Gene Therapy for Neuromuscular Disorders

Dissertation

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ABSTRACT

Neuromuscular disorders affect thousands in the US including both patients as well as caregivers providing physical, emotional, and financial support. The goal of the studies presented here is to gain a further understanding of the pathogenic disease mechanisms of neuromuscular disorders and develop translational therapies to attempt to treat these diseases.

The muscular dystrophies are a group of primary muscle disorders leading to muscle wasting and loss of ambulation. Since muscle wasting is a significant cause of disability in many muscle disorders, the aim of our studies was to develop a strategy to increase muscle mass and strength. We hypothesized that inhibiting myostatin, a negative regulator of muscle growth, would lead to increased muscle mass and strength. To inhibit myostatin, we developed an adeno-associated virus (AAV) gene therapy vector to deliver follistatin, an endogenous myostatin antagonist. A single intramuscular injection of AAV-Follistatin was able to increase muscle mass and strength in a mouse model of muscular dystrophy, even when delivered after the onset of muscle degeneration. Next, we tested AAV-Follistatin in the non-human primate since these animals are biologically closer to humans. AAV-Follistatin delivered by intramuscular injection to the quadriceps
muscle caused increased muscle size and strength, with no adverse effects in any major organ system. Therefore, AAV-Follistatin represents a promising strategy to treat muscle wasting diseases and warrants further clinical translation.

In contrast to the muscular dystrophy field, there are currently few promising therapies reaching the clinic for the treatment of Amyotrophic Lateral Sclerosis (ALS). ALS is a neurological disorder caused by the degeneration of motor neurons leading to paralysis and death within 1-5 years of onset. Since there are presently no predictors for who will develop ALS, one emerging strategy is to target the glial cells which contribute to the progression phase of disease, with the ultimate goal to slow disease progression. The aim of our studies was to investigate how glial cells such as astrocytes contribute to motor neuron death and develop therapies to inhibit this glial toxicity. We hypothesized that co-culturing ALS astrocytes in vitro with motor neurons would result in motor neuron death. Using both astrocytes isolated from the mouse model of ALS as well as from post-mortem ALS spinal cord, we show these astrocytes are toxic to motor neurons in vitro. Our data represents the first report to show a common pathway to motor neuron death mediated by astrocyte dysfunction between familial ALS and the more common sporadic form of ALS. To investigate mechanisms involved in this astrocyte-mediated motor neuron death, we examined whether inflammatory pathways were activated in the astrocytes. We found significant activation of the NF-κB pathway in ALS astrocytes so we designed a gene therapy vector to inhibit this activation. Preliminary data suggests that inhibiting the NF-κB pathway in astrocytes can reduce inflammatory gene expression and may increase survival in the ALS mouse model. Future studies will involve more
rigorous testing of this gene therapy in the ALS mouse model as well as in our co-culture model of sporadic ALS.
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CHAPTER 1

INTRODUCTION

1.1 Introduction

Neuromuscular disorders are a broad class of diseases that involve dysfunction of either the muscles, nerves which control muscles, or a combination of both. Symptoms of a neuromuscular disorder include muscle weakness, spasticity, rigidity, loss of muscle control, and muscle pain depending on the specific muscles or nerves are affected. Many neuromuscular disorders eventually lead to paralysis, loss of ambulation, and ultimately, death if the respiratory or cardiac muscles are affected. Thousands in the US are afflicted by neuromuscular disorders and many succumb to these horrible diseases each year. Obviously, the emotional and psychological impact of being diagnosed with a neuromuscular disease is immense, especially if paralysis is eminent. In addition, the health care costs for these diseases are exorbitant considering many patients will eventually require wheelchair assistance and constant care.

It is discouraging that there are no cures or even effective treatments for the majority of neuromuscular disorders. In fact, although the medical community has been aware of many of these diseases for over a century, the cause of some of these disorders is still relatively unknown. However, emerging therapies offer hope for a variety of these
diseases and include both pharmacological interventions as well as gene and cell therapies. The goal of these studies is to gain a greater understanding for the molecular mechanisms involved in neuromuscular disorders and to develop novel therapies that are both effective and clinically translatable.

1.2 Muscular Dystrophy

The muscular dystrophies (MD) are a group of hereditary disorders characterized by muscle weakness and wasting. The degree of disability depends upon the type of MD, but severe forms can cause loss of ambulation, respiratory problems, and eventual death. While the cause of some MDs is still unknown, genes linked to many forms have been identified as defective structural proteins in the muscle membrane-associated complex [1,2]. This muscle membrane associated complex connects the inner actin cytoskeleton to the extracellular matrix and provides structural support for the muscle fiber. For example, the most common types of MD, Duchenne and Becker MD, are caused by mutations in the dystrophin gene which lead to either absence (Duchenne) or reduction (Becker) of the protein product [3,4]. Although the genetic causes for many types of MD have been identified, there is currently no cure for MD. Patients typically rely on physical therapy and oral corticosteroids for symptom management [5].

Initial symptoms of MD include muscle weakness and loss of coordination. In the case of Duchenne MD, abnormalities may be first noticed early in life between the ages of 2-5 years. This type of MD affects the muscles of the pelvis, upper legs, and upper arms resulting in frequent falls, abnormal gait, and difficulty standing from a sitting position. A common observance is the enlargement of the calf muscles caused by fatty
and connective tissue replacement after muscle wasting. Duchenne patients often lose ambulation in their teenage years and most die in their 20s or 30s due to involvement of respiratory muscles or development of cardiomyopathies.

The dystrophin-linked MDs (Duchenne and Becker MD) account for ~90% of all MD cases [6]. In fact, Duchenne MD is the most common lethal genetic disorder in children and affects about 1 in 3500 newborn males. The high incidence of this disorder is not surprising given that the dystrophin gene is the largest in the human genome encompassing 79 exons [4]. Therefore, the probability of mutations in dystrophin is high and Duchenne MD will likely always continue to exist in the population even with genetic counseling efforts. The inheritance pattern for this disorder is X-linked recessive since the mutated gene, dystrophin, is located on the X chromosome. Females harboring one copy of the mutated dystrophin gene and one wild-type copy will typically not be affected by this disease but instead are considered carriers. Males only inherit one copy of the X chromosome and consequently will develop MD when dystrophin is mutated.

The function of dystrophin in a muscle cell is critical because this protein provides the structural link between the inner cytoskeleton and the outer extracellular matrix. The dystrophin protein is made of four major domains: N-terminal, central rod, cysteine-rich, and C-terminal. The N-terminal and central rod domains connect dystrophin to the actin cytoskeleton and the rod domain contains elastic regions that permit the flexibility of this protein. The cysteine-rich and C-terminal domains contribute important regions that allow for binding of dystrophin to the dystroglycan complex in the plasma membrane. The dystroglycan complex spans the plasma membrane and attaches to laminin, linking the entire dystrophin-dystroglycan complex to the basal lamina [7].
When dystrophin is lost or reduced, the structural support connecting the muscle fiber to the extracellular matrix fails during muscle contraction. It is thought that tears occur in the plasma membrane allowing influx of calcium and subsequent degeneration of the muscle fiber. As muscle fibers undergo necrosis, inflammatory immune cells infiltrate and can contribute to the damage of the muscle. Although the muscle fibers can regenerate, eventually the muscle ends up getting replaced by fibrotic and fatty tissue that cannot maintain the muscle contractions [1].

1.2.1 Gene Therapy for Muscular Dystrophy

Recent research progress has offered great hope for cell and gene therapies to treat MD. Since many forms of MD are monogenic disorders, gene replacement is an attractive therapeutic approach. A wide array of strategies have been developed to deliver dystrophin or other affected genes to muscles, many of which have been highly promising in preclinical studies. Particularly, the first report of viral vector-mediated gene delivery for MD recently showed successful transgene expression in human muscle for up to 3 months [8].

Recombinant adeno-associated virus (AAV) has emerged as the most promising gene delivery vector due to its high tropism for skeletal muscle and low immunogenicity. AAV is a replication-defective parvovirus that can be produced relatively efficiently. Although most adults have been infected with AAV, no pathogenicity has been associated with this virus, making it an ideal vector for gene transfer.

Despite the small packaging capacity of the AAV vector (<5 kb), dystrophin, the largest gene in the human genome (2600kb) [4] has been modified into “mini” and
“micro” forms and packaged [9,10]. These smaller versions of dystrophin retain their binding domains with actin in the cytoskeleton and dystroglycan at the cell membrane, but are without most of the central rod domain. The shortened dystrophin is thought to preserve critical regions for signaling and structural support. It is unclear whether these small forms of dystrophin could provide complete rescue in humans, but preclinical studies in the *mdx* mouse model of Duchenne MD have shown that microdystrophin can reverse the dystrophic phenotype [10].

One challenge for clinical translation is to deliver the vector to all muscle types. Several serotypes of AAV (AAV6, AAV8, and AAV9) have shown considerable preclinical success in targeting muscle after vascular delivery in the *mdx* mouse and dystrophic canine [11-13]. Importantly, AAV9 seems to be particularly efficient in transducing cardiac tissue, which is of high interest considering the prevalence of cardiomyopathy in MD [14,15].

It seems likely that regional vascular delivery will be the first step toward systemic vector delivery. This approach typically targets one muscle or a single muscle group isolated by a tourniquet during administration. It has recently been shown to safely and efficiently transduce the limb muscle of the rhesus macaque model, which has high anatomical similarity to humans [16]. Although AAV has not yet been administered systemically in humans, regional vascular delivery for muscle transduction is on the close horizon, opening the door for increasingly systemic approaches.

Another critical issue in MD gene therapy is evasion of the immune system. The most primary concern is the role of the adaptive immune system in prohibiting delivery of the virus to the targeted cells. Since humans are a natural host for AAV, much of the
population possess binding antibodies to AAV [17,18]. Even low levels of these circulating, neutralizing antibodies can significantly inhibit viral gene delivery. However, by prescreening patients for neutralizing viral antibodies or using rare viral serotypes to deliver genes, this issue can potentially be circumvented.

A more unexpected observation has been the cytotoxic T-cell response to the AAV capsid peptides following vector administration. In a 2006 clinical trial for hemophilia, AAV expressing factor IX was delivered to the liver, but only resulted in transient gene expression [19]. The reduction in gene expression was attributed to T cells forming a cytotoxic response to AAV capsid peptides presented on the surface of transduced liver cells. This observation prompted discussions regarding the potential use of immunosuppressants in patients at least until after capsid peptides are fully cleared from transduced cells [17,20]. Interestingly, this damaging cytotoxic T cell response has not been seen in all clinical trials where AAV has been administered. A recent clinical trial for limb-girdle muscular dystrophy type 2D successfully delivered the missing α-sarcoglycan protein to muscle and showed continued gene expression after 3 months [8]. Out of 3 subjects, only one displayed a minimal cytotoxic T cell response to AAV capsid peptides, which did not preclude gene expression. Differences between the above two trials include the AAV serotype, route of administration, and the targeted tissue. Additional studies will be required to determine whether or not immunosuppression is needed to avoid a cell-mediated T cell response.

In addition to the generation of an immune response against AAV capsid, there may also be an immune response mounted to the therapeutic protein delivered by AAV. In the case of Duchenne MD, patients lack the dystrophin protein and therefore
dystrophin peptides could be seen as a foreign antigen. However, many Duchenne patients have “revertant” fibers where some detectable form of dystrophin is expressed in a small percentage of muscle fibers due to alternative splicing events in the dystrophin gene. This small amount of dystrophin protein may be enough to generate peripheral tolerance so that muscle fibers transduced with an AAV expressing microdystrophin would be recognized as “self” instead of attacked as being “foreign.” It is likely that the level of immune response mounted against the corrective dystrophin is dependent on the patient’s individual mutation as well as the amount of revertant fibers present. The possibility exists that some patients may form an immune response that hinders dystrophin gene replacement therapy; however, they may still be able to benefit from gene delivery of utrophin, an endogenous homologue with some ability to compensate for dystrophin [21].

AAV-mediated gene therapy is presently at the forefront of promising treatments for MD. Certainly, the development of gene therapy for MD has not been without obstacles. Creativity and persistence have led to the development of shortened forms of dystrophin as well as optimized viral vectors for gene delivery. The success in the recent human gene transfer trial for Limb-girdle MD type 2D also leads to way to develop future treatments of enhanced therapeutic efficacy. With the optimization of regional and systemic delivery strategies, these treatments have the potential to significantly change the quality of life for MD patients in the near future.
1.2.2 Myostatin Inhibition for Muscular Dystrophy

While dystrophin gene replacement strategies are certainly promising for MD, the genetic cause for some forms of MD, such as facioscapulohumeral MD, is still unclear. One strategy for muscle wasting diseases is to develop approaches to increase muscle mass and strength. Disruption of the myostatin signaling pathway has been shown to significantly enlarge muscle mass [22]. Myostatin is a member of the transforming growth factor β superfamily and is a negative regulator of muscle growth [23]. Myostatin is expressed specifically in developing and adult skeletal muscle [23]. During development, myostatin expression limits the size of the muscle in concert with multiple factors sculpting the limbs in relation to skeletal, vascular, and ectodermal patterns of growth [24]. Myogenic cells respond to myostatin by down-regulating the expression of Pax-3 and Myf-5, important transcriptional regulators of myogenic cell proliferation, and Myo-D, an early marker of muscle differentiation. In their sentinel report in 1997, McPherron et al. demonstrated the biological effect of targeted disruption of growth and differentiation factor-8 (GDF-8) (later named myostatin) in the mouse [23]. GDF-8 null mice were significantly larger in size than wild-type animals and there was widespread increase in skeletal muscle mass. The increase in mass was the result of a combination of muscle hypertrophy and hyperplasia. These experiments established the GDF-8 peptide as a major player in muscle growth with the designated name, “myostatin”.

Myostatin signaling acts through the activin receptor type IIB (ActRIIB) also known as ACVR2. First, there is presumed recruitment of a type I co-receptor [25]. Activin receptor-like kinases 4 and/or 5 (ALK-4, ALK-5) represent candidate coreceptors that are phosphorylated by ActRIIB [26]. This in turn leads to phosphorylation of TGF-β
specific Smads 2 and 3 that form a complex with Smad 4. The Smad 2/3/4 complex is translocated to the nucleus to regulate expression of targeted genes such as MyoD and myogenic regulatory factors (MRFs) [22,27,28].

