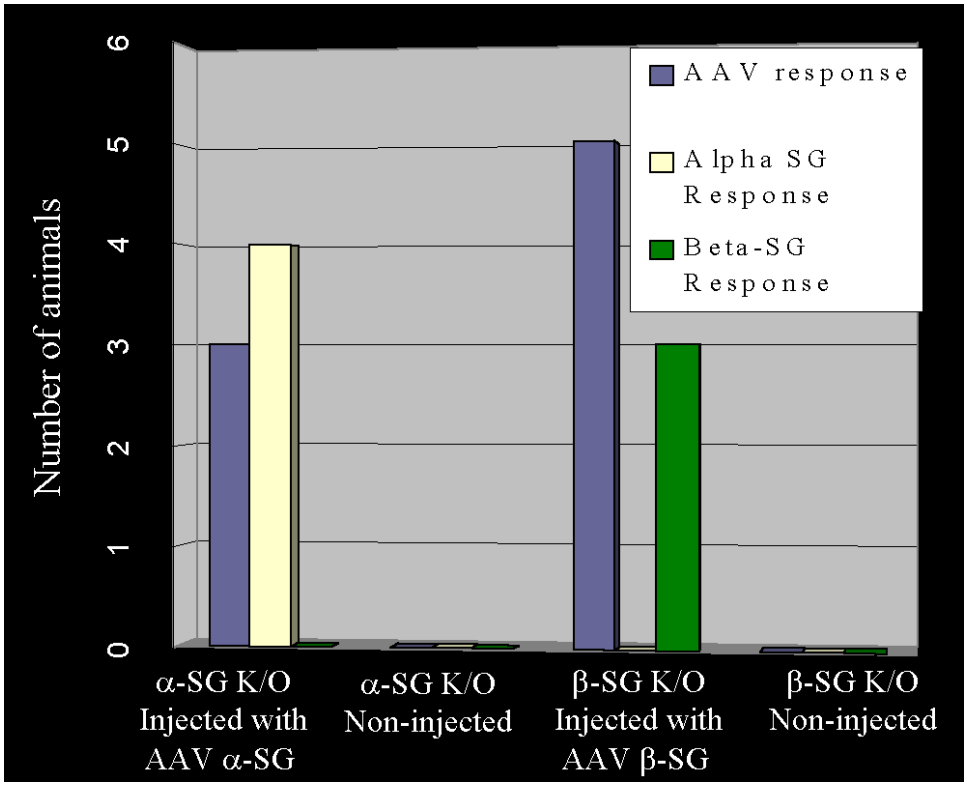

Number of animals
0
1
2
3
4
5
6
AAV response
Alpha SG Response
Beta-SG Response
α-SG K/O Injected with AAV α-SG
α-SG K/O Non-injected
β-SG K/O Injected with AAV β-SG
β-SG K/O Non-injected

Figure 16. AAV CMV α-sarcoglycan biochemical rescue of α-sarcoglycan deficient mouse muscle is not age dependent.

Shown are paired H&E and anti-α-sarcoglycan immunostaining from mice injected at 10 days old (panels A and B) or at 50 days old (panels C and D) and studied at 28 days post injection. Short-term biochemical rescue of the muscle is seen independent of the age of mice at the time of injection although expression is lost by 40 days post-injection (Table 1). Immune cell infiltrate is seen at all times of delivery, often in areas that show rescued myofibers. Arrows in panels A and B show an α-sarcoglycan positive fiber with a central nuclei.

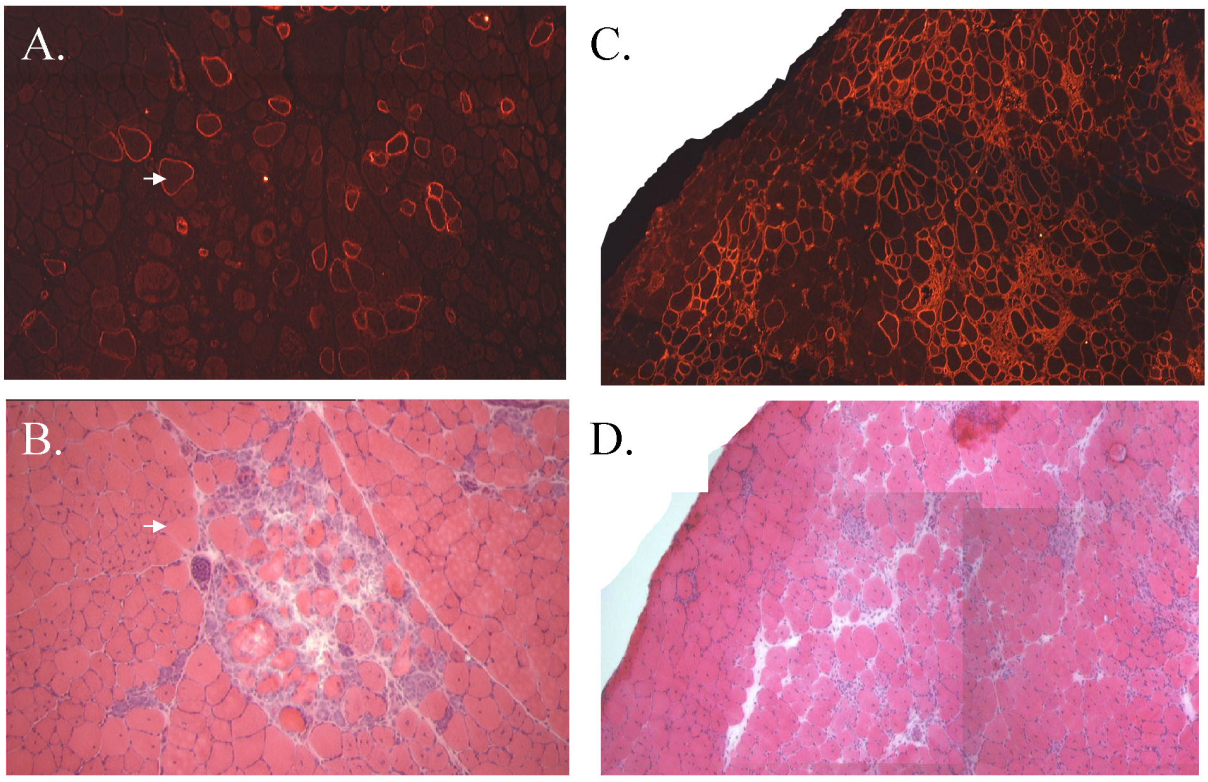
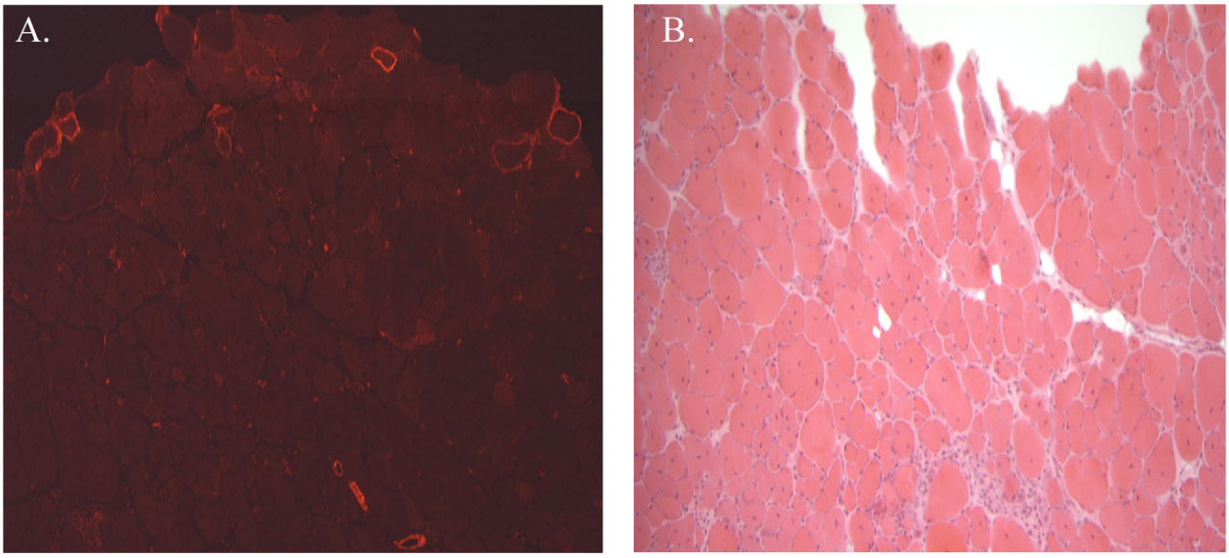

A.
C.
B.
D.

Figure 17. α-sarcoglycan expression is not persistent.

Shown is a region of the TA muscle of an α-sarcoglycan deficient mouse after injection with AAV CMV α-sarcoglycan at 15 days old, studied at 136 days post injection. The immunofluorescent staining (panel A) for α-sarcoglycan is almost entirely absent at this time point, indicating loss of α-sarcoglycan expression over time. In addition, this time point shows no sign of any histological rescue as indicated in the H&E staining (panel B).

Panel C shows the mean percentage (± SE) of transduced fibers of both α-sarcoglycan deficient mice (Alpha) and β-sarcoglycan deficient mice (Beta), studied at either < 30 days post injection (< 30 days post) or > 30 days post injection (> 30 days post) (see Table 1). Injected α-sarcoglycan deficient mice studied at > 30 days post injection show a highly statistically significant loss of transduced fibers, whereas β-sarcoglycan injected mice show no loss of transduced fibers (NS = not significant).


A.
B.

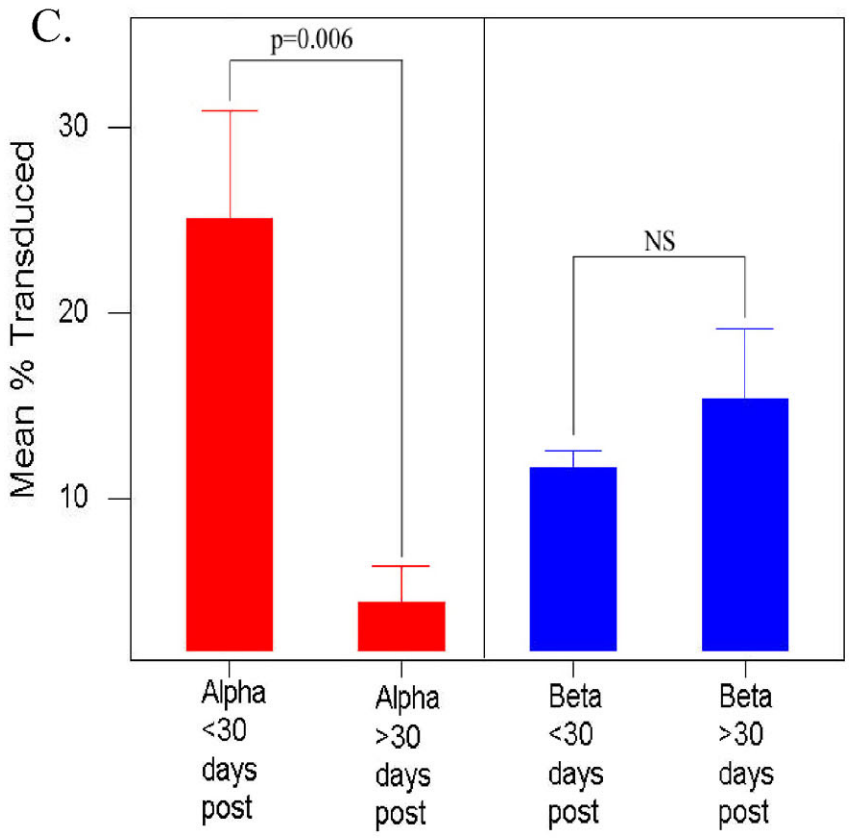
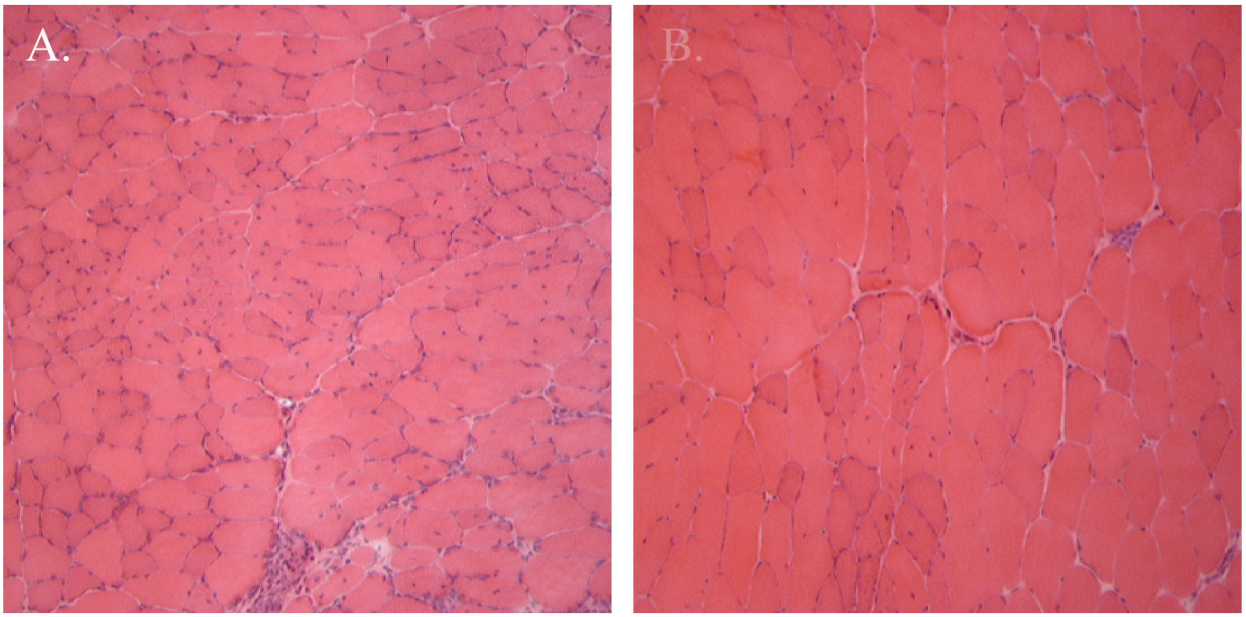
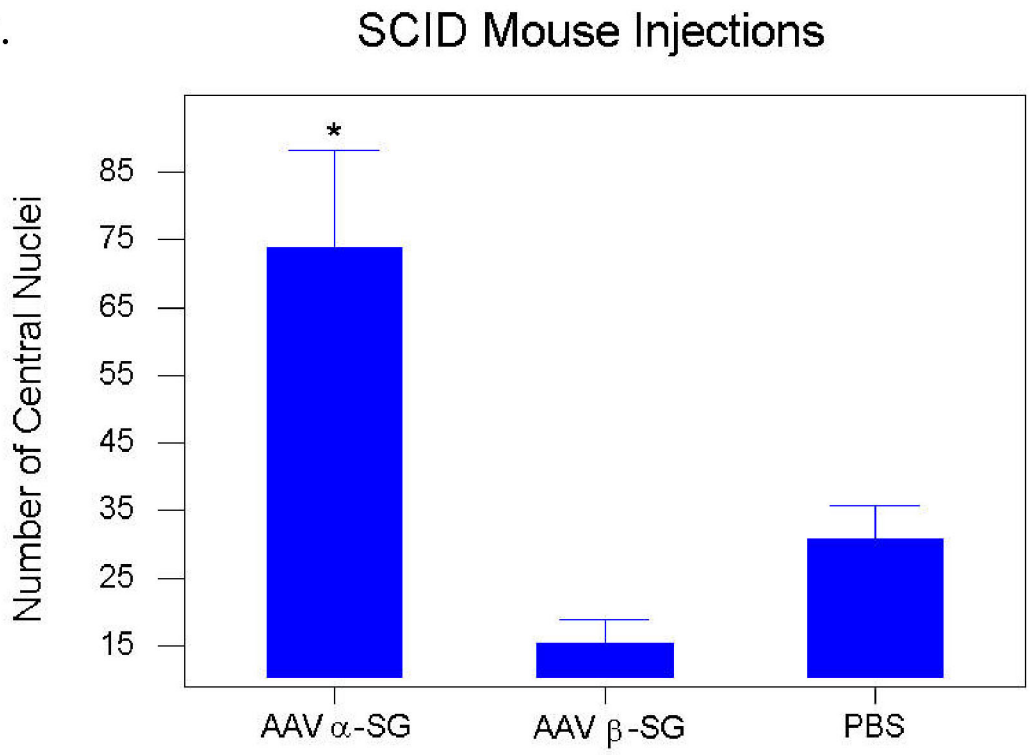

C.
p=0.006
NS
Mean % Transduced
30
20
10
Alpha <30 days post
Alpha >30 days post
Beta <30 days post
Beta >30 days post

Figure 18. SCID mice injected with either AAV α-sarcoglycan, AAV β-sarcoglycan or PBS show that over-expression of α-sarcoglycan is cytotoxic.

