Follistatin Gene Therapy for the Treatment of Muscular Dystrophy

Dissertation

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By

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The muscular dystrophies (MDs) represent a group of inherited myogenic disorders characterized by persistent muscle wasting and progressive functional decline. To date, there are no optimal therapies for MD. Most potential treatments have been targeted towards improving conditions in Duchene Muscular Dystrophy where loss of dystrophin is responsible for the muscle defect. However, a number of emerging therapies are currently under review with particular attention being paid to factors that enhance muscle growth. The myostatin inhibitor follistatin, which results in enhanced muscle growth and function, appears to offer substantial promise. The efficiency of follistatin lies in its ability to bind myostatin thus preventing its receptor binding that would normally allow for the downregulation of genes responsible for myoblast proliferation and differentiation. Follistatin is effective in that it not only circumvents the muscle inhibitory effects of myostatin, but is also able to operate outside of the myostatin pathway to offer muscle protection and improve pathology during instances of muscle wasting. Here, we highlighted the unique muscle enhancing effects of adeno-associated virus (AAV) delivery of follistatin (FS344) by using rodent models and non-human primates. We saw that in young as well as aged dystrophin deficient mdx mice, follistatin was able to improve muscle conditions and decrease dystrophic symptoms. The relatedness of follistatin to muscle disease was also seen in both local and systemic
administration of follistatin gene therapy to severely dystrophic laminin-alpha2 deficient mice that closely recapitulate Merosin Deficient Congenital Muscular dystrophy. To investigate if follistatin was equally effective and safe in larger animals, in that this is an important criterion for clinical evaluations in humans, we administered a one-time injection of an AAV bearing follistatin into the quadriceps muscle of Cynomolgus macaques. Like our observations in mice, we saw significant improvements in muscle size and function as assessed by a physiological measure of muscle strength. To understand the possible mechanism for these follistatin induced changes, we performed a series of binding experiments as well as microarray analysis to study at the protein and transcriptional level the effects of muscle follistatin overexpression. Although not conclusive, these characterization studies set the stage for continued investigations into the follistatin mediated muscle response. Taken together this study not only provided evidence for the ability of follistatin to enhance muscle size and strength in both normal and dystrophic conditions, but also provides new starting points for understanding the molecular and functional relevance of myostatin inhibition by follistatin overexpression.
DEDICATION

This dissertation is dedicated to:

   My family and friends; the source of my strength and stability

The families and individuals affected by Muscular Dystrophy

Science and Total Omniscience

The God of Heaven and Earth- He who saves and sustains me; I call Him JESUS.
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TABLE OF CONTENTS

Abstract .........................................................................................................................ii
Dedication .......................................................................................................................iv
Acknowledgements .........................................................................................................v
Vita .................................................................................................................................vi
List of Tables ..................................................................................................................xi
List of Figures ................................................................................................................xii

Chapter 1: Overview .......................................................................................................1

Chapter 2: Background

  Early Descriptions of Muscular Dystrophy ...............................................................5
  Muscle Structure .........................................................................................................8
  Common Clinically Defined MDs ............................................................................10
  Current MD Therapies .............................................................................................17
  Emergent Treatment Strategies for MD .................................................................19
  Myostatin Inhibition for Enhanced Muscle Growth ..............................................23
  Follistatin for Myostatin Inhibition .......................................................................27
  Gene Therapy for Follistatin Administration .......................................................30
  Figures .......................................................................................................................34
  Tables .......................................................................................................................41
Chapter 3: Methods

Mice ........................................................................................................42

Non-Human Primates ..............................................................................42

Construction of AAV1 and Self-Complimentary AAV9 Expressing Follistatin...43

Viral Vector Production ............................................................................43

Mouse Injections ......................................................................................44

Mouse Behavioral Testing ........................................................................45

  Grip Strength .......................................................................................45

  Coordination ..........................................................................................45

  Ambulatory Movements and Rearing Events .........................................45

Mouse Survival .........................................................................................46

Non-Human Primate Injections .................................................................46

Non-Human Primate Muscle Physiology ..................................................47

Serum Creatine Kinase Assay .................................................................48

Follistatin Enzyme-Linked ImmunoSorbent Assay ..................................48

Non-Human Primate Immune Studies ......................................................49

Muscle Histology .....................................................................................50

Fat Tissue Analysis ..................................................................................50

Muscle Fiber Size Histogram .................................................................51

Production of Recombinant Follistatin .....................................................51

Binding of Follistatin to Laminin-1 and Merosin (Laminin-α2) .................51

Microarray ...............................................................................................52

Statistics ..................................................................................................53
Chapter 4: Single Administration of AAV1-Follistatin-344 Enhances Muscle Mass and Strength in \textit{mdx} Mice

Introduction .............................................................................................................54
Results .....................................................................................................................57
Discussion .............................................................................................................60
Figures ...................................................................................................................64

Chapter 5: AAV1-Follistatin Enhances Muscle Mass and Function in Non-Human Primates

Introduction .............................................................................................................67
Results .....................................................................................................................70
Discussion .............................................................................................................73
Figures ...................................................................................................................77

Chapter 6: Follistatin Gene Therapy Improves Symptoms in a Severe Mouse Model of Congenital Muscular Dystrophy

Introduction .............................................................................................................80
Results .....................................................................................................................84
Discussion .............................................................................................................91
Figures ...................................................................................................................97
Tables .....................................................................................................................105

Chapter 7: Possible Follistatin Mediated Interactions that Promote Muscle Growth and Enhancement

Introduction .............................................................................................................107
Results .....................................................................................................................110
Muscle Growth and Hypertrophy .................................................................112
Muscle Development and Stability ..............................................................113
Muscle Pathology ...............................................................................................114
LIST OF TABLES

Table 2.1. List of Recognized Muscular Dystrophies ........................................41
Table 6.1. Percent Change Differences Among AAV1-Follistatin Treated Laminin-α2
Deficient and Heterozygote Mice at Three Months of Age .........................105
Table 6.2. Percent Change Difference Among scAAV9-Follistatin Treated Laminin-α2
Deficient and Heterozygote Mice at Three Months of Age .........................106
Table 7.1. Differential Gene Expression in C57Bl6 Mice after AAV1-FS Administration
.......................................................................................................................119
LIST OF FIGURES

Figure 2.1. Egyptian Relief Painting From the 18th Dynasty .................................34
Figure 2.2. Drawing on the Wall of a Tomb at Beni Hasan Dating From the Middle
   Kingdom........................................................................................................35
Figure 2.3. Schematic of Skeletal Muscle Structure .............................................36
Figure 2.4. Depiction of the Progression of Duchenne Muscular Dystrophy ..........37
Figure 2.5. Schematic of the Dystrophin Glycoprotein Complex .........................38
Figure 2.6. Schematic of the Inhibition of the Myostatin Signaling Pathway ..........39
Figure 2.7. Schematic of Follistatin Isoforms ......................................................40
Figure 4.1. A Single Injection of AAV1-FS Increases Skeletal Muscle Mass and Strength
   in mdx Mice Injected at Three Weeks of Age ...............................................64
Figure 4.2. mdx Mice Treated With AAV1-FS at Three Weeks of Age and Followed for
   180 Days Demonstrated Myofiber Hypertrophy .........................................65
Figure 4.3. Single Injection of AAV1-FS Increase Muscle and Strength and Reduces
   Pathology in Aged mdx Mice ........................................................................66
Figure 5.1. Cynomolgus macaques Injected with AAV1-FS Show Increased Expression
   and Enhanced Muscle Growth ......................................................................77
Figure 5.2. AAV1-FS Causes Myofiber Hypertrophy and Predominantly Affects Type 2
Myofibers in the Quadriceps of Cynomolgus macaques ..............................78

Figure 5.3. No Cellular Immune Response to Follistatin-344 or the AAV1 Capsid was
Observed from Vector Administration ......................................................79

Figure 6.1. Muscle Enhancement in dy^w/+ After AAV1-FS Administration ..........97
Figure 6.2. Muscle Enhancement in dy^w/dyw After AAV1-FS Administration ......98
Figure 6.3. AAV1-FS Injected Mice Demonstrated Significant Increases in Activity,
Reduced Pathology and Trends Towards Increased Survival ..................99

Figure 6.4. Muscle Follistatin Expression in AAV1-FS Injected Animals..........100
Figure 6.5. H&E stain of scAAV9-FS and PBS-Treated dy^w/+ Mice Three Months Post
Injection ....................................................................................................101
Figure 6.6. Three Months old dy^w/dyw Mice Treated With scAAV9 at One-day of Age
Demonstrated Significant Reductions in Pathology..........................102

Figure 6.7. scAAV9-FS Enhances Activity and Survival in dy^w/dyw Mice ..........103
Figure 6.8. scAAV9-FS Increased Protein Expression in Three Month Old dy^w/+ and
dy^w/dyw Mice.........................................................................................104

Figure 7.1. Follistatin and c-Myc Expression in Transfected HEK293T Cells ........117
Figure 7.2. Evaluation of Follistatin Binding to Laminin and Merosin..............118
CHAPTER 1

OVERVIEW

The muscular dystrophies represent a complex group of disorders largely characterized by progressive muscle weakness marked by myofiber fibrosis and necrosis that can affect respiratory and/or cardiac function leading to premature death (Rando, 2001; McNally, 2007). These disorders are usually caused by mutations that result in loss or decreased expression of select members of the dystrophin associated glycoprotein complex (DGC). This highly specialized protein network helps to provide the signaling scaffold needed for cell survival and the structural foundation to preserve the integrity of the muscle during contractions (Rando, 2001). A disruption in this complex can cause substantial injury that affects muscle growth, tone, function, and regeneration. Furthermore, loss of one of the members of the DGC can lead to the altered expression other membrane components (McNally, 2007). Therefore, the preservation of this highly specialized system is necessary for proper muscle development, maintenance and repair upon injury.

Interest in the molecular and structural framework of this organ system and its relatedness to muscle disease is suggested to date back to ancient times. Archeologists have discovered wall paintings that are believed to depict phenotypic characteristics of
individuals with motor deficits marked by skeletal deformities and muscle pseudohypertrophy reminiscent of the clinical features associated with muscular dystrophy (MD) (Emery, 1987). Nevertheless, the foundation for the modern day study of muscular dystrophy is attributed to Duchenne and Meryon, who charted the progressive development and pathological features of muscular dystrophy to characterize the degenerative nature of the disease (Emery, 1987; Bach, 2000).

Presently, no optimal treatments are available for muscular dystrophy. In the muscular dystrophies, only corticosteroids, which carry a high risk in terms of side effects (bone loss, cataracts, delayed puberty, weight gain, and hypertension) have offered any substantial level of therapeutic intervention (Mendell, 1989). Interestingly, a number of recent studies have suggested a therapeutic potential for the transgenic expression of certain extracellular proteins in the amelioration of key aspects of disease. Specifically, we have demonstrated the biological effects of follistatin (a myostatin inhibitor) to increase myofiber size, muscle strength and body mass in wildtype and the mild-phenotype, dystrophin-deficient $mdx$ mouse (Haidet, 2008). Using adeno-associated viral vector (AAV) gene therapy delivery methods, follistatin overexpression is well tolerated and has been suggested to have a protective effect on muscle even under dystrophic conditions (Haidet, 2008). Its efficacy and safety in non human primates has also been evaluated to determine the therapeutic potential of follistatin to be used clinically, as this is an important criterion for human treatment (Kota, 2009-accepted Science Translational Medicine). As an extension of this work, this dissertation will not only highlight key
features of these studies, but will also address whether follistatin is equally efficient in a clinically relevant and severe animal model of disease where the mouse model effectively recapitulates the human disorder.

Specifically, the overall goal is to undertake a practical, integrative and translatable approach to muscular dystrophy gene therapy. This research pursuit has substantial clinical relevance in that many therapies evaluated in mild models of muscle disease often fail to offer meaningful benefit in more chronic and severe cases of degeneration. Consequently, a therapeutic approach that can result in muscle enlargement, preservation of integrity and enhancement of motor function in diverse models of MD at varying levels of disease severity could be largely effective in advancing the clinical management of muscle wasting.

This dissertation research will be presented in eight chapters. Chapter two is a review of literature establishing the basis upon which these studies were performed. It is divided into several parts that describe early investigations in muscular dystrophy, an appreciation for muscle structure and function, the clinical characteristics of muscle disease when its integrity is compromised, current therapies as well as emerging treatment strategies and the most meaningful methods for their administration with particular attention being made to the relatedness of AAV technology.
Chapter three represents the research design and methodology used to pursue the aims of this thesis work. It details the procedures for data collection and analysis as well as statistical measures to determine significant differences amongst experimental groups. Chapters four through six represent three distinct studies aimed at understanding the relatedness of adeno-associated virus directed expression of the muscle enhancer follistatin. These three chapters are arranged in a logical flow to portray the conceptual events that ultimately led to experiments to investigate the effectiveness of AAV-follistatin in a severe and clinically relevant model of muscular dystrophy. The sequential order of these chapters detail how this potential therapeutic was first investigated in normal conditions, which led to explorations of its effects in dystrophic animals that ultimately generated studies aimed at understanding its safety and tolerability in larger animals as well as investigations into its relevance in chronic conditions of muscle wasting. Chapter seven details experiments made in an attempt to discover new follistatin mediated protein-protein interactions and differential gene expression profiles that could be used to understand the mechanism through with it exerts its muscle regulatory potential. To conclude, the final chapter discusses the overall significance of the research effort highlighting how it can be integrated into the current knowledge and describing its future implications.
CHAPTER 2

BACKGROUND

Early Descriptions of Muscular Dystrophy

Fascination with muscle disease has been suggested to date back to ancient times. In fact, some of the oldest recordings of man, found as wall carvings and paintings on Egyptian caves, suggest that noticeable incidences of muscle disorders may date back as early as 1500 BC (Fig. 2.1) (Emery, 1987). Archeologists have discovered wall paintings depicting individuals with enlarged calves and pictures of boys with flat feet, instead of the more pronounced arches that are usually apparent in ancient Egyptian wall paintings. Moreover, the significant size of the calf muscles, height variations, and pictures of malformed upper extremities have led investigators to believe that these pictures may, to some degree, be representative of individuals who suffered from some form of muscular dystrophy (Fig. 2.2) (Emery, 1987). These early portraits of Egyptian life have stark resemblance to what we have accepted as key characteristic features found in a number of muscle disorders that are presently being investigated.
Modern day scientists attribute the first clinical descriptions to Charles Bell, an English physician most known for his description of the facial nerve abnormality we know as Bell’s palsy (Emery, 1987; Tyler, 2003). However, Bell in his *The Nervous System of the Human Body* (1830), candidly describes the progressive weakness and wasting of the muscles of the lower extremities, absent a sensory or neurological defect, found in an 18 year old man who had been afflicted by this condition beginning at around 10 years of age (Emery, 1987; Tyler, 2003).

Around the same time, Italian physicians Gaetano Conte and L. Gioja described two brothers with progressive wasting of the leg muscles, subsequent joint contractures as well as enlargement of the calf muscles and deltoids (Tyler, 2003). Although the pathology of these muscles was never assessed, it has been speculated that what Conte and Gioja described was pseudohypertrophic muscle disease in which muscles contained excessive connective tissue, a loss of contractile force and a disrupted association with tendons and bones.

Admittedly, these early observations of muscle dysfunction only *highlight* the clinical presentation and symptomology of muscle disease without actually *describing* muscular dystrophy. Without pathology these cases are only speculative. However, this issue was resolved when English physician Edward Meryon examined the cases of eight boys in three families and charted their progressive muscle weakness and loss of function. Of particular interest was the case of a 14-year old boy whose case had been previously
described by English physician, Richard Partridge (Tyler, 2003). By performing post-mortem microscopic examination of the tissue, Meryon was able to identify muscle wasting marked by sarcolemma disruptions, myofiber necrosis and fibrosis, as well as fatty depositions that formed in and around the muscle fibers. Moreover, the lack of a neurological defect associated with these conditions, established that this was a primary muscle disease and has been suggested to be the earliest descriptions of what we know as Duchenne Muscular Dystrophy (DMD).

DMD is a progressively lethal muscle wasting disorder that is characterized by continued muscle wasting, cycles of degeneration/regeneration, myofiber fibrosis and necrosis and a resultant loss of ambulation that occurs usually in the teens (Moser, 1984). With time respiratory and cardiac problems ensue that ultimately result in premature death around the second decade of life (Emery, 1987). Although, initially described by Edward Meryon the name and seminal studies investigating its pathological features are accredited to French neurologist, Guillaume Benjamin Amand Duchenne. Beginning with his hallmark findings published in 1861, Duchenne not only characterized the progressive development of muscular dystrophy, charting its gradual effects beginning in the lower extremities and later affecting the forelimbs, but was also able to chronicle the progressive muscle pathology by performing biopsies on live patients using his needle-harpoon where he took specimens from his patients at different time points throughout the course of the disease (Emery, 1987; Tyler, 2003). This was a shortcoming of Meryon’s studies in that he only analyzed patients upon necropsy. Nevertheless, the
seminal studies of Meryon and Duchenne into the degenerative nature of DMD has set
the stage for continued investigations into the progressive and debilitating changes that
occur in chronic muscle disorders. Because of this, it is now recognized that although
DMD is the oldest investigated and most common muscle disease, there also exists a
number of inherited muscle disorders characterized by variable degrees of muscle
wasting and weakness with various etiologies.

Muscle Structure

The myofiber is the basic cellular component responsible for voluntary movement (Fig.
2.3). Myofibers are formed from the fusion of several singly-nucleated, cells known as
myoblasts. These fusion events result in multinucleated, elongated, cylindrical fibers that
become bundled together and encased in a sheath of connective tissue (McNally, 2007).
These bundles are innervated by motor neurons and vascularized via the abundant nerves
and blood vessels that are found in the connective tissue (McNally, 2007). Each
myofiber contains chains of sarcomeres composed of thick myosin and thin actin
filaments. These proteins allow for muscle contraction and relaxation. Contraction is a
result of calcium release from the sarcoplasmic reticulum due to a depolarizing action
potential. This enables myosin to bind actin. The hydrolysis of ATP provides the energy
for this reaction. The rate of the hydrolysis is proportional to the speed of the muscle
contraction. Thus, the rate of muscle contraction is not the same for all myofibers.
Fibers with a characteristic pattern of contraction can be distinguished using enzyme
histochemistry in an ATPase reaction.
In general, there are two basic types of myofibers: Type I slow-twitch oxidative and Type II fast-twitch glycolytic. The type II fibers can be further distinguished into Type IIA- fast twitch, fatigue resistant; Type IIB-fast-twitch, fatigue non-resistant and Type IIC- a mixture of the two. The twitch properties of the fibers are determined by the innervating motor neuron for the motor unit. The proportions of these fibers change throughout life and due to disease states.

Muscle injury causes a disruption of the myofiber plasma membrane, an influx of inflammatory cells and myofiber necrosis and degeneration. To help repair the muscle, growth factors are released and immature muscle cells with a stem cell like property known as satellite cells differentiate into myoblasts and fuse with the injured myofibers to promote muscle regeneration (Menetrey, 2000; Partridge, 2002). The nucleus of these regenerated fibers becomes centralized rather than being expressed along the periphery as seen in non-injured muscle. An increase in the number of central nuclei indicates ongoing cycles of degeneration and regeneration (McNally, 2007). If there is prolonged and continued muscle injury usually due to muscle disease, the muscle fibers can become replaced with interstitial tissue and fat resulting in fibrosis (McNally, 2007). These conditions are commonly seen in neuromuscular diseases where there is a defect in muscle membrane proteins that result in mechanical instability of the plasma membrane. Membrane stability is important in that myofibrillar force is transmitted across the plasma membrane to the extracellular matrix. Plasma membrane disruption results in increased
permeability and leakage of cytoplasmic proteins. Thus, increased serum creatine kinase and lactate dehydrogenase are used as clinical markers of muscle disease.