A wide variety of both pharmacological and gene therapy approaches have been developed to inhibit myostatin [29-31]. A translational study in the mdx mouse, a model for DMD, provided encouraging results using a monoclonal antibody to block myostatin demonstrating both an increase in muscle size and strength [31]. However, when a neutralizing monoclonal antibody to myostatin (MYO-029) was administered in a double-blind randomized clinical trial, a cutaneous hypersensitivity reaction precluded dose escalation [32]. Another approach to achieve myostatin blockade is to deliver the myostatin propeptide, which keeps the myostatin C-terminal dimer inactive, blocking access to the ActRIIB receptor. In one experimental paradigm the propeptide was stabilized by fusion to IgG-Fc and systemically administered to the mdx mouse resulting in increased muscle size concomitant with treatment [33].

1.2.3 Myostatin Inhibition by Follistatin

Another promising approach to inhibit myostatin is through delivery of myostatin antagonist proteins, such as follistatin. Follistatin, secreted as a glycoprotein, was originally identified in porcine ovarian follicular fluid and received its name because it suppresses synthesis and secretion of follicle-stimulating hormone (FSH) from the pituitary gland [34]. The follistatin gene localizes to chromosome 5q11.2. It is composed of a relatively small 1 kb cDNA consisting of six exons, with an alternative splicing site that generates two major isoforms: one version that encodes a 344-amino acid preprotein
and a carboxy-shortened version containing 317 amino acids [35,36]. Prior to activation, follistatin, undergoes further post-translational modification losing another 29 amino acids by removal of a signal peptide resulting in polypeptides of 315 (FS315), often referred to as the long-isoform and 288 (FS288), called the short isoform.

Further understanding of the functional role for follistatin can be gained from study of genetically modified mice. When the follistatin gene was transgenically introduced under control of a muscle specific myosin light chain promoter, muscle mass increase was significantly greater than observed in the myostatin null mutant mouse [22]. These results suggest that at least part of the effect of follistatin results from impacting a pathway independent of myostatin inhibition. This hypothesis was reinforced by additional studies crossing transgenic follistatin overexpressing mice with myostatin null animals [37]. The resulting phenotype appeared to be additive, with a quadrupling of muscle mass in the Follistatin+/Myostatin−/− mouse compared to either follistatin overexpressing or myostatin null animals alone. These findings emphasize that other signaling pathways could be exploited to increase muscle size and strength [37]. Taking these studies into consideration, development of a gene therapy approach to deliver follistatin represents a promising therapeutic strategy for patients with muscle disease. In contrast to pharmacologic administration of myostatin inhibitors such as neutralizing antibodies [31] or drugs such as trichostatin A [38]), gene therapy offers the potential for a single administration of vector carrying the follistatin gene with persistent expression for many years, even perhaps throughout the lifetime of the individual. Chapters 2 and 3 will discuss the preclinical results of AAV-mediated follistatin delivery in detail [39,40].
1.3 Amyotrophic Lateral Sclerosis

While MD is a neuromuscular disorder affecting primarily muscles, Amyotrophic Lateral Sclerosis (ALS) is caused by degeneration of nerve cells, leading to muscle paralysis. ALS was first described over a century ago in 1869 by the well-known neurologist Jean-Martin Charcot. However, today in the US, ALS is best known as Lou Gherig’s disease named after the famous Yankee baseball player who succumbed to this disease. The incidence of ALS is 1-2 per 100,000 in the US corresponding to about 30,000 Americans living with ALS at any given time. The lifetime risk for developing ALS is 1 in 1000 which highlights the impact of this disease [41,42]. Although ALS was first described over 150 years ago, there is only one FDA-approved drug for the treatment of ALS which marginally extends life by about 3 months [43].

ALS is a disease of mid-age, affecting mostly adults between the ages of 40-60 years. Early symptoms of ALS include muscle weakness in the hands, arms or legs, muscle twitching (fasciculation), spasticity, and slurred speech. As disease progresses, muscle atrophy and paralysis spread and can lead to loss of ambulation and difficulty talking, swallowing, and breathing. As respiratory muscles become more weakened, use of a ventilator is necessary and patients usually die from respiratory failure. The average survival from time of diagnosis to death is between 1-5 years although there is great variability between patients [44].

Pathologically, ALS is a degenerative motor neuron disease affecting both upper and lower motor neurons projecting from the motor cortex, brainstem, and spinal cord. Sensory nerves are relatively spared so patients experience no loss of sight, hearing, taste, touch, or smell. Primarily, the large caliber alpha motor neurons are affected in spinal
anterior horns and oddly, motor neurons controlling bladder function and eye movements are mostly saved [45,46]. Motor neuron degeneration often begins focally and asymmetric and then spreads from the initial site of onset [47]. The motor neuron loss is accompanied by reactive gliosis of astrocytes and microglia in the motor cortex, brainstem, and spinal cord [48]. The mechanisms leading to selective motor neuron death are still largely unknown although some of the current hypotheses include aberrant glial toxicity, abnormal protein aggregation, mitochondrial defects, excitotoxicity, oxidative damage, axonal transport problems, and changes in RNA processing [49].

The majority of ALS cases are classified as sporadic ALS (sALS) which denotes the lack of a family history of ALS. There may be a genetic contribution to sALS, but the etiology of this disease is currently unknown. However, susceptibility and modifier genes have been implicated [50]. In contrast, 10% of ALS cases are classified as familial (fALS), meaning there is a family history with ALS usually inherited in an autosomal dominant fashion. Several loci have been linked to familial ALS and the mutated gene products have been identified in some of these cases. These genes include alsn [51,52], senataxin [53,54], synaptobrevin/VAMP (vesicle-associated membrane protein) [55], and dynactin [56]. The mechanisms whereby these mutated genes lead to ALS are still unclear but the functions of these proteins range from vesicle transport to microtubule assembly. Most recently, two additional genes have been found mutated in both sporadic and familial cases of ALS. These genes are tar-DNA binding protein (TDP43) [57-59] and fused in sarcoma protein (FUS) [60,61] and interestingly, they both encode for DNA/RNA binding proteins.
However, the most well-known and studied gene linked to familial ALS is the Cu/Zn superoxide dismutase 1 (SOD1) which contributes to 20% of familial ALS cases corresponding to about 2% of all ALS [62]. SOD1 is a cytosolic enzyme that converts superoxide anion to hydrogen peroxide and oxygen by the following reaction:

$$2 \text{O}_2^- + 2 \text{H}^+ \rightarrow \text{O}_2 + \text{H}_2\text{O}_2$$

SOD1 functions as a homodimer and each subunit binds one zinc and one copper atom. Through cycles of reduction and oxidation of copper, the reactive oxygen species $\text{O}_2^-$ can be converted into hydrogen peroxide. Over 100 SOD1 mutations have been described in ALS and the majority of these mutations are missense mutations scattered throughout all five exons of the SOD1 gene [63].

Given the antioxidant activity of SOD1, one may hypothesize that mutations cause a reduction in superoxide activity leading to oxidative stress and cell death. However, not all SOD1 mutations affect the enzyme’s dismutase activity. In fact, loss of SOD1 by homozygous knockout in mice does not lead to motor neuron disease, arguing against a loss-of-function mechanism in ALS pathogenesis [64]. In contrast, transgenic mice engineered to express the human mutant SOD1 gene develop motor neuron disease [65]. The presence of a disease phenotype in these mice argues for a gain-of-function mechanism since these mice retain endogenous mouse SOD1 enzyme activity.

1.3.1 Mouse Models of ALS

With the discovery of SOD1 mutations in ALS, the generation of mouse models of familial ALS was soon to follow. Currently, 12 different SOD1 mutations have been expressed in lines of transgenic mice leading to development of some version of motor
neuron disease. These transgenic mice have most commonly been generated using constructs of 12-15 kb of human genome containing the mutant SOD1 gene including the endogenous promoter and regulatory elements leading to ubiquitous expression in the mouse. The most commonly used animal model of ALS is the SOD1<sup>G93A</sup> mutant mouse which contains 25 copies of transgene corresponding to ~10-15 fold increase in SOD1 protein [65,66]. These mice have a clinical disease onset around 3 months of age that includes hindlimb tremor and muscle weakness. Their disease progresses quickly from onset and they become increasingly paralyzed accompanied by muscle atrophy and locomotor deficits. The SOD1<sup>G93A</sup> mice typically die between 4-5 months with endstage marked as the point that they can no longer right themselves in a 30 second time interval. The features of this mouse model closely resemble human ALS both clinically as well as pathologically. Degeneration of motor neurons in the spinal cord, brainstem, and motor cortex is accompanied by retraction of the nerve at the neuromuscular junction. Pathological features of motor neurons in this model include mitochondrial abnormalities [67], aggregates of SOD1 protein [68], and neurofilament inclusions [69] which have also been seen in ALS patients. Importantly, astrogliosis and microgliosis are also prominent hallmarks of this model which increase as disease progresses [70].

### 1.3.2 Non-cell autonomous features of ALS

In recent years, rodent models of ALS have led to significant progress in the field. While it is clearly established that the paralysis associated with ALS results from the death of upper and lower motor neurons, studies utilizing transgenic mouse models of ALS have suggested that the surrounding environment affects motor neuron survival
A range of studies from various groups have expressed the mutant SOD1 protein only in certain cell types instead of ubiquitously. Lines of transgenic mice were generated where mutant SOD1 expression was limited to neurons driven by either the Thy1 promoter or neurofilament light chain promoter [73-75]. In two of these three studies [73,74], the mice did not develop any neurological disease phenotype. A more recent study did report motor neuron disease with neuronal mutant SOD1 expression, however, the disease onset was very late (~500 days of age) and highly variable, emphasizing the important contribution of mutant non-neuronal cells in disease [75]. In addition, reciprocal studies have shown that neuronal mutant SOD1 expression is also required for disease since expression of mutant SOD1 solely in astrocytes does not lead to motor neuron disease [76]. When mutant SOD1 is driven by the prion promoter expressing in both astrocytes and neurons but not microglia, mice develop an ALS-like disease [77]. Overall, these studies have lead to a general consensus in the field that mutant SOD1 must be expressed in multiple cell types in order to cause ALS.

Further studies using chimeric mice and small interfering RNA (siRNA) continued to support this hypothesis [71,78]. In the landmark chimeric mouse study, mice were generated from a mixture of wild-type cells and cells that expressed mutant SOD1 [71]. It was observed that mice with a greater proportion of wild-type cells to mutant SOD1 cells had extended survival. In addition, motor neurons expressing mutant SOD1 that were surrounded by wild-type non-neuronal cells had less severe pathology. Likewise, wild-type motor neurons surrounded by non-neuronal cells expressing mutant SOD1 appeared unhealthy, suggesting that neighboring non-neuronal cells may play a role in the death of MNs. Furthermore, targeting only the motor neurons with a siRNA to
reduce mutant SOD1 levels showed only a transient effect of motor neuron protection, suggesting that other cell types were contributing to the ultimate demise of the motor neurons [78]. Collectively, these studies suggest that ALS is a non-cell autonomous disease, meaning that mutant SOD1-expressing cells (such as non-neuronal cells) can cause other cells (such as motor neurons) to exhibit a mutant phenotype.

More recent studies using the cre-lox system to specifically remove mutant SOD1 expression in specific cell lineages have started to identify the cell types important for various phases of disease. Mice that express reduced levels of mutant SOD1 only in motor neurons but express high levels in all other cell types have a significant delay in disease onset [72,79]. In contrast, mice that express mutant SOD1 in all cell types except peripheral macrophages and microglia show slowed disease progression [72,80]. The reduction of mutant SOD1 in astrocytes by expression of cre via the GFAP promoter similarly slows disease progression [79]. Therefore, the presence of mutant SOD1 in different cell types appears to have distinct roles in the onset and progression phases of disease. The current hypothesis in the field implicates mutant SOD1-expressing microglia and astrocytes as the cells directing disease progression while mutant motor neurons play an unknown role in disease onset.

While focus has been on the involvement of neurons, astrocytes and microglia in ALS pathogenesis, a variety of studies have investigated other cell lineages as well. The removal of mutant SOD1 in Schwann cells seems to modestly accelerate disease progression for unknown reasons [81]. Since motor neurons form close connections with muscle at the neuromuscular junction, the role of muscle in ALS has been extensively investigated. When the mutant SOD1 protein was genetically removed by cre
recombinase from only muscle cells, there was no effect on survival in the SOD1\textsuperscript{G93A} mouse model. Additionally, viral-mediated delivery of a SOD1 siRNA specifically to muscle did not improve the phenotype of these mice; however, viral-mediated delivery of SOD1 siRNA to both muscle and motor neurons resulted in a significant increase in grip strength [82]. Together, these studies present a strong argument against mutant SOD1-expressing muscle playing a primary role in ALS.

1.3.3 In vitro Models of ALS

Transgenic mouse models have elucidated specific cell types that may contribute to ALS disease onset and progression. Within the past couple years, several groups have established in vitro ALS models that are mouse-derived and recapitulate the glial-mediated motor neuron toxicity shown in transgenic ALS mouse models. Two independent in vitro studies have demonstrated that astrocytes expressing mutant SOD1 are toxic to motor neurons in co-culture models of ALS [83] [84]. Chapter 4 of this thesis describes related work supporting that neural progenitor-derived astrocytes isolated from mutant SOD1 mice are toxic to embryonic stem cell-derived motor neurons [85]. Similar work has investigated the toxic effects of primary microglia isolated from mutant SOD1 mice in a co-culture with motor neurons [86]. The motor neurons co-cultured with mutant SOD1-expressing microglia had a reduction in the number and length of neurites and reduced survival in the co-culture. Importantly, this in vitro work supports the findings that mutant SOD1 expression within microglia or astrocytes in vivo has a detrimental effect on motor neuron survival and provides the groundwork for the establishment of reliable in vitro models to investigate ALS disease mechanisms.
While both transgenic rodent models and mouse-derived \textit{in vitro} ALS models have provided invaluable insights into the basic biology of ALS, it is still questioned how closely these mouse models reflect human disease. It is intriguing why the most common SOD1 mutation leading to ALS, the A4V mutation, does not cause motor neuron disease when over-expressed in a transgenic mouse \cite{87}. One strategy to more closely recapitulate human ALS has been to develop \textit{in vitro} co-culture systems based on human cells, instead of mouse cells. Recently, it has been demonstrated that motor neurons can be differentiated from human embryonic stem cells and these human motor neurons are susceptible to toxicity from astrocytes isolated from the SOD1$^{G93A}$ mouse model \cite{88}. Additionally, human motor neurons derived from embryonic stem cells are sensitive to human fetal primary astrocytes overexpressing mutant SOD1 by lentivirus \cite{89}. These \textit{in vitro} models support that mutant SOD1-expressing astrocytes are toxic to motor neurons regardless of the species and provide a way to study familial ALS utilizing human cells. Chapter 5 will discuss the development of novel \textit{in vitro} co-culture models to investigate aberrant glial toxicity in sporadic ALS.