Shown are TA muscles from SCID mice stained with hematoxylin and eosin after injection with AAV α-sarcoglycan (panel A) or AAV β-sarcoglycan (panel B). Both mice were injected at ~ 48 days of age and studied ~ 43 days after injection. AAV α-sarcoglycan injected muscle shows widespread central nucleation of myofibers, indicating cell death and regeneration (panel A). AAV β-sarcoglycan injected muscle shows fewer centrally nucleated myofibers, occurring along the needle track (panel B). Panel C is a graphical representation of the number of central nuclei in the TA of SCID mice that were injected with either AAV α-sarcoglycan, AAV β-sarcoglycan or PBS. The number of central nuclei (a measurement of cytotoxicity in normal muscle) are statistically significantly greater (*) in SCID mice injected with AAV α-sarcoglycan than in SCID mice injected with either AAV β-sarcoglycan or PBS ($p<0.001$). However, AAV β-sarcoglycan and PBS injected muscles are not statistically different from one another. This data suggests that over-expression of α-sarcoglycan is cytotoxic to myofibers.


A.
B.

C.


SCID Mouse Injections
Number of Central Nuclei
85
75
65
55
45
35
25
15
*
AAV α-SG
AAV β-SG
PBS

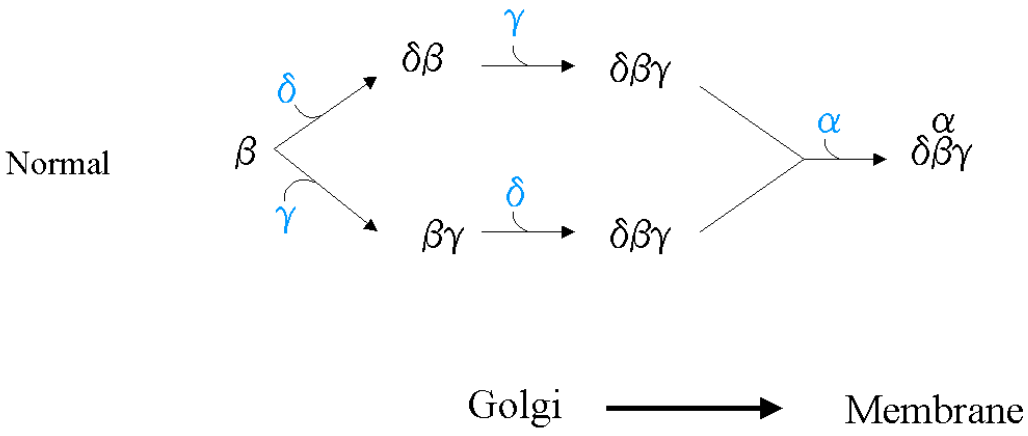


Figure 19. Model for biochemical assembly of the sarcoglycan complex.

In the model based on work by Noguchi *et al.* (2000) diagrammed above, β-sarcoglycan nucleates the sarcoglycan complex, followed by addition of either γ-sarcoglycan or δ-sarcoglycan without apparent preference between the two subunits. Next γ-sarcoglycan binds to the complexes that already have δ-sarcoglycan and δ-sarcoglycan binds to complexes that already have γ-sarcoglycan. The complex formation is complete with the addition of α-sarcoglycan. (β= β-sarcoglycan, δ = δ-sarcoglycan, γ = γ-sarcoglycan, and α = α-sarcoglycan)

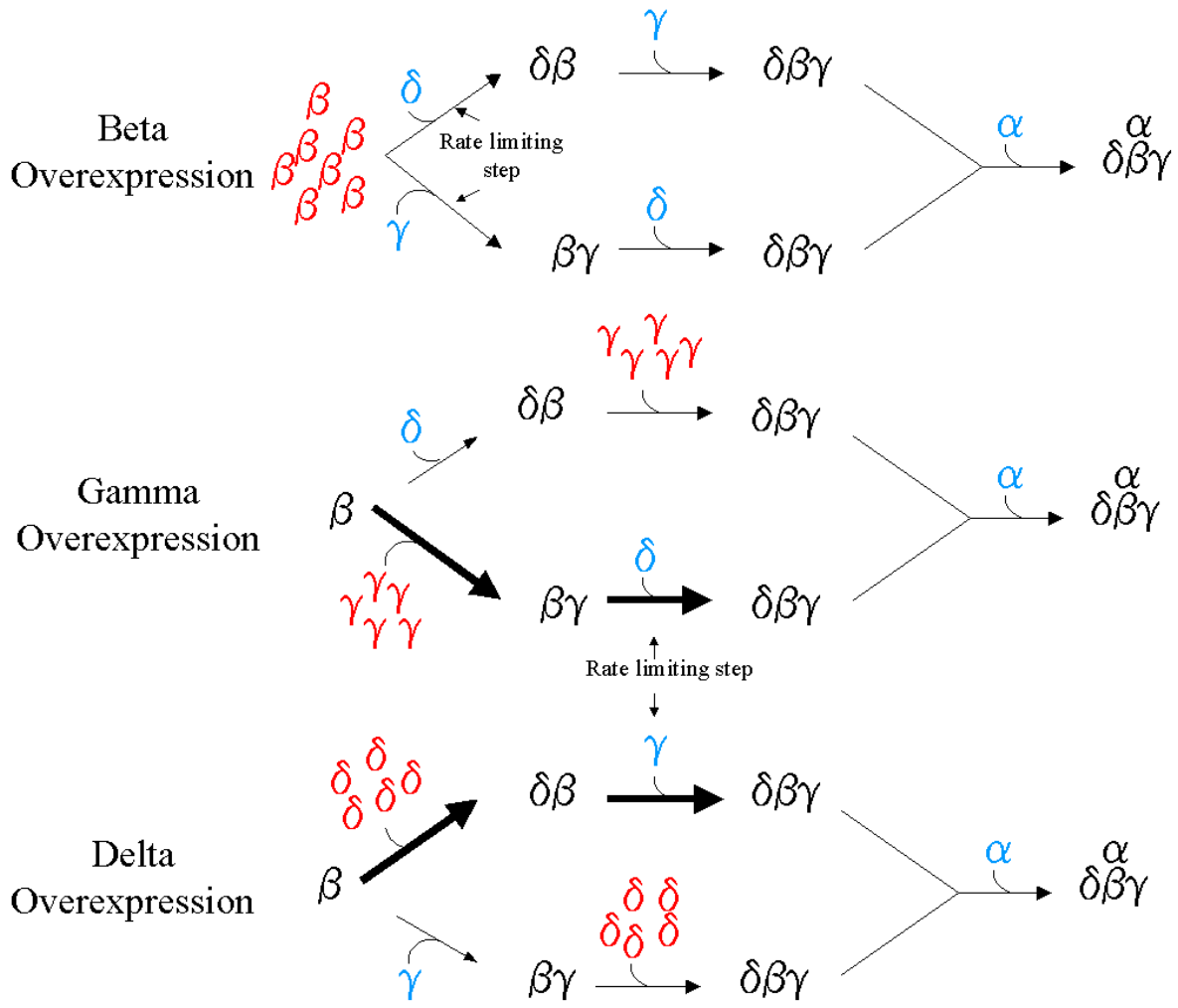


Figure 20. Over-expression of β-, γ-, δ-sarcoglycan should not affect the formation of the sarcoglycan complex.

Over-expression of β-sarcoglycan should not affect the formation of the complex, since it nucleates the complex, and the rate limiting step would be the addition of δ- or γ-sarcoglycan. Over-expression of δ- or γ-sarcoglycan will drive the second addition to the complex toward the over expressed protein, but should not affect formation of the complex, since either protein can be added second in the complex formation. (β= β-sarcoglycan, δ = δ-sarcoglycan, γ = γ-sarcoglycan, and α = α-sarcoglycan)

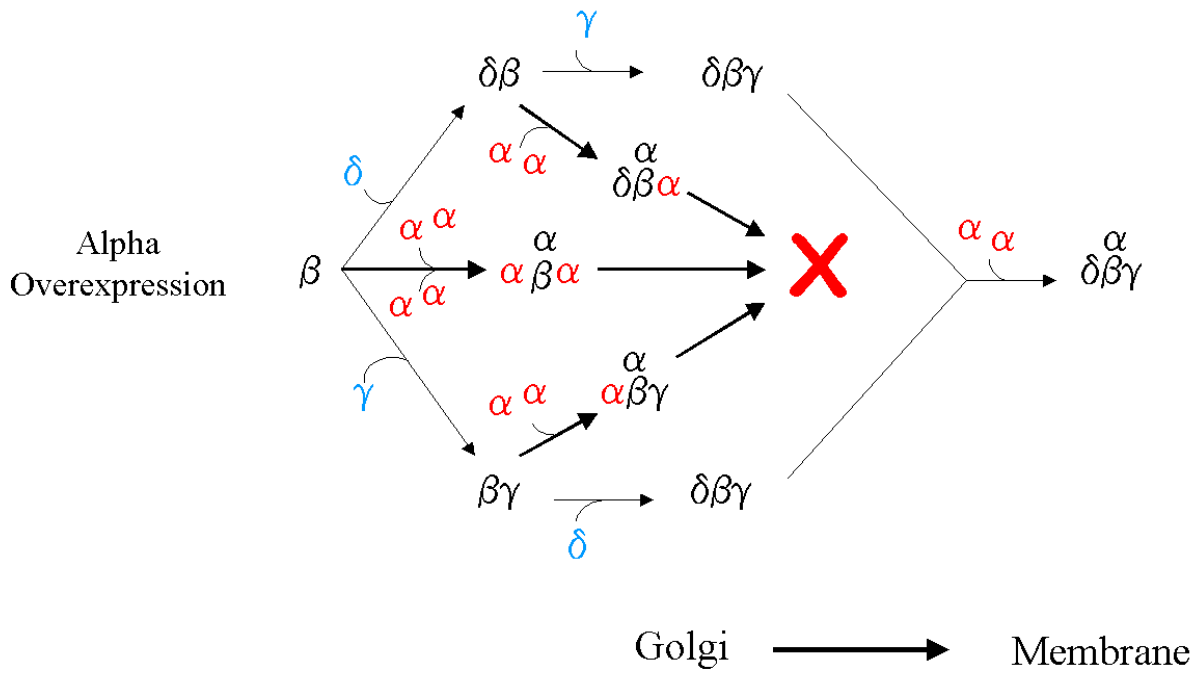


Figure 21. Potential model for over-expression of α-sarcoglycan causing cytotoxicity by improper binding of α-sarcoglycan to β-sarcoglycan.

A potential mechanism for over-expression of α-sarcoglycan causing cytotoxicity is the improper binding of α-sarcoglycan to β-sarcoglycan in the binding positions for either γ- or δ-sarcoglycan. The production of incorrect complex will accumulate over time and will lead to cytotoxicity. It is important to note that due to random association of complex subunits, some correct sarcoglycan complex will be formed, which will account for the initial rescue seen in the α-sarcoglycan deficient mice treated with an AAV α-sarcoglycan. (β = β-sarcoglycan, δ = δ-sarcoglycan, γ = γ-sarcoglycan, and α = α-sarcoglycan)

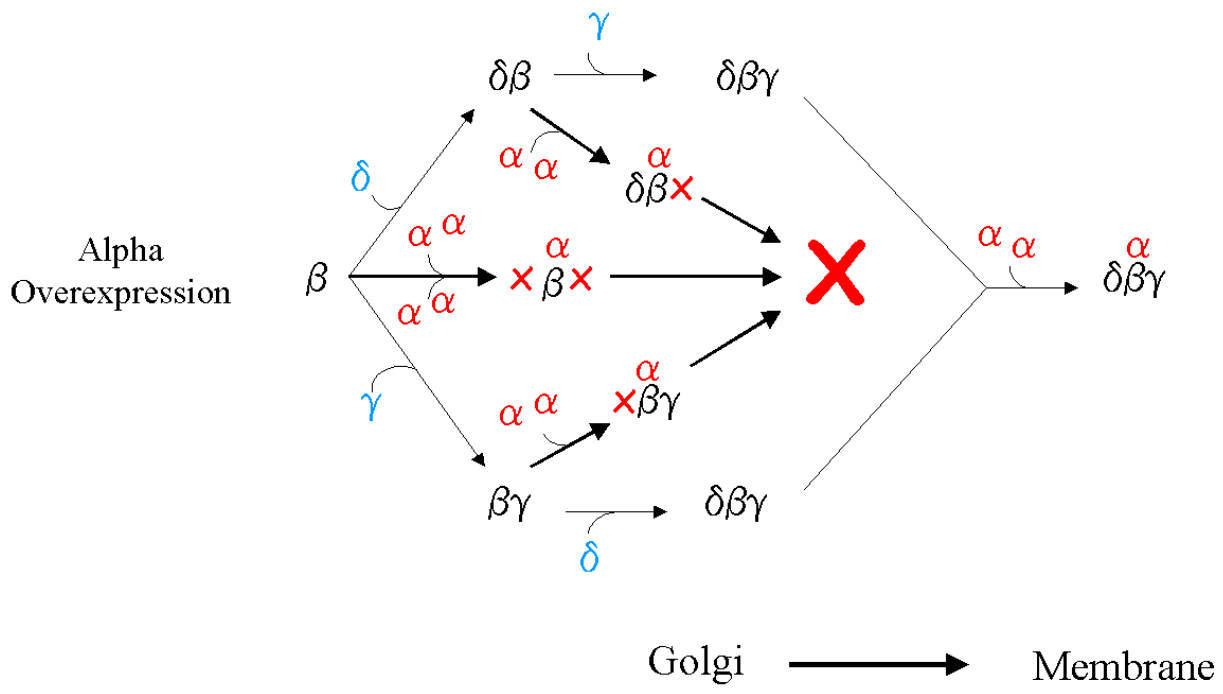


Figure 22. Potential model for over-expression of α-sarcoglycan causing cytotoxicity by incorrect order of assembly of the sarcoglycan complex.