Muscle disease can arise from a number of defects in transmembrane proteins, extracellular matrix proteins, cytoplasmic proteins and proteases as well as nuclear membrane proteins (Rando, 2001). These proteins are components of the dystrophin-glycoprotein complex (DGC) which provides the structural and signaling scaffold for skeletal muscle. It also links the extracellular matrix to the intracellular cytoskeleton. Defects in genes that code for these proteins cause various forms of muscular dystrophy (MD). Historically grouped according to clinical phenotype, MDs of the varying types are now classified based on the specific gene deficiency and the molecular pathogenesis associated with the disorder (Table 2.1).

**Common Clinically Defined Muscular Dystrophies**

Duchenne Muscular Dystrophy (DMD) is the most common X-linked disorder affecting 1 in 3500 live male births (Wagner, 2008). Caused by mutations in the dystrophin gene that result in the absence of dystrophin protein expression, DMD patients usually present with proximal muscle weakness that is progressive (Fig. 2.4) (Emery, 2001; Emery, 2002). However, the clinical features of DMD can be present in a much milder form known as Becker muscular dystrophy, in which a truncated version of dystrophin is produced leading to a relatively more benign disease phenotype (Emery, 1987; Kingston, 1983).
Dystrophin is a member of the dystrophin associated glycoprotein complex (DGC) (Fig. 2.5). This integral protein network is suggested to help protect the muscle from contraction-induced injury and thus helps to maintain muscle integrity during movement. A loss of dystrophin or any member of the DGC affects the expression of other proteins that are linked to it. Dystrophin binds sarcolemmal beta-dystroglycan (β-DG) and intracellular F-actin. These proteins in turn bind other membrane constituents and it helps to link the outside to the inside of the muscle cell. Through the discovery of dystrophin and understanding its membrane associations, considerable advances have been made in DMD research including the generation of animal models of human disease.

In 1984 the dystrophin deficient mdx mouse model was engineered and to date it is the foremost laboratory model for evaluating DMD (Sicinski, 1989). Genetically and biochemically homologous with the human disease, the mdx mouse has a stop codon mutation in exon 23 of the dystrophin gene (Sicinski, 1989; Ryder-Cook, 1988). Histopathological analysis of mdx muscle indicates that like humans, loss of dystrophin results in myofiber necrosis and fibrosis, cycles of degeneration/regeneration as well as increases in serum creatine kinase, a clinical indicator of muscle injury (Bulfield, 1984; Coulton, 1988). However, one of the most devastating drawbacks of this model is that mdx mice display little to no phenotypic characteristics of the disease. The lack of overt functional deficits has halted the evaluation of new potential treatment options as researchers attempt to review existing animal models and/or develop new ones that better correlate with patient symptomology. By studying the pathomechanisms of muscle
disease of a primary defect and understanding the relatedness of the DGC, several other
glycoproteins have been identified that interact with dystrophin (Ohlendieck, 1991). In
accordance, defects in their genetic expression can lead to a clinical presentation quite
similar to that seen in DMD. Therefore, it is necessary to investigate these conditions to
understand the common molecular features that underlie the various conditions of MD.

The limb-girdle muscular dystrophies (LGMD) comprise a heterogeneous group of
muscle disorders that account for up to a third of the cases of MD (Daniele, 2007). The
underlying defects in these orders are usually due to mutations in the genes responsible
for the transmembrane sarcoglycans: alpha (α), beta (β), gamma (γ), and delta (δ)
(Kanagawa, 2006). Nevertheless, the LGMDs are grouped together on the basis of the
focal muscle defects seen in patients primarily affecting muscles of the scapula and
pelvic girdle.

The emergence of the LGMD phenotype can occur as the result of the loss of a number of
sarcomeric, sarcolemmal or enzymatic proteins. LGMDs are classified as either
autosomal dominant, LGMD1 or the more prevalent autosomal recessive, LGMD2.
These two distinguishing groups are further subdivided according to a lettering system
from (A-M) based on the chronology of the identification of the particular loci in which
the mutation occurs (Daniele, 2007). Only three of the causal genes responsible for the
seven dominant forms are known: myotilin for LGMD1A; lamin A/C for LGMD1B;
Caveolin-3 for LGMD1C (McNally, 2007). Thus, for LGMD1D-F, the genes responsible remain unknown.

The autosomal recessive forms of LGMD are far more defined. A loss of Calpain 3 is responsible for LGMD2A; Dysferlin for LGMD2B; γ-sarcoglycan for LGMD2C; α-sarcoglycan for LGMD2D; β-sarcoglycan for LGMD2E; δ-sarcoglycan for LGMD2F; Telethonin for LGMD2G; Tripartite motif-32 (TRIM-32) for LGMD2H; Fukin-related protein for LGMD2I; Titin for LGMD2J; protein-O-mannose β1,2-N-acetylglucosaminyltransferase 1 (POMGnT1) for LGMD2K; Fukutin for LGMD2L; Protein O-mannosyl-transferase 1 for LGMD2M and Protein O-mannosyl-transferase 2 for LGMD2N (Daniele, 2007; Manya, 2009).

Although the prominent features of all LGMDs include weakness of the shoulder and pelvic girdles, there are great variations in terms of the age of onset, the rate of disease progression, the degree of inflammation, respiratory defects, cardiomyopathy and joint contractures (Bushby, 1999; McNally 2007). Moreover, the clinical presentations of the various forms of LGMD display substantial overlap with other common neuromuscular disorders. In fact, certain muscular dystrophies that are congenital and present at birth display a pathological process that is associated with various types of LGMDs. For example, Emery-Dreifuss Muscular dystrophy has been linked to LGMD1B while Congenital Muscular Dystrophy IC and Muscle-Eye Brain disease have genetic defects in the same gene (FKRP) as LGMD2I (McNally, 2007; Longman, 2003). Therefore, it is
also necessary to evaluate these disorders in the context of the pathological events associated with the particular disease.

The Congenital Muscular Dystrophies are a group of clinically and genetically heterogeneous inherited neuromuscular disorders that are present around the time of birth. Clinical features include hypotonia, muscle weakness, degeneration, myofiber fibrosis and necrosis, joint contractures, respiratory problems, spine rigidity and in some cases, neurological defects that can result in mental retardation, epilepsy and white matter changes (Jimenez-Mallebrera, 2005; Mutoni, 2004; Mendell, 2006; Longman, 2003).

The most prevalent form of CMD is laminin-α2 (merosin) deficient. Laminin-α-2 deficient Congenital Muscular Dystrophy (MDC1A) is a progressive neuromuscular disorder characterized by muscle wasting, peripheral nerve demyelination and respiratory problems that result in premature death (Mendell, 2006; Lisi, 2007). A trimer composed of an alpha, beta, and gamma subunit, laminin 2 is an extracellular matrix protein found in the endoneurium of peripheral nerves and the skeletal muscle basal lamina (Wewer, 1996; Jimenez-Mallebrera, 2005; Ryan, 1996). The α2 chain of laminin 2 has near ubiquitous expression in the skeletal muscles (Ryan, 1996; Patton, 2000). Through its binding of alpha-dystroglycan (α-DG), laminin-α2 supports the plasma membrane basal lamina during muscle contraction (Kuang, 1998; Ryan, 1996; Patton, 1999; Yurchenco, 2004). However, to date, the complete functional role of laminin-α2 and the properties that underlie the resultant pathology of its loss are still unknown. Treatment of MDC1A
has been further complicated by the fact that the defects in muscle and nerve appear to
develop independently of each other. This is supported by seminal studies using animal
models in which transgenic expression of laminin-α2 in the muscle basal lamina
improved muscle morphology and muscle function, but did not prevent neurological
defects (Kuang, 1998). Interestingly, these neural defects did not lead to
myodegeneration (Kuang, 1998). For these reasons, efforts have been made to
understand the fundamental properties that underlie the altered expression of other genes
not mutated in MDC1A that might uncover unique ways to combat certain aspects of
disease progression as well as the pathogenic mechanisms found in other congenital
muscle disorders.

It is important to note that conditions of CMD not only result from the loss of members
of the DGC but also abnormal post-translational processing of DGC proteins. Therefore,
conditions caused by mutations that result in aberrant glycosylation of DGC components
with reduced-ligand binding affinity must also be evaluated to get an appreciation of the
heterogeneity of the various forms of MD. A group of CMDs known as
dystroglycanopathies are indeed conditions that are caused by defects in O-linked
glycosylation. α-DG is a central protein in the DGC that undergoes O-linked
glycosylation. α-DG also serves as a receptor for a number of extracellular matrix
proteins such as laminin, perlecan and agrin (Martin, 2005; Michele & Campbell, 2003).
Moreover, O-linked glycosylation of α-DG is necessary for its ligand-binding activity
and disruption of these interactions due to hypoglycosylation likely perturb the DG
mediated linkage between intracellular dystrophin and the extracellular matrix (Kanagawa, 2006; Ervasti & Campbell, 1993). These conditions lead to a wide spectrum of conditions ranging from severe, congenital onset with brain defects such as Walker-Warburg syndrome and muscle-eye brain disease to milder adult onset conditions with no brain involvement like LGMD2N. In fact, mutations in at least six glycosyltransferase genes have been implicated in various forms of CMD and LGMD. All are autosomal recessive and mostly affect the brain, eyes and skeletal muscle. These conditions include Fukuyama congenital muscular dystrophy (FCMD), muscle-eye-brain disease (MEB), Walker-Warburg syndrome (WWS), MDC1C and MDC1D. The primary proteins lost in these disorders are Fukutin, POMGnT1, protein -O-mannosyl transferase 1 (POMT1), FKRP and acetylglucosaminyl-transferase-like protein (LARGE), respectively (Mutoni, 2004; Kanagawa, 2006; Martin, 2005). Remarkably, they all result in a cellular pathology caused by aberrant glycosylation of α-DG. The commonality among the dystroglycanopathies enables them to be evaluated using basic laboratory models that effectively mimic the unique features of human disease. The myodystrophic myd mouse contains a mutation in the murine Large<sup>myd</sup> gene and displays phenotypic characteristics seen in MEB, FCMD, WWS, MDC1C and MDC1D with defects in muscle, eye, brain and the heart (Mutoni, 2004). By utilizing animal models that effectively recapitulate human disease, potential therapeutic strategies can be evaluated aimed at correcting the disease.
Current MD Therapies

Presently, there are no primary cures for MD. In fact, only corticosteroids have offered any substantial level of therapeutic effectiveness. This class of drugs, which includes the widely used prednisone, has been shown to increase muscle strength and stabilize motor function in DMD patients (Kissel, 1993; Mendell, 1989). Although not curative, steroids prolong independent ambulation, preserve lung function, and reduce the incidence of scoliosis (Mendell, 1989). Analysis of dystrophic muscle has revealed transcriptional changes in a number of genes (Angelini, 2007). Therefore, it is possible that by modulating the expression of muscle regulators prednisone has an anabolic effect. Investigating the interaction of prednisone with these factors could uncover how it exerts its therapeutic potential.

Although there are indeed benefits to glucocorticosteroid use, they also carry a high risk in terms of side effects. Steroid use has been associated with the induction of bone loss, cataracts, delayed puberty, weight gain, and hypertension (Kissel, 1993). This necessitates the need for proper monitoring and regulation of adverse effects. Symptom management may include vitamin D supplements, dietetic implementations and the reduction of drug intake (Wagner, 2008).

Respiratory deficits are also experienced in MD sufferers with clinical presentation occurring in the early to mid teens. These complications are often marked by sleep hypoventilation and expiratory muscle weakness that results in an inability to clear
mucosal secretions (Mohr, 1990). These conditions are commonly treated with the use of non-invasive ventilation and cough assist devices (Bach, 2003; Wagner, 2007; Gomez-Merino, 2002).

The cardiomyopathy associated with various conditions of MD also requires therapeutic intervention. Beta-blockers, angiotensin converting enzyme (ACE) inhibitors and diuretics offer some benefit for the cardiac complications associated with MD (Ogata, 2009; Shaddy, 1999; Kaspar, 2009). The aim of these efforts is to enhance survival. However, the improvement of the quality of life requires a multidisciplinary approach that may involve physiotherapy and orthoses.

Achilles’ tendon contractures are often associated with various conditions of MD like the childhood LGMDs (Bushby, 1999). Treatment requires the use of knee-ankle-foot orthoses or orthopedic corrective surgery. However, proactive management like serial casting of the ankles can help reduce the need for these interventions. This combined with modest exercise and passive stretching can improve and maintain muscle strength and prevent joint deformities.

Current therapeutic interventions are indeed able to improve muscle strength and function, helping to prolong survival and improve the quality of life in dystrophic muscles. However, no optimal treatment strategy exists. In most cases, children still lose ambulation in the early teens and succumb to the disease by the second decade of life.
The obvious treatment strategy would be to reintroduce the gene that is lost in the particular dystrophic condition. However, this is not always a feasible approach considering size limitations in gene therapy applications. Most of the studies that have investigated potential treatment strategies for the MDs have utilized animal models of DMD. Given that it is the most prevalent MD, defining ways to circumvent the continuum of disease phenotypes associated with DMD could substantially advance therapeutic approaches that could be implemented in other forms of MD as well.

**Emerging Treatment Strategies for MD**

Most of the current therapies directed towards the population of individuals with muscular dystrophies have been extremely disappointing. Because of the risks associated with steroid use and the fact that their mechanism of action is largely unknown, particular attention has been made in the recent years to develop new treatment strategies that have clinical benefit in the absence of adverse effects. Most studies investigating potential therapeutics have been evaluated in the DMD population. In particular, gene manipulation approaches have been taken aimed at bypassing the defective genetic component to restore functionality in the muscle cell. Also, to increase membrane stability, a number of strategies are being evaluated to reinforce linkages within the DGC.

Exon skipping is an attractive potential treatment for the muscular dystrophies with an emphasis on DMD. Point mutations or exon duplications in the dystrophin gene disrupt the reading frame and result in a loss of protein expression in over 30% of the DMD
population (Aartsma-Rus, 2006). Therefore, if the reading frame can be restored, partial production of the protein can be produced providing for some degree of functional improvement. This approach is highly feasible for DMD therapy in that partial production of dystrophin results in a milder phenotype like that seen in Becker MD where over 50% protein loss leads to only a more modest dystrophic condition. Becker patients are diagnosed much later than DMD patients (Emery, 2002). They often remain ambulant well into adulthood and generally have near normal life expectancies (Emery, 2002). Indeed, exon skipping induced by the use of antisense oligonucleotides has offered significant improvements in muscle strength and function in animal models of DMD and are consequently being evaluated clinically (Aartsma-Rus, 2006; van Deuekom, 2007). Yet, the key drawback of this approach is that persistent expression of the antisense oligonucleotides cannot be achieved due to a short half-life. Therefore, repeat intramuscular or intravenous administration would be required to achieve maximal results. However, these bouts of injection increase the risk of drug toxicity and procedural hazards.

The aminoglycoside gentamicin is an attractive treatment agent for muscle disease with a known gene defect like DMD. Over ten percent of DMD patients have a premature stop codon in the dystrophin gene (Wagner, 2008). This nonsense mutation prevents the proper read through of the fulllength cDNA. Gentamicin has been shown to suppress this mutation and result in proper dystrophin expression in the \textit{mdx} mouse (Barton-Davis, 1999). However, these attempts were unsuccessful in Duchenne and Becker MD patients.
There also exist risks to gentamicin treatment. These therapies have been shown to cause nephrotoxicity by inhibiting protein synthesis in renal cells (Wagner, 2001). Also, like antisense oligonucleotides, they would require multiple doses administered intravenously. To minimize these dangers and to overcome these limitations, a therapy that could be administered orally may be a more suitable approach.

PTC124 is an orally administered small molecule that has been shown to restore dystrophin expression by allowing proper read through of nonsense mutations in cells (Welch, 2007). It not only shows benefit in the *mdx* mouse, but also in DMD patients (Welch, 2007). A Phase 2A clinical trial is ongoing investigating its effectiveness in improving muscle function in dystrophin deficiencies (NCT00592553, clinicaltrials.gov). Presently, PTC124 appears to be well tolerated without adverse events and may have substantial promise. However, this therapy is only applicable to approximately 10% of the DMD population. Therefore, a therapy that replaces the defective gene would circumvent the need for mutation specific genetic approaches offering benefit to a broader DMD population.

The underlying defect in the DMD population is the inability to produce dystrophin. The total absence of dystrophin versus a truncated version dramatically affects the phenotypic outcome of the disease. Becker MD patients, with partial expression of dystrophin protein, present milder characteristics compared to DMD sufferers. Therefore, small variations of the dystrophin gene have been evaluated as possible therapies for DMD and
have been shown to ameliorate disease in the *mdx* mouse (Gregorevic, 2006; Liu, 2005; Wang, 2000; Wang, 2008). These micro and mini dystrophins have overcome the difficulties associated with gene delivery due to the limited capacity of viral vectors. These truncated variations fit well within the packaging capacity of vector constructs like adeno-associated viral (AAV) vectors, the leading viral vectors for human gene therapy. Due to the effectiveness of AAV-mediated gene delivery of micro-dystrophin in animal models of MD, a phase I clinical trial is now ongoing investigating its safety and tolerability in DMD boys (NCT00428935, clinicaltrials.gov).

Current proposed gene manipulation strategies targeted at increasing dystrophin expression are encouraging and have shown some degree of clinical benefit for DMD. However, they offer no hope for MD conditions in which the gene defect is unknown. Therefore, therapies that are applicable to a larger group of MD sufferers may be a more attractive agent. Treatment options that promote muscle growth and regeneration could be beneficial in both genetic and acquired muscle disorders. These agents could help improve dystrophic symptoms in conditions like cancer-related cachexia and sporadic inclusion body myositis as well as moderate and severe forms of MD of varying types.

An emerging theme in the study of the muscular dystrophies is the evaluation of the efficacy and feasibility of gene therapy in producing healthier, stronger muscles. The relatedness of extracellular proteins is a current area under review. Seminal studies have shown that specific alterations of transgenes of the muscle can ameliorate key aspects of
disease (Qiao, 2005; Bentzinger, 2005; Xu, 2007; Burkin, 2001; Meinen, 2007; Bogadanovich, 2002; Nguyen, 2002; Wagner, 2002; Burkin, 2005). Most of this work has been conducted utilizing the dystrophin-deficient \textit{mdx} mouse. Furthermore, \textit{mdx} mouse studies have also shown that the altered expression of a number of other genes \textit{besides} dystrophin is also effective in inhibiting certain aspects of muscle pathology or disease (Wagner, 2002; Bogdanovich, 2002; Nguyen, 2002; Nakatani, 2008; Xu, 2007). These alternative approaches, although not corrective, could enhance the stabilization of the dystrophic muscle, thereby enhancing muscle strength and function making them applicable to a number of diseases.

**Myostatin Inhibition for Enhanced Muscle Growth**

Myostatin \{formerly termed growth and differentiation factor-8 (GDF-8)\} is a member of the transforming growth factor-beta (TGF-\(\beta\)) family. Expressed in the myotome compartment of developing somites at E9.5 with expression continuing throughout adulthood, myostatin is found predominantly in skeletal muscle and tissue (Lee, 2004; Rebbapragada, 2003). Myostatin, like other TGF-\(\beta\) family members, is a secreted protein produced by proteolytic processing of a 375 amino acid precursor protein, resulting in an N-terminal propeptide and the C-terminal dimer, the mature and active ligand (Lee, 2004). The propeptide, when attached to the C-terminal dimer, renders the protein inactive, creating a latent complex preventing myostatin from binding to its receptor (Hill, 2002; Lee, 2001). Upon cleavage, myostatin is able to associate with the activin type IIB receptor (ActRIIB). This binding leads to the recruitment of the ActRIIB co-
receptor followed by the activation and phosphorylation of the TGF-β specific smads, leading to nuclear translocation of the protein complex and subsequent regulation of myogenic regulatory genes to inhibit muscle growth and development (Lee, 2001; Langley, 2002; Massague, 2000). In myogenic cells, myostatin reduces the expression of muscle specific transcription factors myogenin and myogenic differentiation factor (MyoD), as well as inhibits cell proliferation by downregulating Pax-3 and Myf-5 (Massague, 2000).