Development of \textit{in vitro} ALS models provides another tool to investigate disease mechanisms and test therapeutics for ALS. Unfortunately, there has been a disconnect in the translation of drugs from rodent models of ALS to human clinical trials. While various drugs have shown promise in rodent models, there continues to be disappointment in clinical trials which may be a result of various factors including poor preclinical testing regimen, ineffective clinical design and delivery, or use of an animal model that does not accurately reflect human disease \cite{90}. ALS \textit{in vitro} co-culture models could be a helpful tool to identify drugs which modulate glial activity and be
utilized as a complement to the ALS mouse model to select more effective drugs for further clinical development.

### 1.3.4 Neuroinflammatory features of ALS

Glial-mediated motor neuron toxicity has been one of the most studied ALS pathogenic mechanisms over the past five years. However, there is still a debate concerning whether the neuroinflammatory process activated by motor neuron injury is neuroprotective or harmful. It is well known that both microgliosis and astrogliosis occur in the spinal cord and brain of familial and sporadic ALS patients as well as in the ALS mouse model. Additionally, increases in infiltrating T cells, dendritic cells, and macrophages/monocytes characterize sites of motor neuron injury in ALS. Astrocytes become activated early in the mouse model, even before clinical disease onset, and this is followed by microglial activation [70]. Increases in proinflammatory cytokines, chemokines, reactive oxygen species, and prostaglandins are reported to occur in parallel with pathological gliosis [91,92]. While microglia are implicated as being the cell type responsible for producing many of these inflammatory genes, it is still unclear to what degree microglia and astrocytes are responsible.

Astrocytes were initially thought to play solely a structural role in the CNS, yet these cells have been shown to be involved in increasingly complex interactions with other cell types. Some of these roles include regulation of the extracellular environment including synapses, maintenance of cell-cell communication, CNS vascular control, growth factor production, and neurotransmitter metabolism [92]. The initial observation of astrogliosis in ALS patients and in the ALS mouse model led to investigation of the
role of astrocytes in this disease. One early finding was the loss of the astrocyte glutamate transporter EAAT2 (GLT1 in mice) in both familial and sporadic ALS including the ALS mouse model [93-95]. This glutamate transporter is responsible for removing excess glutamate from the extracellular environment. Since glutamate is one of the primary excitatory neurotransmitters in the brain, excess glutamate in synaptic spaces can lead to excitotoxicity and neuronal death. While not likely a primary cause of ALS, drugs are currently in clinical development with the aim to increase expression of EAAT2 on astrocytes in hopes to reduce extracellular glutamate levels. It is also interesting to note that the only FDA approved drug for ALS is thought to act by reducing glutamate levels. In addition to changes in astroglial glutamate transporters, other changes have also been reported in astrocytes isolated from the ALS mouse model. Increases in levels of iNOS and nitric oxide, prostaglandin E$_2$ (PGE$_2$), and COX-II have been found as well as elevated levels of various cytokines including TNF-α and IL-1 [96,97].

Microglia have also been implicated as being a glial cell type of importance in ALS pathogenesis and are likely a primary source of proinflammatory cytokines and chemokines. Typically, microglia are characterized as the resident “immune cells” of the brain and are constantly sampling their environment for potential insults. Microglia, like astrocytes, can become activated and respond to environmental changes by regulation of their morphological shape, surface receptor expression, and production of both growth factors as well as cytokines, chemokines, and reactive oxygen species. Depending on their environment and how other cells interact with microglia, these cells may adopt either an inflammatory “M1” phenotype or a more neuroprotective “M2” phenotype characterized by growth factor release and anti-inflammatory cytokine production [98-
Numerous reports have cited microglia containing the mutant SOD1 protein to be more proinflammatory or of an “M1” phenotype than wild-type microglia [86]. Specifically, mutant SOD1-expressing microglia show increased activation of specific genes involved in the inflammatory response including CCL2, inducible nitric oxide synthase (iNOS), NADPH oxidase (NOX2), TNF-α, and IL-1[91,96,102-107].

A broad assumption in ALS is that the presence of immunological cells in the CNS is generally harmful. Yet, recent studies have shown that SOD1<sup>G93A</sup> mice lacking T cells have accelerated disease progression, arguing for a neuroprotective role for T cells in ALS [108,109]. It has been suggested that infiltrating T cells may cross talk with microglia and macrophages in the CNS and promote differentiation toward a neuroprotective “M2” phenotype.

While T cells play a beneficial role in ALS, numerous reports have cited activation of specific genes involved in the inflammatory response to play a detrimental role in ALS pathogenesis. Surprisingly, genetic approaches to eliminate specific inflammatory proteins in the ALS mouse model have mostly failed. Crossing ALS mice to knockout mice lacking TNF-α [110], IL-1β [104], COX1 [111], or iNOS [112] resulted in no extension in survival. These studies have led to the hypothesis that multiple inflammatory genes are likely contributing to ALS pathogenesis, not just one or two. Therefore, future therapies should be directed toward targeting central mediators of inflammation such as NF-κB or STAT signaling. Chapter 6 of this thesis discusses the role of NF-κB signaling in ALS and the development of strategies to block NF-κB signaling for ALS.
1.3.5 Gene delivery to the CNS for neurodegenerative disorders

One of the largest hurdles in treating disorders of the CNS is the delivery of therapies to the diseased cells. The blood-brain-barrier (BBB) serves as a natural barricade to protect the CNS from potential pathogenic insults as well as maintain concentration gradients in the CNS allowing for a stable environment. However, the BBB also prohibits most systemically administered pharmacologic and gene therapies from reaching their target neuronal or glial cells of the CNS. Various strategies have been developed to bypass the BBB including direct injection into the CNS and the engineering of drugs or gene therapy viruses so that they can enter the CNS.

Both drugs and therapeutic viruses can be injected into the CNS either into the cerebrospinal fluid (CSF) or directly into the parenchyma. Intraparenchymal injection is a viable way to deliver a therapy to a precise region although there are obvious safety concerns. In addition, diffusion from the site of injection may be limited, especially since many drugs have extremely short half-lives. Injection into the cerebrospinal fluid of the ventricles (intraventricular) or the intrathecal space of the the spinal cord (intrathecal) could provide for more systemic diffusion of drug through CSF circulation. In fact, implantation of intrathecal pumps which continuously deliver drug is a routine medical procedure.

Regardless of the delivery route, the in vivo stability of the therapeutic protein is still a large concern. Additionally, drugs delivered by direct injection can rarely be targeted to specific cell types and excluded from others. For these reasons, many have turned to gene therapy as a novel method to deliver therapeutic proteins. By using a virus to deliver a gene, continuous production of the therapeutic protein can be achieved
without constant injections. Viruses can also be targeted to specific cell types either by engineering of the viral capsid so that the virus will only infect certain cells or by design of a cell-type specific promoter to drive gene expression.

While the BBB can block viral entry into the CNS similar to pharmacologic drugs, recent advances have identified ways around this barricade. Certain types of viruses have the ability to be retrogradely transported in neurons. For example, injection of an adeno-associated virus (AAV) expressing therapeutic IGF-1 to the muscle leads to motor neurons absorbing this virus and transporting it back to their cell body for continuous expression of IGF-1 [113]. Osmotic agents such as mannitol have also been used to successfully achieve transport of AAV across the BBB. Mannitol can be injected prior to intravascular virus delivery to cause a temporary opening of the BBB. In fact, a recent study showed that AAV2 can successfully cross the BBB after mannitol treatment and transduce neurons and non-neuronal cells, mainly oligodendrocytes, of the CNS [114]. Similarly, AAV of serotype 9 has been shown to be able to cross the BBB when delivered by an intravascular route [115]. Injection of AAV9 into neonatal mice targeted 60% of lower motor neurons, an important finding for infantile diseases of motor neurons such as spinal muscular atrophy. In addition, intravascular injection of AAV9 into adult mice surprisingly targeted astrocytes and microglia with >60% of grey matter astrocytes being transduced. Obviously, this finding was exciting since so many diseases of the CNS such as ALS have either primary or secondary pathogenesis caused by glial cells. The mechanism whereby AAV9 can cross the BBB is still unclear. Since the cell-type transduced changes between the neonate and the adult despite the same injection route, it has been suggested that developmental changes occur which influence viral delivery. For
example, astrogenesis occurs within the first two weeks of life in the mouse and may provide a reason for astrocyte-specific transduction of AAV9 in the adult [116]. Chapter 6 of this thesis will describe a potential use for AAV9 in targeting astrocytes to deliver an anti-inflammatory protein for ALS.

1.3.6 Neurotrophic factors for ALS

Multiple neurotrophic factors have been investigated in both rodent models and in clinical trials for their ability to ameliorate ALS disease pathogenesis. Two promising candidates are insulin-like growth factor 1 (IGF-1) and vascular endothelial growth factor (VEGF) which have been shown to be the most effective neurotrophic factors in prolonging survival in multiple ALS rodent studies [113,117-120]. Kaspar et al. significantly showed that IGF-1 could be delivered by a retrogradely transported adeno-associated viral vector to motor neurons from muscle [113]. Importantly, this study evaluated the delivery of IGF-1 at the time of disease onset (90 days) and demonstrated a significant increase in survival in the SOD1G93A mouse model. Similarly, Azzouz et al. showed that VEGF delivered to motor neurons by a retrogradely transported lentiviral vector could extend survival when delivered at disease onset in the SOD1G93A mouse [117]. A crucial finding from both of these studies was that IGF-1 and VEGF treatment slowed disease progression when delivered at the time of disease onset. Since glial cells direct disease progression, perhaps motor neurons were secreting IGF-1 or VEGF to the surrounding glia to reduce aberrant activation and further protect the motor neuron. It was shown by Kaspar et al. that IGF-1 treatment reduced astrogliosis and decreased levels of TNF-α in the lumbar spinal cord [121]. From these studies it is difficult to conclude
whether IGF-1 directly had an effect on gliosis or if improved motor neuron pathology led to a reduction in gliosis. Both IGF-1 and VEGF receptors have been found to be expressed on glial cells and VEGF receptors may become upregulated during astrogliosis [122-124]. Although IGF-1 has produced disappointing results in clinical trials, these trials may have failed due to the lack of IGF-1 that reached the desired target given that IGF-1 was injected subcutaneously [125,126]. Indeed, IGF-1 may need to reach both motor neurons as well as glial cells in order to achieve maximal therapeutic benefits. Chapter 4 will directly explore the ability of IGF-1 to protect motor neurons through reducing aberrant glial activity.

1.4 Summary and significance

Neuromuscular disorders affect thousands in the US including both patients suffering from these debilitating diseases as well as caregivers providing physical, emotional, and financial support. Treatments for many of these diseases are on the near horizon. However, in some cases, the etiology for the disease is unknown and little if any therapies are available. The goal of the studies presented here is to gain a further understanding of the pathogenic disease mechanisms of neuromuscular disorders and develop translational therapies to attempt to treat these diseases.

In the case of muscular dystrophy, many of the genes which cause the disorder are well known and have been extensively characterized. Consequently, there are a plethora of promising new therapies reaching clinical trial that aim to correct the defective genes. However, there are some forms of muscular dystrophy as well as other muscle wasting diseases where a genetic target is still unknown.
For these reasons, the **aim** of our studies was to develop a strategy to increase muscle mass and strength. We hypothesized that inhibiting myostatin, a negative regulator of muscle growth, would lead to increased muscle mass and strength. To inhibit myostatin, we developed an AAV gene therapy vector to deliver the gene follistatin, an endogenous myostatin antagonist. We first tested AAV-Follistatin in both wild-type mice and in a mouse model of muscular dystrophy described in **Chapter 2**. A single intramuscular injection of AAV-Follistatin was able to successfully increase muscle mass and strength without any adverse effects for up to 2 years in these mice.

Next, we tested AAV-Follistatin for efficacy and safety in the non-human primate since these animals are biologically and anatomically closer to humans. As presented in **Chapter 3**, AAV-Follistatin was delivered by intramuscular injection to the quadriceps muscle of a *cynomologous macaque*. The injected leg of the macaques showed enlargement in quadriceps size as well as increases in strength sustained until necropsy after one year. Importantly, no adverse effects were noted in any major organ system including the reproductive system, suggesting AAV-Follistatin is a safe and well-tolerated therapy for muscle disorders. We feel that these studies are of particular **significance** given that AAV-Follistatin represents a promising new strategy to treat a variety of muscle wasting diseases. In fact, formal toxicology studies for AAV-Follistatin have already been initiated in mice in preparation for an Investigational New Drug (IND) application and hopefully a clinical trial in the near future.

In contrast to the muscular dystrophy field, the ALS field currently does not have an abundance of promising new therapies reaching the clinic. One reason may be that there is not an identified genetic defect for 90% of ALS cases. Even in cases where genes
have been linked to ALS, no clear pathogenic mechanism has been discovered. Although
it is known that ALS is caused by degeneration of motor neurons, by the time of
diagnosis, many of the patients’ motor neurons have already died. Therefore, one
emerging strategy is to target the cells involved in the progression phase of disease which
are CNS glial cells. The aim of our studies was investigate how glial cells contribute to
motor neuron death in ALS and develop therapies to inhibit this aberrant glial toxicity.