Another potential mechanism for over-expression of α-sarcoglycan causing cytotoxicity is the incorrect order of assembly of the complex with the addition of α-sarcoglycan occurring first or second in the complex formation, which may biochemically change the conformation of β-sarcoglycan and prevent the attachment of γ- or δ-sarcoglycan to complete the complex formation. The production of incorrect complex will accumulate over time and will lead to cytotoxicity. It is important to note that due to random association of complex subunits, some correct sarcoglycan complex will be formed, which will account for the initial rescue seen in the α-sarcoglycan deficient mice treated with an AAV α-sarcoglycan. (β= β-sarcoglycan, δ = δ-sarcoglycan, γ = γ-sarcoglycan, and α = α-sarcoglycan)

4.0

Immunostimulatory properties of dystrophic muscle alter persistence of transgenes delivered by adeno-associated viral vectors.

4.1 Summary

Inherited biochemical defects in patients with muscular dystrophy result in cycles of degeneration and regeneration in muscle. Complementation of the biochemical defects has been the major focus of experimental gene delivery in animal models of dystrophin and sarcoglycan deficiency. We hypothesized that the degeneration/regeneration cycles and immune cell infiltrate in dystrophic muscle would cause an immunostimulatory response, leading to increased immune response to a transgene, whereas histologically normal muscle would not. To test this hypothesis, I delivered both marker and therapeutic genes to dystrophic muscle and to normal muscle using a recombinant adeno-associated virus (AAV). I show that dystrophic muscle does indeed elicit a substantially greater immune response to β-galactosidase, compared to normal muscle. I also show that biochemical rescue of the dystrophic myofibers could mitigate the immunostimulatory properties of dystrophic muscle by co-delivery of a therapeutic transgene (β-sarcoglycan) and the marker transgene (*LacZ*). Our results suggest that full biochemical and histological rescue of dystrophic muscle must be accomplished to avoid strong immune responses against delivered transgenes.

4.2 Introduction

Gene delivery using either adeno-associated viral vectors or adenoviral vectors has shown promise in biochemical rescue in animal model of different types of dystrophies. Adeno-associated virus has garnered the most attention due to the apparent lack of an aggressive immune response against the transgene in muscle, and hence, impressive persistence and high level of expression in AAV transduced myofibers (Duclos *et al.* 1998; Holt *et al.* 1998; Greelish *et al.* 1999; Li *et al.* 1999; Xiao *et al.* 2000; Cordier *et al.* 2000; Durbeej *et al* 2000; Wang *et al.* 2000). We hypothesized that this success of AAV is dependent on the avoidance of losing the transgene from either degeneration of the transduced myofiber or the induction of an immune response to the transgene by the dystrophic muscle. The degeneration/regeneration process in dystrophic muscle has been shown to lead to substantial levels of cellular infiltrate, including high amounts of macrophages, CD4 and CD8 T-cells, with lower amounts of B-cells and natural killer/killer cells (Arahata and Engel 1984; Arahata and Engel 1988). We believe that the degeneration/regeneration process itself may be immunostimulatory. The degeneration of a myofiber will lead to the presence of immune cell infiltrate. The degeneration will also release the cellular contents of the myofiber, including any "foreign" transgene product, into the extracellular space. Because the cellular contents of the myofiber include the foreign transgene product, clearance of it by the immune cells could lead to the immune-dependent clearance of other non-degenerating myofibers that are producing the transgene protein. To date, AAV-mediated marker gene transfer studies resulted in high levels of transgene expression in normal muscle, yet no parallel studies have demonstrated success in dystrophic muscle. This issue is important to resolve, as gene delivery efforts in Duchenne muscular dystrophy (dystrophinopathies) have been recently focused on the construction of dystrophin mini-genes

that can be contained within the size-constrained AAV backbone (Wang *et al.* 2000). These truncated dystrophin proteins are known, from human patients studies, to only partially rescue the biochemical defect (Hoffman *et al.* 1988; Hoffman *et al.* 1989). While biochemically and histologically rescued myofibers using a normal protein delivered by AAV would not be expected to elicit any immune response against the transgene, a myofiber rescued with a truncated semi-functional protein might retain some dystrophic characteristics and the muscle would still be immunostimulatory.

A recent report showed impressive long-term rescue of dystrophin deficient (*mdx*) mice with a dystrophin mini-gene (Wang *et al.* 2000). This report showed that partial biochemical rescue using a highly truncated form of dystrophin had long-term persistence, with no obvious immune response. One reason why gene transfer studies by Wang *et al.* (2000) did not elicit an immune response with the semi-functional dystrophin may be that murine muscle is more "tolerant" of dystrophin abnormalities than higher vertebrates (dog and human). The higher vertebrates show constant degeneration/regeneration, while *mdx* muscle functionally recovers to some extent (Bulfield *et al.* 1984; Sicinski *et al.* 1989; Partridge 1991; Infante and Huszagh 1999; Hoffman 2001). Thus, the same highly truncated dystrophin construct might be less protective against dystrophic features of muscle in humans than in mice and, therefore, could elicit an immune response in humans.

We believe that dystrophic muscle may contain immunostimulatory factors, due to muscle fiber degeneration/regeneration. The resulting immune cell infiltrate, may act to boost the immune response to delivered transgenes if the transgenes do not fully rescue the primary biochemical defect. To test if there are immunostimulatory properties of dystrophic muscle, we used a dystrophic mouse model. Here, we use delivery of marker genes and therapeutic genes by

AAV, both singly and together, in both normal and dystrophic muscle. Important to the interpretation of these results is the finding that co-injected AAV vectors form concatamers, thus co-infecting the same cells (Yang *et al.* 1999; Duan *et al.* 2000; Yan *et al.* 2000). We show that dystrophic muscle does indeed elicit a dramatic immune response against a marker gene protein, and that this response is attenuated by biochemical rescue of the myofibers by a therapeutic transgene.

4.3 Materials and methods

4.3.1 Virus production

Construction of AAV vectors containing *LacZ* was previously described (Li *et al.* 1999). The construction of an AAV containing the human β-sarcoglycan cDNA driven by the CMV promoter was completed as follows. The human β-sarcoglycan cDNA was amplified using the following primer set: forward 5' ACAGTCGGGCGGGGAGCT 3' and reverse 5' CATGTTGGTGACCTCTGGG 3'. AAV was produced using a triple plasmid co-transfection method previously described (Xiao *et al.* 1998). Viral titers were determined by DNA dot-blot analysis and found to be in a range of 2-5 x 10^{12} particles per mL.

4.3.2 Mouse strains and delivery of virus

β-sarcoglycan gene knockout mice were obtained from Dr. Eijiro Ozawa, and were previously described (Araishi *et al.* 1999). Breeding colonies of these animals were established, and mice at 108 days old were used for intramuscular injection of AAV. Normal C57BL/6NHsd mice were obtained from Harlan, and were injected at 90 days old.

Each mouse received a single intramuscular injection into the tibialis anterior (TA) muscle. Those mice receiving only one virus received an injection containing 11 μL of AAV *LacZ* (5 x 10^{10} viral particles) and 11 μL of PBS for a total of 22 μL. Those receiving two viruses received a single injection containing 11 μL of AAV *LacZ* (5 x 10^{10} viral particles) and 11 μL of AAV β-sarcoglycan (5 x 10^{10} viral particles) for a total of 22 μL (1 x 10^{11} viral particles). All mice were sacrificed at 43 days post injection.

4.3.3 β-galactosidase and immunofluorescent analysis

Each tibialis anterior (TA) muscle was carefully dissected from both tendon insertion sites and immediately flash frozen in liquid nitrogen-cooled isopentane. Muscles were stored in

hydrated, airtight containers at –80° prior to sectioning. β-galactosidase staining was done on 8 μm frozen sections, while all immunostaining was done on 4 μm frozen sections.

β-galactosidase staining was performed on fresh sections, which were immediately fixed using 0.5% gluteraldehyde for 10 min. Slides were then rinsed 3 times for 10 minutes with PBS, and stained with the X-gal Staining Set (Roche Molecular Biochemicals) overnight. Finally, slides were rinsed with PBS and mounted with Gel/Mount (Biomeda Corp.). Once the staining was completed, the number of cells producing β-galactosidase in the entire TA muscle (cross-section at belly [center of muscle]) was counted under 10X magnification (Nikon FXA microscope).

For immunostaining, sections were thawed on Superfrost Plus slides (Fisher Scientific Inc.) then immediately incubated with 10% horse serum in PBS for 30 minutes. The sections were incubated with primary antibody at room temperature for an hour. The primary antibody used was a rabbit anti-β-SG antibody previously described (Araishi *et al.* 1999). Sections were washed 3 to 4 times with 10% horse serum in PBS and incubated with a Cy3-conjugated secondary antibody diluted in 10% horse serum and PBS for an hour, and subsequently washed an additional three times. Sections were then mounted with Gel/Mount (Biomeda Corp.) and analyzed using a Nikon FXA microscope.

4.3.4 ELISA assay

ELISA assays were done by first adding 50 μL of purified β-galactosidase from *E. coli* (Sigma) at 0.4mg/mL in Carbonate/Bicarbonate buffer pH 9. 6 (Pierce Chemical Company), to each well of a 96 well assay plate for 2 hours at room temp. Plates were washed, and 200 μL of Superblocker PBS (Pierce Chemical Company) was added to each well and incubated for 30 minutes at room temp. Each well was then rinsed with 3 x 100 μL of wash buffer (0.05% of

Surfact-Amps Tween 20 (Pierce Chemical Company) diluted in Superblocker PBS). Each ELISA plate was freshly made for each experiment.

Assays for antibodies to β-galactosidase in mouse serum were done by first collecting serum from whole blood collected at 43 days post-injection from AAV injected mice. Serum was first diluted 1:1 with PBS and 0.1% BSA and added to the first well of the ELISA plate. Those samples that were initially determined to have a higher antibody titer serum were diluted at 1:20 for each of the 8 remaining wells, while those with lower antibody titer serum were diluted at 1:5 over a total of 8 wells. Diluted serum was incubated on the plates for 1 hour at room temperature, then washed three times. Diluted secondary antibody (anti-mouse HRP 1:6000) was added for 1 hour, and washed. 100 μl of TMB substrate solution (Pierce Chemical Company) was added to each well and incubated for approximately 20 minutes. The reaction was stopped by adding 100 μl of 2 M H_2SO_4 to each well. Absorbance was read using a Thermo Max plate reader (Molecular Devices Corp.) at 450 nm, and the data was analyzed using Soft Max Pro version 2.6.1 (Molecular Devices Corp.).

4.3.5 Statistical analysis

Data was determined to be parametric; therefore all statistical comparisons were performed using Student's t-tests. Statistical analysis of the ELISA data was performed at an absorbance reading of 1, where values for each sample were determined by interpolation from the absorbance curve (Figures 25, 26). In this data, one data point was omitted from the analysis after further examination (i.e. linear regression of absorbance in response to serum dilution and subsequent calculation of Cook's distance) defined it as a statistical outlier.

4.4 Results

4.4.1 AAV gene transfer of *LacZ*, or *LacZ* and β-sarcoglycan to both normal and β-sarcoglycan deficient mice

Recombinant AAV containing either the *LacZ* gene (Goodman *et al.* 1994), or the cDNA for β-sarcoglycan (Dressman *et al.* submitted) was produced by a triple plasmid transfection method (Xiao *et al.* 1998). Both transgenes were driven by a CMV promoter and were flanked by AAV ITRs. Each construct was verified by DNA sequencing.

Normal mice and β-sarcoglycan deficient mice received either an injection of AAV *LacZ* alone or a combination of AAV *LacZ* and AAV β-sarcoglycan**.** Five β-sarcoglycan deficient mice and 3 normal mice received a single intramuscular injection into the TA containing an equal mix of both AAV *LacZ* and PBS. Five β-sarcoglycan deficient mice and 4 normal mice received a single intramuscular injection into the TA containing an equal mix of AAV *LacZ* and AAV β-sarcoglycan.

Mice were sacrificed 43 days post-injection, and each TA muscle was analyzed for β-galactosidase positive myofibers. A comparison between the normal and β-sarcoglycan deficient mice receiving the AAV *LacZ* virus alone showed a statistically significant difference ($p<0.05$) between the number of β-galactosidase producing myofibers, with the β-sarcoglycan deficient mice having many fewer β-galactosidase positive myofibers. The mean number of β-galactosidase positive myofibers in the β-sarcoglycan deficient mice was 14 ± 24 (SEM), while the normal mice had a mean of 282 ± 157 myofibers (Figure 23).

The comparison between normal mice and β-sarcoglycan deficient mice that received the mixture of both AAV *LacZ* and AAV β-sarcoglycan showed no statistically significant difference in the number of β-galactosidase positive myofibers. The mean number of β-

galactosidase producing cells in the β-sarcoglycan deficient mice receiving both viruses was 167 ± 202 myofibers, and the mean number of myofibers for the normal mice receiving both viruses was 63.5 ± 42.8 (Figure 23).

To confirm that there was co-transduction of myofibers by the two viruses, β-sarcoglycan deficient muscle that received both viruses was sectioned and double-stained for both β-galactosidase and for β-sarcoglycan production. Analysis of this muscle confirmed that the majority of myofibers were positive for both β-galactosidase and β-sarcoglycan (Figure 24).

4.4.2 ELISA analysis of serum antibody titers against β-galactosidase

To assay antibody production against β-galactosidase, serum from each injected mouse was analyzed by ELISA. Serum dilutions were plotted in a log-linear dilution curve for each of the β-sarcoglycan deficient animals (Figure 25) and without outliers (Figure 26). All normal animals injected with either AAV *LacZ* virus alone or both AAV *LacZ* and AAV β-sarcoglycan showed no detectable serum antibodies to β-galactosidase (Figure 25 and data not shown).

β-sarcoglycan deficient mice showed considerably more serum antibodies to β-galactosidase (Figure 25), with the highest titers in animals receiving the AAV *LacZ* alone. Co-transduction of AAV *LacZ* and AAV β-sarcoglycan showed attenuation of the immune response against β-galactosidase (Figures 23, 26), suggesting that biochemical rescue of myofibers from the dystrophic phenotype could, in part, mitigate the immunostimulatory effect of dystrophic muscle.

To determine the antibody titer differences between β-sarcoglycan deficient animals receiving AAV *LacZ* virus alone or both AAV *LacZ* and AAV β-sarcoglycan, the dilution value of animal's serum was interpolated at an absorbance value of 1. When these interpolated dilution values, minus one outlier as described below, were compared, the mean dilution value of

the β-sarcoglycan deficient animals receiving AAV *LacZ* virus only was significantly higher (p=0.010) than the mean dilution value of the group receiving both AAV *LacZ* and AAV β-sarcoglycan (Figure 26).