Myostatin-induced changes in muscle growth are not just a developmental phenomenon. In fact, myostatin overexpression in adult mice leads to muscle wasting. This suggests that myostatin elimination could enhance muscle growth. Indeed, myostatin inhibition results in an increase in myofiber size (hypertrophy) and number (hyperplasia) (McPherron, 1997; Wagner, 2005; Lee, 2004). These effects, however, are developmental-stage specific in that embryonic myostatin elimination results in hypertrophy and hyperplasia, while postnatal inhibition has no effect on fiber number, but increases myofiber size. These effects have been evaluated in a number of animals and are relevant in that myostatin is conserved among species. In fact, inactivation mutations have been observed in mice, dogs, cattle, sheep and humans (McPherron, 1997; McPherron, 1997; Shelton, 2007; Mosher, 2007; Bellinge, 2005).

Although exploratory efforts have been effective in highlighting the functional relevance of myostatin in helping to maintain the integrity of muscle under normal conditions, to
date little is known about the mechanism through which it exerts its muscle regulatory potential or the fundamental properties that underlie its aberrant functioning in disease states. Of particular importance is the fact that myostatin has been shown to be implicated in cachexic disorders like AIDS, as well as the progression of mild to severe muscular dystrophies (Bogdanovich, 2002, Wagner, 2002; Zimmers 2002; McFarlane, 2006; Bogdanovich, 2007; Patton, 2006; Bartoli, 2007). Thus, studies directed towards understanding the benefits of myostatin modulation in conditions of muscle wasting could offer unique ways to combat muscle disease.

Utilizing the dystrophic \textit{mdx} mouse, it has been shown that myostatin inhibition results in increased muscle growth, enhanced motor function and decreased pathology (Haidet, 2008; Bogdanovich, 2002; Bogdanovich 2005). This attenuation of disease results from both embryonic and postnatal myostatin elimination (Haidet, 2008; Nakatani, 2008; Wagner, 2002). Indeed, neutralizing antibodies directed towards myostatin result in improved conditions in adult \textit{mdx} and wild-type mice (Bogdanovich, 2002). These studies have translated to the clinic and the safety and tolerability of a monoclonal antibody to myostatin (MYO29) has been evaluated to improve treatment of various muscular dystrophies, including Becker, facioscapulohumeral and limb-girdle (Wagner, 2008). However, its benefits were minimal and marked by cutaneous hypersensitivity. Moreover, another caveat of this approach is that, antibody-directed myostatin inhibition would require repeated injections. This suggests that alternative methods for regulating myostatin expression would likely offer more promise.
Several other myostatin regulators have been identified: follistatin, follistatin-like related gene (FLRG) and growth and differentiation factor-associated serum protein-1 (GASP-1). The proteins have been shown to bind myostatin, thereby preventing it from activating the ActRIIB receptor (Fig. 2.6) (Sidis, 2006; Hill, 2002; Hill 2003; Amthor, 2004). Indeed, increased expression of these proteins results in increased muscle mass that has in some cases been more substantial than that which has been observed in myostatin null mice (Lee, 2001). The effectiveness of these myostatin regulatory proteins is not limited to muscle growth. Recent studies have indicated a role of myostatin in skeletal muscle fibrosis (Li, 2008; Bogdanovich, 2002). By binding to the ActRIIB receptor on fibroblasts, myostatin activates the canonical Smad signaling pathway leading to excessive formation of connective tissue that ultimately replaces muscle fibers. Interestingly, these unique myostatin inhibitors have been shown to result in decreased fibrosis and improved muscle pathology in dystrophic conditions (Haidet, 2008; Bogdanovich, 2002). These results suggest that myostatin inhibition may be an effective treatment approach for muscle enhancement in conditions of muscle wasting. However, the most effective means for myostatin inactivation should be identified and properly characterized to achieve optimal clinical efficiency.

To this end, one of the goals of this dissertation has been to study the most promising muscle modifiers to find an optimal therapy for stimulating muscle growth and strength in different dystrophic microenvironments. These efforts are aimed at highlighting a
functional relevance for myostatin inhibition in both moderate and severe forms of muscular dystrophy, thereby supporting the clinical evaluation of boosting muscle mass by targeted myostatin inactivation.

**Follistatin for Myostatin Inhibition**

Follistatin is an attractive agent for myostatin postnatal inhibition. An extracellular regulatory protein, follistatin has been shown to bind myostatin and members of the transforming growth factor- beta family with high affinity (Welt, 2002). Moreover, transgenic follistatin overexpression results in increased muscle mass and improved muscle function *in vivo* (Nakatini, 2007; Amthor, 2004; Thompson, 2005). A potent inducer of muscle growth, follistatin gene ablation results in skeletal abnormalities and reduced muscle mass at birth (Matzuk, 1995). However, the developmental abnormalities observed in follistatin gene disruption are not limited to skeletal muscle. In fact, mice deficient for follistatin display hair loss, XX sex reversal, bone deformities and premature neonatal death (Matzuk, 1995). This indicates that follistatin is essential for proper mammalian development.

Follistatin is produced by a number of tissues such as liver, gonads and muscle and exerts its regulatory potential to maintain liver homeostasis, wound repair, spermatogenesis, and ovarian follicle maturation (Sidis, 2006). Originally isolated from gonadal fluids, the earliest characterization of follistatin identified it as a follicle-stimulating hormone inhibitor. Thus, it was named for this function. However, the identification of follistatin
mRNA and protein expression in a number of tissues has demonstrated that its biological and biochemical properties exceed its initially proposed role in the endocrine system.

Follistatin protein is generated from the alternative splicing of the primary follistatin transcript (Fig. 2.7). The mRNA that is produced codes for two distinct forms: a full length cDNA (follistatin 344) that codes for 315 amino acids and a shorter polypeptide of 288 amino acids (follistatin 317) (Rodino-Klapak, 2009). A third isoform is also produced by the proteolytic cleavage of the 315 isoform and is suggested to be an intermediate between 315 and 288 (Lin, 2003). The shorter isoforms possess heparin surface binding activity that allows them to attach to cell surfaces (Innis & Hyvonen, 2003; Sidis, 2006). This property has been suggested to be the main mechanism through which follistatin affects reproductive physiology with activins and inhibins of the hypothalamic-pituitary-gonadal axis (Lee, 2004, Shimasaki, 1988, Hashimoto, 1997, Sumitomo, 1995; Shimonaka, 1991). However, this same activity is not exhibited by the 315 isoform which possesses a C-terminal acidic tail that prevents it from localizing to cell surface proteoglycans (Sugino, 1993). Therefore, it is the predominant serum-based type which lacks the ability to influence reproductive functions (Schneyer, 2004).

The biological activity of follistatin and its regulatory functions are implicated in a number of organ systems. Of particular importance is its role in growth enhancement. Indeed, transgenic follistatin overexpression results in muscle enhancement and growth (Nakatini, 2007; Amthor, 2004; Thompson, 2005). However, it appears that follistatin
doesn’t induce these changes by operating solely as a myostatin inhibitor. This is evidenced by studies in which follistatin transgenic animals exhibited increases in muscle mass significantly greater than those observed in myostatin null mice (Lee, 2001). The fact that follistatin is capable of operating independent of the myostatin pathway is also evidenced in studies in which the intercrossing of follistatin transgenic mice with myostatin knock-out mice resulted in a quadrupling of muscle mass compared to the double muscling effect observed in mice deficient for myostatin alone (Lee, 2007). This indicates that follistatin may play a role in mediating the activity of other muscle mass regulators with a function similar to that of myostatin. These findings suggest that other signaling pathways may be investigated to enhance various aspects of muscle disease.

Follistatin has also been shown to possess an activin binding property. Multifunctional cytokines, activins not only regulate the hypothalamic-pituitary-gonadal axis but also modulate proliferation of extracellular matrix proteins like muscle fibroblasts as well as the release of pro-inflammatory cytokines. Interestingly, follistatin overexpression leads to decreased tissue fibrosis and down-regulation of inflammatory response (Aoki & Kojima, 2007; Jones, 2007). This implicates a functional role of follistatin not limited to muscle enlargement in disease states.

Many members of the TGF-β family utilize the ActRIIB receptor to activate the Smad signaling pathway. Therefore, by preventing activation of the pathway associated with ActRIIB receptors, follistatin may possess a number of functional roles that are not
limited to its effects on any one particular ActIIB receptor ligand and could have a vast therapeutic potential in neuromuscular disease. This is important considering a myostatin inhibiting antibody approach failed to provide any substantial benefit to either aged delta-sarcoglycan deficient or late-stage dystrophin-deficient animals (Parsons, 2006). The tempered enthusiasm to targeted myostatin inhibition was also seen when myostatin gene knockout in an animal model of laminin-α2 deficient CMD (dy<sup>w/dyw</sup> mouse) resulted in increased postnatal mortality (Li, 2005). These observations suggest that myostatin inhibition may not be effective in combating muscle deficits in more severe disease states. However, follistatin overexpression has been shown to promote tissue growth and regeneration, thus enhancing muscle mass and function in young as well as aged mdx mice (Haidet, 2008; Lee, 2004). Therefore, a clinical measure that promotes muscle growth by regulating key transcriptional factors known to negatively impact muscle function could offer substantial benefit at varying levels of disease severity.

**Gene Therapy for Follistatin Administration**

Gene therapy is emerging as a promising and viable approach to alter the genetic expression of potent muscle regulators. With improvement of viral vector design over the years, our understanding of them has substantially been increased. As for muscular disorders, first generation Adenoviral vector (AV) studies involving AV-carrying truncated dystrophin have been shown to effectively reverse dystrophic features of the mdx mouse at various stages of disease (Yuasa, 1998; Kumar-Singh, 1996). Although there was high reward with the use of Adeno (Ad) vectors, there was also a high risk that
warrants mention. These first generation Ad vectors had limited cloning capacity and high immunogenicity. Consequently, it became necessary to develop viral vectors that could overcome these pitfalls. Investigators have now turned their attention towards recombinant adeno-associated viral vectors (AAV), a nonpathogenic virus that can persist in healthy tissue for years (Lai, 2002). AAV has fundamental properties that make it an attractive gene therapy vector. It is safe, non-pathogenic, can be introduced into multiple cell types, and has long-term expression (Lai, 2002; McCarty, 2001). It has a decreased risk of immunogenicity due to the elimination of all viral sequences except the 145-bp inverted repeats (ITRs) (Thomas, 2003). Furthermore, AAV has no etiologic association with any known diseases (Thomas, 2003).

Many AAV serotypes have been discovered with distinct efficacies in targeting certain tissues. Although serotype 2 has been the most extensively investigated, studies implicate that other serotypes such as AAV-1, 6, 8 and 9 may more effectively transduce skeletal muscle (Kwon, 2008; Coura Rdos, 2007; Zincarelli, 2008). The transduction efficiency of the varying serotypes depends on a number of factors. Tissue integrity, route of administration, animal age, dose and cellular immune response may determine how effectively the tissue is transduced (Kwon, 2007; Bostick, 2007). Moreover, the activity and efficiency of the promoters used to drive the gene of interest may also play a role in determining the expression profiles of the viral vectors (Gregorevic, 2004; Kota, 2009- accepted Science Translational Medicine). Another factor for consideration is that an important rate limiting step for viral vectors is the conversion of single-stranded
genomes to double-stranded DNA for gene expression. This is considered to be the rate-limiting step in viral vector applications. The use of self-complementary AAV (scAAV) vectors bypasses this step by allowing for earlier genetic expression for viral vectors (McCarty, 2008). However, the cloning capacity of these already size-limited vectors is greatly reduced in scAAV vectors. Therefore, careful attention must be made to determine the most effective means for gene administration when considering a potential therapy for the MDs.

The potential of AAV-mediated follistatin expression has been evaluated. Using an AAV1-driven follistatin approach under the control of the strong cytomegalovirus (CMV) promoter, it has been shown that follistatin overexpression results in increased muscle mass and function with decreased pathology in \textit{mdx} mice. These effects were seen in the injected hindlimbs as well as in muscles remote from the site of injection (Haidet 2008). Following vector administration, follistatin was secreted into the serum and was well tolerated with persistent expression for approximately 1 year after treatment (Haidet, 2008). Interestingly, there was also a significant reduction in serum creatine kinase, indicating that follistatin had a protective effect on muscle despite its dystrophin deficiency.

This dissertation takes a logical and translatable approach to understanding the efficacy and feasibility of using AAV technology to drive the expression of the potent myostatin antagonist, follistatin to improve disease symptoms in both moderate and severe animal
models of muscular dystrophy. These improvements will be evaluated based on
enhancement of motor growth and function coupled with a decrease in pathology.
Another aim of this thesis work is to determine the optimal route for vector
administration to result in the highest level of sustained expression with particular
attention being devoted to understanding changes in transcription and transduction
following gene delivery.
Figure 2.1: Egyptian relief painting from the 18th Dynasty.

Picture of an ancient Egyptian relief painting found on the wall of the tomb of the Queen of Punt in the Temple of Hatsheput. The picture dates from around 1500 BC. In the picture the queen appears to have calf enlargement which may have been drawn in an attempt to depict muscular dystrophy.
Fig. 2.2: Drawing on the wall of a tomb at Beni Hasan dating from the Middle Kingdom.

The three pictures were found on the wall of a tomb at Beni Hasan dating from the Middle Kingdom (2800 – 2500 BC). The picture of the far left seems to portray an individual with bilateral club foot and skeletal deformity. The drawing in the middle appears to depict an individual with enlarged calf muscles and flat feet as well as hypertrophy of the upper extremities. The picture on the right, however, appears to be a healthy individual with the characteristic pronounced arches of the feet and proportional features more characteristic of those commonly displayed in Egyptian wall paintings.
Muscles contain multinucleated, elongated fiber bundles that are encased in connective tissue. These bundles are highly vascularized and extensively innervated. They contain sarcomeric chains of thick myosin and thin actin that allow for muscle contraction and relaxation.
Fig 2.4: Depiction of the progression of Duchenne Muscular Dystrophy.

Patients with Duchenne muscular dystrophy usually present with proximal muscle weakness that is progressive. With time and continued muscle wasting, various other muscle groups are affected. These include intercostals, the diaphragm and cardiac tissue. A pseudohypertrophic effect can also be seen in muscles of the extremities where skeletal muscle has become replaced by adipose and fibrotic tissue.
Fig 2.5: Schematic of the dystrophin glycoprotein complex (DGC).

The DGC is a protein network comprised of transmembrane, extracellular matrix, nuclear membrane and cytoplasmic proteins and proteases. These proteins provide the structural and signaling scaffold for skeletal muscle. A number of muscle conditions can arise from defects in DGC components resulting in various forms of muscular dystrophy.
Figure 2.6: Schematic of the inhibition of the myostatin signaling pathway.

Muscle regulatory proteins like follistatin, follistatin-related gene (FLRG) and growth and differentiation serum factor-1 (GASP-1) bind myostatin to prevent its association with the activin type IB receptor and the initiation of a series of events that downregulate transcription factors responsible for muscle growth and development.
Figure 2.7: Schematic of follistatin isoforms.

Follistatin is produced by the alternative splicing of the mRNA to produce two distinct isoforms: follistatin 344 which codes for 315 amino acids and the shorter polypeptide follistatin 317 which codes for 288 amino acids. The 288 isoform represents the tissue bound form due to its heparin surface binding ability while the 325 isoform lacks this ability due to its acidic tail and is therefore the predominant serum circulating form.
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Table 2.1: List of recognized muscular dystrophies.

Several muscular dystrophies have been characterized and described. This table lists these recognized diseases, their pattern of inheritance as well as the gene defect responsible for them and the gene locus associated with them. MD = muscular dystrophy, CMD = congenital muscular dystrophy, LGMD = limb girdle muscular dystrophy, XR = X-linked recessive, AD = autosomal dominant, AR = autosomal recessive.
CHAPTER 3

METHODS

Mice

C57Bl/6, C57BL/10 and C57BL/10ScSn-DMD\textsuperscript{mdx}/J were purchased from The Jackson Laboratory. \(\text{dy}^w/\text{dy}^w\) mice were generated by intercrossing \(\text{dy}^w/+\) (Lama2+/-) mice, a gift from Eva Engvall (Burnham Institute). These mice have lacZ inserted into the Lama2 gene and have reduced levels of laminin-alpha2 protein. All control mice were heterozygote littermates derived from the same litters. All animals were housed in standard mouse cages with food and water ad libitum.

Non-Human Primates

Non-human primates (\textit{Cynomolgus macaques}) were a generous gift from Batelle (Columbus, Ohio) and housed at The Research Institute at Nationwide Children’s Hospital Non Human Primate facility (Columbus, Ohio). All animals were housed individually in standard primate cages. All studies were approved by and conducted in accordance with the standards set forth by the Institutional Animal Care and Use Committee and the institutions Vivarium Standard Operating Procedures (SOP) for the Husbandry of Non-human Primates.
Protocols for all studies were approved by the Institutional Animal Care and Use Committee and conducted in accordance with the Department of Agriculture Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals.

**Construction of AAV1 and self-complementary (sc) AAV9 expressing Follistatin**

The cDNA for human follistatin-344 was purchased from Origene (Rockville, MD) and subcloned into the AAV1 or scAAV9-ITR vector plasmids. For AAV1-MCK-FS production the human follistatin was subcloned into the KpnI/XbaI sites of an AAV-ITR vector plasmid containing the muscle creatine kinase promoter. For AAV1-CMV-FS production the human follistatin was subcloned into the EcoRI/XbaI sites into an AAV vector plasmid containing the cytomegalovirus (CMV) promoter. For scAAV9-CB-FS production the human follistatin was subcloned into the NotI sites of a double stranded AAV vector plasmid containing the chicken β-actin hybrid (CB) promoter.

**Viral Vector Production**

Recombinant AAV vectors were produced by triple transfection calcium phosphate precipitation in human embryonic kidney carcinoma HEK293 cells. AAV1 was produced using a single-stranded AAV2-ITR-based MCK or CMV vector with a plasmid encoding Rep2Cap9 sequence. AAV9 was produced using a double-stranded AAV2-ITR-based CB vector, with a plasmid sequence coding Rep2Cap9 sequence. For virus purification, clarified 293 cell lysates were obtained by sequential iodixanol gradient purification and ion exchange column chromatography using a linear NaCl salt gradient using vector
services from Virapur, LLC (San Diego, CA). Virus was dialyzed against phosphate buffered saline (PBS) and formulated with 0.001% Plutonic-F68 to prevent virus aggregation and stored at 4°C. Viral vector genome (vg) titers were determined by quantitative polymerase chain reaction (qPCR) techniques using Taq-Man technology.

**Mouse Injections**

C57Bl/6, C57BL/10 and C57BL/10ScSn-DMD<sup>mdx</sup>/J mice (n=10-15 per group) received bilateral intramuscular injections of a total dose of either $1 \times 10^{10}$ or $1 \times 10^{11}$ of DNase Resistant Viral Particles of an AAV1 expressing follistatin or AAV1-expressing green fluorescent protein (GFP) into the quadriceps (3-4 weeks of age) or gastrocnemius muscles (6.5 months of age) using a 0.3-cc insulin syringe.