We hypothesized that co-culturing ALS astrocytes in vitro with motor neurons
would result in motor neuron cell death. As described in Chapter 4, mutant SOD1-
expressing astrocytes proved to be toxic to motor neurons in vitro. In addition, motor
neurons could be rescued by treatment with the growth factor IGF-1 which has also
shown to be effective in the ALS mouse model. Using this in vitro co-culture model, we
were able to identify one mechanism of action of IGF-1 which was to suppress astrocyte-
mediated motor neuron toxicity.

While mouse models of familial ALS can be useful, SOD1 ALS only accounts for
2% of the total ALS cases. It is still unknown whether the same mechanisms lead to
motor neuron death in fALS compared to sALS. We hypothesized that astrocytes from
sporadic ALS patients would also cause motor neuron death in an in vitro co-culture
system. To test this hypothesis, we derived astrocytes from human sporadic ALS patients
and co-cultured these cells with motor neurons. The results presented in Chapter 5 show
astrocytes derived from sporadic ALS patients were toxic to motor neurons through an
unknown secreted factor. Our data highlights a common pathway to motor neuron death
between sporadic and familial ALS mediated by astrocyte dysfunction. Significantly, we
also introduce the first model system to investigate molecular disease mechanisms and evaluate therapies for sporadic ALS.

Aberrant glial activation is a prominent feature in human ALS and can be recapitulated in *in vitro* co-culture models of ALS. Since glial cells are involved in the progression of disease, it is likely that a therapy targeted to these cells would slow disease progression although this sort of therapy will most likely not be a “cure”. We hypothesized that suppressing a central mediator of inflammation in glial cells would decrease glial activation and slow disease progression. As described in Chapter 6, we found NF-κB to be activated in astrocytes isolated from the ALS mouse model. By suppressing NF-κB, reduction of a variety of inflammatory cytokines and chemokines could be achieved. Future studies will involve testing inhibition of NF-κB in both *in vitro* co-cultures of ALS as well as in the ALS mouse model with the ultimate goal to slow disease progression.
CHAPTER 2

LONG-TERM ENHANCEMENT OF SKELETAL MUSCLE MASS AND STRENGTH BY SINGLE GENE ADMINISTRATION OF MYOSTATIN INHIBITORS

2.1 Introduction

Muscle-enhancing strategies have been proposed for a number of neuromuscular disorders, including muscular dystrophies and age-related muscle disorders, and have shown promising results to build or regenerate stronger, healthier muscles [127]. These strategies have mainly focused on the use of trophic factors, such as insulin-like growth factor-1 that induce myocyte precursor proliferation and myofiber hypertrophy [128]. Attention has recently highlighted the potential benefit for inhibiting myostatin, resulting in the doubled muscle phenotype of myostatin deficient cattle[129-131] and myostatin knockout mice [29,132]. Myostatin is a transforming growth factor-β (TGF-β) family member that plays a crucial role in regulating skeletal muscle mass [23,25]. Myostatin appears to function in two distinct roles: to regulate the number of myofibers formed in development and to regulate the postnatal growth of muscles. The regulation of muscle growth postnatally is being explored by various pharmacological methods for a number of muscle disorders. Delivery of neutralizing antibodies against myostatin has shown
promise in dystrophic mdx mice,[31] yet there have been varying reports on the efficacy to enhance muscles when delivered in aged animals [133]. Furthermore, recent data demonstrated muscle mass enhancement and morphological recovery in muscular dystrophy mice treated with deacetylase inhibitors. The resulting muscle enhancement was attributed to an increase in the protein follistatin, which has been shown in part to inhibit the activity of myostatin [38]. Trichostatin A (TSA) treatment required daily administration and was not evaluated in aged animals where off target effects may exist.

The identification of myostatin binding proteins capable of regulating myostatin activity has led to potential new approaches for postnatal muscle enhancement and expanded the potential for gene therapy to be considered as a method to inhibit myostatin activity. Follistatin (FS) has been shown to bind to some TGF-β family members and can function as a potent myostatin antagonist. Overexpression of follistatin by transgenic approaches in muscle has been shown to increase muscle growth in vivo,[22] and a lack of follistatin results in reduced muscle mass at birth [134]. Recent data has also shown that follistatin is capable of controlling muscle mass through pathways independent of the myostatin signaling cascade. In these studies, myostatin knockout mice were crossed to mice carrying a follistatin transgene. The resulting mice had a quadrupling of muscle mass compared with the doubling of muscle mass that is observed from lack of myostatin alone, confirming a role for follistatin in the regulation of muscle mass beyond solely myostatin inhibition [37]. In addition to follistatin, two other proteins have been identified that are involved in the regulation of the myostatin. Follistatin-related gene (FLRG) is highly similar to follistatin and has been shown to inhibit activin and multiple bone morphogenic proteins in vitro [135,136]. Growth and differentiation factor-
associated serum protein-1 (GASP-1) is a protein that has been discovered to contain multiple domains associated with protease-inhibitor proteins and a domain homologous to the 10-cysteine repeat found in follistatin. GASP-1 was shown to bind directly to the mature myostatin and myostatin propeptide and inhibits myostatin’s activity [137]. Although recombinant protein injections or myostatin blocking antibodies are feasible strategies, gene therapy to express these myostatin inhibitor genes may prove a more efficacious therapeutic route for numerous reasons, including the lack of potential immune response to antibody treatment and the requirement for multiple injections.

Here, we report that a one-time postnatal intramuscular injection of adeno-associated virus (AAV) encoding myostatin inhibitor proteins resulted in long-term improvement of muscle size and strength in wild-type animals. Delivery of a myostatin inhibitor protein in dystrophic mdx animals reversed muscle pathology and improved strength, even when administered in 6.5-month-old animals. Specifically, we show here that follistatin-344 resulted in the greatest effects on muscle size and function and was well tolerated with no untoward effects on cardiac pathology or reproductive capacity in either male or female treated animals.

2.2 Materials and Methods

Animals. C57BL/6, C57BL/10, and C57BL/10ScSn-DMDmdx/J were purchased from The Jackson Laboratory. All studies were approved by Institutional Animal Care and Use Committee.

Cloning and AAV Production. The cDNA for human follistatin-344 (FS) was obtained from Origene, follistatin-related gene (FLRG) was obtained from American Type Culture
Collection, and growth and differentiation factor associated serum protein 1 (GASP-1) was cloned from a human cDNA library (Clontech). Recombinant AAV serotype 1 vectors were produced by a contract manufacturing company (Virapur).

**AAV Injections and Testing.** Mice received bilateral intramuscular injections of a total dose of $1 \times 10^{11}$ viral particles (high = $1 \times 10^{11}$, low = $1 \times 10^{10}$) ($n=10–15$ per group) at 3–4 weeks of age or at 6.5 months of age. Muscle strength was assessed weekly, using a grip strength meter [82]. Force measurements were recorded in three separate trials and averaged. Mouse coordination was tested by using the accelerating rotarod (Columbus Instruments).

**Histological Analysis.** Muscles were dissected, weighed, snap-frozen in liquid nitrogen-cooled isopentane, cryostat sectioned, and stained by hematoxylin-eosin (H&E) or succinic dehydrogenase (SDH) for analysis of fiber diameters. Five animals per group were chosen randomly for muscle fiber size morphometry. For each analysis, five representative pictures (one central and four peripheral) were taken of muscle sections, compounding to 0.7 mm$^2$. Images were captured at x20 magnification, and myofiber diameters measured with a calibrated micrometer, using the AxioVision 4.2 software (Zeiss). Fiber size-distribution histograms were generated and expressed as percentage of total fibers analyzed.

**Creatine Kinase and Follistatin Assay.** Serum creatine kinase measurement was performed by using a creatine kinase test kit (Pointe Scientific) and expressed as units/liter. Serum was collected at 90 days after injection and assayed by using the human follistatin quantikine ELISA kit (R&D Systems) with normalization to controls.
**Statistical Analysis.** All statistical analysis was performed in Graph Pad Prizm software, using one- and two-way ANOVA with Bonferroni post hoc analysis.

### 2.3 Results

AAV-mediated gene delivery to muscle provides a system to generate high levels of protein in the target tissue or by a secreted product carried to remote sites through the circulation [138]. We cloned the known secreted myostatin-inhibiting genes, including growth and differentiation factor-associated serum protein-1 (GASP-1),[137] follistatin-related gene (FLRG),[136] and follistatin-344 (FS)[22] into AAV serotype 1, which have demonstrated high muscle transduction capabilities. There are two isoforms of follistatin generated by alternative splicing. The FS-344 variant undergoes peptide cleavage to generate the FS-315 isoform and the other FS-317 variant produces the FS-288 isoform after peptide cleavage. We used the human FS-344 variant, which exclusively generates the serum circulating FS-315 isoform of FS and includes a C-terminal acidic region [35]. We chose FS-344 (FS), because the other FS-317 isoform, lacking the C terminus, shows preferential localization to the ovarian follicular fluid and high tissue binding affinity through heparin sulfate proteoglycans, which may affect reproductive capacity and bind to other off-target sites [139]. FS-288 represents the membrane-bound form of follistatin,[140] is a potent suppressor of pituitary follicle stimulating hormone,[141] is found in the follicular fluid of the ovary and in the testes, and demonstrates a high affinity for the granulosa cells of the ovary.

We sought to determine the efficacy of these proteins to increase muscle mass in normal and dystrophic mice. We administered $1 \times 10^{11}$ AAV1 viral particles per animal
encoding FS, FLRG, GASP-1, or GFP bilaterally into the quadriceps and tibialis anterior muscles of 4-week-old wild-type C57BL/6 mice. All animals treated with the myostatin inhibitors demonstrated an increase in body mass with an observable gross enhancement of muscles when analyzed at 725-days of age compared with GFP-treated controls (Fig. 2.1 a and b).

Figure 2.1. Myostatin inhibitor proteins increase muscle mass and strength in wild-type C57BL/6 mice. (a) Gross hindlimb muscle mass is increased in all myostatin-inhibitor-protein treated mice at 725 days of age compared with AAV1-GFP injected controls. (b)
Total body mass is significantly increased in AAV1-FS-injected (**, $P < 0.01$) and AAV1-GASP1-injected (*, $P < 0.05$) mice compared with AAV1-GFP controls at 725 days of age ($n = 10$). (c) The mass of individual hindlimb and forelimb muscles is increased in mice injected with AAV expressing myostatin inhibitor proteins ($n=10$) (*, $P < 0.05$). (d) Hindlimb grip strength improves for over 2 years in all treated mice with the greatest differences in AAV1-FS treated animals compared with AAV1-GFP controls ($n=10$). Error bars represent standard error.

Evaluation of individual muscle weights showed an increase in muscle mass for all myostatin inhibitor-treated animals, with the greatest increase in FS-treated animals. The increased muscle mass was found in the injected hindlimb muscles and remote muscles to the injection site, such as the triceps. Thus, these inhibitors were secreted into the circulation from the site of muscle injection, enhancing skeletal muscle mass at remote sites (Fig. 2.1c). The enlarged muscle mass was accompanied by functional improvement demonstrated by an increase in hindlimb grip strength (Fig. 2.1d). There was no effect on heart mass or histological appearance of cardiomyocytes, indicating that myostatin inhibition was selective to skeletal muscle tissue (data not shown). There has been concern that FS adversely effects gonadal function. We found no change in reproductive capacity in mice treated with our AAV1 carrying the FS344 transgene (Table 2.1).
Table 2.1. Reproduction was normal in animals treated with AAV1-FS.

<table>
<thead>
<tr>
<th>Reproductive study group</th>
<th>n</th>
<th>Mean litter size (SD)</th>
</tr>
</thead>
<tbody>
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<td></td>
<td></td>
</tr>
<tr>
<td>AAV1-FS-treated male × untreated female</td>
<td>4</td>
<td>9.0 (2.582)</td>
</tr>
<tr>
<td>AAV1-FS-treated female × untreated male</td>
<td>4</td>
<td>9.25 (1.708)</td>
</tr>
<tr>
<td>Untreated male × untreated female</td>
<td>4</td>
<td>9.0 (2.160)</td>
</tr>
<tr>
<td>mdx</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AAV1-FS-treated male × untreated female</td>
<td>3</td>
<td>4.5 (0.707)</td>
</tr>
<tr>
<td>AAV1-FS-treated female × untreated male</td>
<td>2</td>
<td>2.0 (0)</td>
</tr>
<tr>
<td>Untreated male × untreated female</td>
<td>6</td>
<td>3.83 (1.169)</td>
</tr>
</tbody>
</table>

Male and female C57BL/10 and mdx mice treated for either 2 years or 6 months, respectively, were able to breed normally with no differences in the litter size of treated animals compared with noninjected controls. ($P > 0.05$.)

Furthermore, we found no histological/pathological alterations in the gonadal tissue of FS treated-mice compared with controls (data not shown).

Given the robust effects of FS delivery, we next tested the potential for AAV1-FS delivered postnatally in a clinically meaningful paradigm to increase muscle mass and strength and delay muscle deterioration in the mdx mouse model of Duchenne muscular dystrophy (DMD). DMD is an X-linked recessive disease resulting in the wasting of skeletal muscles and cardiac function, ultimately resulting in death. Recently, FS was investigated in mdx animals overexpressing a duplicated domain of the follistatin gene.
Results demonstrated increased muscle mass and attenuated pathology, although the results were only documented to 15 weeks of age [142]. In our studies, *mdx* animals were injected bilaterally in the quadriceps and tibialis anterior muscles with a low (1 x 10^{10} viral particles) or high dose (1 x 10^{11} viral particles) of AAV1-FS at 3 weeks of age and followed for 5 months before necropsy. Increased levels of circulating FS were detected in the serum of both low and high dose treated animals with the high dose expressing the greatest levels of serum detected FS (high dose, 15.3 +/- 2.1 ng/ml; low dose, 6.8 +/- 0.4 ng/ml; GFP controls, 0 +/- 0.1 ng/ml; *n* = 8 per group; *P* <0.01). We demonstrated that AAV1-FS increased body mass compared with GFP treated controls, with the greatest increase in the high dose FS group (data not shown). Gross observation of AAV1-FS treated mice displayed a significant increase in muscle size compared with AAV1-GFP treated animals (Fig. 2.2a), with the greatest individual muscle weight increase in high dose FS-treated animals (Fig. 2.2b).
Figure 2.2. Single injection of AAV1-FS increases muscle mass and strength in young mdx mice. (a) Gross hindlimb muscle mass is increased in AAV1-FS injected mdx animals at 180 days of age compared with AAV1-GFP-injected controls. (b) The mass of individual hindlimb and forelimb muscles is increased at 180 days of age in mice injected
at 3 weeks of age with AAV1-FS compared with AAV1-GFP controls (n = 15). *, P < 0.05. (c) Grip strength is improved in a dose-dependent manner in young mdx mice injected at 3 weeks of age with AAV1-FS followed for 180 days (n = 15). Red, high-dose AAV1-FS; blue, low-dose AAV1-FS; green, AAV1-GFP controls. Error bars represent standard error.