One β-sarcoglycan deficient animal that received both AAV *LacZ* and AAV β-sarcoglycan showed a noticeably higher serum dilution value at an absorbance of 1 than the other 4 mice receiving both viruses. This value was further examined and determined to be a statistical outlier. A simple linear regression was performed with serum dilution as the dependent variable and injection group as the categorical independent variable. From this regression line, a Cook's distance, which measures the influence of an observation on the regression equation, was calculated for each observation. Observations with a Cook's distance greater than 4/n can be considered suspect and unduly influencing the equation estimation. (Kleinbaum *et al.* 1998; StataCorp. 2001). One data point was found to have an increased Cook's distance, and therefore was omitted from the analysis. Comparison between the β-sarcoglycan deficient mice receiving both AAV *LacZ* and AAV β-sarcoglycan and mice receiving AAV *LacZ* alone using all animals exhibited only partial significance (p=0.0545), with mice receiving only one virus having a higher mean serum dilution than those receiving two viruses. This change in significance level from 0.0545 to 0.010 by removing one data point further shows that point's undue influence on the statistics.

4.5 Discussion

Experimental gene delivery research has generally focused on either marker gene studies in normal tissues, or delivery of therapeutic genes to abnormal tissue. Given the pathological nature of many diseased tissues, such as dystrophic muscle, it is likely that the abnormal state of the muscle could lead to an exacerbation of immune responses to transgenes. True biochemical and histological rescue of cells by a therapeutic transgene might mitigate the potential immunostimulatory effect of dystrophic muscle. We felt that it was important to begin to address this possibility, given that increasing attention is being placed on the use of modified therapeutic genes in muscular dystrophy research. One example is the use of highly truncated dystrophin proteins, which are deliverable by the size-limited AAV vectors and are able to retain some of the biological functions needed to rescue muscle histologically and functionally. It is possible that these semi-functional therapeutic proteins will not completely rescue the dystrophic phenotype, and thus have potential for eliciting an increased immune response against the transgene.

To test this, I carried out a series of experiments *in vivo*, using both a marker gene delivered by AAV (*LacZ*), and a therapeutic gene (β-sarcoglycan) to both normal mouse muscle, and dystrophic mouse muscle (β-sarcoglycan deficient). I found that normal mice did not elicit detectable immune responses against the marker transgene, despite the high expression level in muscle, the potential high antigenic nature of this bacterial-derived protein, and co-injection and expression of human β-sarcoglycan vector. This data is consistent with previously published reports showing high levels of expression of novel transgenes in normal mouse and rat muscle, with no detectable immune response, and consequent long-term persistence of the transduced myofibers (Xiao *et al.* 1996; Snyder *et al.* 1997; Li *et al.* 1999).

In contrast, parallel studies of dystrophic muscle (β-sarcoglycan deficient) showed an aggressive immune response against the marker gene (Figures 25, 26), as evidenced by high serum titers to purified β-galactosidase. This data shows that the milieu of the dystrophic muscle, due to infiltration of macrophages and T cells, or due to increased antigen presentation of the biochemically abnormal myofibers, or both, is conducive to development of immune responses against the transgene. In this respect, the dystrophic muscle appears to have an immunostimulatory effect.

We and others have shown that biochemical rescue of dystrophic muscle leads to long-term persistence of therapeutic transgenes delivered by AAV, with little or no evidence of inflammatory cell infiltrate (Duclos *et al.* 1998; Holt *et al.* 1998; Greelish *et al.* 1999; Li *et al.* 1999; Xiao *et al.* 2000; Cordier *et al.* 2000; Durbeej *et al* 2000; Wang *et al.* 2000; Dressman *et al.* submitted). This suggests that dystrophic muscle that has been biochemically and histologically rescued may no longer have immunostimulatory properties. To test this hypothesis, I co-injected both marker and therapeutic AAV into dystrophic muscle, with the goal of showing that the co-delivery of the two genes would lead to biochemical rescue of the co-transduced fibers, and mitigation of the immunostimulatory effect of dystrophic muscle. Consistent with our model, co-injection of the two vectors statistically significantly lowered the serum antibodies against β-galactosidase, even though twice as much AAV was injected into these animals. Importantly, the biochemical rescue did not completely ablate the immune response to the marker gene, but only attenuated it.

Interpreting these findings together with previous data, the bacterial β-galactosidase marker protein is probably a particularly stringent test of the potential immunostimulatory effect of dystrophic muscle. Delivery to dystrophic muscle of therapeutic genes, even those that are

from human into murine muscle, are likely less antigenic, and therefore lead to less severe immune response, and greater persistence than what we observed here (Li *et al.* 1999; Cordier *et al.* 2000; Xiao *et al.* 2000). However, a corollary to our findings is that *partial* biochemical rescue of dystrophic muscle using highly truncated *semi-functional* proteins is likely to lead to a more aggressive immune response than would *normal* therapeutic proteins. Human muscle is recognized to have a more stringent demand for the dystrophin protein when compared with murine muscle. This is seen with Duchenne muscular dystrophy patients have a more severe phenotype and rapid disease progression when compared to the *mdx* mouse model's disease progression (Bulfield *et al.* 1984; Sicinski *et al.* 1989; Partridge 1991; Infante and Huszagh 1999; Hoffman 2001). We anticipate that delivery of partially functional dystrophin proteins to patient muscle may require more attention to immune modulatory influences on transgene persistence, and subsequent gene delivery success.

Figure 23. AAV reporter construct shows greater transduction in normal versus dystrophic muscle, but the difference is mitigated by co-transduction with a therapeutic gene.

Shown is graphical representation of a comparison (at 43 days post-injection) of β-galactosidase positive myofibers in the tibialis anterior of normal and β-sarcoglycan deficient animals injected with either an AAV containing *LacZ* alone or co-injected with an AAV containing *LacZ* and an AAV containing β-sarcoglycan. Injection of AAV *LacZ* alone shows poor transduction/persistence in dystrophic muscle (Beta SG K/O AAV *LacZ*) compared to normal muscle (Normal AAV *LacZ*). This difference is partially ablated by co-transduction of AAV *LacZ* and an AAV expressing a therapeutic gene, β-sarcoglycan (Beta SG K/O AAV *LacZ* and Beta SG). This suggests that partial rescue of the biochemical defect mitigates the immunostimulatory properties of dystrophic muscle.

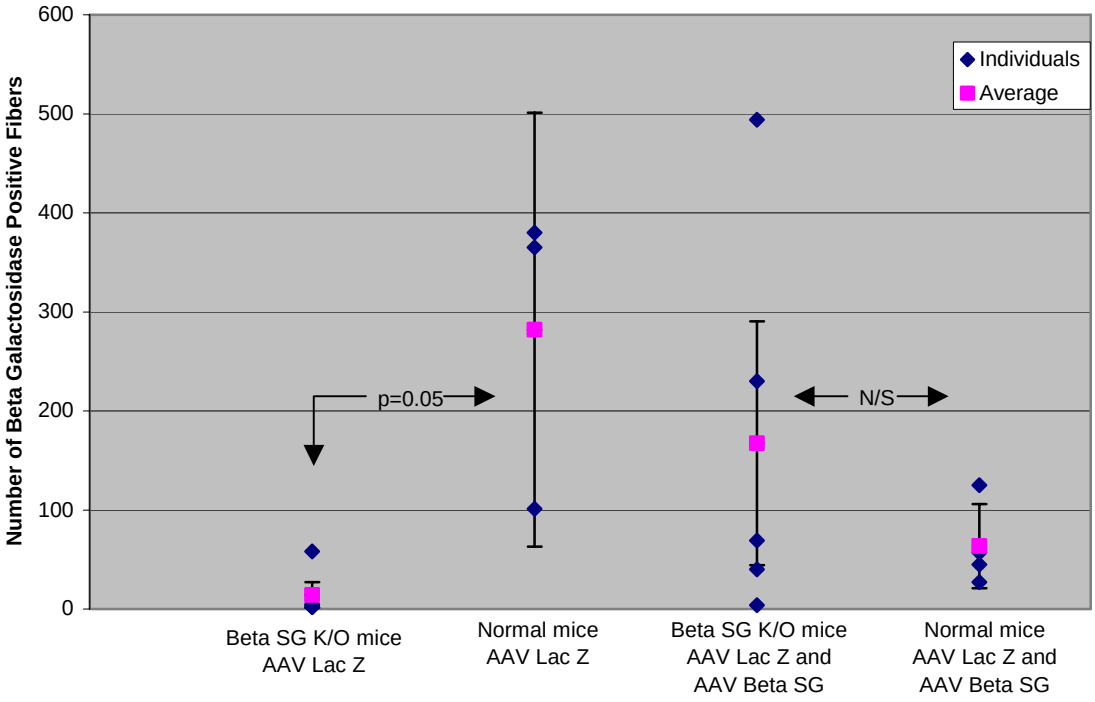
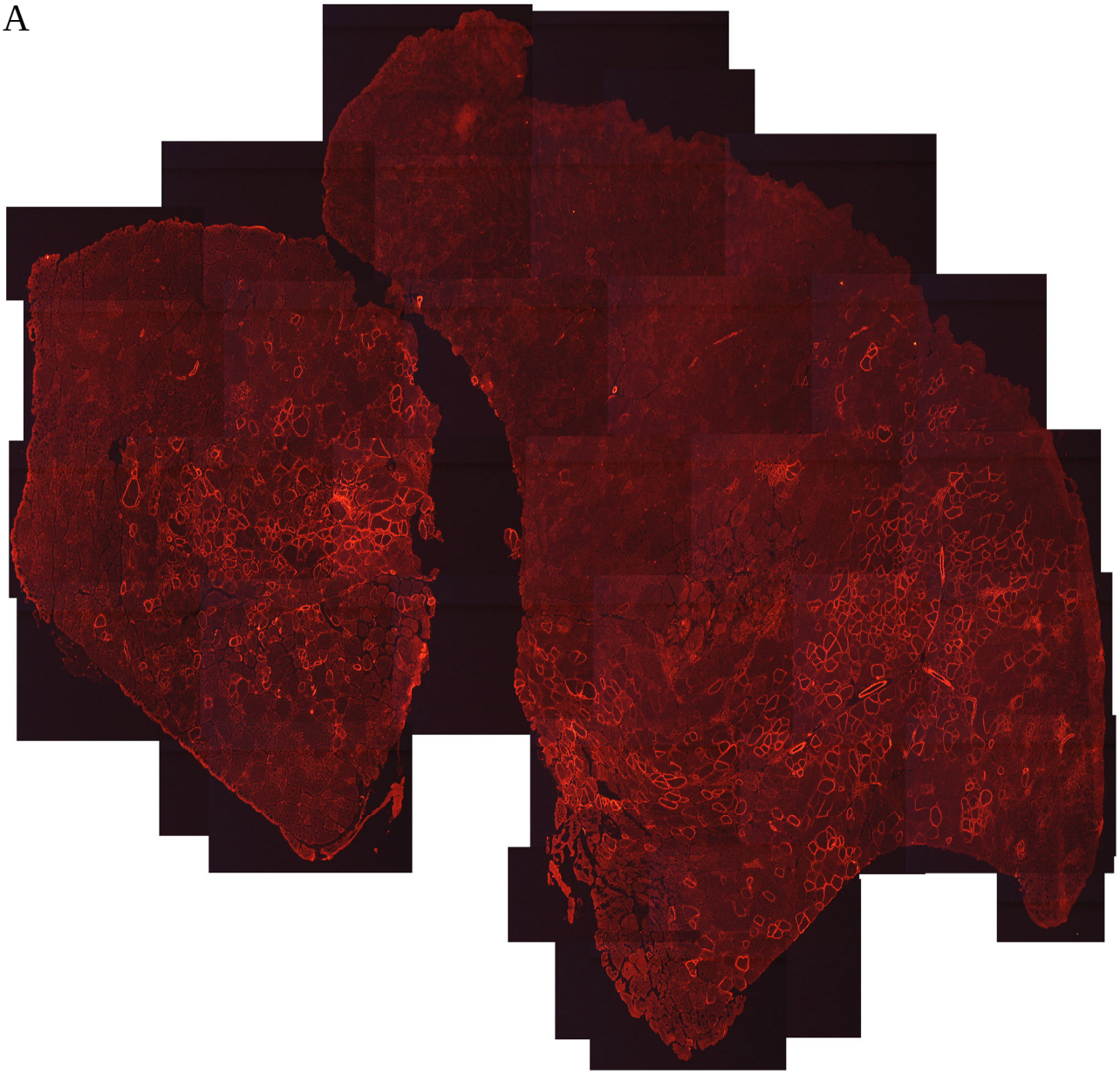

Number of Beta Galactosidase Positive Fibers
600
500
400
300
200
100
0
Individuals
Average
p=0.05
N/S
Beta SG K/O mice AAV Lac Z
Normal mice AAV Lac Z
Beta SG K/O mice AAV Lac Z and AAV Beta SG
Normal mice AAV Lac Z and AAV Beta SG

Figure 24. β-sarcoglycan deficient animals that receive an injection with two viruses, express both viral transgenes in the same cells.

Shown are photomontages of the entire TA muscle of a mouse co-injected with AAV *LacZ* and AAV β-sarcoglycan. Panel A shows the TA stained with anti-α-sarcoglycan antibody to indicate which cells are sarcoglycan positive. Panel B shows the TA stained with β-galactosidase. The pattern of staining of myofibers that stain positive for the sarcoglycan (panel A) overlaps with those that are β-galactosidase positive (panel B). This suggests that myofibers that were co-injected with an AAV *LacZ* and AAV β-sarcoglycan, which are co-expressing the *LacZ* transgene and the β-sarcoglycan transgene, are protected from the dystrophic phenotype of the β-sarcoglycan deficient muscle.