$dy^w/dy^w$ and $dy^w/+-$ mice (n= 10-12 per group) were injected using a 0.3-cc insulin syringe with a total dose of either $5 \times 10^{10}$ DNase Resistant Particles of the AAV1 expressing follistatin into both gastrocnemius muscles at 7 days of age or $1 \times 10^{11}$ DNase Resistant Particles of the scAAV9 expressing follistatin via the facial (temporal) vein at P1. Control animals in each experimental group received 50µl of sterile PBS to either the gastrocnemius muscles or the facial vein. For all muscle injections, each respective muscle received half the total dose. Muscles were always injected into the mid-belly of the muscle.
**Mouse Behavioral Testing**

**Grip Strength**

All animals were tested for baseline hindlimb grip strength at 4 weeks of age using the grip strength meter (Columbus Instruments). For grip strength detection, mice were allowed to grasp a square metal grid connected to a force transducer with their hindlimbs and gently pulled down the length of the grid until the grip was broken. The transducer recorded the maximal resistance value of three trials. An average score of hindlimb grip strength performance was recorded.

**Coordination**

Animals were tested for baseline motor function at 3 weeks of age. Once baseline function was determined, motor function assessments were performed once a week. For rotarod performance assessment, each animal was subjected to 3-4 trials on an elevated accelerating rotarod beginning at 5rpm/min with continued acceleration until the animal can no longer maintain balance and coordination. An average score of rotarod performance was recorded.

**Ambulatory Movement and Rearing Events**

Ambulatory movements and rearing events were determined using the open field test. This measure consisted of one 30 min trial per animal. Mice were placed in the center of a 36” x 36” plexiglass box consisting of 36 equal squares with laser beams aligned evenly with the squares. Mouse movement is monitored and recorded based on beam/line
crosses and rearing events by advanced motion recognition software. All testing is carried out in a temperature, noise, and light controlled room.

**Mouse Survival**

To determine mortality in a reliable and humane fashion, an artificial endpoint will be used, defined by the inability of mice to right themselves 30 seconds after being placed on their sides. Moribund mice will be scored as “dead”, euthanized, and muscle tissues will be collected.

**Non-Human Primate Injections**

Briefly, animals were prescreened for antibodies to AAV1 and AAV8. Antibody-negative animals were divided into three groups: Control/No Treatment, AAV1-MCK-FS or AAV1-CMV-FS (n=3 animals/group). Primates in the AAV1-MCK and AAV1-CMV groups each received three intramuscular injections (5 mm apart) of a total dose of $1 \times 10^{13}$ viral particles of follistatin in 1.5 ml PBS (500μl/injection) into the right quadriceps muscle. Animals in each follistatin injection group were given immunosuppression by oral Tacrolimus (2.0 mg/kg) and Mycophenylate Mofetil (50 mg/kg) daily throughout the duration of the study beginning one week prior to rAAV1-follistatin administration.

For all AAV1-FS injections, animals were sedated with intramuscular Telazol (3-6 mg/kg) and maintained on 1 - 4% Isoflurane (in Oxygen) via tracheal intubation. Non-
human Primates also received intramuscular Buprenorphine (0.01mg/kg) to minimize pain due to injections. A warming blanket was secured to the animal to maintain an appropriate temperature of 37°C. The quadriceps muscle was shaved and sterilized with 95% EtOH and povidine®. Intramuscular injections were then made using a 26-guage needle. Immediately following injections, tattoos (Indian ink) were introduced at the injection sites to allow for tracing of injection sites in muscle biopsies. Primate breathing and vitals were persistently monitored before, during and after injections as well as during recovery from the procedure and anesthesia. No adverse reactions to anesthesia or injections were observed. Animals in all groups were routinely observed throughout the course of the study. Monkeys were euthanized by intravenous Sodium pentobarbital (1ml/10 lbs) at 5 or 14 months post injection and tissue was collected for biopsy.

Non-Human Primate Muscle Physiology

*Cynomolgus Macaques* were sedated with intramuscular injections of Telazol (3-6 mg/kg), intubated and secured to a procedural table as described above. Deep anesthesia by Isoflurane in Oxygen (4-5%) was administered and intramuscular Buprenorphine (0.1mg/kg) was given to minimize pain. The femoral nerve exciting the quadriceps muscle will be dissected and miniature platinum electrode clips will be affixed directly onto the nerve. These electrodes were connected to a stimulator (Scientific Instruments, STIM2) used to stimulate muscle contractions. Quadriceps muscle was then prepared free of skin, facia, and connective tissue. The hip was immobilized using restraining straps around the lower waist and upper thigh. Thereafter, the tendon connecting the
muscle near the knee was tied to a force-transducer (Imada, DS-2) using a metal hook. The connection between the tendon and the transducer was reinforced with suture. The tendon below this connection was then severed and the muscle was stimulated to contract via the platinum electrode. The force of contraction was measured by the force transducer and signals were recorded. Throughout the procedure deep anesthesia was maintained and primate breathing and vitals were persistently monitored. Immediately following skeletal muscle physiology, animals were sacrificed by intravenous Sodium pentobarbital (1ml/10 lbs) and tissue was collected for biopsy.

**Serum Creatine Kinase Assays**

Creatine Kinase activity assays were done using an enzyme-coupled absorbance assay kit (CK-SL; Diagnostic Chemicals Limited; Charlottetown PEI, Canada) according to the manufacturer’s instructions. Blood was collected from the tail vein of animals and allowed to clot for 30 minutes hour at room temperature. Clotted cells were centrifuged at 1500 x g for 10 minutes, and serum was collected and analyzed. Absorbance was measured at 340 nm every 30 seconds for 4 minutes at 25°C to calibrate enzyme activity. All measurements were done in triplicate and expressed as U/L (units/liter).

**Follistatin Enzyme-Linked Immunosorbent Assays**

Serum and muscle follistatin levels were evaluated using the human follistatin quantikine ELISA kit (R & D systems) according to the manufacturer’s instructions with normalization to controls. For serum ELISA assays, blood was collected from the tail
vein and allowed to clot for 30 minutes at room temperature. Clotted cells were centrifuged at 1500 x g for 10 minutes and serum was collected. For muscle follistain ELISAs, total soluble protein was isolated from the muscle using CellLytic MT Mammalian Tissue Lysis Agent (Sigma). A total of 100 µg of protein as determined by BCA assay (Pierce) was loaded per well and follistatin levels were estimated against a standard curve derived from a recombinant human follistatin provided by the manufacturer.

**Non-Human Primate Immune Studies**

Peripheral blood mononuclear cells (PBMCs) from cynomolgus macaques were collected before AAV1-follistatin administration and at monthly intervals throughout the duration of the study. An interferon-γ ELISpot assay was used to detect reactivity to human follistatin and AAV1 capsid antigens. To do so, 96-well PVDF microtiter plates (Millipore) were pre-coated with antibodies to monkey interferon-γ (U-Cytech, Utrecht, Netherlands) and 2 x 10^5 PBMCs in AIM-V medium containing 2% heat-inactivated human serum were added to each well. Duplicate wells received pools of overlapping synthetic peptides at a concentration of 1µg/ml prepared for the AAV1 capsid and the human follistatin. Plates were incubated at 37°C for 36 hours and developed for spot formation using a second antibody to interferon-γ conjugated to enzyme followed by substrate. Spot forming colonies (SFCs) in the microtiter wells were quantified using a CTL analyzer (CTL, Cleveland, CT). Green fluorescent peptide pool served as a negative control and concanavalin A was used as the positive control for cell viability.
For calculating the number of spots, negative control well spots were subtracted from each well and results were expressed as SFC per million PBMCs.

**Muscle Histology**

Muscles were dissected, weighed, snap frozen in liquid nitrogen-cooled isopentane and cryostat sectioned at 8 to 10 µm. Sections were either stained with hematoxylin and eosin (H &E) or immunostained with follistatin or laminin antibodies. Quantitation of central nuclei and myofiber diameters were done at or near the midsection of the infected skeletal muscle near their widest diameter as previously described. All myofibers were counted in each section analyzed, and all data was used in determinations of significance. Images were captured at 20X and diameters measured with a calibrated micrometer, using the AxioVision 4.2 software (Zeiss). All immunostaining was done as previously described.

**Fat Tissue Analysis**

The intra-abdominal fat pads (retroperitoneal, mesenteric, and gonadal) and the femoral fat pad were harvested from mice of all genotypes. Brown fat was collected from the scapular area. A percentage of total body fat (brown and white) was determined by weighing fat collected and expressing it as a percentage of total body weight.
**Muscle fiber size histogram**

Muscle fibers will be separated according to type by succinic dehydrogenase (SDH) or myofibrillar ATPase stain (pH 4.6). Referential type effects were evaluated (slow twitch oxidative, fast twitch oxidative, glycolytic, and fast twitch gylcolytic) for animals in each group. Muscle fiber size (diameter in \( \mu m \)) distribution histograms were generated from 8 randomly selected areas captured with a Zeiss AxioCam MRC5 camera under 20X objective and measured with a calibrated micrometer using AxioVison 4.2 software (Zeiss).

**Purification of recombinant follistatin**

cDNAs encoding recombinant, secreted and c-myc-tagged follistatin was transfected into HEK293T cells and secreted recombinant c-myc-tagged proteins were purified from the supernatant using chromatography in the presence of added protease inhibitors. Purified proteins were eluted from the column in Tris-buffered saline (TBS). Production of recombinant epitope-tagged protein was verified by immunoblotting using antibodies to follistatin and to the epitope tag and by silver staining of SDS-PAGE gels (for overall purity).

**Binding of follistatin to laminin-1 and merosin (laminin-\( \alpha 2 \))**

Either laminin or merosin were immobilized on ELISA plates at a concentration of 1\( \mu g \)/well in carbonate/bicarbonate buffer (50 mM, pH 9.5) overnight at 4°C. Wells were blocked with ELISA-buffer for 1 hr and descending concentrations (0 - 80 nM) of
purified c-myc-tagged follistatin were added and incubated overnight at 4°C. Respective wells were washed using ELISA-buffer and incubated with antibodies specific to the c-myc epitope for 2 hours. Respective wells were washed and incubated with respective secondary antibodies for 30 min. Wells were then washed with ELISA-buffer and substrate (SIGMA Fast™ OPD) was added to each well. Wells were read at absorbance 450

**Microarray**

RNA was prepared from the gastronemius muscles of 2 month old C57Bl6 mice that had been treated with AAV1-FS into the gastrocnemius muscle at 1 month of age. RNA isolated from the gastrocnemius muscle of non-injected, littermate controls was used for comparison. For microarray analysis, RNA from each was isolated using TRIZOL and further purified using the RNeasy kit (Qiagen); Valencia, CA). All samples were prepared in triplicate. Microarray was performed by the Microarray Core facility in The Research Institute at Nationwide Children’s Hospital (Columbus, OH) using 430 2.0 GeneChips® (Affymetrix Inc.; Santa Clara, CA). Gene expression of profiles from follistatin injected and control mice were obtained. Genetic changes of 1.5 fold or more were considered significant. Genes with significant changes in expression were grouped using DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov/).
Statistics

All statistical analysis was performed in Graph Pad Prizm software using a paired Student’s t-test; \(^\text{3P} < 0.05\); \(^\text{xxP} < 0.01\); \(^\text{xxxP} < 0.001\) or by multi-way ANOVA followed by Bonferroni post-hoc analysis of mean differences between groups. If needed, consultation with an onsite statistician was used.
CHAPTER 4

SINGLE ADMINISTRATION OF AAV1-FOLLISTATIN-344 ENHANCES MUSCLE MASS AND STRENGTH IN MDX MICE

Introduction

Duchenne Muscular Dystrophy (DMD) is an X-linked, progressively, lethal myogenic disorder that affects proximal limb, axial, cardiac and respiratory muscles. DMD is caused by mutations in the dystrophin gene that result in little to no dystrophin production. Hallmark characteristics of DMD include continual muscle wasting, delays in motor function, Gower’s sign, kyphoscoliosis, and some degree of cognitive impairment (Wagner, 2008; Emery, 2001; Emery, 2002). The progressive muscle weakness and degeneration leads to loss of ambulation and wheelchair dependency in the early teens (Emery, 2001). With time and continual loss of muscle, cardiac and respiratory failure ensues leading to death. Although investigations into DMD pathophysiology span more than a century, no definitive mechanisms for disease development and/or progression have been identified. Thus, DMD remains the most common youth myogenic disease and presumably the most detrimental with a high incidence of 1 in 3500 live male births (Aartsma-Rus, 2006; Wagner, 2008). Devastatingly, no optimal treatment options are available. In the muscular dystrophies,
only corticosteroids have, to some degree, altered the natural course of disease. Unfortunately, the benefit of steroids comes at a high cost in terms of side effects (bone loss, cataracts, delayed puberty, weight gain, and hypertension) (Angelini, 2007; Wagner, 2008). Therefore, exploratory efforts into alternative treatment options are warranted.

An emerging theme in the study of the muscular dystrophies is the evaluation of the efficacy and feasibility of gene therapy in producing healthier, stronger muscles. The relatedness of extracellular proteins is a current area under review. Seminal studies have shown that specific alterations of transgenes of the muscle can ameliorate key aspects of disease (Qiao, 2005; Bentzinger, 2005; Xu, 2007; Li, 2007; Nakatani, 2007; Moll, 2001; Bogadanovich, 2002; Meinen, 2007; Nguyen, 2002; Parsons, 2006; Moghadaszadeh, 2003; Barton, 2002; Wagner, 2002; Burkin, 2005). Of particular importance is the potential benefit of inhibiting myostatin to enhance muscle size and function. Expressed in the myotome compartment of developing somites at E9.5 with expression continuing throughout adulthood, myostatin is found predominantly in skeletal muscle and tissue (Lee, 2004; Rabbapragada, 2003). This secreted signaling molecule has been suggested to restrict skeletal muscle growth and myostatin inhibition results in increased muscle mass, from both hypertrophy (postnatal inhibition) and hyperplasia (embryonic and postnatal inhibition) (McPherron, 1997; Wagner, 2005; Lee, 2004). Although little is known about the mechanism through which it exerts its muscle regulatory potential, observations of myostatin deficient mice indicate a fundamental role of myostatin as a negative regulator of key myogenic differentiation factors of muscle development like
MyoD, Myf-5, and myogenin, which are usually downregulated in muscle wasting disorders (Massague, 2005). Therefore, a therapeutic strategy that inhibits myostatin expression could offer substantial benefit for DMD.

The \textit{mdx} mouse is the foremost laboratory model for studying dystrophin deficiency. With biochemical and pathological features similar to those found in DMD patients, lack of dystrophin results in cycles of degeneration-regeneration that not only decrease myofiber size but also impair functional ability (Bogdanovich, 2002). The delivery of neutralizing antibodies to myostatin has shown promise in mdx animals. However, the benefits of these pharmacological agents in advanced disease states are still questionable. Nevertheless, the identification of myostatin binding proteins that can inhibit its receptor-interactions and expression has expanded the potential for gene therapy to be considered as a method to inhibit myostatin activity. Amongst these binding proteins is the extracellular regulatory protein follistatin.

A myostatin interacting protein, follistatin binds myostatin with high affinity preventing its association with the ActRIIB. Moreover, follistatin overexpression results in increased muscle size and function in normal and dystrophic animals that is superior to that caused by other myostatin regulators such as follistatin-related gene (FLRG) and growth and differentiation factor-associated serum protein-1 (GASP-1) (Haidet, 2008). Furthermore, the potential for adeno-associated viral vector (AAV)-delivery of this “muscle booster gene” could greatly enhance MD gene therapy as these findings offer
implications for possible AAV serotype-1 (AAV1) delivered follistatin in humans. This work will provide the exploratory data to develop Muscular Dystrophy gene therapy treatments and provide novel tools to investigate the mechanism of myostatin inhibition postnatally. We have demonstrated the potential for follistatin to inhibit the effects of myostatin and shown that AAV1 is a suitable vector for muscle directed gene therapy. We now propose to evaluate the effects of follistatin in aged dystrophic \textit{mdx} mice. This effort will provide insight into the possible benefits of follistatin gene therapy in various muscular degenerative disorders.

\textbf{Results}

Follistatin (FS) is a naturally occurring glycoprotein that is produced by alternative splicing of the pre-mRNA to generate two different isoforms: FS-317 and FS-344. Peptide cleavage of these variants results in two active products with distinct functions and expression properties. FS-317 is cleaved to produce FS-288 which is the predominant type found to be associated with activins and inhibins of the hypothalamic-pituitary-gonadal axis and has the ability to bind tissue surface heparin sulfate proteoglycans (Lin, 2003; Rodino-Klapac, 2009). However, the FS-344 variant is cleaved to generate the serum-based circulating isoform, FS-315 which lacks the ability to interact with cell surface receptors due to its acidic C-terminal tail (Sugino, 1993). We chose the FS-344 variant to prevent any possible off-target effects that could be associated with reproductive functions. Moreover, we have previously demonstrated that
FS-344 delivered by AAV1 has no adverse effect on reproductive capacity in normal or dystrophic mice (Haidet, 2008).

We sought to determine the efficacy of FS-344 in a clinically meaningful paradigm of muscular dystrophy by utilizing the dystrophin deficient \textit{mdx} mouse. Recently, \textit{mdx} mice engineered to overexpress the follistatin transgene have been shown to exhibit increased muscle mass and an attenuation of pathology up to 15 weeks of age (Nakatani, 2008). However, we wanted to demonstrate the effectiveness of follistatin after postnatal administration in \textit{mdx} animals over a more sustained period of time. To do so, \textit{mdx} animals received bilateral injections of an AAV1 expressing follistatin driven by the cytomelagous (CMV) promoter into the quadriceps and tibialis anterior muscles. In these studies, 3 weeks old \textit{mdx} animals received either a low dose or a high dose of a total of 1 x 10^{10} or 1 x 10^{11} viral particles, respectively. Age matched control mice received similar injections bearing equal doses of AAV1-CMV expressing green fluorescent particles (GFP) into the respective muscles. Animals were evaluated for grip strength abilities over the course of 5 months. Immediately prior to necropsy serum was collected and analyzed for circulating FS expression levels. Serum FS levels were significantly higher in both groups with the high dose group expressing the greatest levels of detectable FS (high dose, 15.3 \pm 2.1 ng/ml; low dose, 6.8 \pm 0.4 ng/ml; GFP controls, 0 \pm 0.1 ng/ml; \( n = 8 \) animals per group; \( P < 0.01 \)).
This dose dependent response to FS treatment was also seen in grip strength abilities of treated mice when compared to GFP-treated controls. In AAV1-FS-injected animals followed for 180 days, increases in both hindlimb and forelimb grip strength abilities were improved after vector administration with the greatest effect seen in the high dose group (Fig. 4.1C). This increase in grip strength indicated an increase in overall muscle size. Indeed, gross observation as well as muscle wet weights of individual muscles demonstrated a significant increase in overall muscle mass in AAV1-FS treated animals (Fig. 4.1A & B). Histological and morphometric analyses demonstrated myofiber hypertrophy with no increase in myofiber number in injected as well as remote muscles (Fig. 4.2 A-D). This hypertrophic effect was further confirmed by analysis of total fibers per square millimeter area an indicator of myofiber density (Fig. 4.2 E). Therefore, fewer myofibers were observed per square millimeter area due to increased size of individual myofibers. The effectiveness of AAV1-FS was also seen upon pathological analysis of myofibers and serum enzyme markers. High dose AAV1-FS treated animals exhibited significant reductions in serum creatine kinase levels (Fig. 4.3 A) as well as reduced focal areas of fibrosis, necrotic muscle fibers and mononuclear cell infiltrates compared to GFP-treated controls (Fig. 4.1 A).