Effects were not restricted to the injected muscles; they were also found at sites remote from directly targeted muscles (Fig. 2.2b). Increased muscle mass translated to a dose-dependent improvement in muscle strength in the hindlimbs and forelimbs of treated animals compared with GFP treated controls (Fig. 2.2c). Histological and morphometric analyses of AAV1-FS injected muscles and at remote sites demonstrated myofiber hypertrophy, supporting gross observations made at the time of necropsy (Fig. 2.3a–c).
Figure 2.3. *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 with AAV1-GFP control. (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 hatched line) is significantly increased in mice injected with AAV1-FS compared with AAV1-GFP-injected controls. (P < 0.001; n = 5). (c) The mean diameter of intermediate and light myofibers (indicated by hatched line) in the triceps is significantly increased in mice injected with AAV1-FS compared with AAV1-GFP-injected controls. (P < 0.001; n=5). (d) The distribution of dark, intermediate, and light fibers as determined by succinic dehydrogenase (SDH) staining is not changed by treatment with high or low doses of AAV1-FS. (P < 0.05 between all groups; n = 5.) (e) The mean number of fibers counted per an unbiased 0.14 mm² counting frame is decreased in the tibialis anterior of AAV1-FS-treated mice, given that the mean diameter of myofibers is increased. (*, P < 0.01; n = 5.) Error bars represent standard errors.
Furthermore, there was no shift in muscle fiber types in AAV-FS treated animals; however, there were fewer total fibers per square millimeter of area in the tibialis anterior muscle in animals treated with the high dose AAV-FS (Fig. 2.3d and e). Strikingly, FS-treated mice demonstrated a significant reduction in serum creatine kinase compared with GFP-treated controls (Fig. 2.4a).
Figure 2.4. *Mdx* mice treated with AAV1-FS show decreased markers of muscle damage and aged *mdx* mice are responsive to FS treatment with functional benefit. (a) Serum creatine kinase levels (units/liter) are decreased at 3 months after injection with AAV1-FS compared with AAV1-GFP-injected controls. (*, *P* < 0.05; *n* = 10.) Error bars represent standard errors. (b) Hindlimb grip strength is significantly increased (*P* < 0.05) at 275 days and beyond in aged *mdx* mice treated with AAV1-FS at 210 days of age (*n* =
15). Red, high-dose AAV1-FS; green, AAV1-GFP controls. (c) H&E stain of aged gastrocnemius demonstrates reduced pathology when injected at 210 days of age with FS compared with GFP-injected controls. (Original magnification, x40.) (d) H&E stained diaphragm of animals injected at 210 days of age with FS shows less fat replacement than GFP-injected controls at late stage. (Original magnification, x20.)

We also evaluated the potential for AAV1-FS to increase muscle strength in *mdx* animals when treated at an older age. We found that AAV1-FS injection at 210 days of age increased muscle strength <60 days after administration and that the increased strength persisted long-term throughout the 560 days evaluated in this study (Fig. 2.4b). As early as 180 days of age, before AAV1-FS treatment, there was evident pathology in muscles of untreated *mdx* animals, with prominent endomysial connective tissue proliferation and inflammation (Fig. 2.4c and d). Pathological evaluation of gastrocnemius and diaphragm muscles at 560 days of age demonstrated that AAV1-FS treated animals had substantially fewer focal groups of necrotic muscle fibers and mononuclear cell infiltrates. Importantly, AAV1-FS treated animals had significantly reduced focal areas of endomysial connective tissue proliferation, which were pronounced in GFP treated animals, demonstrating that fibrosis, a hallmark of muscular dystrophy, was decreased in FS-treated animals (Fig. 2.4c). Pathology in the diaphragm also showed that FS-treatment reduced inflammation and fatty replacement compared with GFP-treated animals (Fig. 2.4d). Furthermore, AAV1-FS treatment demonstrated significant increases in muscle fiber diameters at this age compared with control GFP-
treated animals (Fig. 2.4c and d). These results demonstrated that myostatin inhibition by
FS treatment was beneficial in aged mdx animals that had undergone multiple rounds of
muscle degeneration and regeneration.

2.4 Discussion

In this study, delivery of myostatin inhibitor genes by AAV to mouse muscle led
to significant increases in muscle mass and strength. Several myostatin inhibitor proteins
were tested (FLRG1, GASP1, and FS) and the most potent muscle-enhancing protein was
found to be follistatin (FS). Although AAV1-FS was delivered by intramuscular injection
to defined muscle groups, effects of myostatin inhibition were observed in remote
muscles as well. We hypothesized this was due to the secretion of follistatin into the
serum, allowing for systemic administration. We did detect increased levels of follistatin
in the serum of treated mice, explaining the remote muscle-enhancing effect. Others have
shown that postnatal myostatin inhibition leads to an increase in muscle mass through a
mechanism of myofiber hypertrophy. In our study, we confirmed that delivery of AAV1-
FS caused myofiber hypertrophy. In addition to myofiber hypertrophy, we reported a
decrease in inflammation, fatty tissue replacement, and serum creatine kinase. This is of
interest, because FS was protective despite its lack of correction of the underlying
dystrophin deficiency. The exact mechanism is not clear, but one might speculate that
increasing the strength of individual fibers makes them less susceptible to damage from
the stress of normal activities. Collectively, these results suggest that follistatin would be
an ideal therapy for muscle diseases characterized by muscle wasting, regeneration and
degeneration, and inflammation.
In our studies, we chose to specifically use the 344 isoform of follistatin which is thought to be the serum circulating isoform. The other isoform, follistatin 317, is found in the follicular fluid of the ovary and in the testes, and demonstrates a high affinity for the granulosa cells of the ovary. Since follistatin plays an endogenous role in reproductive physiology, we investigated reproduction in treated animals even though we used the follistatin 344 isoform exclusively. The gonads of mice treated with AAV-FS344 showed no pathological abnormalities and these mice were able to breed normally and deliver average-sized litters. In addition to the reproductive system, we noticed no other abnormalities in treated mice, indicating that follistatin was well tolerated.

Importantly, follistatin was effective in both wild-type mice as well as in the *mdx* mouse model of MD. Our results show that even if follistatin is delivered at an older age in the *mdx* mouse, this therapy can still improve muscle strength and pathology. Translation to a clinical parallel suggests that AAV-mediated FS gene therapy could have potential for the older DMD patient independent of replacing the missing gene and may have a potential role in combinational therapy similar to that demonstrated for IGF-1 and minidystrophin gene replacement [143]. These results suggest that inhibition of myostatin by FS-344, delivered by a single AAV1 injection can enhance muscle size and strength and is well tolerated for >2 years. AAV1-FS treatment may offer a more powerful strategy than those targeting solely myostatin because of additive effects, such as follistatin’s involvement in multiple signaling pathways, and the recent finding demonstrating a reduction in inflammation in a model of endotoxemia [37,144]. The striking ability of FS to provide gross and functional long-term improvement to
dystrophic muscles in aged animals warrants its consideration for clinical development to treat musculoskeletal diseases, including older DMD patients.
CHAPTER 3

FOLLISTATIN GENE DELIVERY ENHANCES MUSCLE GROWTH AND STRENGTH IN NONHUMAN PRIMATES

3.1 Introduction

Severe weakness of the quadriceps is a defining feature of several neuromuscular disorders, including sporadic inclusion body myositis, Becker muscular dystrophy, and myotonic dystrophies. Despite progress in understanding the pathophysiological basis of these conditions, few treatment strategies have produced satisfactory results. Androgen steroids, popular among athletes, offer a means to enhance strength but pose long-term risks [145]. Glucocorticosteroids, used in patients with Duchenne muscular dystrophy (DMD)[5,146] improve muscle strength and function in the short term, but their long-term benefits remain unclear [147]. Gene manipulations to treat genetic muscle disease, including gene replacement [8,12,148,149], exon skipping [150], and mutation suppression [151,152], are being assessed in early clinical trials, but lasting benefits have yet to be established. Moreover, these approaches are not applicable to muscle disorders that lack a defined gene defect, such as facioscapulohumeral muscular dystrophy, characterized by weakness and degeneration of voluntary muscles [153]. An alternative strategy, inhibition of the myostatin pathway, has shown substantial promise in preclinical studies, in which significant enlargement of muscle mass and increased
muscle strength have been noted [22,25,29,30,154]. Myostatin is a member of the transforming growth factor–β (TGF-β) superfamily of signal peptides that is expressed specifically in developing and adult skeletal muscle [23]. In myogenic cells, myostatin induces down-regulation of Myo-D, an early marker of muscle differentiation, and decreases the expression of Pax-3 and Myf-5, which encode transcriptional regulators of myogenic cell proliferation [27].

Myostatin signaling acts through the activin receptor type IIB (ActRIIB) on skeletal muscle, triggering a cascade of intracellular events [26]. After recruitment of a co-receptor, followed by sequential phosphorylation of TGF-β–specific Smads, the protein complex translocates to the nucleus, where it controls the expression of specific myogenic regulatory genes [26-28]. Inhibition of this pathway results in muscle hypertrophy and increased strength. Indeed, injection of a neutralizing monoclonal antibody to myostatin led to increased skeletal muscle mass in mice without undue side effects [31]. This method was found to be safe in a subsequent clinical trial, although dose escalation was limited by cutaneous hypersensitivity restricting potential efficacy [32].

Several myostatin-binding proteins capable of regulating myostatin activity have been discovered. Follistatin is an especially robust antagonist of myostatin and has even shown muscle-enhancing effects beyond those predicted by myostatin inhibition alone [37]. This naturally occurring glycoprotein, which prevents myostatin from binding to ActRIIB receptors on muscle cells [22], occurs in two distinct isoforms (FS288 and FS315), a result of alternative splicing of the precursor messenger RNA [35,36]. FS288, but not FS315, functions in reproductive physiology collaboratively with activin and
inhibins of the hypothalamic-pituitary-gonadal axis [155,156], which suggests that FS315 would be the more reliable isoform for exclusively targeting muscle. In a previous study, we tested the effects of follistatin in a small animal model by injecting normal and dystrophic mice intramuscularly with an adeno-associated virus serotype 1 (AAV1) expressing the human FS344 transgene, which encodes the FS315 protein isoform. This treatment led to significant increases in muscle mass and strength even when given to older mice showing repeated cycles of muscle degeneration and regeneration [39]. Thus, using a relatively noninvasive strategy in mice, we were able to achieve long-term gene expression with positive effects on degenerating muscle.

Despite the safety and potent myostatin-inhibitory effects of AAV1-FS344 in mice, such results are not necessarily applicable to humans. We therefore extended our FS344 gene transfer technology to a nonhuman primate, the cynomolgus macaque (Macaca fascicularis), to establish a paradigm for strengthening the quadriceps muscle that could serve as the basis for testing in patients. We report here that injection of AAV1-FS344 was well tolerated by all macaques and led to increased muscle mass and strength.

### 3.2 Materials and Methods

**Animals.** The cynomolgus macaques were a gift from Battelle and were housed at the nonhuman primate facility of the Research Institute at Nationwide Children’s Hospital. Protocols for all animal 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.

**Cloning and AAV production.** The cDNA for the human FS344 gene was obtained from Origene and cloned by KpnI/XbaI restriction into an AAV inverted terminal repeat vector plasmid containing the MCK promoter or by XhoI/BamHI restriction into a second AAV vector plasmid containing the CMV promoter. Recombinant AAV1 vectors were produced by a standard triple-transfection calcium phosphate precipitation method using human embryonic kidney 293 cells [157]. The production plasmids were (i) pAAV.CMV-FS or pAAV.MCK-FS, (ii) rep2-cap1-modified AAV helper plasmid encoding the cap serotype 1, and (iii) an adenovirus type 5 helper plasmid (pAdhelper). Viruses were purified from clarified 293 cell lysates by sequential iodixanol gradient purification and ion exchange column chromatography with a linear NaCl salt gradient for particle elution. Vector genome titers were determined by quantitative polymerase chain reaction (QPCR) as described [158].

**Follistatin enzyme-linked immunosorbent assay (ELISA).** Muscle follistatin was evaluated with a human follistatin immunoassay kit (Quantikine; R&D Systems) according to the manufacturer’s instructions. Briefly, total soluble protein was isolated from the muscle tissue with CellLytic MT Mammalian Tissue Lysis reagent (Sigma). A total of 100 mg of protein was loaded per well, and muscle follistatin concentrations were determined against a standard curve made with recombinant human follistatin provided by the manufacturer.

**Morphometrics.** During necropsy, skeletal muscles were dissected and snap-frozen in liquid nitrogen–cooled isopentane. Cryosections, 8 to 10 μm thick, were stained with
either H&E or myofibrillar ATPase (pH 4.6) for analysis of fiber diameters. For each animal, 12 random 20X images were captured with a Zeiss AxioCamMRC5 camera. For each fiber type, the smallest diameter was measured with a calibrated micrometer using AxioVision 4.2 software (Zeiss). A frequency distribution was used to determine percentage of fibers within 20-µm intervals. Cardiac muscle was collected during necropsy, and formalin-fixed tissues were sectioned at 4 µm and stained with H&E for analysis of cardiomyocyte diameter. About 400 cardiomyocytes were measured for each animal.