A



B

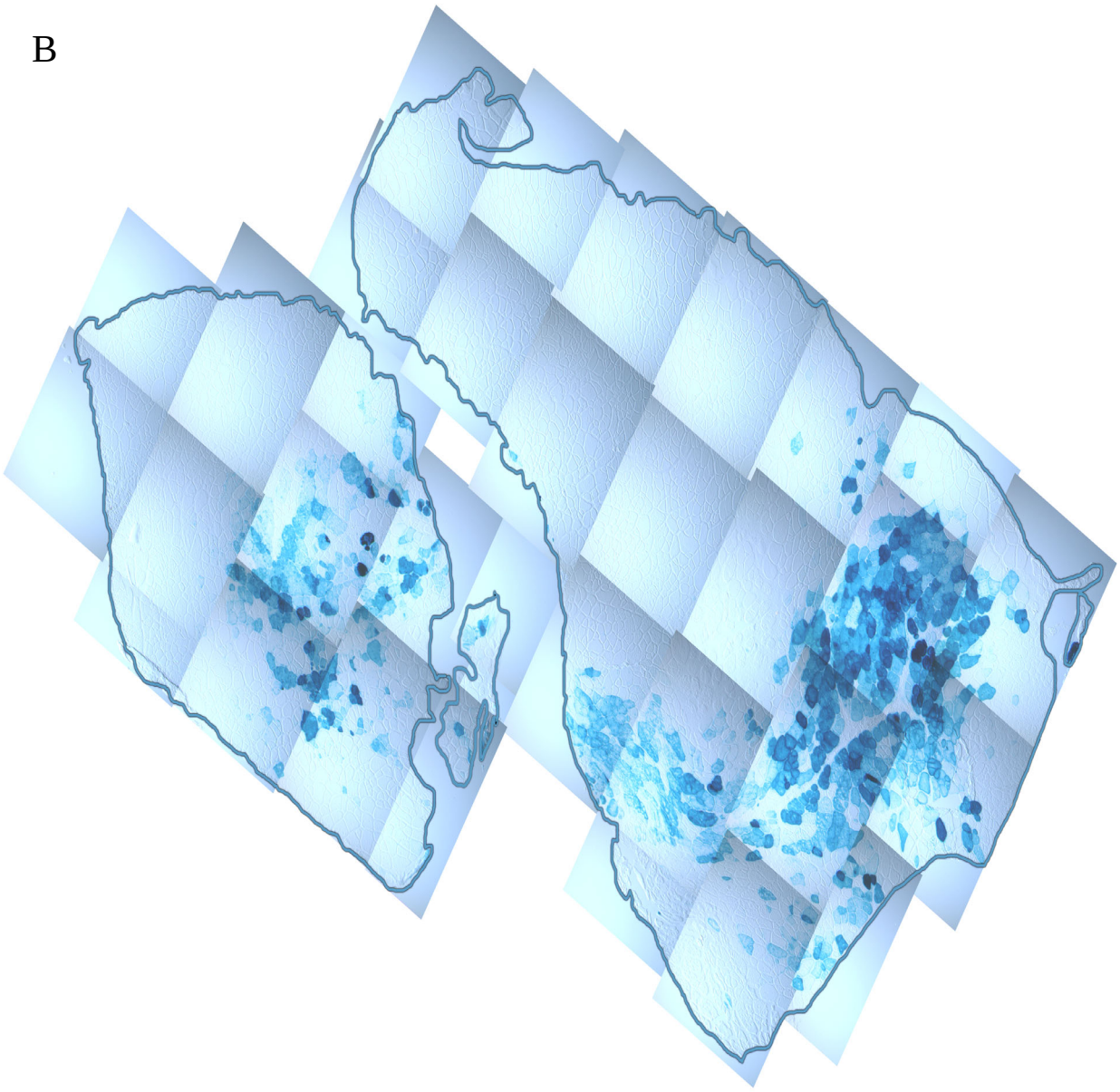
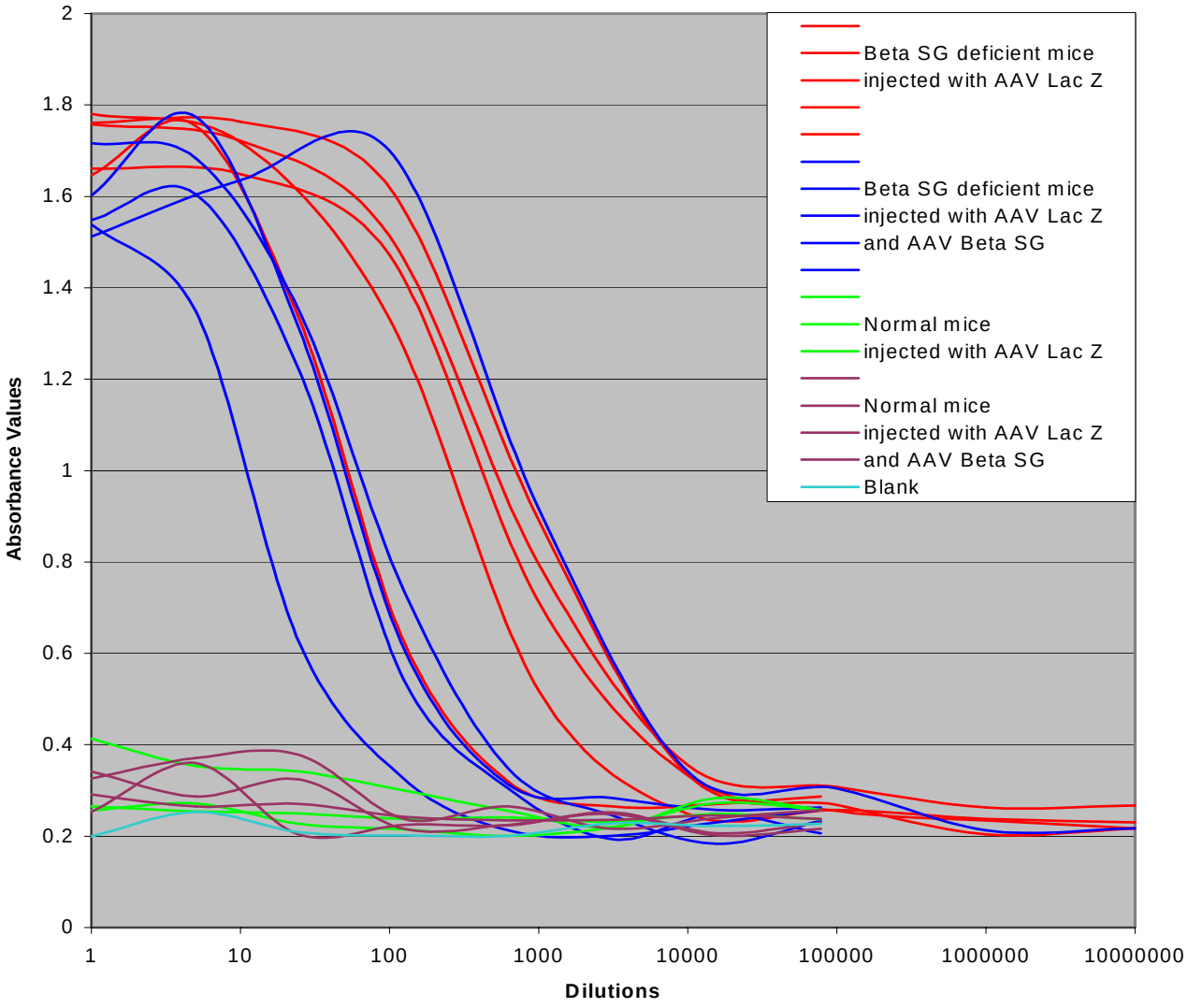


Figure 25. Log-linear plot of serum antibody levels to β-galactosidase in all injected animals shows that β-sarcoglycan deficient mice elicit an immune response to the marker transgene, while normal mice elicit little to no immune response to the transgene.

Shown is a log-linear plot of serum antibody titers in all animals injected with either AAV-*LacZ* alone or co-injected with both an AAV-*LacZ* and an AAV-β-sarcoglycan. The plots are based on absorbance readings from the ELISA plates on diluted sera from mice. All of the β-sarcoglycan deficient mice elicit an immune response, while the normal mice sera exhibit similar patterns to the plate background readings.


Absorbance Values
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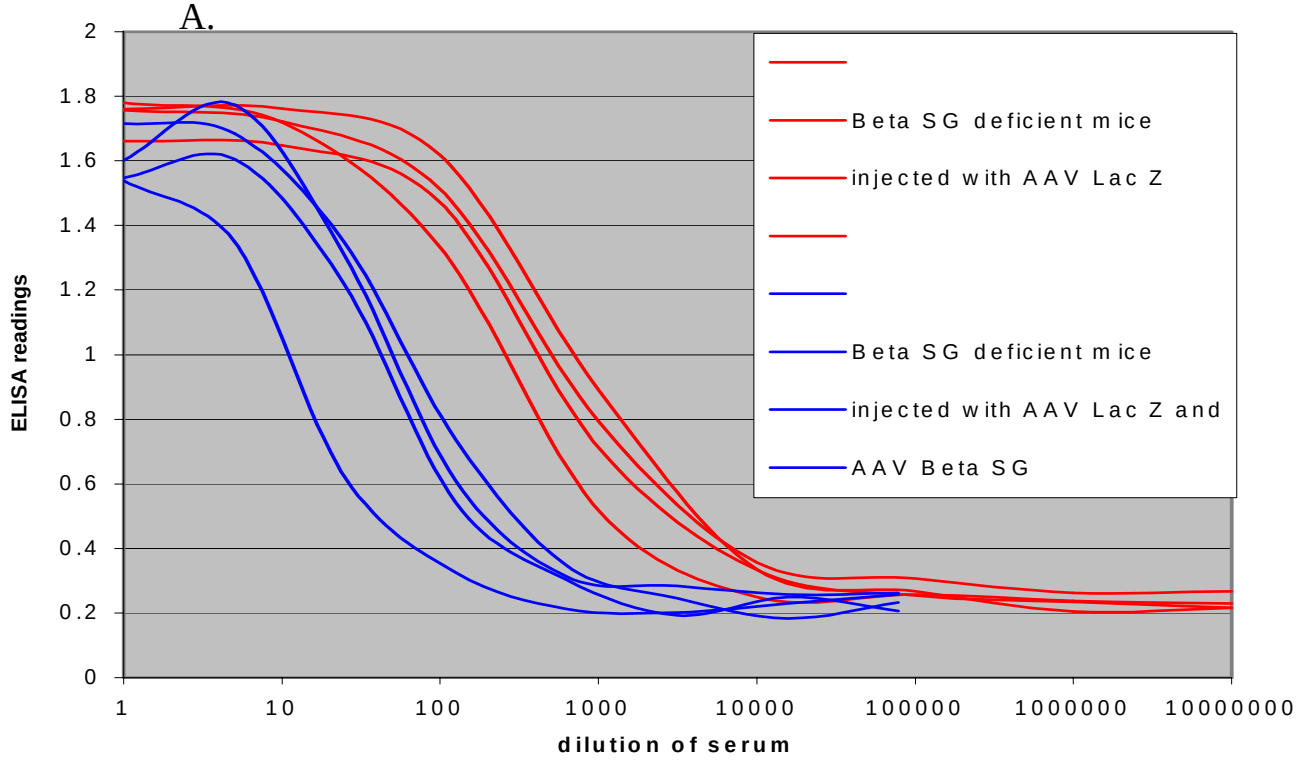
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
10000
100000
1000000
10000000
Dilutions
Beta SG deficient mice
injected with AAV Lac Z
Beta SG deficient mice
injected with AAV Lac Z
and AAV Beta SG
Normal mice
injected with AAV Lac Z
Normal mice
injected with AAV Lac Z
and AAV Beta SG
Blank

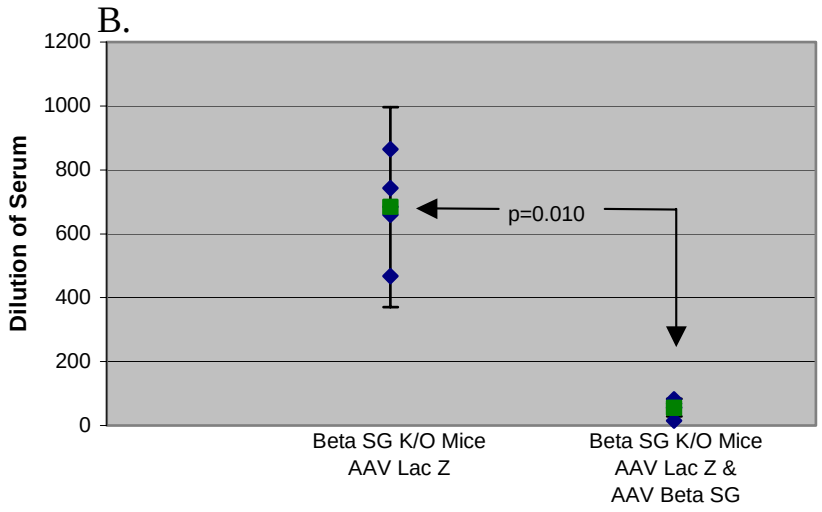
Figure 26. Log-linear plots of only the β-sarcoglycan deficient mice indicate there is quenching effect of antibody production to the *LacZ* transgene when the dystrophic muscle phenotype is corrected.

Panel A shows a log-linear plot of serum antibody titers in β-sarcoglycan mice injected with either AAV-*LacZ* alone or co-injected with both an AAV-*LacZ* and an AAV-β-sarcoglycan, excluding the outlier. Panel B shows the dilution values interpolated at an absorbance value of 1, which shows that the animals, which receive both AAV-β-sarcoglycan and AAV-*LacZ*, have a significantly lower serum dilution to have the same absorbance reading. This indicates that correction of the dystrophic phenotype is accompanied by a significant decrease in antibody production to the *LacZ* transgene.

A.


ELISA readings
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
1
10
100
1000
10000
100000
1000000
10000000
dilution of serum
Beta SG deficient mice
injected with AAV Lac Z
Beta SG deficient mice
injected with AAV Lac Z and
AAV Beta SG

B.


Dilution of Serum
1200
1000
800
600
400
200
0
p=0.010
Beta SG K/O Mice
AAV Lac Z
Beta SG K/O Mice
AAV Lac Z &
AAV Beta SG

5.0

Utilization of a 10-needle injection manifold for delivery to large muscle groups for the treatment of muscular dystrophies.

5.1 Summary

Recombinant adeno-associated virus (AAV) vector shows the most promise of any gene therapy vector for gene delivery to diseased muscle. There have been a number of successful reports demonstrating use of AAV to deliver genes to rodent models of muscular dystrophy (Holt *et al.* 1998; Greelish *et al.* 1999; Li *et al.* 1999; Wang *et al.* 2000; Xiao *et al.* 2000; Cordier *et al.* 2001). However, there are some major hurdles that need to be overcome in order for gene delivery to show clinical efficacy in human trials. One major hurdle is the limited packaging capacity of AAV (~5 kb), which is too small to package the gene causing the most common form of muscular dystrophy, Duchenne muscular dystrophy. However, recent reports have shown biochemical efficacy of a highly truncated "mini" dystrophin gene containing only ~30% of the normal coding sequence (Wang *et al.* 2000). A second hurdle is overcoming the problem of widespread delivery of AAV to muscle tissue, which accounts for ~30% of the body mass. A recent promising report has described the use of Extra Corporeal Membrane Oxygenation (ECMO) system to isolate an entire limb of a rat and a hamster and perfuse it with histamine and the AAV vector (Greelish *et al.* 1999). Whether this highly invasive protocol can be employed in higher vertebrates or humans remains to be seen. In order to address the problem of large scale delivery in a controlled manner, we designed a multi-needle injection manifold to consistently deliver virus to a 2mm x 1cm area of muscle. Using this injection manifold we have successfully delivered AAV to the quadriceps, the largest muscle group, in a hamster model of muscular dystrophy.

5.2 Introduction

The muscular dystrophies are a heterogeneous group of diseases which have traditionally been subdivided into various types based on the clinical distribution of muscle involvement, severity of muscle weakness, age of onset, or inheritance pattern. The most common form is Duchenne muscular dystrophy (DMD), which is caused by a mutation in the dystrophin gene (Hoffman *et al.* 1987). DMD affects approximately 1 in 3500 males in all populations studied to date, making it one of the most common genetically inherited diseases worldwide. The limb-girdle muscular dystrophies (LGMD), on the other hand, are a subset of the muscular dystrophies with much lower prevalence (1 in 100,000) (Emery 1991). A molecular classification of the limb-girdle muscular dystrophies is now possible with the identification of the causative genes for most types.