We also evaluated the histological, pathological, and behavioral effects of AAV1-FS on aged \( mdx \) mice. With time and continued use, muscles would have undergone more cycles of degeneration/regeneration and exhibit the appearance of more immune infiltrations and microtrauma. Therefore, evaluating the ability of FS to increase muscle
mass and function in a more advanced disease state could have a more meaningful therapeutic relevance than simply observing these changes in younger animals. To investigate these conditions, 210-day old *mdx* mice were injected with AAV1-FS and monitored up to 560 days of age. Muscle damage and pathology was clearly evident before treatment at 180 days of age (Fig 4.3 C, left panel). However, pathological evaluation of gastrocnemius (Fig. 4.3 C, middle and right panels) and diaphragm muscles revealed that a 560 days of age (Fig. 4.3 D), AAV1-FS-treated animals exhibited fewer focal areas of endomysial connective tissue, immune infiltrates and necrosis as indicated by H&E stain. Follistatin-induced hypertrophy was also evident in aged treated *mdx* mice, with increases in the size of individual myofibers (Fig 4.3 C). Furthermore, this increase in size translated to an increase in muscle in function with significant increases in hindlimb grip strength in AAV1-FS-treated animals compared to GFP-treated controls (Fig.4.3 B). These results suggest that myostatin inhibition by FS-344 could be effective in instances of moderate as well as advanced disease states.

**Discussion**

Increasing muscle mass and strength to attenuate the pathological features of conditions of chronic muscle wasting represent an important goal for a number of proposed treatment strategies. The relatedness of muscle regulatory proteins is a current area under review. From hallmark studies into the unique ability of negative muscle regulators to suppress muscle growth and development, it is apparent that the altered expression of certain muscle signaling molecules can have substantial effects on determining the
progression of muscle disease. The use of follistatin, a potent antagonist of the muscle suppressor myostatin, appears to be a powerful treatment paradigm that has benefit in both moderate and advanced cases of muscle wasting. This muscle booster gene coupled with AAV technology offers a logical and feasible approach for sustained gene expression of follistatin that circumvents the use of repeat pharmacological administration which would be necessary for small molecule or neutralizing antibody-based therapies. Therefore, AAV1-FS could result in long-term improvement of muscle size and strength that evades the possibility for immune response to antibody treatment and the requirement for multiple injections.

Follistatin delivered to \textit{mdx} animals in this study represents the serum-based circulating isoform, FS-344. This isoform primarily affect skeletal muscle with no observed changes in cardiac tissue. Because, this isoform lacks the ability to bind heparin sulfate proteoglycans, we suspect it to have minimal if any effect on reproductive physiology and function. This correlates with previous studies indicating no change in reproductive ability of AAV1-FS injected animals (Haidet, 2008).

The efficacy of AAV-FS to increase muscle mass and function was demonstrated in both young and aged dystrophic animals. However, the effectiveness of FS appeared to be dose dependent with the high dose group achieving the greatest muscle size and functional improvement. These effects were seen in injected muscles as well as remote muscles. This suggests that follistatin was in fact secreted into the serum and was able to
target non-injected muscle groups. These increases in size translated to increases in function with increases in both hindlimb and forelimb grip strength abilities. This suggests that FS was able to preserve muscle integrity and sustain muscle tone. This is important considering the lack of correction of dystrophin deficiency which has been shown to result in reduce muscle functional abilities. The effectiveness of follistatin also appeared to prevent certain pathological features of disease associated with muscle wasting. Injected animals demonstrated fewer instances of immune cellular infiltrates, fibrosis and necrosis, hallmark features of muscle disease and DMD.

The fact that FS had such a striking effect in aged animals suggests that it could offer clinical benefit in more advanced stages of DMD independent of dystrophin replacement. Moreover, the use of FS in conjunction with other proposed therapies such as microdystrophin or insulin-growth factor 1 (Igf-1) could set the stage for combination therapies aimed at gene correction or satellite cell enhancement. Microdystrophin represents a gene replacement strategy to restore dystrophin expression and function in DMD. Coupled with follistatin it could produce bigger, stronger, dystrophin expressing muscles offering a more profound therapeutic benefit than either therapy alone. Moreover, as a natural defense against muscle disease, satellite cells are activated. However, degeneration-regeneration cycles exhaust satellite cell functional abilities. Nevertheless, Igf-1 stimulates satellite cell differentiation and proliferation (Philippou, 2007). Therefore, follistatin gene therapy coupled with enhanced myofiber production could promote tissue growth and regeneration.
Follistatin delivered by a single injection of AAV1 is well tolerated with sustained expression for more than 2 years. Because of its involvement in multiple signaling pathways, including in attenuating inflammation (Aoki & Kojima, 2007; Jones, 2007) it could prove to be a more useful treatment strategy than mere myostatin inhibition alone. Its ability to provide gross and functional improvement without adverse events at varying stages of muscle disease warrants its consideration for clinical evaluation for musculoskeletal disorders, including those without a known genetic defect.
Figure 4.1: A single injection of AAV1-FS increases skeletal muscle mass and strength in mdx mice injected at 3 weeks of age.

(A) Gross hindlimb muscle mass of mice injected with AAV1-FS at 3 weeks of age is increased at 180 days of age compared to AAV1-GFP-injected controls. (B) The mass of individual hindlimb and forelimb muscles is increased at 180 days of age compared with AAV1-GFP controls. (C) Grip strength is increased at 180 days of age in a dose-dependent manner in young mdx mice injected at 3 weeks of age with AAV1-FS. Red = high dose AAV1-FS, Blue = low dose AAV1-FS, Green = AAV1-GFP controls. Error bars represent standard errors.
Fig 4.2: *mdx* mice treated with AAV1-FS at 3 weeks of age and followed for 180 days demonstrate myofiber hypertrophy.

(A) H&E staining of the tibialis anterior reveals myofiber hypertrophy in AAV1-FS injected muscle compared to AAV1-GFP treated controls. (Original magnification, X40).
(B) The mean diameter of dark (slow-twitch, oxidative), intermediate (fast-twitch oxidative glycolytic) and light (fast-twitch glycolytic) myofibers in the tibialis anterior (indicated by dotted line) is significantly increased in mice injected with AAV1-FS compared with AAV1-GFP-treated controls. (C) The mean diameter of intermediate and light myofibers (indicated by dotted line) in the triceps is significantly increased in mice injected with AAV1-FS compared with AAV1-GFP-injected controls. (D) The distribution of dark, intermediate and light fibers as determined by fiber typing is not changed by treatment with high or low doses of AAV1-FS. (E) The myofiber densities are decreased in the tibialis anterior of AAV1-FS injected mice, given that the mean diameter of myofibers is increased. Error bars represent standard errors.
Figure 4.3: Single injection of AAV1-FS increases muscle strength and decreases pathology in aged mdx mice.

(A) Serum creatine kinase levels (units/liter) are decreased at 3 months after AAV1-FS injection compared to AAV1-GFP treated controls. Error bars represent standard errors. (B) Hindlimb grip strength is significantly increased at 275 days and beyond in aged mdx mice treated with AAV1-FS at 210 days of age. Red = high dose, Green = AAV1-GFP-injected controls. (C) H&E stain of the gastrocnemius muscles of age mdx mice injected at 180 days of age demonstrated reduced pathology at 560 days. Error bars represent standard error.
CHAPTER 5

AAV1-FOLLISTATIN ENHANCES MUSCLE MASS AND FUNCTION IN NON-HUMAN PRIMATES

Introduction

Cachexic conditions that result in loss of muscle mass, tone and function have few satisfactory treatments. These genetic and acquired disorders that include diseases such as acquired immunodeficiency syndrome (AIDS), cancer, aging and the muscular dystrophies are often treated with androgen steroids, which pose long term risks. Although gene manipulation strategies are currently being investigated as potential therapies for genetic muscle disorders such as Duchenne Muscular Dystrophy (DMD), these approaches are not clinically relevant to disorders without a known gene defect such as sporadic inclusion body myositis (sIBM) or fasioscapulohumeral muscular dystrophy (FSHD). However, recent work investigating the regulatory mechanism that skeletal muscle uses to control tissue mass has identified a transforming growth factor β (TGF-β) superfamily member as a negative regulator of muscle growth. Myostatin, formerly known as growth and differentiation factor-8 (GDF-8) has been shown to restrict muscle size and development by suppressing key transcriptional regulators for myogenic cell proliferation and differentiation (Massague, 2000). Indeed, targeted
myostatin disruption results in increased skeletal muscle mass. Consequently, the use of
differential antagonists to inhibit myostatin is a current area under review.
Several approaches have been attempted to alter the myostatin regulatory pathway and
thus increase muscle mass. Several new proteins have been identified and shown to be
capable of binding and inhibiting the activity of the myostatin C-terminal dimer. A
neutralizing antibody approach to myostatin has been tested in mdx mice and has been
shown to result in an increase in body weight, muscle mass, muscle size and absolute
muscle strength along with a significant decrease in muscle degeneration and
concentrations of serum creatine kinase (Bogdanovich, 2002). On the basis of these
studies, a clinical protocol has been approved by FDA for a phase II study of MYO29
(antibody to GD8 or myostatin) in muscular dystrophies of various types, including
Becker, facioscapulohumeral, and limb-girdle dystrophies (Wagner, 2008). However, the
dose escalation studies into the clinical potential of MYO29 were limited by cutaneous
hypersensitivity (Wagner, 2008). Moreover, this antibody approach is also limited by the
fact that it would require repeated injections over time.

The discovery of certain myostatin regulators has expanded the potential for gene therapy
to be considered as a method to inhibit myostatin activity. Follistatin, which binds to
some TGF-B family members with high affinity and functions as a potent myostatin
antagonist. Overexpression of follistatin in muscle has been shown to increase muscle
growth in vivo (Nakatani, 2008; Amthor, 2004; Thompson, 2005). Furthermore, lack of
follistatin results in reduced muscle mass at birth (Matzuk, 1995).
The effectiveness of follistatin in conditions of muscle wasting has been evaluated using the dystrophin deficient \textit{mdx} mouse. With pathological features similar to those seen in DMD patients, follistatin overexpression has been shown to result in increased muscle mass, enhanced overall strength and reduced pathology in both young and aged dystrophic animals. These observations set the stage for the clinical evaluation of follistatin gene therapy in humans. However, before the safety and tolerability of this potential therapeutic can be considered for the treatment of neuromuscular disease in humans, it must first be tested in larger animals.

Non-human primates (NHPs) represent an ideal group for preclinical investigations into the therapeutic potential of AAV1-FS to enhance muscle mass and function. Given that the human FS-344 used in these studies has a 98% homology to non-human primate follistatin, the evaluation of this group is a relevant model for investigating the potential benefits of follistatin in large animals. Moreover, as for any potential therapeutic, the experimental assessment of follistatin in sophisticated biological systems is an important criterion for its advancement to clinical trials. In this study, we extend our knowledge into the effectiveness of follistatin by adopting a paradigm that could be directly applied to patients. To do so we investigated the potential of follistatin to increase muscle size and physiological functioning in the quadriceps muscle of \textit{Cynomolgus macaques}. The targeting of this muscle group is relevant in that the quadriceps muscle is severely affected in conditions of sIBM. By targeting this muscle group, we set the stage for the
clinical feasibility of this gene therapy approach in diseases where preferential weakness of knee extension is a prominent feature. The primary outcome variables for this study were demonstration of safety and efficacy.

Results

The AAV1 vector bearing the FS-344 variant was administered to the right quadriceps muscle of normal *Cynomolgus macaques* under the control of the cytomegalous (CMV) or muscle creatine kinase (MCK) promoter. The left quadriceps muscle served as the contralateral control. Prior to injections, all animals were prescreened for antibodies to AAV1 and AAV8. Antibody-negative animals were divided into three groups: Control/No Treatment, AAV1-MCK-FS or AAV1-CMV-FS. Non-Human Primates (NHPs) in the AAV1-MCK and AAV1-CMV groups each received three intramuscular injections (3.0 cm apart) in a diagonal line extending from proximal (7 cm below the inguinal ligament) to the midpoint of the quadriceps muscle of a total dose of $1 \times 10^{13}$ viral particles of follistatin in 1.5 ml PBS (500 μl/injection) (Fig. 5.1 A). Injection parameters were designed to allow for vector administration to the bellies of all branches of the quadriceps muscle. Immediately following vector administration, Indian ink was administered to tattoo the injection site (Fig 5.1 A). Animals in both the AAV1-MCK-FS and AAV1-CMV-FS groups received on tacrolimus (2.0 mg/kg) and mycophenylate mofetil (50 mg/kg) two weeks prior to vector administration and were maintained on immunosuppression throughout the study.
To evaluate increased levels of follistatin expression after vector administration, one animal from each group was necropsied at 5 months (20 weeks) and 15 months (60 weeks) after follistatin injection. Muscle sections taken from the quadriceps muscle were analyzed for human specific follistatin expression. Analysis of total soluble protein extracted from muscle sections revealed a significant increase in follistatin expression for CMV-FS treated NHPs at both 5 and 15 months post gene transfer (>20 ng/mg tissue) (Fig. 5.1 B). However, only a minimal amount of follistatin could be detected in MCK-FS treated animals. This suggested that the CMV promoter may be a better tool for more robust follistatin expression and thus muscle enhancement.

To assess increases in muscle size after vector administration, external circumference of the thigh muscles at the midpoint of the quadriceps muscle was measured monthly after gene transfer up to 20 weeks post vector administration. Animals in both the MCK-FS and CMV-FS groups demonstrated increases in muscle size with the greatest effect seen in the CMV-FS group (CMV-FS = 15% increase over baseline, \( P = 0.01 \); MCK-FS = 10% increase over baseline, \( P = 0.02 \)) with a peak in increase by week 8, followed by a plateau in growth through week 20 (Fig. 5.1 C). Upon necropsy at 20 weeks, animals in both the CMV-FS and MCK-FS treated groups demonstrated significant increases in the size of myofiber diameters compared to non-treated controls (CMV-FS = 87.7 µm ± 18.9 µm; \( P = 0.001 \); MCK-FS = 73 µm ± 12.6 µm; \( P = 0.04 \); Control = 65.5 ± 11.9) (Fig. 5.2 A&B).
Morphometric analyses of muscles in both CMV-FS and MCK-FS treatment groups suggest that follistatin has a more pronounced effect on the myofiber diameters of select fiber types. Myofibrillar ATPase stain (pH 4.6) of myofibers in both groups demonstrated that follistatin treatment primarily affected fast-twitch-oxidative type 2a (CMV-FS = 85.4 µm ± 12.6 µm; MCK-FS = 71.4 µm ± 14.7 µm; Control = 66.3 µm ±10.5 µm) and fast-twitch-glycolytic type 2b fibers (CMV-FS = 102.3 µm ± 11.0 µm; MCK-FS = 79.3 µm ± 8.3 µm; Control = 77.0 ± 11.9 µm) (Fig. 5.2 C-E). This change in fiber size did not change the proportion of fiber types found in the muscles of either of the groups indicating that this increase in size was due to myofiber hypertrophy with no fiber type switch (Fig. 5.2D). The increase in circumference was sustained over the course of 60 weeks without significant loss. Due to this sustained growth, muscle enlargement could be appreciated by gross observation at the time of necropsy at 5 months after injection in both the MCK-FS and CMV-FS treated groups (Fig. 5.1 D).

Muscle enlargement has been correlated with increases in muscle function (Haidet, 2008). To determine if the follistatin-induced increases in muscle size translated to an enhancement of strength, muscle twitch force was assessed in two MCK-FS-treated and one CMV-FS–treated animal. To do so, the quadriceps muscle was prepared free of skin, fascia, and connective tissue. The hip was immobilized using restraining straps positioned around the lower waist and upper thigh and the distal tendon was disconnected at the patella and secured to a force-transducer (Imada, DS-2). Upon stimulation twitch and tetanic contractions were recorded and compared to that exhibited by the
contralateral quadriceps muscle. Therefore, each animal served as its own control.

Twitch force measures for the two MCK-FS-treated macaques were analyzed. One MCK-FS treated animal exhibited an 11.8% increase while the other NHP had a 35.7% increase when compared to the contralateral untreated leg. These same animals were evaluated for increases in tetanic force and they demonstrated 12.3% and 77.9% increases (respectively) when compared to the opposite leg. The CMV-FS treated macaque showed a 26.3% increase in twitch force and a 12.5% enhancement of tetanic force when compared to its untreated side.

To determine if follistatin treatment elicited an adverse immune response, peripheral blood mononuclear cells (PBMCs) were isolated monthly and the immune response to AAV capsid and follistatin was determined in both MCK-FS and CMV-FS treated NHPs. There were no detectable antigen-specific IFN-γ responses found in any treatment group (Fig. 5.3 A&B). Moreover, cardiac, lung, liver, spleen, kidney, testis, ovary and uterus tissue were not affected as assessed by gross observation and histological examination in either the MCK-FS or CMV-FS treatment groups.

Discussion

Follistatin administration in NHPs represents a unique way to assess any potential adverse events that could result from transgene administration in large animals. The preclinical evaluation of these conditions is a necessary criterion for testing this therapy in humans. From these studies it appears that AAV1 directed follistatin expression is
well tolerated and was effective in increasing muscle mass, individual myofiber size as well as enhancing function in the quadriceps muscle of *Cynomolgus macaques* at both 5 and 15 months post vector administration. Skeletal muscle was exclusively affected and no adverse effects on other organ systems were detected. This is believed to be due to the diminished ability of the FS-344 variant to bind heparin sulfate proteoglycans found on cell surfaces therefore reducing its ability to affect non-muscle tissue.

Follistatin expression coupled with the robust nature of the AAV vectors resulted in sustained expression over the course of the study. Although, there was a peak in expression around 8 weeks after vector delivery and a relative plateau during the first 5 months, significant levels of follistatin could be detected up to 15 months post gene transfer. This suggests that a muscle regulatory system was able to maintain follistatin expression, yet prevent uncontrollable growth. The exact nature of this mechanism is unclear. However, these observations are consistent with those seen in spontaneous myostatin inhibition in cattle, mice and dogs (McPherron, 1997; McPherron, 1997; Shelton, 2007; Mosher, 2007).

The differences in follistatin levels achieved by CMV and MCK promoter-driven expression led us to conclude that there are differences in the activity profiles of different promoter systems. From our studies it appears that the CMV promoter was more effective in achieving high levels of gene expression. These observations are consistent with findings of studies investigating CMV vs MCK driven gene expression in *mdx* mice.
However, there is a degree of concern regarding the use of the ubiquitous CMV promoter in that studies have shown that these viral promoters may elicit an immune response in non-muscle tissue (Wang, 2008). These issues were not encountered in our studies. Gross and histological examination of various non-muscle tissues indicated no adverse events, toxicity or initiation of immune response to either the vector or the transgene. Therefore, the CMV promoter appears to be a suitable medium for driving follistatin gene expression \textit{in vivo}.

We have demonstrated the effectiveness of AAV1–FS muscle wasting conditions of a known genetic defect in previous studies using the \textit{mdx} mouse (Haidet, 2008). However, these studies allude to its therapeutic in acquired diseases where the origin is unknown such as sIBM or FSHD. Although we’ve demonstrated the effectiveness of follistatin in normal muscle using NHPs, this does not take away from the clinical relevance of follistatin as a beneficial agent for chronic inflammatory conditions. Our previous work addressing myostatin inhibition in aged \textit{mdx} mice, where immune infiltrations are prevalent and there are repeat cycles of degeneration/regeneration, we show that FS expression is sustained for periods greater than 1 year after vector administration. Moreover, the reduction in serum creatine kinase levels and decreased pathology in these animals demonstrate the efficacy of follistatin despite the waves of muscle regeneration.
known to characterize this model (Haidet, 2008). We therefore conclude that AAV1-FS is a suitable treatment option for addressing muscle wasting conditions associated with active muscle regeneration and inflammation in costly and severe cases of muscle disease.
Figure 5.1: *Cynomolgus macaques* injected with AAV1-FS show increased expression and enhanced muscle growth.