Muscle physiology. These experiments were performed immediately before necropsy. Macaques were sedated with Telazol (3 to 6 mg/kg) and maintained on isoflurane and oxygen (4 to 5%). Buprenorphine (0.1 mg/kg) was given to minimize pain. The femoral nerve was dissected and affixed with miniature platinum-stimulating electrodes connected to a stimulator (STIM2; Scientific Instruments). Quadriceps muscle was prepared free of skin, fascia, and connective tissue. The hip was immobilized with restraining straps around the lower waist and upper thigh. The distal tendon was disconnected at the patella and secured to a force transducer (Imada, DS-2) for assessment of twitch and tetanic contractions after muscle stimulation.

Immune response studies. Peripheral blood mononuclear cells (PBMCs) were collected at baseline and monthly intervals after vector delivery and tested in an IFN-γ ELISpot assay for reactivity to human follistatin and AAV1 capsid antigens. Briefly, 96-well polyvinylidene difluoride microtiter plates (Millipore) were precoated with antibodies to macaque IFN-γ (U-Cytech), and $2 \times 10^5$ PBMCs in AIM-V medium containing 2% heat-inactivated human serum were added to each well. Duplicate wells contained pools of
overlapping synthetic peptides at a concentration of 1 µg/ml (18 amino acids in length with an 11-residue overlap) prepared for the AAV1 capsid (104 peptides) and human follistatin (48 peptides; Genemed Synthesis). Plates were incubated at 37°C for 36 hours and developed for spot formation by using a second antibody to IFN-γ conjugated to enzyme followed by substrate. Spot forming colonies (SFCs) in the microtiter wells were quantified with a CTL analyzer. Green fluorescent peptide pool served as the negative control, whereas concanavalin A was used as the positive control for cell viability. The number of spots was calculated by subtracting the number of negative control spots from each well.

**Statistical analysis.** We used means and SEM or SD to summarize results obtained with more than two macaques; otherwise, individual values are reported, and all statistical analyses were performed with GraphPad Prism software and paired t tests.

### 3.3 Results

We injected the vector AAV1-FS344 under the control of the ubiquitously strong cytomegalovirus promoter (CMV-FS) or the muscle creatine kinase promoter (MCK-FS), which provides muscle-specific gene expression, into the right quadriceps muscle of six normal cynomolgus macaques. Briefly, each animal received three 500-µl doses of the vector injected at 3-cm intervals in a diagonal line extending from a proximal site (7 cm below the inguinal ligament) to the midpoint of the quadriceps muscle. This pattern was intended to provide vector to adjacent bellies of the vastus medialis, rectus femoris, and vastus lateralis muscles (**Fig. 3.1a**).
Figure 3.1. Injection of AAV1-FS344 into the quadriceps increases muscle mass in cynomolgus macaques. (a) AAV1-FS344 was administered by three direct unilateral injections into the right quadriceps muscle (total dose of $1 \times 10^{13}$ vector genomes in 1.5 ml). Indian ink tattoos, drawn immediately after the injections, allowed the vector to be localized at necropsy. (b) Concentrations of human follistatin in muscle, as measured by ELISA at 5 and 15 months after injection. The values represent individual macaques with
four muscle samples analyzed per macaque. (c) Increases in quadriceps size after injection of AAV1-FS344, driven by either the CMV-FS or the MCK-FS promoter. Mean ± SEM values for three macaques per treatment are shown. Asterisks indicate a 15% increase over baseline at 8 weeks in the CMV-FS group ($P = 0.01$) and a 10% increase in the MCK-FS group ($P = 0.02$). (d) Quadriceps enlargement seen at necropsy of MCK-FS and CMV-FS macaques.

A total dose of $1 \times 10^{13}$ vector genomes was administered per treatment. The product of the human FS344 transgene used in these studies has 98% homology with follistatin in nonhuman primates. In all comparisons, the contralateral quadriceps served as a noninjected control. To control for any remote effects of naturally occurring follistatin, we conducted parallel studies in age-matched, untreated macaques. Because gene transfer can be compromised by the immune system, as demonstrated in both preclinical studies [159-161] and human gene therapy trials [19], we maintained the animals with the immunosuppressants tacrolimus (2.0 mg/kg) and mycophenylate mofetil (50 mg/kg) beginning 2 weeks before vector administration.

In both the CMV-FS (n = 3) and the MCK-FS (n = 3) cohorts, we necropsied one macaque at 5 months and two at 15 months after vector administration and then measured concentrations of human follistatin in the quadriceps muscle of each animal. The three CMV-FS–treated primates had marked increases of follistatin (>20 ng per milligram tissue) at both 5 and 15 months after gene transfer, in contrast to the minimally elevated concentrations in the MCK-FS–treated group (Fig. 3.1b). We also measured the external
circumference of the thigh muscles at the midpoint of the quadriceps muscle every 4 weeks after gene transfer through week 20 (before any animals were necropsied). Macaques in the CMV-FS group showed a 15% increase in quadriceps circumference over baseline (P = 0.01) at 8 weeks after treatment, followed by growth stabilization through week 20 (Fig. 3.1c). Although quadriceps size increased by 10% (P = 0.02) in the MCK-FS group, this beneficial effect was delayed relative to the CMV-FS group (12 weeks versus 8 weeks), and by week 16, there was an overlap between quadriceps measurements in the MCK-FS group and the untreated controls. The gains in quadriceps circumference achieved with AAV1-FS344 were maintained from week 20 to week 60 after treatment for CMV-FS [20.5 ± 1.15 cm (SEM) at 20 weeks (n = 3) versus 21.75 cm at 60 weeks (n = 2)] and MCK-FS [18.17 ± 0.46 cm (SEM) at 20 weeks (n = 3) versus 19.75 cm at 60 weeks (n = 2)] without any appreciable loss. Enlargement of the quadriceps muscle in these animals was sufficient to be appreciated by gross inspection of the excised quadriceps at the time of necropsy (5 months after injection) (Fig. 3.1d). Together, the results show that expression of the FS344 transgene under control of the CMV promoter results in more muscle follistatin than does the MCK promoter, leading to more robust increases in quadriceps size. Histologic sections comparing control and follistatin-treated quadriceps muscles showed an increase in mean fiber size diameter at 20 weeks in both the CMV-FS [87.7 ± 1.30 mm (SEM), P = 0.001]) and the MCK-FS (73.0 ± 0.74 mm, P = 0.04) groups (n = 3 each) relative to untreated controls (65.5 ± 0.62 mm) (Fig. 3.2a and b).
Figure 3.2. AAV1-FS344 treatment causes myofiber hypertrophy and affects fast-twitch type 2 myofibers in the quadriceps muscle. (a) H&E staining of quadriceps muscle reveals myofiber hypertrophy in CMV-FS macaque (right) compared to untreated control (left). Scale bars, 20 μm. (b) Morphometric analysis of quadriceps muscle at 5 months after AAV1-FS344 injection demonstrates a significant increase in mean fiber diameter (dotted lines) of both MCK-FS ($P < 0.04$, $n = 3$ muscle samples) and CMV-FS ($P < 0.001$, $n = 3$ muscle samples) animals relative to untreated controls ($n = 3$ muscle samples). (c) Representative fiber types determined by ATPase staining (pH 4.6) of quadriceps muscle from CMV-FS macaques (right) and untreated controls (left). Scale
bars, 20 μm. (d) Fiber type ratios in control, CMV-FS, and MCK-FS muscles. Mean ± SEM values for three animals are shown. (e) Mean ± SEM fiber diameters (dotted lines) of fast-twitch oxidative glycolytic type 2a and fast-twitch glycolytic 2b fibers show significant increases in three CMV-FS animals relative to untreated controls ($P < 0.001$); slow-twitch oxidative type 1 fibers are not affected. Similar but not statistically significant trends are apparent in the MCK-FS group ($n = 3$).

Follistatin treatment primarily affected fast-twitch oxidative glycolytic type 2a and fast-twitch glycolytic type 2b fibers. Myofibrillar adenosine triphosphatase (ATPase) staining (pH 4.6) revealed increases in the mean diameters of both type 2a [CMV-FS: 85.4 ± 2.13 μm (SEM), $P < 0.001$; MCK-FS: 71.4 ± 2.53 μm, $P = $ not significant (n.s.), versus controls, 66.3 ± 1.15 μm] and type 2b [CMV-FS: 102.3 ± 1.13 μm (SEM), $P < 0.001$; MCK-FS: 79.3 ± 0.56 μm, $P = $ n.s., versus controls, 77.0 ± 0.95 μm] fibers without alterations in the ratio of muscle fiber types (types 1, 2a, and 2b each represented about one third of total fibers) (Fig. 3.2c to e). Therefore, the enhancement of type 2 fibers by CMV-FS treatment is likely to augment high force–generating fibers, used for getting to a standing position or compensating for knee weakness.

In principle, the muscle enlargement we observed would be expected to correlate with increases in muscle strength. This prediction can be technically challenging to test in macaques, requiring in situ quadriceps muscle measurements in anesthetized animals. Nonetheless, we assessed muscle strength in two CMV-FS and two MCK-FS macaques.
by comparing measurements for the treated leg with those for the contralateral untreated side (technical problems prevented data interpretation for the second CMV-FS macaque).

Twitch force measurements in treated muscles of the two MCK-FS macaques showed an 11.8% and a 35.7% increase, respectively, relative to findings in the opposite untreated side (Table 3.1).

Table 3.1. Strength measurements of AAV1-FS344-treated quadriceps in macaques measured in newtons (N).

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<th>Twitch force (N)</th>
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<td>19.0</td>
</tr>
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<td>MCK-FS #2</td>
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</tbody>
</table>

These same animals had 12.3% and 77.9% increases in tetanic force over measurements of the untreated side. In the single CMV-FS macaque, the twitch force was increased by 26.3% and the tetanic force by 12.5%. The greatest percent increases in twitch and tetanic forces were found in the quadriceps muscle of an MCK-FS macaque (MCK-FS #2, Table 1) whose untreated leg showed the lowest force generation among all of the legs tested. This result may indicate that increased muscle size after follistatin treatment does not necessarily result in a proportional stronger force generation, as has been seen with other approaches to myostatin inhibition [162]. Additional study will be required to resolve this
issue, yet the enhancement of force generation after treatment with both CMV-FS and MCK-FS supports the clinical effort to improve muscle mass and strength.

Despite administering immunosuppressive drugs to both the MCK-FS–treated and the CMV-FS–treated macaques, we could not be certain that we had avoided immune responses to the AAV capsid and human follistatin. Thus, we used the enzyme-linked immunospot (ELISpot) assay to detect antigen-induced secretion of cytokines by T cells isolated monthly from peripheral blood mononuclear cells (PBMCs). At no time during the study did we observe antigen-specific interferon-γ (IFN-γ) responses to either follistatin or the AAV1 capsid (Fig. 3.3) in either the MCK-FS or the CMV-FS group; this finding suggests that, under the condition of this experiment, therapy with the FS344 gene expressed by an adeno-associated viral vector did not elicit an immune response.
Figure 3.3. The FS344 transgene and the AAV1 capsid do not produce cellular immune responses. (a) PBMCs were isolated before injection and at monthly intervals thereafter and then were stimulated with human follistatin peptide pools (I and II) and AAV1 capsid peptide pools (I, II, and III) or with concanavalin A (ConA; positive control). IFN-γ release was measured with an ELISpot assay; representative data obtained at 5 months after injection are shown. (b) Mean ± SEM spot-forming colonies (SFCs) for each antigen per 1 × 10⁶ PBMCs are shown over 5 months for each treatment group (n = 3). Increases in SFC did not exceed 25, the threshold (red dashed line) for significance with this assay in our laboratory.
To assess the long-term safety of this treatment, we performed a panel of hematologic and biochemical tests every 4 weeks for the duration of the 15-month study. Neither the MCK-FS nor the CMV-FS group (n = 3 each) showed abnormal changes from baseline in liver, kidney, or muscle function or in hematopoiesis (Table 3.2).
Table 3.2. Blood and serum chemistry profiles of AAV1-FS344-treated macaques.

Hematology and serum chemistries were monitored throughout the study monthly after gene transfer. Data from 5 and 15 months are shown. The values are means +/- SD (n=3); there were no significant changes at any time. CK, creatine kinase, ALT, alanine aminotransferase, AST, aspartate aminotransferase, BUN, blood urea nitrogen, GGT, γ-glutamyltranspeptidase.
Table 3.2 Blood and serum chemistry profiles of AAV1-FS344-treated macaques.

<table>
<thead>
<tr>
<th></th>
<th>MCK-FS</th>
<th></th>
<th>CMV-FS</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>5 Months after injection</td>
<td>15 Months after injection</td>
<td>Baseline</td>
</tr>
<tr>
<td>Hemoglobin (mg/dl)</td>
<td>11.7 ± 1.2</td>
<td>12.3 ± 0.7</td>
<td>13.5</td>
<td>12.9 ± 0.9</td>
</tr>
<tr>
<td>Leukocytes (10^3/mm^3)</td>
<td>9.4 ± 3.6</td>
<td>11.0 ± 1.8</td>
<td>7.5</td>
<td>13.2 ± 1.7</td>
</tr>
<tr>
<td>Platelets (10^3/mm^3)</td>
<td>444.7 ± 78.6</td>
<td>473.7 ± 101.5</td>
<td>448.5</td>
<td>475.3 ± 21.2</td>
</tr>
<tr>
<td>CK (U/liter)</td>
<td>282.3 ± 123.3</td>
<td>103.3 ± 34.0</td>
<td>261.0</td>
<td>315.1 ± 436.8</td>
</tr>
<tr>
<td>ALT (U/liter)</td>
<td>29.7 ± 12.9</td>
<td>19.7 ± 2.1</td>
<td>31.5</td>
<td>28.7 ± 10.3</td>
</tr>
<tr>
<td>AST (U/liter)</td>
<td>35.3 ± 3.51</td>
<td>34.7 ± 9.9</td>
<td>37.5</td>
<td>44.3 ± 11.4</td>
</tr>
<tr>
<td>BUN (mg/dl)</td>
<td>19.0 ± 1.0</td>
<td>12.3 ± 1.5</td>
<td>16.0</td>
<td>16.3 ± 4.9</td>
</tr>
<tr>
<td>Creatinine (mg/dl)</td>
<td>0.5 ± 0.1</td>
<td>0.8 ± 0.1</td>
<td>0.7</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>GGT (U/liter)</td>
<td>72.0 ± 28.8</td>
<td>92.0 ± 38.7</td>
<td>77.5</td>
<td>77.0 ± 20.7</td>
</tr>
</tbody>
</table>
Normal serum concentrations of creatine kinase confirmed histologic studies of skeletal and cardiac muscle, demonstrating that gene transfer does not cause muscle fiber breakdown (Table 3.2 and Fig. 3.4).