Among the autosomal recessive limb-girdle muscular dystrophies, the sarcoglycanopathies are some of the best characterized at the molecular level. Mutations in the sarcoglycan genes α-, β-, γ-, and δ- are responsible for LGMD 2D, 2E, 2C, and 2F, respectively (Hoffman 1999). Clinically, patients with primary sarcoglycanopathies are indistinguishable from those with primary dystrophin abnormalities.

Much of the research on the sarcoglycanopathies has been possible with the discovery that the Bio 14.6 hamster is a spontaneous loss-of-function animal model for δ-sarcoglycanopathy (LGMD2F) (Nigro *et al.* 1997). This model has served as an important test system in a series of genetic, biochemical, histological and functional studies that have proven the utility of recombinant AAV as a gene delivery vector for muscle (Holt *et al.* 1998; Greelish *et al.* 1999; Li *et al.* 1999; Xiao *et al.* 2000). Animal models for the remaining sarcoglycanopathies have been developed as well (Duclos *et al.* 1998; Hack *et al.* 1998; Araishi

et al. 1999; Coral-Vazquez *et al.* 1999; Liu and Engvall 1999; Durbeej *et al.* 2000), and have been used in gene therapy research using either adenovirus or adeno-associated virus (AAV) (Duclos *et al.* 1998; Allamand *et al.* 2000; Durbeej *et al.* 2000). Despite recent advances, major obstacles stand in the way of using of viral vectors as effective modalities in the treatment of muscular dystrophies. Muscles comprise approximately 30% of the body mass in humans, and introducing an AAV transgene to a number of different muscle groups presents a daunting challenge. Intramuscular injection, for instance, will require multiple injections. Additionally, many muscle groups are inaccessible to injection percutaneously, e.g. the diaphragm. Given that muscle is a highly vascularized tissue, systemic delivery using the vascular route would appear to be a good means of delivery. Unfortunately, reports indicate that AAV vectors appear to have substantial liver-tropism *in vivo*, with most intravenously injected AAV vectors accumulating in liver with almost no expression in muscle (Ponnazhagan *et al.* 1997; Koeberl *et al.* 1999). Alternatively, access to the arterial side, bypassing the liver, might be a more appropriate approach. A recently published report claims that administration of AAV vector through the arterial supply of an isolated limb via an Extra-Corporeal Membrane Oxygneation (ECMO) circuit resulted in widespread expression of the transgene in hamsters (Greelish *et al.* 1999). Unfortunately, the exact nature of the procedure and its consequences has not been adequately studied, including the potential risks of using histamine to induce endothelial permeabilization.

We have developed a multi-needle injection manifold that we envision as a safer alternative for large-scale delivery of vectors to large areas of muscle. Here, we report the successful delivery of a complementary gene construct, an AAV-δ-sarcoglycan vector, to the Bio14.6 hamster (δ-sarcoglycan deficient) quadriceps muscles.

5.3 Materials and methods

5.3.1 AAV vector production

An AAV vector was produced containing the human cDNA for δ-sarcoglycan under control of a CMV promoter, as we have previously described (Li *et al.* 1999). The human δ-sarcoglycan cDNA was amplified using the following primers: δ-SG-For (5' TCCTTCAGAGCTGCTGCTCAGCACGCCC 3') and δ-SG-Rev (5' CCCGTTTGTTCATTGCCCATCAGGCC 3'). The AAV was produced using a triple plasmid co-transfection method previously described (Xiao *et al.* 1998). Viral titers were determined by DNA dot-blot analysis and found to be in the range of 2-5 x 10^{12} particles per mL.

5.3.2 Viral delivery to muscle

Bio 14.6 hamsters were purchased from Bio Breeders (Fitchburgh, MA). Hamsters were anesthetized with an intraperitoneal administration of 2.5% Avertin (Aldrich, Milwaukee, WI) at a dose of ~20mL/kg body weight, prior to viral vector injection. Each hamster received a 100 μL intramuscular injection of AAV δ-sarcoglycan utilizing the injection manifold (10μl/needle). Each quadriceps was exposed by an incision in the leg of each animal prior to injection of AAV.

5.3.3 10-needle injection manifold

Hamilton (Reno, NV) constructed the custom 10-needle injection manifold. The MicroLab 501A dispenser is connected to ten 50 μL Hamilton glass syringes that each have a 12 inch 30 gauge needle with a 30° bevel. The needles are aligned using an aluminum needle holder such that all needles are 2 mm apart from one another in a 2 x 5 pattern covering a 2mm x 1 cm area (Figure 27).

5.3.4 Western blot analysis

Injected hamster quadriceps were dissected at 86 days post-injection, halved, then serially sectioned as follows. One half of each quadricep was sectioned every 300 μm for a total of ~18 regions covering a total of 5.4 mm. Each of these regions of muscle sections were solubilized in 100 μL of muscle solubilization buffer (10% SDS, 0.1 M Tris pH 8, 10 mM DTT) and boiled for 2 min. 25 μL of this solution was then loaded onto a 10% SDS PAGE gel. The gel was processed for western blotting as previously described. The post-transfer gel was stained with Coomasie Blue to visualize the myosin heavy chain protein, which was used as a control for the amount of protein loaded and as an indicator for efficiency of transfer. δ-sarcoglycan specific antibodies from Novocastra Laboratories (Newcastle, UK) were used for staining.

5.4 Results

In our preliminary study (Li *et al.* 1999), we empirically observed the distance of AAV dissemination from a single needle injection point to be approximately 1 mm. We therefore designed an injection manifold with the spacing of the needles at 2 mm apart. The 10 needles cover a 2mm x 1 cm area, which is able to completely cover the largest muscle group of the hamster, the quadriceps. The utilization of the MicroLab 501A from Hamilton Industries (Reno, NV) allows for specific control of injection speed and the volume of the vector being delivered in each needle (Figure 27). Additionally, this injection manifold can be controlled with a foot pedal device allowing for more flexibility with the use of both hands during the procedure.

Using this device, we injected 5 Bio 14.6 hamsters in one quadriceps with a AAV-δ-sarcoglycan vector at 10 μl/injection site (100 μl total; 2-5 x 10^{11} viral particles). At 86 days post injection, the animals were sacrificed, and their quadriceps dissected and flash-frozen. After serial sectioning the samples and immunostaining for δ-SG, we found consistent homogeneous staining of the entire quadriceps (Figure 28). These results were confirmed by western blot analysis, which showed equal amounts of δ-sarcoglycan throughout the entire quadriceps of the Bio 14.6 hamster at approximately 3 times more than normal (Figure 29).

5.5 Discussion

Our results indicate that consistent, homogeneous, and long-term expression of a transgene vector over a wide area of muscle can be achieved using an evenly spaced multi-needle injection manifold device. The device, the design of which is detailed in this report, has many advantages. First, the device provides a reproducible, accurate, and consistent method to deliver transgene vectors to wide areas of diseased muscle. Second, the device utilizes removable, metal needles that can be autoclaved, preventing any cross contamination of the needles between experiments and/or animals. If this device were to be utilized in human trials in the future, a larger needle platform and disposable needles would need to be used. Third, the speed and amount of vector delivery can be precisely controlled. Fourth, this device can be operated by a foot petal, freeing up both hands for ease of operation. Lastly, this method should be considerably safer than the previously described ECMO delivery, and has yielded highly promising results.

Some of the potential problems that can be encountered when using this manifold are the following. First, while the manifold does correct for two-dimensional error by user, i.e. where the needles are placed in the muscle, it does not correct for the third-dimension of the muscle, i.e. how deep each injection is into the muscle. Second, while theoretically there is no loss of vector during delivery, in reality there is always the potential that some of the vector will be lost by not being fully evacuated from the needle or the syringe.

We envision the design concept of this device leading to the development of an increased number of needles for injection of human patients in a staggered injection schedule. Immunosuppressive agents could also be used so that readministration of more viral vectors could occur at a later date. We would target those muscles from which the patient can derive the

most benefit. In the case of the limb-girdle muscular dystrophies, which present initially with proximal muscle weakness, we would target the limb muscles, allowing the patient to ambulate longer.

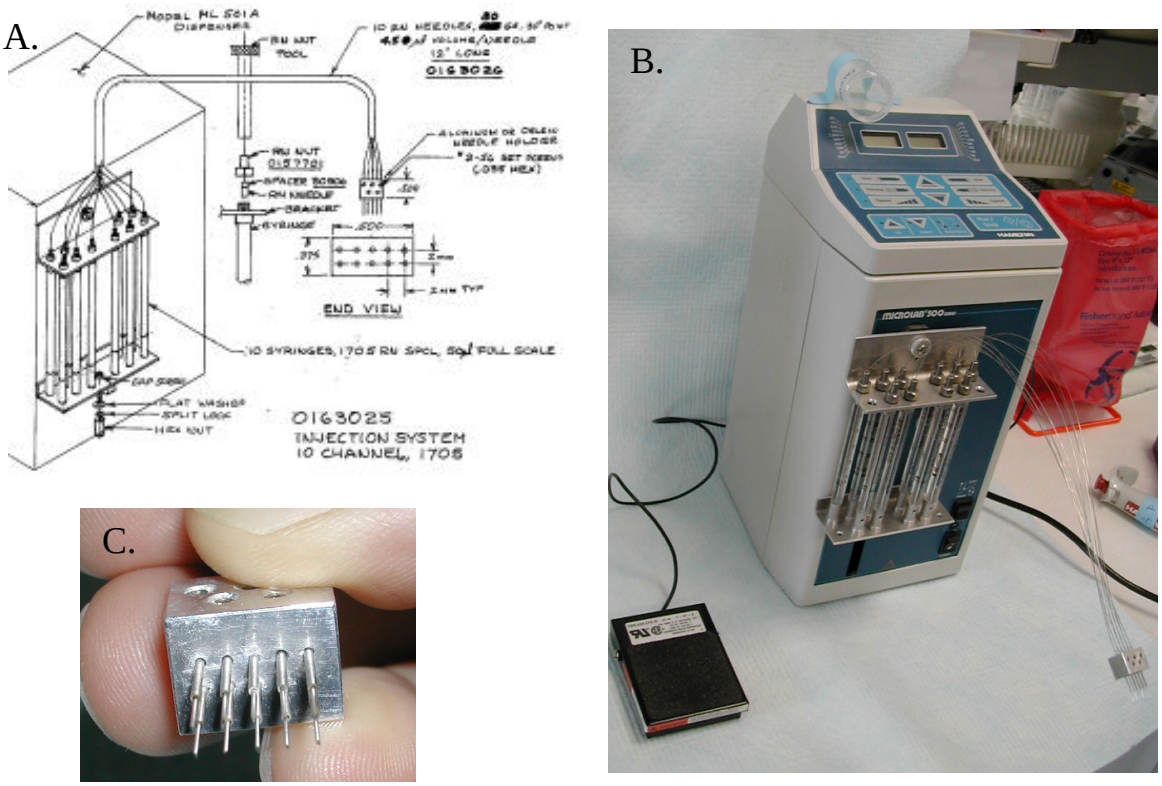


Figure 27. Schematic and photograph of 10-needle injection manifold.

Panel A shows a Schematic drawing of the 10-needle injection manifold. Panel B is a picture of the actual 10-needle injection manifold, with foot pedal. Panel C shows a close up photograph of the needle holder.

Figure 28. Widespread expression of δ-sarcoglycan via intramuscular injection using the 10-needle injection manifold delivering an AAV containing δ-sarcoglycan.

α-sarcoglycan staining of the Bio 14.6 hamster quadriceps after intramuscular injection using the 10-needle injection manifold indicates that there is widespread expression of the transgene throughout the entire muscle section.

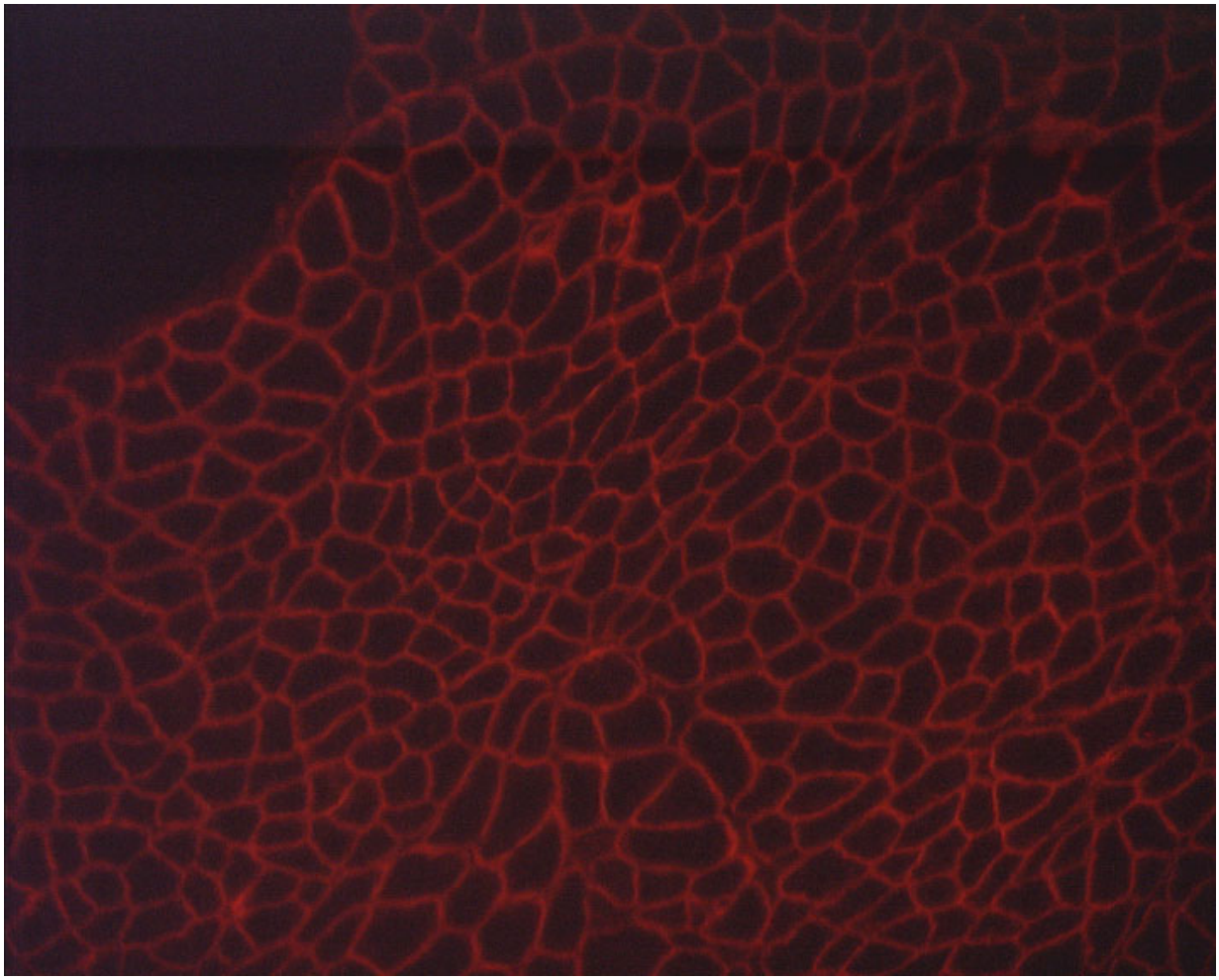
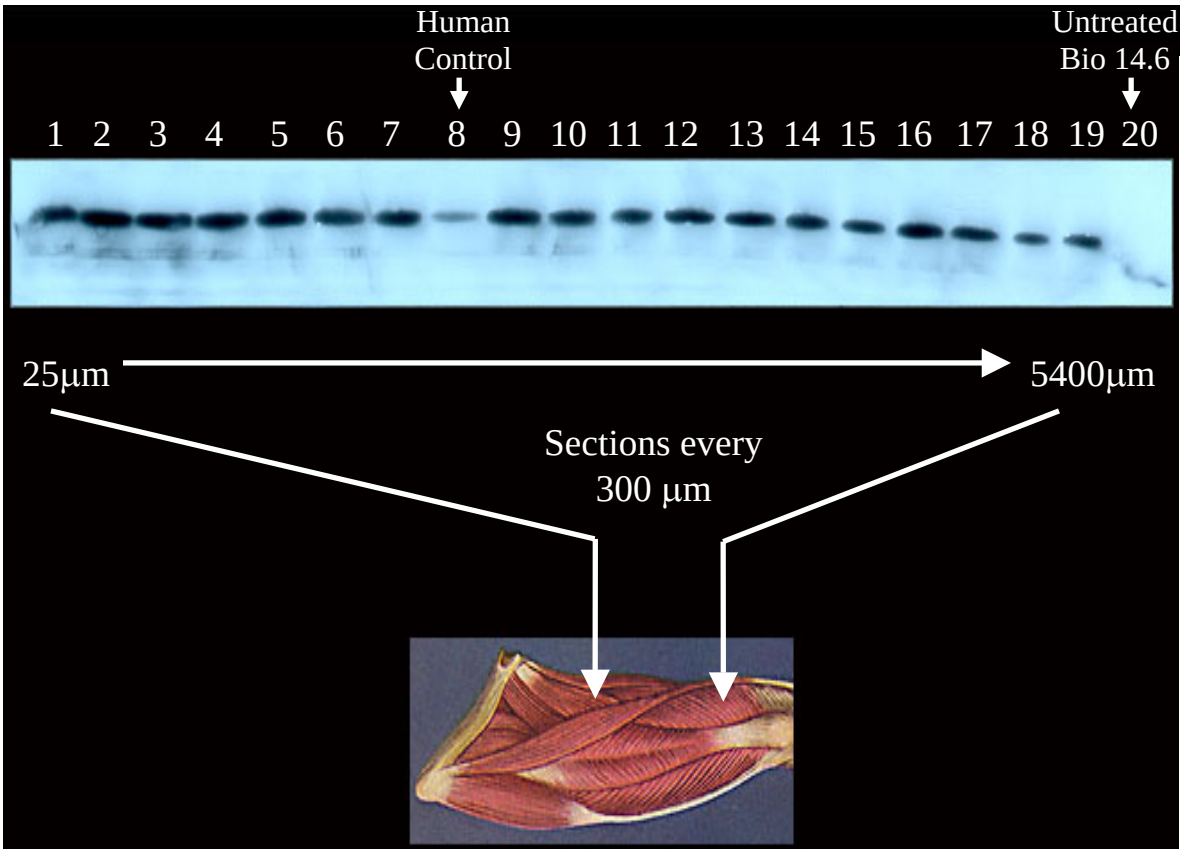