(A) Non-human primates were injected with either AAV1-MCK or AAV1-CMV into the right quadriceps muscles. For treatment, three equally spaced injections were administered. (B) Muscle follistatin expression was analyzed by ELISA at 5 and 15 months post vector transfer. (C) The circumference of the thigh following muscle injections was increased in both treatment groups with the greatest effect seen in AAV1-CMV injected animals. (D) Gross quadriceps mass is increased after AAV1-FS treatment. Error bars represent standard deviation.
Figure 5.2: AAV1-FS causes myofiber hypertrophy and predominately affects type 2 myofibers in the quadriceps muscle of *Cynomolgus macaques*.

(A) H&E stain of the injected (right) quadriceps muscle of AAV1-FS treated *macaques* (right, representative picture from the CMV-FS group) compared to untreated control (left).  (B) Morphometric analysis of the quadriceps muscle 5 months after injection demonstrated a significant increase in mean diameter (indicated by dotted lines) in both MCK-FS and CMV-FS injected animals compared to controls.  (C) Representative fiber types demonstrated by ATPase stain (pH 4.6) of CMV-FS injected animals (right) and controls (left).  (D) Fiber type ratios in control, CMV-FS and MCK-FS treated muscles.  (E) The mean diameter (indicated by dotted line) of type 1, type 2a and type 2b fibers for control, CMV-FS and MCK-FS treated quadriceps muscles.  Error bars represent standard deviation.
Figure 5.3: No cellular immune response to follistatin-344 or the AAV1 capsid was observed after vector administration.

(A) PBMCs were isolated prior to muscle injection and at monthly intervals after injection for stimulation with human follistatin peptide pools (I and II) and AAV1 capsid peptide pools (I, II or III) or concanavalin A (positive control). IFN-gamma release was measured by ELISpot assay and representative data are shown (3 months after injection). (B) The mean number of spot forming colonies (SFCs) for each antigen is shown over time for each treatment group (n = 3). The increase in SFCs did not exceed 25, (red dashed line) the threshold for significant in the assay. Error bars represent standard deviation.
CHAPTER 6

FOLLISTATIN GENE THERAPY IMPROVES SYMPTOMS IN A SEVERE MOUSE MODEL OF CONGENITAL MUSCULAR DYSTROPHY

Introduction

Congenital Muscular Dystrophy (CMD) is a group of autosomal recessive, muscle wasting disorders that often result in premature death (Mendell, 2006; Lisi, 2007). The most common of the CMDs, merosin-deficient CMD (MDC1A) is caused by mutations in the laminin-alpha(α)2 gene that result in altered protein expression. Laminin-α2 is important because through its binding with the dystroglycans, which in turn bind the intracellular protein dystrophin, it is believed to help anchor the inside to the outside of the muscle cell (Kuang, 1998; Ryan, 1996; Patton, 1999). This protein network which merosin helps to create preserves the integrity of the muscle and protects the muscle fiber from contraction-induced damage. Consequently, loss of laminin-α2 results in disruption of the basal lamina and severe muscular dystrophy.

To date, there are no effective treatments for MDC1A in patients. However, its pathogenesis has been studied utilizing several mouse models including the dyw/dyw mouse (Kuang, 1998; Guo, 2005; Guo, 2003). Deficient for laminin-α2, these mice have a severe phenotype, which can result in death in as little as 3 weeks (Guo, 2003). The
chronic muscle loss, poor regeneration, and inflammatory pathology of the $\text{dy}^w/\text{dy}^w$ mouse make it a suitable model to investigate potential therapeutics for severe forms of muscular dystrophy.

In the muscular dystrophies, muscle enhancing agents appear to offer great promise. Specifically, the inhibition myostatin has been shown to result in increased muscle mass and reduced pathology in dystrophic animals (Bogdanovich, 2002; Bogdanovich, 2008; Bartoli, 2007). Most of these studies have been conducted utilizing the dystrophin-deficient $\text{mdx}$ mouse (Haidat, 2008; Nakatani, 2008; Wagner, 2002). These efforts have been demonstrative in highlighting the effectiveness of muscle enhancers in muscle wasting diseases with a mild phenotype. This in turn led to investigation of these same factors in more severe animal models of muscular dystrophy. However, myostatin null $\text{dy}^w/\text{dy}^w$ mice experienced more pre-weaning deaths and had decreased essential fat (Li, 2005). These early postnatal deaths are reminiscent of cattle studies in which there were more premature deaths before weaning depending on the level of reduced myostatin copies (Casas, 1999; Casas, 2004). These studies reiterated the fact that prenatal knockout studies carry a high risk that may not always outweigh the rewards.

Recently, Martin and colleagues have reported that embryonic transgenic overexpression of a glycosyl transferase that, in turn, increases expression of an extracellular matrix protein results in inhibition of pathology, but decreased myofiber size and inhibition of muscle development (Nguyen, 2002). However, adeno-associated virus (AAV) vector
delivery of the same transgene resulted in increased muscle mass and growth (Xu, 2007; Chandrasekeharan, 2009). Their results demonstrated that the unwanted effects of protein transgenic expression could be divorced from its therapeutic benefit if the transgene is expressed in the early postnatal period before the onset of disease pathology (Xu, 2007). Also, the beneficial effects of prenatal vs postnatal gene inactivation may be disease-specific. Antibody-mediated myostatin blockade in gamma-sarcoglycan-deficent (Sgceg -/-) mice resulted in increased muscle mass, force and myofiber size (Bogdanovich, 2008). However, there were no improvements in pathology. This is quite different from effects seen in mdx studies in which prenatal and postnatal myostatin inhibition resulted in increased muscle mass and reduced dystrophic pathology with no negative effects on lifespan (Nakatani, 2008; Haidet, 2008).

Follistatin is an attractive agent for myostatin postnatal inhibition. It has been shown to bind members of the transforming growth factor-beta family and transgenic overexpression results in increased muscle growth in vivo (Welt, 2002). Originally identified as a follicle-stimulating hormone inhibitor, follistatin has also been shown to possess an activin binding property that has been suggested to lead to decreased tissue fibrosis and down-regulation of the inflammatory response (Aoki & Kojima, 2007; Jones, 2007). This implicates a functional role of follistatin not limited to myostatin inhibition in disease states. The fact that follistatin is capable of operating independent of the myostatin pathway is also evidenced in studies in which the intercrossing of follistatin transgenic mice with myostatin knock-out mice resulted in a quadrupling of muscle mass
compared to the double muscling effect observed in mice deficient for myostatin alone (Lee, 2007). This indicates that follistatin may play a role in mediating the activity of other muscle mass regulators that possess a function similar to that of myostatin.

The use of AAV driven transgene expression has shown substantial promise in recent clinical studies (Worgall, 2008; Kaplitt, 2007). The attractiveness of AAV lies in the fact that it is a nonpathogenic virus that can be introduced into multiple cell types providing for long-term transgene expression and can persist in healthy tissue for years. Furthermore, AAV has no etiologic association with any known diseases (Thomas, 2003). Many AAV serotypes have been shown to have distinct efficacies in targeting certain tissues. Although serotype 2 has been the most extensively investigated, several studies implicate that serotype 1 may more effectively transduce skeletal muscle while more recently discovered serotypes such as AAV6, AAV8, and AAV9 are efficient in transducing a broader range of tissues such as liver, lung, the central nervous system and cardiac and skeletal muscle (Zincarelli, 2008; Coura Rdos, 2007).

In this study we show that a single injection of AAV vectors of varying serotypes carrying the follistatin transgene resulted in increased body weight, muscle growth and strength in severely dystrophic dyw/dyw mice and non-dystrophic dyw/+ controls. To investigate the unique tropism of AAV1 driven follistatin for postnatal myostatin elimination we administered bilateral intramuscular injections into the gastrocnemius muscles of 7 day old mice and compared its effectiveness to AAV9 directed follistatin
expression after intravenous administration into the facial vein of neonates. We found that the greatest benefit to the severely dystrophic laminin-α2 deficient mouse was seen after AAV9 systemic follistatin delivery, which allowed for a larger biodistribution of expression. Moreover, follistatin-treated dy^w/dy^w mice in both treatment groups exhibited decreased fibrosis and pathology as well as increased survival. Here, we show that follistatin treatment may be an effective therapy for not only increasing muscle mass, but also improving motor functional deficits in both moderate and severe forms of muscular dystrophy.

**Results**

To determine the effectiveness of follistatin in non-dystrophic muscle, mice heterozygote for laminin-α2 (dy^w/+ ) were given a total dose of 5 x 10^{10} AAV1 viral particles encoding follistatin (AAV1-FS) or 50µl of PBS bilaterally into the gastrocnemius muscle at 7 days of age. All animals treated with AAV1-FS demonstrated significant increases in whole body mass beginning at around two months of age with continued significant growth up to three months post injection (Fig. 6.1A). Histological analysis of the gastrocnemius muscles reveals myofiber hypertrophy in AAV1-FS injected animals (Fig. 6.1B). Evaluation of the effects of the follistatin transgene on individual muscles (wet weights) of the hindlimbs of mice heterozygote for laminin-α2 showed an increase in muscle mass for all AAV1-FS injected mice when compared to PBS-treated controls (P < 0.001 for gastrocnemius and tibialis anterior and P < 0.0001 for quadriceps) (Fig. 6.1C). However, there were no significant differences in the size of the triceps muscles of FS-treated mice.
when compared to PBS-injected animals (Fig. 6.1C). Accordingly, myofiber diameters were significantly larger only in the hindlimb muscles of FS treated animals (Fig. 6.1D). Increases in the size of hindlimb muscles were due to hypertrophy as indicated by significant reductions in myofiber densities (Fig. 6.1 E). Because increased growth post AAV1-FS administration has been shown to correlate with increased muscle strength, we next analyzed the extent of muscle functional improvement using grip strength analysis. Indeed, the enlarged muscle mass was accompanied by significant increases in hindlimb grip strength with AAV1-FS treated animals exhibiting nearly 4 times the force of controls (Fig. 6.1F).

Since there were no adverse events to AAV1-FS administration and no premature deaths observed in treated dy^w/+ animals who are of the same background as the severely dystrophic laminin-α2 deficient mice (Fig. 6.3 D), we next sought to determine the efficacy of AAV1-FS in dy^w/dy^w animals. To determine the overall consequences of postnatal myostatin blockade on dy^w/dy^w animals, AAV1-FS injected mice were monitored for changes in growth based on weekly measurements. By 3 months of age, AAV1-FS treatment resulted in a 25% increase in body weight in dy^w/dy^w treated mice compared to PBS-treated controls (Fig. 6.2A). Moreover, the injected muscle (gastrocnemius) of AVV1-FS treated animals also appeared to have larger fibers with more uniform myofiber sizes as evidenced by H&E stain of the injected muscle (Fig. 6.2B).
To determine if there were effects on remote muscles, we analyzed individual muscle size and myofiber diameters of the injected gastrocnemius and other hindlimb muscles of dy\(^w\)/dy\(^w\) mice. We found that AAV1-FS treated animals displayed significant increases in both injected muscles and remote tissues. dy\(^w\)/dy\(^w\) mice exhibited significant increases in the gastrocnemius, tibialis anterior, and quadriceps muscles after follistatin administration compared to the same representative muscles of PBS-treated controls (Fig. 6.2 C). Furthermore, histological and morphological analysis of injected and remote muscles suggests AAV1-FS had a hypertrophic effect. This was assessed by examining the myofiber diameters and densities of individual muscles (Fig. 6.2 D & E). dy\(^w\)/dy\(^w\) myofiber diameters were significantly larger in three out of four muscles analyzed in AAV1-FS injected animals (Gastroc: 35.0 µm ± 4.2 µm; TA: 29.2 µm ± 1.8 µm; Quads: 35.3 µm ± 0.9 µm) compared to littermate controls (Gastroc: 29.4 µm ± 1.1 µm; TA: 24.3 µm ± 0.9 µm; Quads: 30.1 µm ± 1.6 µm) (Fig. 6.2D).

Increased body weight and the enhancement of muscle size are good indicators of improved overall well-being and physical health of dy\(^w\)/dy\(^w\) animals. Because of the substantial increases in body weight and muscle size, we evaluated the effects of AAV1-FS treatment on animal strength. We found that at 3 months of age, there was a 30% increase in hindlimb grip strength in dy\(^w\)/dy\(^w\) mice compared to controls (Fig. 2F). AAV1-FS treated mice were also more active and exhibited more exploratory behaviors than non-treated dy\(^w\)/dy\(^w\) controls as evidenced by open field assessment of ambulatory movements and rearing events (Fig 6.3 A & B). Moreover, these significant
improvements in motor function could be attributed to decreased pathology and follistatin having a protective effect on muscle fibers, making them less susceptible to injury. Indeed, there were significant differences in percent central nuclei between treated and control dy^w/dy^w mice (Fig. 6.3 C) as well as fewer mononuclear cell infiltrations and fibrosis as indicated by H&E staining of the gastrocnemius muscle (Fig. 6.2 B).

It has been previously reported that myostatin inactivation leads to decreases in fat accumulation (McPheron, 1997; Wagner, 2002). Also, because reduction in fat has been suggested to cause increased mortality in myostain null dy^w/dy^w mice (Engvall, 2005), we next sought to determine if AAV-FS had any effects on total body fat. We collected both brown and white fat from the abdominal fat pads (retroperitoneal, mesenteric, and gonadal) and the femoral fat pads of mice from the all groups and expressed it as a percentage of total body weight. We confirmed that myostatin inhibition did not lead to a significant reduction in percent body fat in dy^w/dy^w mice although it was significant in heterozygotes (Table 6.1). Moreover, these reductions in fat had no adverse effect on survival (Fig 6.3 D). Therefore, it appears from these data that the negative effects of myostatin inactivation on essential brown fat accumulation and lifespan in dy^w/dy^w mice are not a concern with postnatal follistatin overexpression.

Despite these significant changes in muscle mass, strength and improved pathology, the overall survival of dy^w/dy^w AAV1-FS treated mice was only improved by 8% compared to PBS-injected controls (Fig. 6.3 D). Furthermore, the muscle enhancement induced by
follistatin administration in dy²/dy² mice was not as pronounced as that observed in injected heterozygotes. We therefore sought to determine if there were differences in follistatin expression that could be attributed to the unparallel changes in muscle enhancement. We verified follistatin transgene protein expression by measuring serum circulating and muscle expression levels by ELISA and immunostaining. Follistatin ELISA analysis of 3 month old dy²/+ mice showed that AAV1-FS treated animals had a 211.0% ± 24.3% increase in serum follistatin expression over non-treated controls and a 2216.0% ± 607.3% increase in expression of the injected muscle (gastrocnemius) (Table 6.1). However, follistatin injected dy²/dy² mice only exhibited a 19.3% ± 2.2% increase in serum circulating levels and a 26.8% ± 7.3% in muscle expression (Table 6.1). To further evaluate protein expression in the injected muscle, at 3 months of age mice in both groups were sacrificed and injected muscles were immunostained with anti-follistatin antibodies. Indeed, protein overexpression was observed in the gastronemius muscle of AAV1-FS injected mice with the greatest effect was seen in heterozygotes (Fig. 6.4).

These significant differences in serum and muscle expression between injected dy²/dy² and heterozygotes led us investigate if increasing serum follistatin levels and targeting more muscles could produce more uniform changes in muscle enhancement amongst the groups. Many AAV serotypes have been shown to have distinct efficacies in targeting certain tissues. Although AAV serotype 1 effectively transduces skeletal muscle, more recently discovered serotypes such as AAV9 appear to be highly efficient in transducing
a broader range of tissues such as liver, lung, the central nervous system and cardiac and skeletal muscle (Schaffer, 2004). Coupled with the ability of the self-complementary vector (scAAV) to by-pass the rate limiting step of second-strand synthesis, scAAV9 has been shown to result in robust muscle expression of the transgene (Foust, 2009; Bostick, 2007). We therefore, investigated the ability of scAAV9 bearing follistatin driven by the CAG promoter (scAAV9-FS) to increase transgene expression, muscle mass and function in laminin-alpha2 deficient and heterozygote mice.

One-day-old dyw/dyw and dyw/+ mice received temporal vein injections of 1 x 10\(^{11}\) vg of scAAV9-FS and were monitored for three months post injection. Littermate controls for both genotypes received similar injections of 50 µl PBS. H&E analysis of the gastrocnemius muscles of injected mice revealed increases in myofiber size for both heterozygotes (Fig. 6.5) and laminin-α2 deficient mice (Fig. 6.6 A). Moreover, there were significant improvements in pathology as determined by decreases in percent central nuclei in the gastrocnemius muscle of scAAV9-FS injected dyw/dyw mice compared to PBS-treated controls (Fig. 6.6 B). The general health of dystrophic animals also appeared to be improved above levels seen with intramuscular injections. In fact, scAAV9-FS intravenous injection resulted in increased exploratory behaviors and rearing events (Fig. 6.7 A & B) as well as a significant increase in percent survival (Fig. 6.7 C).

AAV9 has been shown to be effective in targeting the muscle. Therefore, systemic administration of scAAV9 should result in body wide muscle enhancement. Indeed,
injected animals of both genotypes exhibited significant improvements above PBS-treated controls. dy\textsuperscript{w/+} mice showed a 23.7% ± 0.9% increase in body weight as well as an improvement in both hindlimb and forelimb grip strength {(81.4% ± 5.6% and 71.0% ± 7.2%, respectively) Table 6.2}. There were also substantial improvements in individual muscle weights for all evaluated muscle groups (Table 6.2) with a 78.7% ± 3.5% increase in the gastrocnemius muscle (P = 0.03), a 74.7% ± 3.0% increase in the tibialis anterior (P = .006), a 79.6% ± 4.0% increase in the quadriceps muscles (P = 0.04) and a 130.0% ± 5.0% increase in the triceps (P = 0.01). Like other follistatin injection studies, scAAV9-FS had a hypertrophic effect on myofibers as evidenced by increased myofiber diameters in the muscles of treated animals in relation to PBS-injected controls (P < 0.001 for all muscle groups) (Table 6.2).

Follistatin mediated changes in the dy\textsuperscript{w}/dy\textsuperscript{w} mouse were evaluated and expressed as the average percent increase over the mean values of controls. In comparison to PBS-treated littermates, at three months post injection, scAAV9-FS treated resulted in a 44.4% ± 1.8% increase in body weight and a 39.0% ± 2.4% increase in hindlimb grip strength accompanied by a 45.3% ± 4.6% increase in forelimb grip strength (Table 6.2). These enhancements of body weight and muscle performance are consistent with significant improvements in the gastrocnemius muscles {43.3% ± 1.9% (P = 0.04)}; the tibialis anterior {62.5% ± 2.5% (P = 0.04)}; the quadriceps {53.1% ± 2.7% (P 0.02)}; and the triceps {78.7% ± 3.0% (P = 0.03)} (Table 6.2). There were also significant changes in the myofiber diameters of these muscles (P <0.001 for all muscle groups). Importantly,
the enhancement of body weight and individual muscle mass did not have substantial
effects on overall body fat or cardiac tissue as assessed by gross inspection and organ wet
weights (Table 6.2).

To determine if the effectiveness of scAAV9 directed follistatin administration was due
to increased serum circulating expression of the transgene, muscle and serum levels were
evaluated. Serum circulating levels were substantially increased over those of controls
for both dyw/+ (2534.8% ± 268.6%) and dyw/dyw mice (1628.8% ± 172.6%) (Table 6.2).
Similarly, gastronemius follistatin levels were increased in both dyw/+ (459.7% ±
146.5%) and dyw/dyw mice (330.4% ± 105.0%) (Table 6.2). However, since muscle
expression levels were substantially lower than those seen after intramuscular follistatin
administration, only focal areas of follistatin expression were observed by immunostatin
with antibodies directed against human follistatin (Fig. 6.8).