**Figure 3.4.** AAV1-FS344 treatment does not cause hypertrophy of cardiomyocytes. (a) H&E staining of cardiac muscle from both the MCK-FS and the CMV-FS groups reveals normal cardiac muscle histology compared with nontreated controls. Scale bars, 50 μm. (b) Morphometric analysis shows similar mean ± SEM diameters of cardiomyocytes.
from AAV1-FS344–treated macaques (MCK-FS and CMV-FS groups) compared with untreated controls ($n = 3$ per group).

Female macaques in the CMV-FS or MCK-FS group ($n = 1$ each) maintained normal menstrual cycles throughout the study, and sperm isolated from a CMV-FS male macaque showed normal motility and counts (Tables 3.3 and 3.4).
Table 3.3. Menstrual cycles in untreated control and AAV1-FS344 treated cynomolgus macaques. Menstrual cycles were monitored in female macaques following gene transfer. Both CMV-FS and MCK-FS treated macaques showed normal menstrual cycle duration compared to untreated control macaque. Data from 6 months prior to necropsy are shown.

<table>
<thead>
<tr>
<th>Months Post Injection</th>
<th>Control</th>
<th>MCK-FS</th>
<th>CMV-FS</th>
</tr>
</thead>
<tbody>
<tr>
<td>10 months</td>
<td>+ (27)</td>
<td>+ (26)</td>
<td>+ (26)</td>
</tr>
<tr>
<td>11 months</td>
<td>+ (24)</td>
<td>+ (31)</td>
<td>+ (28)</td>
</tr>
<tr>
<td>12 months</td>
<td>+ (28)</td>
<td>+ (24)</td>
<td>ND</td>
</tr>
<tr>
<td>13 months</td>
<td>ND</td>
<td>+ (29)</td>
<td>+</td>
</tr>
<tr>
<td>14 months</td>
<td>+</td>
<td>+ (31)</td>
<td>+ (30)</td>
</tr>
<tr>
<td>15 months</td>
<td>+ (29)</td>
<td>+</td>
<td>+ (26)</td>
</tr>
<tr>
<td>Mean ± SD menstrual cycle duration (days)</td>
<td>27.0 ± 2.2</td>
<td>28.2 ± 3.1</td>
<td>27.5 ± 1.9</td>
</tr>
</tbody>
</table>

Abbreviations: ND, Not detected; SD, Standard Deviation

“+” indicates menstrual cycle detected. A value in parenthesis indicates days between two consecutive menstrual cycles.
Table 3.4. Sperm motility and morphology of untreated control and AAV1-FS344 treated cynomolgus macaques. Prior to necropsy, sperm was collected from untreated control and AAV1-FS344 treated macaques. There was no significant change in sperm motility and morphology in the CMV-FS macaque compared to untreated control macaques.

<table>
<thead>
<tr>
<th>Group</th>
<th>Motility (%)</th>
<th>Normal Morphology (%)</th>
<th>Abnormalities (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control #1</td>
<td>60</td>
<td>91</td>
<td>9</td>
</tr>
<tr>
<td>Control #2</td>
<td>90</td>
<td>77</td>
<td>23</td>
</tr>
<tr>
<td>Mean (Control)</td>
<td>75</td>
<td>84</td>
<td>16</td>
</tr>
<tr>
<td>CMV-FS</td>
<td>65</td>
<td>76</td>
<td>24</td>
</tr>
</tbody>
</table>

Additionally, concentrations of follicle-stimulating hormone (FSH), luteinizing hormone (LH), estradiol, and testosterone remained within normal physiological concentrations compared to the baseline values. We did find a slight increase in estradiol and LH concentrations in one macaque (MCK-FS #2); however, these were within normal ranges and within the normal fluctuations seen in vivarium-housed nonhuman primates (Table 3.5).
Table 3.5. Hormonal findings in AAV1-FS344–treated cynomolgus macaques.

Hormonal concentrations were followed in each cynomolgus macaque after gene transfer. FSH, LH, estradiol, and testosterone showed little change from baseline. Concentrations from each individual animal can be traced by number. Empty slots at 15 months were the result of previous necropsies (PN). The widest variations were seen in the male testosterone concentrations. The baseline values of testosterone were low as seen in some vivarium-housed macaques. Those with low values remained in the same range after gene transfer. Each value represents a single animal, reported in the same order at each test interval. The broad fluctuations of some hormone concentrations are consistent with findings in macaques held in vivariums.
<table>
<thead>
<tr>
<th>Time point</th>
<th>Animal no.</th>
<th>FSH (ng/ml)</th>
<th>LH (ng/ml)</th>
<th>Estradiol (pg/ml)</th>
<th>Testosterone (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Males</td>
<td>Females</td>
<td>Males</td>
<td>Females</td>
</tr>
<tr>
<td>Baseline</td>
<td>1</td>
<td>0.53</td>
<td>1.7</td>
<td>0.34</td>
<td>0.57</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.74</td>
<td>1.39</td>
<td>2.21</td>
<td>1.03</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.39</td>
<td>0.35</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.34</td>
<td>0.18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 Months</td>
<td>1</td>
<td>0.52</td>
<td>1.65</td>
<td>0.68</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.42</td>
<td>1.39</td>
<td>0.78</td>
<td>2.35</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>0.5</td>
<td>0.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.36</td>
<td>0.25</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 Months</td>
<td>1</td>
<td>PN</td>
<td>1.21</td>
<td>PN</td>
<td>0.64</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>0.29</td>
<td>2.42</td>
<td>0.23</td>
<td>2.34</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>PN</td>
<td>PN</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>0.45</td>
<td>0.42</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
A complete necropsy was performed on all macaques at either 5 or 15 months after treatment. To further evaluate the effects of gene transfer on key organs, we collected heart, liver, lung, spleen, kidney, testis, ovary, and uterus and processed the tissue sections by staining with hematoxylin and eosin (H&E) for a blinded histopathologic analysis by a board-certified veterinary pathologist. Examination of the heart showed that this organ was of normal size in both treated and untreated macaques. Moreover, in stained sections of cardiac tissue, cardiomyocytes did not show evidence of hypertrophy (Fig. 3.4a). Morphometric analysis revealed no differences in cardiomyocyte diameter among CMV-FS, MCK-FS, and control animals [28.78 ± 0.71 µm (SEM) versus 28.17 ± 0.50 µm versus 29.7 ± 1.03 µm, respectively] (Fig. 3.4b). Similarly, none of the other organs examined had abnormalities that could be attributed to gene transfer. These findings suggest the long-term safety of AAV1-FS344 therapy controlled by the CMV or MCK promoter in nonhuman primates.

3.4 Discussion

In this study, therapy with an isoform of human follistatin delivered by an AAV1 vector to quadriceps muscle in cynomolgus macaques resulted in increased muscle size and strength. Expression of the transgene, FS344 complementary DNA (cDNA), which encodes the soluble circulating 315–amino acid follistatin protein isoform [163], affected skeletal muscle exclusively; the likelihood of an effect on nonmuscle tissue is low, owing to the poor affinity of the FS344 product for heparan sulfate proteoglycan–binding sites on cell surfaces [140]. We did not observe any treatment-related pathologic changes in
major organ systems by analysis of serum chemistries or by direct histologic examination. Our results also indicate that AAV1-FS344 treatment does not appear to interfere with reproductive physiology in primates, as serum estradiol, testosterone, LH, and FSH concentrations remained similar to baseline throughout the study.

The FS344 transgene exhibited long-term expression for up to 15 months after gene transfer. Muscle growth was apparent in both the MCK-FS and the CMV-FS groups for the first 12 weeks after treatment, after which the growth rates appeared to stabilize. This result suggests that, after a peak increase in growth, a feedback loop sustains the enlarged muscle fibers while preventing uncontrolled growth. This model is consistent with observations of spontaneous myostatin inhibition seen in cattle or in mice lacking the myostatin gene [23,131]. The CMV-FS had the largest effect on muscle size, which suggests that dosage of follistatin is important and that the CMV promoter is superior to the MCK promoter for high-level expression of transgenes in the muscle.

The potential use of AAV1-FS344 for muscle strengthening has important implications for patients with muscle diseases. In DMD, the most common severe form of childhood muscular dystrophy, experimental and clinical gene replacement strategies use a small dystrophin cDNA designed to fit into an AAV vector [10]. Co-delivery of the small dystrophin fragment with a second growth-enhancing agent insulin-like growth factor 1 resulted in full functional recovery in the mdx mouse [143]. We suggest that FS344 could fill a similar role when co-delivered with small dystrophins. Other forms of muscular dystrophy might also respond to FS344 gene therapy. In facioscapulohumeral muscular dystrophy, for example, in which gene replacement or other types of gene manipulation are not feasible because of the absence of a specific gene defect, one might
achieve a clinically significant result by targeting AAV1-FS344 to the focal muscle
groups that are affected by the disease [153]. Finally, the gene therapy we describe could
be useful against acquired disorders, such as sporadic inclusion body myositis, in which
quadriceps muscle weakness is an important cause of disability [164].

Despite the beneficial effects of follistatin gene therapy that we demonstrated in
macaques, these animals did not have a degenerative muscle disease, and so our findings
may not translate successfully to clinically effective treatments for such diseases. In
particular, genetic disorders of muscle are characterized by cycles of degeneration and
regeneration, which could lead to loss of the nonintegrating AAV1 vector, hence
diminishing therapeutic efficacy. Nevertheless, there are data suggesting that this may not
be a problem and that AAV1-FS344 therapy may in fact be useful in patients. First,
myostatin inhibition with AAV1-FS344 leads to increased muscle size and strength and
lowers serum creatine kinase in the mdx mouse model of DMD [39] despite the
pronounced cycles of muscle degeneration-regeneration in this animal model. The
benefits of AAV1-FS344 treatment persist for more than a year in these mice, which
suggests that as muscle strength and size increased, a protective shield developed to
guard muscle from further degeneration-regeneration [39]. In addition, in human DMD,
the number of muscle fibers undergoing regeneration at any one time is very limited
relative to the mdx mouse [165]. Second, in another disease that might be treated with
AAV1-FS344 therapy, sporadic inclusion body myositis, the pathologic process is very
slow, with ambulation persisting for a mean 13 ± 8 years after onset [166]. The primary
focus for our gene therapy approach is the quadriceps, as loss of strength in this particular
muscle group is minimal over any 6-month period [167], predicting only minor loss of
the viral vector. Finally, by establishing that follistatin can inhibit myostatin with an adeno-associated virus, our studies provide the basis for tests of other integrating viruses, such as lentivirus, which transduce muscle and stem cells effectively and may be less affected by rounds of muscle degeneration and regeneration.
CHAPTER 4

DELIVERY OF AAV-IGF-1 TO THE CNS EXTENDS SURVIVAL IN ALS MICE THROUGH MODIFICATION OF ABERRANT GLIAL CELL ACTIVITY

4.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disease characterized by a loss of motor neurons in the motor cortex, brain stem, and spinal cord. Approximately 20% of diagnosed familial cases of ALS are due to dominantly inherited mutations in superoxide dismutase-1 (SOD1) [62]. Transgenic mice that express the mutant human SOD1 protein recapitulate many pathological features of ALS and are currently the best available animal model to study the disease [65].

Trophic factors, such as insulin-like growth factor-1 (IGF-1), have potent effects on motor neuron survival and have been investigated extensively as potential treatments for ALS [49,168,169]. Recently, it has been shown that simultaneous delivery of IGF-1 to the neuromuscular junction, muscle, and spinal cord by intramuscular injection of an IGF-1-expressing viral vector leads to extended survival in SOD1^{G93A} mice [121]. Although it is evident from this experiment that IGF-1 is beneficial, it is difficult to conclude which component of the neuromuscular axis IGF-1 is primarily acting on to delay disease progression. Interestingly, existing evidence suggests that muscle may be
the principle target of IGF-1 as double transgenic SOD1\textsuperscript{G93A} and MLC/mIgf-1 mice show improved survival [170].

The mechanism by which IGF-1 slows disease progression when delivered at the time of disease onset remains unclear. It is likely that IGF-1 is delaying motor neuron cell death through the stimulation of anti-apoptotic pathways. However, it is also possible that IGF-1 may be attenuating the pathological activity of non-neuronal cells (i.e., astrocytes and microglia) that have been reported to modulate both disease onset and progression in ALS mice [71,72].

In this study, we report that central nervous system (CNS)-restricted delivery of IGF-1 is sufficient to modify disease progression in ALS mice. We demonstrate that delivery of AAV-IGF-1 vectors to the deep cerebellar nuclei (DCN) of symptomatic SOD1\textsuperscript{G93A} mice resulted in axonal transport of vector and/or expressed IGF-1 protein throughout the brain stem and spinal cord [171-174]. Concomitant with IGF-1 expression within the CNS was a profound reduction in neuropathology throughout the CNS, increased motor neuron survival, improved motor function, and a significant extension of life span. In addition, the results of our in vitro studies indicate that IGF-1 may be delaying disease progression through attenuation of glial cell–mediated release of factors [i.e., tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) and nitric oxide (NO)] known to initiate motor neuron cell death.

### 4.2 Materials and Methods

**Animals.** Transgenic male and female littermate mice that expressed the mutant SOD1\textsuperscript{G93A} transgene at high levels were divided equally among groups. SOD1 gene copy
number and SOD1 protein expression were confirmed with PCR and western blot analysis. Animals were housed under light/dark (12:12 hour) cycle and provided with food and water ad libitum. All procedures were performed using a protocol approved by the Columbus Children’s Research Institutional Animal Care and Use Committee.

**Stereotaxic surgery.** Eighty-eight- to ninety-day-old mice anesthetized with isoflurane were bilaterally injected into the DCN (A–P: −5.75; M–L: −1.8; D–V: −2.6 from bregma and dura; incisor bar: 0.0) with 3 µl/site of AAV1-GFP (n = 26), AAV1-IGF-1 (n = 25), AAV2-GFP (n = 26), and AAV2-IGF-1 (n = 27) using a beveled 10-µl Hamilton syringe (rate of 0.5 µl/min for a total of $2.0 \times 10^{10}$ DNase Resistant Particles (DRP)/injection site) [175].