Figure 29. Western blot analysis of δ-sarcoglycan deficient hamster quadriceps injected with an AAV containing the human δ-sarcoglycan gene using the 10-needle injection manifold shows consistent delivery throughout the entire muscle.

Shown is a western blot where each well represents 250 μm of muscle, with all 18 wells covering a total of ~5400 μm of the quadriceps. Lane 8 is a control human muscle using approximately the same amount of muscle. Lane 20 is an uninjected control δ-sarcoglycan deficient hamster. All lanes show more δ-sarcoglycan production than is seen in normal human muscle, with fairly consistent staining throughout the entire muscle. The slight decrease in the staining toward the tendon may be due to smaller muscle diameter towards the tendon insertion.


Human
Control
Untreated
Bio 14.6
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
25µm
5400µm
Sections every
300 µm

6.0

Discussion

6.1 Complex protein biochemistry alters efficacy of gene delivery

Here I report the biochemical and histological rescue of both α- and β-sarcoglycan deficient mice with an AAV vector containing the corresponding sarcoglycan gene. This was done in an effort to better understand the interactions of the sarcoglycan complex, as well as to advance progress in therapeutic gene delivery to the muscular dystrophies. I was able to successfully deliver and express transgenes to complement both types of sarcoglycan deficiency. While both α- and β-sarcoglycan delivery at early time points exhibited primary and secondary biochemical and histological correction of the correspondingly dystrophic muscle, only β-sarcoglycan AAV transduction resulted in long-term biochemical and histological rescue. The results from the β-sarcoglycan data are consistent with the data that were previously generated using δ-sarcoglycan AAV and the hamster model (Li *et al.* 1999, Xiao *et al.* 2000). Our data is also consistent with a report on AAV gene complementation of γ-sarcoglycan deficiency in a mouse model (Cordier *et al.,* 2000).

The α-sarcoglycan data I obtained was not consistent with the other 3 sarcoglycans, as α-sarcoglycan deficient mice, treated with an AAV containing the human α-sarcoglycan gene at 30 days post-injection, exhibited large-scale loss of α-sarcoglycan positive cells. Histological examination of injected muscle showed infiltration of immune cells in areas where α-sarcoglycan positive fibers were located. The immune infiltration was analyzed by immunophenotyping and was found to be primarily macrophages with some T- and B-cells. We interpreted these results to indicate that macrophage infiltration was a secondary response to myofiber necrosis, due to over-expression of α-sarcoglycan and subsequent cytotoxicity.

To test our hypothesis of α-sarcoglycan toxicity, we designed experiments to determine if the immune response was causative for the clearance of α-sarcoglycan positive fibers, or if it is not necessary for loss of α-sarcoglycan positive myofibers. This was done by injecting α-sarcoglycan and β-sarcoglycan expressing AAV into Severe Combined Immune-Deficient (SCID) mouse muscle; these mice lack T- and B-cells and therefore should lose transduced myofibers only if loss is mediated by cytotoxicity. Since these mice have normal sarcoglycan expression, I was not able to monitor transgene sarcoglycan expression in muscle by immunofluorescence. Instead I assayed for centrally nucleated cells as the endpoint; central nucleation is an accurate marker for the amount of myofiber degeneration/regeneration that has occurred. The number of myofibers undergoing degeneration/regeneration in normal (non-dystrophic) muscle is very small. Comparison of the number of central nucleated fibers in SCID mice injected with either AAV α-sarcoglycan, AAV β-sarcoglycan or PBS showed that the AAV β-sarcoglycan and PBS groups did not have a statistically different number of centrally nucleated fibers, but the group injected with AAV α-sarcoglycan had statistically more centrally nucleated fibers than the other two groups. These results suggest that over-expression of α-sarcoglycan causes cytotoxicity. They also indicate that the immune response seen in the α-sarcoglycan deficient mice is likely secondary to the cytotoxic response, and does not reflect immune-mediated loss of transduced myofibers.

These *in vivo* results corroborate recent biochemical studies of the sarcoglycan complex. The current model of sarcoglycan complex assembly is that β-sarcoglycan nucleates the complex in the golgi, followed by the addition of both γ- and δ-sarcoglycan in either order, with α-sarcoglycan addition completing the tetrameric complex (Noguchi *et al.* 2000) (Figure 19). One explanation for apparent variable order of γ- and δ-sarcoglycan attachment is that these two

proteins are highly homologous and are probably the result of a relatively recent gene duplication event. The hierarchical assembly of the complex appears in agreement with *in vivo* studies, where over-expression of α-sarcoglycan causes cytotoxicity while over-expression of β-sarcoglycan, δ-sarcoglycan (from our previous results), and γ-sarcoglycan (Cordier *et al.* 2000) do not (Figure 20). There are two possible models that we believe integrate the biochemical and *in vivo* AAV findings. The first possibility is that α-sarcoglycan subunits inappropriately fill the γ- or δ- binding sites on β-sarcoglycan, causing the formation of abnormal tetramers. The $\beta\alpha_2$ or $\beta\alpha_3$ complexes may form initially, but accumulate in the golgi or are unstable, leading to loss of the complex and eventual cytotoxicity (Figure 21). Another possibility is that the over-expression of α-sarcoglycan causes an incorrect order of assembly of the complex, with α-sarcoglycan binding prematurely to β-sarcoglycan. This premature binding could cause conformational changes in β-sarcoglycan, which could prevent binding of γ or δ-sarcoglycan. Again, the resulting complex might not be transported to the cell surface and could accumulate in the golgi, or may be unstable; each possibility may result in cytotoxicity (Figure 22). However, with both of these models, there will be low levels of proper assembly of the sarcoglycan subunits that will form normal sarcoglycan complex, and only accumulation over time of the improper complexes will lead to cytotoxicity.

There are additional explanations of our data that do not depend on the altered assembly of the complex. First, I delivered the human α-sarcoglycan gene to a murine model, and the human protein product may not be sufficiently similar to the murine orthologue to assemble or function appropriately. Consistent with this interpretation, the murine and human α-sarcoglycan proteins show the greatest sequence divergence, with α-sarcoglycan protein showing 88% identity between mouse and human, and β-sarcoglycan protein showing 95% identity (Appendix

A, B). In addition, α-sarcoglycan is distinct from the other three sarcoglycans in that the N terminus is extracellular, while the other three have intracellular N termini (Figure 2). The signaling needed for correct post-translational processing and orientation in the membrane may have diverged sufficiently between the human so as to incorrectly process α-sarcoglycan. The major argument against this explanation as the cause of cytotoxicity is the finding of successful short-term biochemical and histological rescue of α-sarcoglycan deficient muscle. We believe that a lack of function of human α-sarcoglycan in murine muscle would most likely not show time-dependent loss of transduced fibers. Also, our observation of cytotoxicity in SCID mice indicates that with co-expression of the murine α-sarcoglycan and human α-sarcoglycan, the human α-sarcoglycan has a dominant cytotoxic effect on transduced muscle, likely due to over-expression (i.e. co-expression of murine α-sarcoglycan was not protective).

A second potential reason for α-sarcoglycan induced cytotoxicity is that α-sarcoglycan contains a particularly large cytoplasmic domain relative to the other three sarcoglycans. This cytoplasmic domain may be involved with protein-to-protein interactions, and over-expression of α-sarcoglycan could lead to inappropriate interactions of some intracellular factors. However, the available data on the timing of AAV driven expression of transgenes may argue against this model. Studies have shown that AAV expression levels are highest at 2 weeks post-injection (Vincent-Lacaze *et al.* 1999), and one would expect that the highest levels of expression of α-sarcoglycan would lead to the most inappropriate protein interaction and therefore the highest level of cytotoxicity. However, I observed biochemical and histological rescue of muscle at this 2 week post-injection time point arguing against a model where cytotoxicity is caused by improper interaction of intracellular molecules.

Finally, α-sarcoglycan is distinct from the other sarcoglycans in that there is a closely related molecule, ε-sarcoglycan, that is also expressed in skeletal muscle, and is thought to be somewhat interchangeable with α-sarcoglycan in the sarcoglycan complex. The over-expression of α-sarcoglycan could result in the exclusion of ε-sarcoglycan from binding to the sarcoglycan complex, and this might mediate toxicity via a dominant-negative effect on ε-sarcoglycan function. However, ε-sarcoglycan expression does not change (compensate) in α-sarcoglycan-deficient mice (Liu and Engvall 1999), and immunostaining for the sarcoglycan complex showed that the other three sarcoglycan subunits (β-, γ-, or δ-) were not present at the membrane. Taken together, these data suggest that ε-sarcoglycan does not play a protective or compensatory role in α-sarcoglycan deficiency.

In conclusion, I have shown that delivery of closely related proteins by AAV can lead to very different outcomes, with complete, long-term rescue of muscle by β-, γ-, or δ-sarcoglycan, but significant cytotoxicity and muscle damage by α-sarcoglycan. Therefore, there are aspects of protein biochemistry that do in fact alter efficacy of gene therapy, even in closely related biochemical defects.

6.2 Disease pathology alters immune responses to transgenes in muscle

Dystrophic muscle has many histological hallmarks, including fiber size variation, scattered areas of degeneration/regeneration, hypercontracted fibers, connective tissue proliferation, and cellular infiltration. Each of the cellular pathologies has a potential to modulate the transduction rate and persistence of transgenes delivered to dystrophic muscle. Fiber size variation reflects the presence of both mature and immature myofibers, both of which show differential transduction by specific viral particles. The different maturity of these fibers does not appear to affect AAV transduction as much as adenovirus transduction (Cao *et al.* 2001). Connective tissue proliferation is likely to inhibit the spread of virus in muscle. Degeneration/regeneration could lead to the loss of positive fibers, as they are degenerated with the regeneration of a fiber that does not contain the transgene. In addition, degenerating fibers would allow for the cellular content of the myofibers, including the transgene product, to be released into the intercellular space where it would be surveyed by immune cells. Immune cell infiltrate due to necrotizing myofibers may create a milieu that promotes an immune response to the delivered transgene. The infiltrating bone marrow-derived cellular infiltrate will present antigens (Arahata and Engel 1984; Arahata and Engel 1988; Emslie-Smith *et al.* 1989), including those produced by the transgene. In addition, myofibers in pathological muscle, such as hypercontracted fibers, express antigen-presenting molecules (Nagaraju *et al.* 1999) and become more efficient at antigen presentation. Thus, a myofiber itself may present a transgene product as an antigen that can be recognized by activated T-cells in pathological muscle.

We were particularly interested in the degeneration/regeneration followed by immune cell infiltration, as we hypothesized that this process would impart immunostimulatory properties to dystrophic muscle, and thus elicit more aggressive immune responses than normal muscle.

We tested this hypothesis by injecting both normal muscle and dystrophic muscle (β-sarcoglycan deficient muscle) with an AAV containing a bacterial reporter gene (*LacZ*). In agreement with our hypothesis, we found that the dystrophic muscle is considerably more prone to eliciting an immune response to the transgene, indicated by statistically fewer cells expressing the transgene (*LacZ*) 43 days post-injection, and by dramatic rises in serum titers of antibodies against the transgene. Normal mice did not generate detectable antibody titers to the transgene and also showed persistent expression of the transgene at 43 days post injection. We conclude that dystrophic muscle does indeed possess immunostimulatory properties.

We further hypothesized that complementation of the biochemical defect in dystrophic myofibers could attenuate the immunostimulatory response; either due to a decrease in inflammatory cell infiltrate (mitigation of necrosis), or due to decreased antigen presentation by transduced myofibers, or both. To test this, I injected both normal and β-sarcoglycan deficient mice with a combination of two AAV viruses, one carrying the *LacZ* reporter gene and the other a therapeutic β-sarcoglycan gene. Indeed, even though I injected twice as many viral particles into the muscle in the co-transduction experiments, I found a 10 fold decrease in the immune response against the reporter gene. This indicates that biochemical rescue of myofibers does mitigate the immunostimulatory response, either by decreasing necrosis and subsequent macrophage and T-cell infiltration, and/or by decreasing the antigen-presenting ability of rescued myofibers.