Discussion

The experiments presented here demonstrate the ability of a single gene delivery of
AAV-FS344 to enhance skeletal muscle mass, strength and survival in a severe mouse
model of congenital muscular dystrophy. By overexpressing follistatin for both local and
global myostatin inhibition in the laminin-α2 deficient (dyw/dyw) mouse, we were able to
show that follistatin is well tolerated and effective in chronic cases of muscular dystrophy
where muscle regeneration is markedly impaired. Moreover, the fact that there were no
adverse events related to postnatal myostatin inhibition demonstrates that follistatin gene
delivery may be a viable approach for enhancing muscle growth, evading the dangers associated with embryonic myostatin elimination in the dy	extsuperscript{w/dy	extsuperscript{w}} mouse.

Several other approaches have offered benefit to the laminin-α2 deficient mouse. Examples of these include corticosteroids, the growth enhancing agent insulin-like growth factor 1 (Igf-1), the cytotoxic T cell (CT) GalNAc transferase and mini-agrin (Connolly, 2002; Dominov, 2005; Lynch, 2001; Bentzinger, 2005; Xu, 2007). However, agents used for improving symptoms in laminin-α2 deficiency aren’t always applicable to other forms of MD. This is evident from studies where the apoptosis inhibitor BCL2 failed to produce any significant improvements in pathology due to dystrophin deficiency (Dominov, 2005). Accordingly, approaches that were effective in the mdx mouse have also fallen short when applied to laminin-α2 deficient animals. Studies involving the transgenic overexpression of ADAM12 resulted in no meaningful alterations of muscle pathology or disease progression in dy	extsuperscript{w/dy	extsuperscript{w}} animals (Guo, 2005). These studies suggest that all muscular dystrophies are not equal and careful attention should be paid to understanding the clinical relevance of a proposed therapeutic to a number of MDs before concluding it to be an effective agent for combating muscle disease. Our follistatin based therapeutic approach is unique in that it is able to offer benefit in young mdx mice, more adversely affected aged mdx animals as well as in the severely dystrophic dy	extsuperscript{w/dy	extsuperscript{w}} mouse (Haidet, 2008).
AAV-gene delivery of follistatin represents a way to obtain high levels of protein expression in both the infected tissue and remote sites. Because follistatin is secreted, intramuscular AAV1-FS gene delivery resulted in increases in muscle mass in not only the gastrocnemius (injected muscle) but also in a number of hindlimb muscles. These enhancements in muscle size were also seen after intravenous scAAV9-FS treatment. However, because scAAV9-FS resulted in a more global spread of the transgene and higher levels of protein expression, we saw changes in both hindlimb and forelimb muscles after follistatin administration. This seemed to be attributed to the ability of the systemic approach to target more muscles and greatly enhance protein levels. Indeed, there was about ~85 times more detectable serum follistatin in scAAV9-FS injected dy\(^w/dy^w\) mice compared to the AAV1-FS treated ones. Accordingly, although to a lesser degree, scAAV9 injected dy\(^w/+\) mice exhibited nearly 12 times more serum follistatin expression than what had been seen after AAV1-FS treatment. We believe that the lack of a parallel response between AAV1-FS injected dy\(^w/dy^w\) and dy\(^w/+\) mice could be attributed to the integrity of the muscle tissue in the severely dystrophic animals. Changes observed in laminin-\(\alpha2\) heterozygotes were similar to what had been seen in AAV-FS injected wild-type and \(mdx\) mice (Haidet, 2008). Also, the profound increases observed in non-human primates after intramuscular injection further demonstrate the potent ability of follistatin to enhance muscle mass in cases where skeletal muscle is not severely compromised (Kota, 2009- accepted Science Translational Medicine). Because follistatin is not curative and wasn’t able to ameliorate the disease process, it was ineffective in the absolute prevention of muscle wasting. However, due to the
improvements in pathology observed in dy^w/dy^w animals after both local and systemic injection, we believe that by follistatin acting the myofibers spared by the disease it was able to make the muscle less susceptible to damage from the sheer stress of normal activities. Moreover, because of its proposed role in the attenuation of the inflammatory response, it could be providing some degree of muscle protection to guard against further damage (Aoki, 2007). Therefore, by allowing for a greater spread of follistatin by increasing its levels in the circulation, our systemic scAAV9 approach proved to be the most effective method that would enable follistatin to target more skeletal muscles in the severely dystrophic dy^w/dy^w mouse. Thus, offering a greater range of protection and muscle enhancing response.

Unlike AAV1-FS intramuscular injections, muscle follistatin expression was not easily detected by immunostatining after scAAV9 treatment. In fact, only focal areas of expression were noticed even though scAAV9-FS treatment resulted in 330% and 450% increases in protein expression of the gastrocnemius muscles of dy^w/dy^w and dy^w/+ mice, respectively. This is not surprising considering follistatin expression was observed only in the gastrocnemius of AAV1-FS injected dy^w/+ mice who exhibited muscle expression levels that were ~2000% higher than those of normal animals (Table 6.1). Follistatin expression was never detected in non-injected muscles of these animals despite the profound increases in hindlimb muscle mass, strength and myofiber diameters. These enhancements were also accompanied by an increase in overall body weight as well as an improvement in hindlimb (dy^w/dy^w and dy^w+/+) and forelimb grip strength (dy^w+/+).
Although there were observed differences in percent body fat and cardiac size, these changes were not significant in dy<sup>w</sup>/dy<sup>w</sup> animals and did not have a negative effect on the overall health of the mice. These results are much like what has been reported in the mdx mouse where AAV1-FS did not adversely affect heart mass or the histological appearance of cardiomyocytes (Haidet, 2008). Likewise, the significant reductions in fat seen in heterozygotes did not influence mortality. The fact that myostatin is expressed in adipose tissue explains how alterations in myostatin expression could affect fat accumulation (McPherron, 2002; Rabbapragada, 2003). Moreover, although these changes in fat were observed, they were not to the degree of that which had been seen in myostatin null mice or animals engineered to overexpress IGF-1, where in some cases mice appeared to be devoid of fat; however, quantitative analyses were not performed (McPherron, 2002; Musaro, 2001). Therefore, the follistatin-induced reduction in fat posed no detriment to the general health of the injected animals. In fact, it appears that follistatin overexpression actually enhanced animal well-being. Exploratory behaviors assessed by open field analysis of ambulatory movements and rearing events were substantially increased in follistatin injected dy<sup>w</sup>/dy<sup>w</sup> mice.

There has been concern about follistatin effects on reproductive function. Although we did not address if follistatin affected gonadal function due to the inability of the dy<sup>w</sup>/dy<sup>w</sup> mouse to reproduce, we do not believe that it would pose any detriments to the reproductive system. This is due to the lack of histological and pathological alterations in
gonadal tissue of normal and dystrophic mice treated with AAV1-FS in earlier reports (Haidet, 2008). In these studies, there were no changes in the reproductive capacity in either wild-type or mdx mice treated with the AAV1-FS transgene (Haidet, 2008). These same results were also seen in non-human primates treated with AAV1-FS where hormonal levels of follicle-simulating hormone, leuteinizing hormone, estradiol and testosterone remained within the normal physiological range up to 15 months after treatment (Kota, 2009- accepted Science Translational Medicine). Furthermore, sperm morphology and motility were also unchanged following follistatin gene transfer (Kota, 2009- accepted Science Translational Medicine).

Follistatin appears to be a promising therapeutic option for a number of diseases that involve muscle wasting and a loss of muscle tone and function. By utilizing the severely dystrophic dy<sup>w</sup>/dy<sup>w</sup> mouse we not only show that follistatin has therapeutic implications in cases of chronic muscle loss, but also that the increased mortality caused by excessive loss of adipose tissue due to embryonic myostatin elimination can be evaded when it is inhibited postnatally. Moreover, the treatment paradigm employed in these studies have direct clinical relevance in that AAV is a practical agent for driving transgenic expression due to its lack of immungenity and prolonged pattern of expression. The ability of follistatin to increase muscle size and function in dystrophic muscle with different characteristic features of disease severity makes it an attractive candidate for the clinical treatment of musculoskeletal diseases of varying types.
Figure 6.1: Muscle enhancement in dyw/+ after AAV1-FS administration.

(A) AAV1-FS treated laminin-α2 heterozygote mice exhibited significant increases in body weight by 3 months of age. (B) H&E stain of the gastrocnemius muscle indicates that AAV1-FS increases myofiber size in dyw/+ mice by three months of age. (C) Three month old AAV1-FS treated mice exhibited increases in muscle wet weights in all hindlimb muscles examined. (D) Significant increases in myofiber diameters of hindlimb muscles were observed in AAV1-FS treated mice. (E) AAV1-FS treated gastrocnemius muscles demonstrated myofiber hypertrophy indicated by significant reductions in the number of myofibers per mm² area. (F) Hindlimb grip strength was significantly increased in AAV1-FS treated mice. Error bars represent standard errors.
Figure 6.2: Muscle enhancement in dyw/dyw after AAV1-FS administration.

(A) AAV1-FS treated laminin-α2 deficient mice exhibited significant increases in body weight by 3 months of age. (B) H&E stain of the gastrocnemius muscle indicates that AAV1-FS increases myofiber size in dyw/dyw mice by three months of age. (C) Three month old AAV1-FS treated mice exhibited increases in muscle wet weights in all hindlimb muscles examined. (D) Significant increases in myofiber diameters of hindlimb muscles were observed in AAV1-FS treated mice. (E) AAV1-FS treated gastrocnemius muscles demonstrated myofiber hypertrophy indicated by significant reductions in the number of myofibers per mm² area. (F) Hindlimb grip strength was significantly increased in AAV1-FS treated mice. Error bars represent standard errors.
Figure 6.3: AAV1-FS injected mice demonstrate significant increases in activity, reductions in pathology and trends toward increased survival.

(A) Seven-day-old dy^w/dy^w mice injected with AAV1-FS exhibited significant increases in ambulation at 3 months of age. (B) Seven-day old dy^w/dy^w mice injected with AAV1-FS exhibited significant increases in rearing events at 3 months of age. (C) dy^w/dy^w mice injected with AAV1-FS at seven days of age exhibited significant decreases in percent central nuclei at three months after injection. (D) dy^w/dy^w mice injected with AAV1-FS at seven days of age demonstrated trends toward increased survival compared to PBS-injected controls. Error bars represent standard errors.
Figure 6.4: Muscle follistatin expression in AAV1-FS injected animals.

AAV1-FS induced muscle protein expression in 3 month old dy^{w/+} and dy^{w/\text{w}} mice with the greatest effect seen in mice heterozygote for laminin-\(\alpha_2\). Seven-day-old mice were given bilateral intramuscular injections of a total of 5 x 10^{10} of AAV1-FS or 50\mu\text{l} PBS into the gastrocnemius muscles. Muscle follistatin expression was assessed by histioimmunostatin at three months of age. Laminin staining was used to label the myofiber membrane.
Figure 6.5: H&E stain of scAAV9-FS and PBS-treated dy<sup>w</sup>/+ mice three months post injection.

(A) H&E staining of the gastronemius muscle of three month old dy<sup>w</sup>/+ mice reveals myofiber hypertrophy in scAAV9-FS injected animals compared to PBS treated controls.
Figure 6.7: Three month old dy<sup>w</sup>/dy<sup>w</sup> mice treated with scAAV9-FS at one-day of age demonstrate significant reductions in muscle pathology.

(A) H&E staining of the gastronemius muscle of three month old dy<sup>w</sup>/dy<sup>w</sup> mice reveals myofiber hypertrophy in scAAV9-FS injected animals compared to PBS treated controls. 
(B) By three months of age significant decreases in percent central nuclei were observed in the gastrocnemius muscles of dy<sup>w</sup>/dy<sup>w</sup> mice that had been treated with scAAV9-FS. Error bars represent standard errors.
Figure 6.7: scAAV9-FS enhances activity and survival in dy^w/dy^w mice.

(A) One-day-old dy^w/dy^w mice injected with scAAV9-FS exhibited significant increases in ambulation at 3 months of age.  (B) One-day old dy^w/dy^w mice injected with scAAV9-FS exhibited significant increases in rearing events at 3 months of age.  (C) dy^w/dy^w mice injected with scAAV9-FS at one day of age demonstrated significant increases in percent survival compared to PBS-injected controls.  Error bars represent standard errors.
Figure 6.8: scAAV9-FS induced muscle protein expression in 3 month old dy<sup>w</sup>/+ and dy<sup>w</sup>/dy<sup>w</sup> mice.

One day old mice were given intravenous injections of a total of 1 x 10<sup>11</sup> of scAAV9-FS or 50µl PBS into the temporal vein. Muscle follistatin expression was assessed by histoimmunostatin at three months of age. Laminin staining was used to label the myofiber membrane.
Table 6.1: Percent change differences among AAV1-FS treated laminin-α2 deficient and heterozygote mice at 3 months of age. “+” indicates increased levels while “–” denotes decreases.

<table>
<thead>
<tr>
<th>Metric</th>
<th>dy\textsuperscript{w}/dy\textsuperscript{w}</th>
<th>dy\textsuperscript{w}/+</th>
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<tr>
<td>Body Weight</td>
<td>+ 15.6 ± 0.8</td>
<td>+ 30.0 ± 1.6</td>
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<td>Hindlimb Grip Strength</td>
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<td>+ 521.8 ± 65.9</td>
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<tr>
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<td>+ 165.4 ± 17.4</td>
</tr>
<tr>
<td>Quads Muscle Weight</td>
<td>+ 22.1 ± 1.2</td>
<td>+ 87.8 ± 4.7</td>
</tr>
<tr>
<td>Triceps Muscle Weight</td>
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<td>+ 8.9 ± 0.7</td>
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<tr>
<td>Gastroc Myofiber Diameters</td>
<td>+ 21.7 ± 1.0</td>
<td>+ 68.0 ± 3.2</td>
</tr>
<tr>
<td>TA Myofiber Diameters</td>
<td>+ 20.0 ± 0.4</td>
<td>+ 36.6 ± 0.8</td>
</tr>
<tr>
<td>Quads Myofiber Diameters</td>
<td>+ 17.4 ± 0.3</td>
<td>+ 34.4 ± 0.6</td>
</tr>
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<td>Triceps Myofiber Diameters</td>
<td>+ 0.8 ± 0.1</td>
<td>+ 2.3 ± 0.2</td>
</tr>
<tr>
<td>Percent Body Fat</td>
<td>- 13.4 ± 1.9</td>
<td>- 32.2 ± 2.3</td>
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<tr>
<td>Heart</td>
<td>+ 2.3 ± 0.2</td>
<td>+ 5.5 ± 0.5</td>
</tr>
<tr>
<td>Serum FS Expression</td>
<td>+ 19.3 ± 2.2</td>
<td>+ 211.0 ± 24.3</td>
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<tr>
<td>Gastroc FS Expression</td>
<td>+ 26.8 ± 7.3</td>
<td>+2216.0 ± 607.3</td>
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Table 6.2: Percent change differences among scAAV9-FS treated laminin-α2 deficient and heterozygote mice at 3 months of age. “+” indicates increased levels while “–” denotes decreases.

<table>
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<th></th>
<th>dy&lt;sup&gt;w&lt;/sup&gt;/dy&lt;sup&gt;w&lt;/sup&gt;</th>
<th>dy&lt;sup&gt;w&lt;/sup&gt;/+</th>
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<td>Body Weight</td>
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<td>+ 23.7 ± 0.9</td>
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<td>Hindlimb Grip Strength</td>
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<td>+ 81.4 ± 5.6</td>
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<td>Forelimb Grip Strength</td>
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<td>+ 71.0 ± 7.2</td>
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<td>+ 79.6 ± 4.0</td>
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<td>Triceps Muscle Weight</td>
<td>+ 78.7 ± 3.0</td>
<td>+ 130.0 ± 5.0</td>
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<td>Gastroc Myofiber Diameters</td>
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<td>+ 18.9 ± 0.2</td>
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<td>TA Myofiber Diameters</td>
<td>+ 19.2 ± 0.3</td>
<td>+ 24.2 ± 0.4</td>
</tr>
<tr>
<td>Quads Myofiber Diameters</td>
<td>+ 16.2 ± 0.2</td>
<td>+ 16.7 ± 0.2</td>
</tr>
<tr>
<td>Triceps Myofiber Diameters</td>
<td>+ 31.3 ± 0.4</td>
<td>+ 36.2 ± 0.5</td>
</tr>
<tr>
<td>Percent Body Fat</td>
<td>- 6.0 ± 0.2</td>
<td>- 14.9 ± 0.5</td>
</tr>
<tr>
<td>Heart Weight</td>
<td>+ 2.4 ± 0.0</td>
<td>+ 4.3 ± 0.6</td>
</tr>
<tr>
<td>Serum FS Expression</td>
<td>+ 1628.8 ± 172.6</td>
<td>+ 2534.8 ± 268.6</td>
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<tr>
<td>Muscle FS Expression</td>
<td>+ 330.4 ± 105.0</td>
<td>+ 459.7 ± 146.5</td>
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CHAPTER 7

POSSIBLE FOLLISTATIN MEDIATED INTERACTIONS THAT PROMOTE MUSCLE GROWTH AND ENHANCEMENT

Introduction

Muscle hypertrophy is a natural response to stretch overload, tension or injury. Increased muscle growth is a result of the activation of a number of signaling pathways that control the proliferation and differentiation of myogenic cells. Unfortunately, the exact mechanism by which muscle size is increased still remains unclear. Nevertheless, the understanding of the transcriptional events that allow for muscle development are essential for identifying potential therapeutics to increase muscle size and function in cases of aging, disuse or musculoskeletal disease. It is generally accepted that satellite cells play a substantial role in modulating skeletal muscle hypertrophy (Partridge, 2002; Karalaki, 2009). However, the transcriptional framework that regulates myogenesis, particularly differentiation and hypertrophy has been linked to the action of insulin-like growth factors and the phosphatidylinositol 3-kinase (PI3K)-Akt pathway (Philippou, 2007). Interestingly, recent work has shown that this signaling system can induce myofiber hypertrophy absent satellite cell activation (Blaauw, 2009). These along with a number of knockout and transgenic studies suggest that a number of genes, proteins and
transcriptional regulators are involved in the generation of enlarged muscles (Blaauw, 2009).

Of particular importance are the resultant effects of myostatin inactivation. Myostatin is a negative regulator of muscle growth and down regulates a number of genes responsible for myoblast proliferation and differentiation. Gene elimination studies report that myostatin disruption results in a double muscling phenotype (McPherron, 1997). Consequently, a number of studies have investigated this pathway in an attempt to understand the cellular mechanisms involved in inducible muscle growth. However, recently it has been shown that effectors of the myostatin pathway aren’t specific to myostatin alone. In fact, follistatin a potent myostatin inhibitor has been shown to not only act via the myostatin associated pathway, but also inhibit other repressors of muscle growth. This is evident from myostatin knockout studies in which myostatin null animals were engineered to overexpress the follistatin transgene (Lee, 2007). The results were mice with quadrupled muscle size twice that of what had been observed in by myostatin blockage alone (Lee, 2007). Therefore, the exploration of the specific actions of myogenic regulators is important to understanding the mechanistic properties that underlie inducible systems of muscle growth and function.

Follistatin is an attractive candidate for investigations into genes and proteins that may be affected by induced muscle hypertrophy. Follistatin has been shown to increase muscle mass and strength in normal and dystrophic animals at varying degrees of disease
severity (Nakatani, 2008; Haidet, 2008). A naturally occurring glycoprotein, follistatin has been shown to alter muscle growth, inhibit pathology and offer muscle protection in instances of persistent muscle wasting. These actions have been attributed to protein-protein interactions involving myostatin, activin, decorin and histone deacetylase inhibitors (Gilson, 2009; Zhu, 2007). Although it is unclear whether the effects of follistatin are a result of its direct interaction with all of these proteins, it is accepted that its expression does, in some way, help regulate the actions of these cellular components. Therefore, it is necessary to investigate the gene expression patterns that are associated with follistatin overexpression as well as to explore possible muscle membrane interactions that follistatin may have which would allow for muscle preservation in cases of muscular dystrophy.

Here we investigate these conditions using ELISA-based binding experiments and microarray-based gene expression studies. With particular interest on the basement membrane, we investigated the ability of follistatin to interact with laminin and merosin, basement membrane glycoproteins that mediate cell survival, adhesion and cell-cell interactions. These studies were performed to provide a possible explanation for how follistatin enhances muscle growth and function to protect the muscle during dystrophic conditions. Here, we show that follistatin does not exhibit any meaningful interaction with laminin or merosin. Disappointingly, only minute demonstrations of association were observed. Nevertheless, these studies are important in that they for the first time provide insight into a possible association between follistatin and critical members of the
muscle membrane that are largely responsible for the development and viability of myogenic cells.

Gene profiling of normal animals injected with an adeno-associated viral (AAV) vector expressing follistatin identified the differential expression of a number of genes that are involved in muscle membrane stability, growth and signaling. These changes highlight key systems that are involved in follistatin mediated hypertrophy. Moreover, these changes provide information about possible protein-protein interactions that follistatin might have that would explain its ability to provide muscle stability during dystrophic conditions. Although, no relevant interactions were observed, they open the door for continued explorations into the mechanisms that underlie the muscle enhancing effects of follistatin.

Results
In these studies we sought to determine if follistatin-mediated enhancements were due to an association with the basal laminin components laminin and merosin. To test these effects, we performed a series of binding studies, we produced a c-myc-tagged human follistatin. This tagged follistatin was transfected into Hek293T cells which stably secreted and expressed follistatin and c-myc (Fig. 7.1A). Supernatant collected from follistatin overexpressing Hek293T was collected and purified. Using antibodies that were specific to c-myc, we demonstrated that c-myc-tagged follistatin was able to be extracted from the Hek293T supernatant (Fig. 7.1B).
To test the binding of follistatin to laminin and merosin we used an ELISA-based assay where 80nM of follistatin was added to an ELISA plate containing 1µg of either immobilized laminin or merosin. At 120 min. after the addition of the HRP substrate (Sigma Fast OPD), no meaningful binding activity between follistatin and laminin or follistatin and merosin was observed (Fig. 7.2).

Because of the limited interaction of follistatin the two very prominent members of the basal lamina, laminin and merosin, we next sought to identify differentially expressed genes in follistatin infected skeletal muscle that could help explain the mechanism through which it exerts its muscle enhancing potential. To identify unique differential gene expression as a result of follistatin treatment, RNA was isolated from the gastrocnemius muscle of eight-week-old C57Bl6 mice that had been injected there at four weeks of age with 1 x 10^{11} of an AAV1 expressing human follistatin. Using Affymetrix Genome Array, gene expression of AAV1-FS treated muscle was compared to the gastrocnemius muscle of non-injected litter mate control mice (n= 3 animals/group). Animal samples were analyzed in triplicate. Using Affymetris gene arrays, we identified a number of genes found to be differentially expressed amongst the treatment and control groups. For both groups, fold differences ≥ 1.5 with p-values < 0.01 between injected and non-injected muscle were considered significant. The clustering result performed by using DAVID Bioinformatics Database (http://david.abcc.ncifcrf.gov/) demonstrated that
a number of genes responsible for muscle growth and development exhibit different patterns of expression following follistatin injection (Table 7.1).

**Muscle Growth and Hypertrophy**

As expected, the follistatin exhibited the highest fold change in gene expression compared to controls. However, a number of other genes that have been associated with hypertrophy and satellite cell activation demonstrated differential gene expression after follistatin treatment. Heparan sulfate proteoglycans (HSPGs) are cellular macromolecules that mediate cell signaling, adhesion, growth and development.

Sulfation of these proteins is important for their proper action. Notably, the modification of heparin sulfates by heparan sulfate 6-O-sulfotransferase 2 (HS6ST2) has been shown to facilitate the action of two very potent inhibitors of myoblast differentiation, fibroblast growth factor 2 (FGF2) and syndecan-1 (Lum, 2006). Thus, the observation that both HS6ST2 and syndecan-1 exhibited decreased expression after follistatin administration could indicate a follistatin-mediated influence on muscle growth.

Insulin–like growth factor 2 (IGF-2) gene expression has been shown to be increased in hypertrophied muscle (Kocamis, 2002; Armand, 2004). Likewise, the IGF-2 transcript has been shown to be upregulated in regenerating muscle where there is a myostatin deficiency. This effect corresponds with the inverse relationship that appears to exist between myostatin and IGF-2 in muscle atrophy (Lalani, 2000). Although, no
meaningful changes were observed in myostatin levels in our microarray studies, significant increases in IGF-2 and its mRNA binding protein were observed in follistatin overexpressing animals.

Decorin is leucin-rich proteoglycan that has been shown to affect muscle growth. It has been shown to enhance myoblast proliferation and differentiation by modulating myostatin activity (Kishioka, 2008; Miura, 2006). This activity has been suggested to be due to an upregulation of the myostatin inhibitor follistatin as well as other myogenic promoting genes (Li, 2007). Therefore, the observed increase in decorin expression in follistatin-treated muscle could demonstrate the influence of an extracellular matrix protein on muscle growth and hypertrophy.

**Muscle Development and Stability**

Several genes that encode LIM domain containing proteins were found to be differentially expressed between FS-treated animals and controls. LIM domains are cystein rich regions that are known to have a zinc finger-like motif and mediate protein-protein interactions (Willmann, 2001). LIM domain containing proteins are believed to play a role in myogenesis in that differences in certain LIM proteins have been shown to cause myopathy that is believed to be due to disruption of the architecture of the cytoskeleton (Arber, 1994; Arber, 1997).
Titin (connectin) is a large myofibrillar protein that has been shown to be important for the stabilization of sarcomeres and mediates the contractile machinery of the muscle fiber. It also contains binding sites for a number of muscle associated proteins such as calpain-3, α-actinin and telethonin (Hackman, 2002). Defects in the titin gene result in muscular dystrophy (Hackman, 2002). The observed increase in titin after follistatin therapy could indicate a role for it in helping to stabilize the myofiber by enhancing the expression of an important regulator of muscle function.

**Muscle Pathology**

Fibrosis is a common pathological feature of muscular dystrophy. Certain potent stimulators of skeletal muscle fibrosis have been identified. Among these is transforming growth factor-β1 (TGF-β1). Indeed, increased TGF-β1 levels are observed in dystrophic muscle. This response is associated with an increase in myostatin which is suggested to also promote fibrosis. Decorin has been shown to downregulate tissue fibrosis by augmenting the TGF-β1/myostatin response. Also, decorin increased expression results in decreased fibrosis and improvements in muscle repair (McCroskery, 2005). Therefore, observed increases in decorin in follistatin injected muscle suggests a possible follistatin-mediated effect on muscle pathology in conditions of muscle injury.

Tenascin-C is an extracellular matrix protein that is generally not expressed in normal adult muscle. However, during instances of muscle injury and fibrosis increased expression is observed (Gullberg, 1997; Ringelmann, 1999). In fact, increased tenascin C
expression has been observed in patients with DMD and Becker muscular dystrophy (Gullberg, 1997). The decreased in tenascin C exhibited by follistatin injected animals could suggest that follistatin is able to attenuate the fibrotic response by modulating the effects of tenascin-C.

Discussion

Using an ELISA-based system we studied possible interactions of follistatin protein with prominent ECM components. However, we were unsuccessful in finding any meaningful interactions of follistatin with either laminin or merosin. This suggests that follistatin does not regulate the action of these proteins due a direct association with them. This does not exclude the possibility of a follistatin-associated interaction with the ECM. It is not known whether or not follistatin possesses a self-binding property. If so, it could have possible interactions with ECM proteins that contain follistatin-like domains. Among these is agrin, a HSPG with nine follistatin-like repeats. Agrin overexpression has been shown to ameliorate muscle disease in laminin-α2 deficient mice enhancing animal body weight, lifespan and motility (Bentzinger, 2005; Qiao, 2005). Investigations directed towards understanding if follistatin associates with agrin by forming a network of self assembly with the follistatin-like repeats could shed new light on how it enhances muscle growth and function.

Follistatin mediated muscle enhancement is due mainly to hypertrophy. The evaluation of the differential genetic expression of certain muscle regulators as a result of follistatin
overexpression is necessary for understanding the mechanism through which it exerts its potential. Follistatin administration to skeletal muscle resulted in the altered expression of a number of genes responsible for proteins associated with muscle ECM, cytoskeleton, growth and survival. By affecting satellite cell proliferation and differentiation follistatin is able to regulate muscle size and development. Also, by promoting the increased expression of key genes responsible for proteins that augment in the inflammatory response, follistatin may be able to decrease tissue fibrosis in cases of muscle insult. Its effectiveness in increasing the expression of muscle fiber stabilizers like titin that are pivotal for preventing contraction–induced injury, follistatin may utilize this mechanism to protect muscle fibers from damage during motility.

Our analyses of follistatin’s possible mechanism for muscle mass control help to set the stage for future explorations into its muscle enhancing abilities. The findings of these studies will shed new light on the study of the biological roles of follistatin in conditions of muscle wasting. These effects are yet to be clarified and properly understood. Nevertheless, the lessons learned from investigations into the possible cell-cell associations and differential genetic expression as a result of follistatin administration, provide new clues for deciphering mechanistic properties that mediated muscle enhancement under hypertrophic conditions.
Figure 7.1: Follistatin and c-myc expression in HEK293T transfected cells.

(A) HEK293T cells were transfected with c-myc-tagged follistatin. Immunostatin for c-myc and follistatin shows colocalization in follistatin expressing cells. (B) Production of recombinant c-myc-tagged protein was verified by immunoblotting using antibodies to follistatin and to the epitope tag.
Figure 7.2: Evaluation of follistatin binding to laminin and merosin

Recombinant laminin or merosin were immobilized on ELISA plates at a concentration of 1µg/well in carbonate/bicarbonate buffer and tested for their ability to bind a 80 nM concentration of follistatin. No meaningful associations between follistatin and either laminin or merosin were observed.
Transcripts Differentially Expressed in AAV1-FST injected C57Bl6 mice.

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* Positive numbers represent an increase in gene expression in relation to non-injected C57Bl6 mice.
** Negative (-) numbers represent a decrease in gene expression in relation to non-injected C57Bl6 mice.
Continued

Transcripts Differentially Expressed in AAV1-FST injected C57Bl6 mice.

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</table>

* Positive numbers represent an increase in gene expression in relation to non-injected C57Bl6 mice.
** Negative (‐) numbers represent a decrease in gene expression in relation to non-injected C57Bl6 mice.

Table 7.1: Differential gene expression in C57Bl6 mice after AAV1-FS administration.
CHAPTER 8

DISCUSSION

The muscular dystrophies represent a complex group of heterogeneous disorders that are characterized by continued muscle wasting, the infiltration of immune cells and myofiber necrosis and fibrosis. These conditions can affect cardiac and respiratory function leading to early death. A number of therapies have been proposed to alleviate symptoms associated with muscular dystrophy (MD). However, to date steroids, which carry a high risk in terms of side effects, are still the most commonly used and most effective treatment regimen. Therefore, the development of effective therapies that enhance muscle growth and performance, yet evade the potential dangers associated with steroid use are warranted. This dissertation work has characterized the potent effects of myostatin inhibition by follistatin to increase muscle mass and function, offering an alternative approach to treating neuromuscular diseases that would bypass the dangers of anabolic steroids. The primary strengths of these studies lie in the fact that follistatin therapy is not only relevant for the muscular dystrophies but also for cachexic conditions such as AIDS, ALS, and SMA. Coupled with the advantages of AAV technology, follistatin gene transfer allows for persistent expression that circumvents the use of repeat
pharmacological administration which would be necessary for small molecule or neutralizing antibody-based therapies.

Follistatin is not the only method that could be employed for targeted myostatin inhibition. However, from comparative studies involving a number of myostatin inhibitors, it was found to be the most effective (Haidet, 2008). This is believed to be due in large part to its ability to affect alternative pathways that regulate muscle development outside of myostatin signaling. This is evidenced by the fact that myostatin deficient mice that overexpress follistatin demonstrate a quadrupling effect of muscle. A possible hypothesis for this could be that follistatin possesses the ability to bind other TGF-β family members that have been shown to be negative regulators of muscle growth (Welt, 2002). The TGF-β family is a large family of proteins that regulate tissue homeostasis, affecting cell growth, proliferation and differentiation (Lee, 2005). TGF-β family members are structurally similar and use a common signaling mechanism. In general they bind to cell membrane receptors which initiate a series of events that ultimately result in the activation, phosphorylation and translocation of smad proteins (Lee, 2005).

Myostatin has been shown to bind the activin type 2B (ActRIIB) membrane receptor. This binding mediates its ability to downregulate muscle regulatory factors responsible for myoblast proliferation and differentiation. However, in addition to myostatin a number of TGF-β family members have been shown to bind this receptor (Lee, 2005). Therefore, it is conceivable that other TGF-β family members may display a myostatin
redundant function in regards to muscle growth and development. This is evident from studies where the administration of a soluble ActRIIB receptor resulted in enlargement of muscle that more extensive than that which had been seen using myostatin-specific inhibitors. Moreover, like in follistatin overexpressing mice, myostatin null animals that received the soluble ActRIIB receptor exhibited more profound increases in muscle mass when compared to animals that lacked myostatin alone (Lee, 2005). Therefore, therapies that possess functional properties not limited to the attenuation of myostatin could offer substantial benefit for a number of muscle diseases.

Follistatin has been suggested to offer some degree of muscle protection during cases of muscular dystrophy. This has been demonstrated in published studies utilizing the mdx mouse and this dissertation extended this work by highlighting its clinical relevance in the laminin-α2 deficient, dyw/dyw mouse (Haidet, 2008; Nakatani, 2008). Undoubtedly, muscle structural and functional changes in response to physical activity involve the alteration of a number of genes. These changes allow for the expression of myofibrillar and metabolic proteins that regulate skeletal muscle plasticity. The observation that follistatin injected muscle displays reduced muscle damage and pathology suggests that follistatin may be operating in concert with myogenic systems that positively control and respond to environmental changes to result in meaningful metabolic and morphological adaptations. By investigating possible associations with the muscle basal lamina which bears a great deal of the tensile forces of the muscle fiber, we attempted to identify key patterns of activity that could offer insight into how follistatin is able to offer protection
against shear stresses imposed during muscle contraction. Moreover, the investigation of the differential expression of genes that are responsible for muscle growth, hypertrophy, development and pathological response was necessary to understand if there are known or novel pathways that are affected by follistatin overexpression. Admittedly, these studies did not provide explicit answers to address these concerns. However, they did provide groundwork for future studies directed at the identification of a list of candidates that may be affected by follistatin overexposure as well as setting the stage for additional investigations aimed at understanding how follistatin physically interacts with itself or other signaling molecules.

Our demonstration that follistatin is effective in enhancing muscle size in larger species helps to establish translational links for its clinical application. The use of non-human primates to investigate plausible therapeutics provides insight into how a potential treatment may operate in larger mammalian species. By extending our gene transfer studies to this group, we adopted a paradigm that can be directly applied to patients. The fact that we were able to identify the most effective promoter system used to drive follistatin gene expression as well as highlight muscle systems that display the greatest reaction to transgene administration provided clues into regulatory processes that allow for a predictable follistatin specific response. Of particular interest are follistatin’s fiber type specific effects. Using myofibrillar ATPase staining, we saw that follistatin primarily increased the size of type II fast-glycolytic fibers, yet resulted in no fiber-type shift. This could be explained by the patterns of myostatin expression amongst the
varying fiber types. It has been shown that myostatin mRNA is higher in fast-twitch than in slow-twitch muscle (Matsakas, 2005). Therefore, myostatin may be acting more on fast-twitch fibers to inhibit their muscle growth. Follistatin circumvents these effects resulting in bigger type II fibers.

Of the key goals of this dissertation was to investigate if follistatin was relevant at varying stages of disease severity. By utilizing aged dystrophin deficient and severely dystrophic dy"+/dy" mice, we were able to resolve this issue. We found follistatin to provide for increased muscle strength, size and stability of dystrophic muscle under both moderate and extreme instances of muscle wasting. Moreover, because it is not a therapy aimed at correcting the gene that is causative for the disease, follistatin gene therapy is applicable to a number of disorders both with and without a known gene defect such as facioscapulohumeral muscular dystrophy or inclusion body myositis. Nevertheless, an ideal treatment regimen for disorders such as DMD would be the co-administration of follistatin with gene replacement therapies to allow for the most meaningful clinical outcome.

Our investigations into the effectiveness of follistatin have been advantageous in highlighting how it works in normal systems and in mouse models of muscle disease. However, future work should be focused on evaluating its benefits in larger models of dystrophic animals such as the golden retriever dystrophic dog. Also, considering the complexities of the follistatin mediated pathway and the fact that we are uncertain as to
which systems it operates in, it is necessary to evaluate any potential follistatin-induced changes on other organ systems outside of muscle regulation. Although we didn’t observe nor do we expect any treatment-related organ system pathology, due to the fact that the FS344 isoform used in our studies display little to no affinity for heparin sulfate proteoglycan binding on membranes, more extensive analysis of possible follistatin cell surface associations should be performed.

Myostatin has been suggested to operate as a local inhibitor of muscle growth as well as working as a circulating regulator of the fat and muscle metabolic processes that respond to physiological changes such as nutritional status. Therefore, future investigations into the therapeutic potential of follistatin should be directed towards understanding the relationship between follistatin inhibition of myostatin locally vs. its suppression of myostatin’s global metabolic processes. Our systemic scAAV9 treatment of dystrophic mice resulted in no adverse events on survival or gross organ pathology, yet continued investigations into the effects of increased global follistatin expression in more sophisticated organ systems are necessary to more explicitly assess the possible risks associated with globally manipulating the myostatin pathway.

The understanding of the possible dangers of modulating myostatin signaling is not limited to the treatment of muscle disease. There is the possibility that myostatin inhibitors may be used as life-style drugs. Nutritional supplements such as Myostat, Myozap and Myoblast are currently being advertised as myostatin inhibitors. These
products are suggested to be performance enhancers aimed at attracting athletes and the aging population with the claim that they can alter the muscle to fat ratio. Yet, no evidence exists that the active ingredient in them (CSP-3) affects myostatin activity or enhances muscle growth (Matsakas, 2004). Considering the long-standing issues with steroid misuse among this populace, it can be feared that so-called myostatin inhibiting substances may someday be among the list of commonly abused muscle enhancers. Another justifiable concern lies in the fact that some people may have already begun taking these supplements thus posing incalculable risks to their health. Therefore, great scientific integrity lies in our ability to address possible detriments associated with these therapies aimed at highlighting adverse outcomes due to altered myostatin activity in the metabolic processes of humans.

This dissertation was a collection of comprehensive studies aimed at understanding the benefits of myostain inhibition by follistatin gene therapy in the treatment of muscle disease. Our analysis of follistatin-induced changes at varying levels of disease severity highlighted how increasing muscle mass without gene correction can still result in functional improvement and decreased disease symptoms. This work also presented a therapeutic paradigm that is feasible and translatable for the clinical investigation of follistatin’s effectiveness in humans. By exploring possible physical interactions and alterations in gene expression profiles, our studies set the stage for continued
investigations into the characterization of follistatin-induced changes in muscle growth and function. This knowledge provides new starting points for understanding the molecular and functional relevance of myostatin inhibition by AAV-directed follistatin overexpression.