I also measured the immune response using an ELISA assay to monitor the amount of antibody production in β-sarcoglycan deficient mice receiving a *LacZ* transgene. A significantly higher antibody response to β-galactosidase was observed in the β-sarcoglycan deficient mice that received only AAV *LacZ* virus compared with the β-sarcoglycan deficient mice that

received both a corrective AAV β-sarcoglycan virus and the AAV *LacZ* virus. While the trend was clearly evident, statistical significance ($p=0.010$) was attained only when one statistical outlier was eliminated from the assay. When the statistical outlier was included in the analysis, only partial significance was found ($p=0.0545$). The outlier was defined statistically using the methods of Cook, i.e. calculation of a Cook's distance statistic, and was from the group of β-sarcoglycan deficient mice that received both the AAV *LacZ* and the AAV β-sarcoglycan. Importantly, this outlier was also the animal that exhibited a lower number of β-galactosidase positive cells relative to others in its group. We attribute this data point outlier to variability in transduction efficiencies based upon intra-muscular injection techniques. The ELISA data also suggest that antibody levels are correlated with the number of β-galactosidase positive cells, i.e. animals with higher antibody titers had a lower number of β-galactosidase positive fibers, whereas animals with lower antibody titers had a higher number of β-galactosidase positive fibers. The normal mice did not vary significantly from the background readings of the ELISA plates, indicating that the normal mice did not show a significant antibody response to the β-galactosidase reporter protein. Again, these results indicate that dystrophic muscle itself can stimulate an immune response to the transgene. In addition, disease muscle pathology can also alter gene transfer efficacy. One consequence of these results is that a transgene that can fully biochemically and histologically rescue the biochemical defect will show less of an immune response against the transgene, while a transgene that only partially rescues a dystrophic muscle may still evoke a strong immune response.

6.3 Large scale gene delivery

The muscular dystrophies are among the most common and clinically devastating of the inherited monogenic disorders. Worldwide, 1:3500 males are born with the Duchenne muscular dystrophy type alone. Adeno-associated virus (AAV) has emerged as the most promising delivery vector to muscle. However, with muscle consisting of a large portion (30%) of the body mass there needs to be major advances in large scale gene delivery if clinical efficacy of gene delivery can ever be achieved. Here we present one step towards this goal with the development of a 10 needle-injection manifold for efficient and consistent gene delivery to a 2 mm x 1 cm region of muscle (the largest muscle group in the hamster).

Muscle is a highly vascularized tissue, which makes intravenous (IV) delivery of genes a promising route of administration. However, reports have shown that IV delivery of AAV does not efficiently reach the muscle target tissue, but rather accumulates in the liver (Ponnazhagan *et al.* 1997). Another group attempted to bypass the liver by delivering an AAV vector along with histamine using an Extra Corporeal Membrane Oxygenation (ECMO) system, which isolated the blood supply for an entire limb (Greelish *et al.* 1999). While this report does show considerable promise, there are major risks associated with the use of an ECMO model for perfusion of an entire limb. In addition, there is an inherent danger of using histamine to induce endothelial permeabilization. We believe that the injection manifold method is a safer and more predictable way by which to deliver genes.

The injection manifold provides a consistent and reproducible means of vector delivery. We have shown expression of the transgene at 3 times the normal level as compared to human control muscle, and delivery of the transgene and expression level was consistent throughout the entire quadriceps. The stable needle holder ensures 2 mm spread between each of the needles for

consistent injections. The footpad operation of the injection enables the use of both hands during the injection procedure. The concept of this device could be further expanded to develop a method to deliver genes to all muscles in human patients in a safe and effective manner by increasing the number of needles for injection.

6.4 Future Studies

The biochemical and histological rescue of β-sarcoglycan deficient mouse muscle with AAV-mediated gene delivery of the β-sarcoglycan gene has led us to pursue a collaboration with a company to produce AAV for clinical trials. We hope to test the safety of this delivery method for therapeutic purposes in humans. In addition, based on our results with the successful use of the multi-needle injection manifold, we are also proposing to use the 10-needle injection manifold for delivery of the β-sarcoglycan AAV vectors in the safety trials, to test this delivery method in human muscle.

Based on our results, we hypothesize that the cytotoxicity seen with the AAV gene delivery of α-sarcoglycan was due to its over-expression because of the use of the CMV promoter to drive expression. We will test the effectiveness of using the α-sarcoglycan endogenous promoter for gene expression, in the hopes that the production of α-sarcoglycan is diminished to normal levels of expression, which should not cause cytotoxicity and therefore have therapeutic potential. In addition, we hope to determine what sarcoglycan subunits are present in animals that are over-expressing α-sarcoglycan, and to help better define which of the potential models for α-sarcoglycan cytotoxicity may be correct. We will attempt to accomplish this by immunoprecipitate the sarcoglycan complex from muscle that has been injected with an AAV α-sarcoglycan.

Our results with immune cell involvement of the clearance of AAV α-sarcoglycan expressing myofibers has led us to pursue some other experiments to better define some specific types of immune cells involved. It is necessary to determine if macrophages are present in the SCID mice receiving an injection of AAV α-sarcoglycan, so experiments will be performed to

quantitated macrophages in the injected SCID mice. We also would like to determine what role natural killer (NK) cells are playing in the clearance of α-sarcoglycan positive fibers from cells that are over-expressing α-sarcoglycan, NK beige mice will be injected with an AAV α-sarcoglycan and studied for evidence of cytotoxicity. Also an α-sarcoglycan deficient mouse muscle injected with AAV α-sarcoglycan will be analyzed for the presence of NK cell infiltrate.

Our findings of the immunostimulatory effect that dystrophic muscle has on delivered transgenes, has led us to propose the experiment of delivering the truncated "micro-dystrophin" genes using an AAV vector to a higher vertebrate model, such as the dog, prior to any safety testing in human patients. Finally, to help further understand the immunostimulatory properties of dystrophic muscle, we will also attempt to determine, with expression profiling, the role of antigen presentation in the dystrophic muscle receiving a marker transgene.

APPENDICES

Appendix A

Mouse α-sarcoglycan vs. human α-sarcoglycan

```
Percent Similarity: 90.698   Percent Identity: 88.889

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   2
                    . =   1

red   = N-terminal signal sequence
green = N-linked glycosylation
blue  = Transmembrane domain

                    .         .         .         .          .
       1 maaavtwipllagllaglrdtkaqqttlhllvgrvfvhplehatflrlpe 50
         ||  . | |||  ||||| ||.||||||| |||||||| |:| ||| |||
       1 maetlfwtpllvvllaglgdteaqqttlhplvgrvfvhtldhetflslpe 50
                 .         .         .         .         .
      51 hvavpptvrltyhahlqghpdlprwlhytqrspynpgflygsptpedrgy 100
         |||||| | :|||||||||||||||| ||||||:.||||||| ||||||
      51 hvavppavhityhahlqghpdlprwlrytqrsphhpgflygsatpedrgl 100
                 .         .         .         .         .
     101 qvievtaynrdsfdttrqrllligdpegprlpyqaeflvrshdveevlp 150
         |||||||||||||||||||.| ||||||| |||||||||||| |||||
     101 qvievtaynrdsfdttrqrlvleigdpegpllpyqaeflvrshdaeevlp 150
                 .         .         .         .         .
     151 ttpanrfltalgglwepgelqllnitsaldrggrvplpiegrkegvyikv 200
         .|||.|||.|||||||||||||||:||||||||||||||||||||||||
     151 stpasrflsalgglwepgelqllnvtsaldrggrvplpiegrkegvyikv 200
                 .         .         .         .         .
     201 gsatpfstclkmvaspdsyarcaqgqppllscydtlaphfrvdwcnvslv 250
         |||.|||||||||||||:|||||||||||||||||||||||||||.||
     201 gsaspfstclkmvaspdsharcaqgqppllscydtlaphfrvdwcnvtlv 250
                 .         .         .         .         .
     251 dksvpepldevptpgdgilehdpffcppteatdrdfltdalvtllvpllv 300
         ||||||| |||||||||||||||||||| ||||| ||||||||||||
     251 dksvpepadevptpgdgilehdpffcppteapdrdflvdalvtllvpllv 300
                 .         .         .         .         .
     301 allltlllayimcfrregrlkrdmatsdiqmfhhcsihgnteelrqmaas 350
         ||||||||||:|| |||||||||:||||||| |||.||||||||||||||
     301 allltlllayvmccrregrlkrdlatsdiqmvhhctihgnteelrqmaas 350
                 .         .         .
     351 revprplstlpmfnvrtgerlpprvdsaqmplildqh 387
         |||||||||||||| ||||||||||||.|||||||
     351 revprplstlpmfnvhtgerlpprvdsaqvplildqh 387
```

Appendix B

Mouse β-sarcoglycan vs. human β-sarcoglycan

```
Percent Similarity: 95.912   Percent Identity: 94.969

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   2
                    . =   1

green = N-linked glycosylation
blue  = Transmembrane domain

                 .         .         .         .         .
       1 maaaaaaaaateqqgsngpvkksmrekaverrnvnkehnsnfkagyipid 50
           ||||||| ||| |||||||||||||||.||||||||||||||||||
       1 ..maaaaaaaaeqqssngpvkksmrekaverrsvnkehnsnfkagyipid 48
                 .         .         .         .         .
      51 edrlhktglrgrkgnlaicvivllfilavinllitlviwavirigpngcd 100
         ||||||||||||||||||||:||||||||||:||||||||||||||||||
      49 edrlhktglrgrkgnlaicviillfilavinliitlviwavirigpngcd 98
                 .         .         .         .         .
     101 smefhesgllrfkqvsdmgvihplykstvggrrnenlvitgnnqpivfqq 150
         ||||||||||||||||||||||||||||||||||||||||||||||||||
      99 smefhesgllrfkqvsdmgvihplykstvggrrnenlvitgnnqpivfqq 148
                 .         .         .         .         .
     151 gttklsveknktsitsdigmqffdprthnilfstdyethefhlpsgvksl 200
         |||||||| |||||||||||||||| ||||||||||||||||||||||||
     149 gttklsvennktsitsdigmqffdprtqnilfstdyethefhlpsgvksl 198
                 .         .         .         .         .
     201 nvqkasteritsnatsdlnikvdgraivrgnegvfimgktiefhmggdve 250
         ||||||||||||||||||||||||||||||||||||||||||||||..|
     199 nvqkasteritsnatsdlnikvdgraivrgnegvfimgktiefhmggnme 248
                 .         .         .         .         .
     251 lkaensiilngtvmvsptrlpssssgdqsgsgdwvryklcmcadgtlfkv 300
         ||||||||||.|||| |||||||||| ||||||||||||||||||||||
     249 lkaensiilngsvmvsttrlpssssgdqlgsgdwvryklcmcadgtlfkv 298
                 .         .
     301 qvtghnmgcqvsdnpcgnth 320
         |||  |||||:||||||||
     299 qvtsqnmgcqisdnpcgnth 318
```

Appendix C

Mouse γ-sarcoglycan vs. human γ-sarcoglycan

```
Percent Similarity: 87.629   Percent Identity: 83.849

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   2
                    . =   1

green = N-linked glycosylation
blue  = Transmembrane domain

                 .         .         .         .         .
       1 mvreqyttvtegthierpenqhiykigiygwrkrclylfvlllailvvn 50
         |||||||| |||  |||||||::||||||||||||||||||| |||||
       1 mvreqyttategicierpenqyvykigiygwrkrclylfvllliilvvn 50
                 .         .         .         .         .
      51 laltiwilkvmwfspigmghlhvtadglrlegeseflfplyakeirsrvd 100
         |||||||||||||| ||||| || |||||||||||||||||| ||||
      51 laltiwilkvmwfspagmghlcvtkdglrlegeseflfplyakeihsrvd 100
                 .         .         .         .         .
     101 sslllqstqnvtvsarnsegevtgrvkvgaqmvevqsqhfqinsedgkpl 150
         |||||||||||||.||||||||||.||| .|||||.| ||||| |||||
     101 sslllqstqnvtvnarnsegevtgrlkvgpkmvevqnqqfqinsndgkpl 150
                 .         .         .         .         .
     151 fsaeeqdvvvgtgrlrvtgpegalfehsvetplvradpfqdlrlesptrs 200
         |. :|.:||||| :|||||||||||||||||||||||||||||||||||
     151 ftvdekevvvgtdklrvtgpegalfehsvetplvradpfqdlrlesptrs 200
                 .         .         .         .         .
     201 lsmdaprgvhvkanagklealsqmdiilqssegvlvldaetvgltklkqg 250
         ||||||||||:.|.|||:||||||||:  ||:|.|||||||| | || ||
     201 lsmdaprgvhiqahagkiealsqmdilfhssdgmlvldaetvclpklvqg 250
                 .         .         .         .
     251 tqgpagssngfyeicacpdgklylsmagevttceehshvcl 291
         | ||.|||   |||| |||||||||.||  |||:||||:||
     251 twgpsgssqslyeicvcpdgklylsvagvsttcqehshicl 291
```

Appendix D

Hamster δ-sarcoglycan vs. human δ-sarcoglycan

```
Percent Similarity: 96.540   Percent Identity: 94.810

        Match display thresholds for the alignment(s):
                    | = IDENTITY
                    : =   2
                    . =   1

green = N-linked glycosylation
blue  = Transmembrane domain

                 .         .         .         .         .
       1 .mpqeqyshhrstmpssegphiykvgiygwrkrclyffvlllmililvnl 49
          ||||||.|||||||  |  ||  :|||||||||||||||||||||||||||
       1 mmpqeqythhrstmpgsvgpqvykvgiygwrkrclyffvlllmililvnl 50
                 .         .         .         .         .
      50 amtiwilkvmnftidgmgnlritekglklegdseflqplyakeiqsrpgn 99
         |||||||||||||||||||||||||||||||||||||||||||||||.|||
      51 amtiwilkvmnftidgmgnlritekglklegdseflqplyakeiqsqpgn 100
                 .         .         .         .         .
     100 alyfksarnvtvnilndqtkvltrlvtgpkaveaygkkfevktvsgkllf 149
         |||||||||||||||||||||.|:|||||||||||||||||||||||||||
     101 alyfksarnvtvnilndqtkvltqlitgpkaveaygkkfevktvsgkllf 150
                 .         .         .         .         .
     150 saddnevvvgaerlrvlgaegtvfpksietpnvradpfkelrlesptrsl 199
         |||.|||||||||||||||||||||||||||||||||||||||||||||||
     151 sadnnevvvgaerlrvlgaegtvfpksietpnvradpfkelrlesptrsl 200
                 .         .         .         .         .
     200 vmeapkgveinaeagnmeatcrselrleskdgeiklddaakiklprlprgs 249
         ||||||||||||||||||||.||||||||||||||||:||||| ||
     201 vmeapkgveinaeagnmeatcrtelrleskdgeiklddaakirlprlphgs 250
                 .         .         .         .
     250 ytptgtrqkvfevcicangrlflsqagtgstcqintsvcl 289
         ||||||||||||:|:||||||||||| ||||||||||
     251 ytptgtrqkvfeicvcangrlflsqagagstcqintsvcl 290
```

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