**Production of recombinant vectors.** Recombinant AAV1 and AAV2 vectors were produced as described earlier [176,177]. A contract manufacturing company (Virapur, San Diego, CA) was used for some virus preparations. Titers were determined to be $3 \times 10^{12}$ DRP/ml using quantitative PCR. The cDNA for the human IGF-1 encoded the Class 1 IGF-1Ea with a portion of the 5′-untranslated region of IGF-1 [113]. The human cDNA for either wild-type or mutant G93A SOD1 was cloned into cytomegalovirus-based expression vector for lentiviral production by the quadruple plasmid transfection method with CaCl$_2$ [178].

**Reverse transcriptase-PCR analysis of IGF-1 mRNA and enzyme-linked immunosorbent assay (ELISA) of IGF-1.** RNA was isolated as described earlier. No reverse transcriptase controls were performed to evaluate for DNA contamination with no detectable products (data not shown). Primers against the 5′-untranslated region of the rAAV transcript (5′-GTGGATCCTGAGAACTTCAG-3′) were used along with the 3′-
primer (5′-ATTGGGTTGGAAGACTGCTG-3′), which is homologous to IGF-1 and PCR performed. Amplified products were confirmed by sequencing to be specific for the human IGF-1 transcript.

Protein was isolated by rapidly dissecting the spinal cord on ice and immediately homogenizing using Tissue Protein Extraction Reagent (Pierce, Rockford, IL) with Complete protease inhibitor (Roche, Palo Alto, CA). Enzyme-linked immunosorbent assays for human IGF-1 were performed in quadruplicate using the Quantikine kit (R&D Systems, Minneapolis, MN). Protein homogenates were diluted 1,000-fold using the assay diluent and the assays performed following the manufacturer’s recommendations.

**Immunostaining.** Brain and spinal cord sections from 110-day-old mice treated with AAV1-IGF-1, AAV2-IGF-1, and AAV-GFP (n = 8/group) were stained with rabbit anti-hIGF-1 antibody (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti-chAT antibody (1:500; Chemicon International, Temecula, CA), rabbit anti-glial fibrillary acidic protein antibody (1:2,500; Dako, Glostrup, Germany), rabbit anti-NO synthase antibody (1:1,000; Chemicon International), mouse anti-nitrotyrosine antibody (1:2,000; Upstate, Temecula, CA), or rat anti-F4/80 antibody (1:10; Genzyme, Cambridge, MA). Secondary antibodies used were donkey anti-species–specific antibodies conjugated with fluorescein isothiocyanate or Cy3. Sections were visualized using a Nikon Eclipse E800 fluorescent microscope and evaluated for the percent reduction in fluorescent deposits using a MetaMorph Image Analysis System (Universal Imaging, Downingtown, PA) as described earlier [175].

**Rotarod, grip strength, and survival analysis.** Testing of motor function using rotarod and grip strength was performed as reported earlier [39]. A “death event” was entered
when animals could no longer “right” themselves within 30 seconds after the animal was placed on its back. “Death event” classification was performed by two individuals who were blinded to treatment during assessment.

**In vitro ALS models.** Mouse embryonic stem cells that express GFP driven by the Hb9 promoter (HBG3 cells, gift from Tom Jessell, Columbia University) were cultured on primary mouse embryonic fibroblasts (Chemicon International) and differentiated into motor neurons as described earlier [179]. After 5 days of differentiation as embryoid bodies, ~50 embryoid bodies were infected with $2 \times 10^9$ viral particles of lentivirus expressing human SOD1$^{G93A}$ or wild-type SOD1.

Neural progenitors were harvested from the spinal cords of B6SJLTg (SOD1G93A) mice and wild-type B6SJL mice at 8 weeks of age by the Percoll density gradient centrifugation method as described earlier [180]. Spinal cord progenitors were cultured on poly-ornithine-/ laminin-coated plates in fibroblast growth factor/endothelial growth factor/heparin containing media. To induce astrocytic differentiation, fibroblast growth factor-2 and endothelial growth factor were removed and 10% fetal bovine serum was added to the media for 7 days. Twenty-four hours after infection of the motor neurons with lentivirus, motor neurons were plated on top of the astrocytes. In some experiments, astrocytes were infected 24 hours before starting the coculture with either Adenovirus-CMV-Akt1(Myr) (cA) or Adenovirus-CMV-Akt1 (dN) (Vector Biolabs, Philadelphia, PA) at 100 plaque forming units/cell. Before motor neurons were plated on the astrocytes, they were washed five times with phosphate-buffered saline and then allowed to incubate with fresh media for 30 minutes before motor neuron plating. The coculture of motor neurons and astrocytes was cultured in conditioned media from
HEK293 cells transfected with either an IGF-1-expressing plasmid or mock-transfected and conditioned for 24 hours. The coculture media were replaced daily with fresh conditioned media. Cultures were fixed in 4% paraformaldehyde, blocked in 10% donkey serum with 0.1% Triton X-100, and stained with primary antibodies to guinea pig glial fibrillary acidic protein (1:1,000; Advanced ImmunoChemical, Long Beach, CA) and rat cleaved caspase-9 (1:100; Cell Signaling, Danvers, MA). All images were collected using a laser scanning confocal microscope while maintaining the same exposure time, magnification, and gain. For HB9-GFP + and cleaved caspase-9 + counts, embryoid bodies were selected at random for quantification.

BV2 microglia (gift of Dr Phil Popovich, Ohio State University) were plated at 100,000 cells/well to three wells of a six-well dish and infected with $1 \times 10^9$ viral particles of either wild-type- or G93A SOD1 expressing lentivirus in the presence of polybrene (4 ng/ml; Sigma, St Louis, MO). Two days after infection, the BV2 cells were analyzed for TNF-α and NO production. BV2 microglia expressing SOD1 WT or SOD1 G93A were serum-starved overnight and then treated for 30 minutes with conditioned media from HEK293 cells transfected with IGF-1 or mock transfected. BV2 cells were then stimulated with lipopolysaccharide (055:B5, 100 ng/ml; Sigma) and the media were harvested 5 hours after stimulation for the TNF-α enzyme-linked immunosorbent assay (R&D Systems) or 3 days after stimulation for the nitric oxide (NO) assay. Total NO levels were determined by measuring nitrite and nitrate levels which are the breakdown products of NO metabolism. Nitrate is converted to nitrite by nitrate reductase and total nitrite levels are measured by a colorimetric assay utilizing the Griess reaction (R&D Systems).
Statistics. Survival analysis was performed by Kaplan–Meier analysis which generates a \( \chi^2 \) value to test for significance. The Kaplan–Meier test was performed using the log-rank test equivalent to the Mantel–Haenszel test. In addition, two-tailed P values were calculated. When comparing survival curves, median survival times were calculated with a 95% confidence interval. All other statistical tests not involved in survival analysis were performed by multiway analysis of variance followed by a Bonferroni post hoc analysis of mean differences between groups (GraphPad Prizm Software, San Diego, CA).

4.3 Results

Figure 4.1a illustrates the connections between the DCN and the spinal cord. The medial and interposed nuclei receive input from each division of the spinal cord whereas the lateral nucleus receives input primarily from the thoracic division [172-174,181,182]. All of the cerebellar nuclei have been reported to send input to the cervical division of the spinal cord [183].
Figure 4.1. Delivery of viral vectors capable of axonal transport results in transgene delivery throughout the spinal cord. (a) Diagram illustrating afferent and efferent connections between the deep cerebellar nuclei (DCN) and spinal cord. The DCN is composed of three separate nuclei: the lateral (orange), interposed (purple), and medial (yellow). The medial and interposed nuclei receive input from every region (i.e., cervical, thoracic, lumbar, and sacral) of the spinal cord whereas the lateral receives input only from the thoracic division. All the three nuclei send projections to the cervical division of the spinal cord. Bilateral stereotaxic injections of viral vectors were made between the
medial and interposed nuclei. (b) Insulin-like growth factor-1 (IGF-1) staining in AAV-GFP- and AAV-IGF-1-treated mice throughout each segment of the spinal cord.

To determine the potential for targeting multiple regions of the CNS via the afferent and efferent projection pathways of the DCN, we tested two adeno-associated virus (AAV) serotypes, AAV2 and AAV1. AAV2 was chosen because most clinical studies to date have used this serotype vector. AAV1 was selected because it has been previously demonstrated to express high levels of transgenes in the brain [175,184]. We stereotaxically injected $2 \times 10^{10}$ DNase resistant particles (DRP) of AAV1-IGF-1 into the DCN of 90-day-old SOD1$^{G93A}$ mice and evaluated IGF-1 expression 20 days after injection. Positive IGF-1 signal was observed throughout the hindbrain, brain stem, and spinal cord after bilateral delivery of the IGF-1-expressing AAV vectors to the DCN. Positive IGF-1 staining was detected in the cerebellar cortex, brain stem (i.e., pontine nucleus, facial nucleus, locus ceruleus, and vestibular nuclei), and spinal cord. Within the spinal cord (Figure 4.1b), positive IGF-1 staining was most widely distributed within the cervical and thoracic divisions with detectable expression found in the lumbar and sacral regions, demonstrating that IGF-1 was expressed in all regions of the spinal cord at levels that may provide trophic support to motor neurons.

AAV1-IGF-1 and AAV2-IGF-1 were tested for their ability to enhance motor neuron survival in SOD1$^{G93A}$ mice compared with control AAV1-GFP and AAV2-GFP when injected at disease onset (88–90 days old). All regions of the spinal cord were evaluated at 110 days of age for the number of ChAT positive cells. AAV1-IGF-1-treated
animals (17.86 ± 1.91) showed a significant ($P < 0.01$) preservation of motor neurons in the cervical region of the spinal cord compared with AAV2-IGF-1-treated mice (11.8 ± 1.83) or AAV-GFP-treated controls (12.74 ± 1.08) (Fig. 4.2a).

**Figure 4.2.** AAV-IGF-1-promoted motor neuron survival and delayed death in amyotrophic lateral sclerosis mice. Motor neuron counts in (a) cervical, (b) thoracic, (c) lumbar, and (d) sacral regions of the spinal cord. Kaplan–Meier survival analysis of
AAV1-IGF-1-, AAV2-IGF-1-, AAV1-GFP-, and AAV2-GFP-treated animals (e). Mice were scored as dead when they could no longer right themselves within 30 seconds of being placed on their back. Green fluorescent protein (GFP)-treated mice are indicated in green and insulin-like growth factor-1 (IGF-1)-treated mice are indicated in red. AAV, adeno-associated virus; WT, wild type.

Both AAV1-IGF-1- (19.96 ± 0.39) and AAV2-IGF-1-treated animals (15.94 ± 1.21) displayed significantly (P < 0.01) higher numbers of motor neurons per section compared with AAV-GFP-treated animals (11.74 ± 0.762) in the lumbar region of the spinal cord (Fig. 4.2c). There was no difference in the mean numbers of ChAT-positive cells between AAV1-IGF-1- and AAV-GFP-treated animals in the thoracic or sacral regions of the spinal cord at this time point (Fig. 4.2b and d).

In a separate cohort of animals, survival was assessed by Kaplan–Meier survival curves (Figure 4.2e). AAV-IGF-1 delivered to the DCN resulted in a ~14-day increase in median survival compared with AAV-GFP-treated animals (n = 25 animals/group, χ² = 17.16, P = 0.0007). Median survival of AAV1-IGF-1-treated animals was similar to AAV2-IGF-1-treated animals (133.5 days versus 134 days, respectively) and the median survival of AAV1-GFP- and AAV2-GFP-treated animals was comparable (121.5 days versus 120 days). There was no difference in survival between untreated controls and AAV-GFP-treated animals (data not shown).
A battery of motor function tests was used to monitor disease progression. Forelimb grip strength measurements demonstrated that AAV-IGF-1-treated animals maintained statistically ($P < 0.05$) greater grip strength from 103 days of age through 131 days of age compared with those administered AAV-GFP (Fig. 4.3a).
Figure 4.3. AAV-IGF-1 significantly prolonged motor function in amyotrophic lateral sclerosis mice. Mice were tested weekly for (a) forelimb grip strength, (b) hindlimb grip strength, and (c) rotarod coordination. Insulin-like growth factor-1 (IGF-1)-treated mice had increased performance compared with green fluorescent protein (GFP)-treated mice. GFP-treated mice are indicated in green and IGF-1 treated mice are indicated in red.
In addition, animals treated with IGF-1 showed remarkable, statistically significant ($P < 0.05$) increases in hindlimb grip strength (Fig. 4.3b). Rotarod tests also demonstrated that AAV-IGF-1-treated animals maintained their ability to coordinate their movement for a longer period than AAV-GFP-treated animals from 110 days of age until end stage ($P < 0.05$ from 110 days of age onward, $n = 25$ animals/group). In all of the motor function tests, there were no statistical differences observed between animals treated with AAV1-IGF-1 and AAV2-IGF-1 other than one time point at 124 days of age in the rotarod test (Fig. 4.3c).

To determine whether IGF-1 was expressed at similar levels by the two serotype vectors, we measured the levels of the trophic factor using an enzyme-linked immunosorbent assay that recognized the expressed human IGF-1 and not the endogenous murine counterpart. Detectable levels of human IGF-1 were noted in all of the regions of the brain and spinal cord of mice injected with AAV-IGF-1. Highest levels were found in the cerebellum and cervical region of the spinal cord, which were at or near the site of injection with no statistical differences between AAV1 and AAV2 (Fig. 4.4a).
Figure 4.4. Deep cerebellar nuclei delivery of AAV-IGF-1 resulted in insulin-like growth factor-1 (IGF-1) expression in all regions of the spinal cord along with retrograde transport of vector to various regions of the spinal cord. (a) Human IGF-1 levels in
homogenates of various regions of the central nervous system were measured by enzyme-linked immunosorbent assay. (b) Retrograde transport of the viral vectors was detected by reverse transcriptase-PCR analysis of various regions of the spinal cord and brain after administrating AAV1-IGF-1 and AAV2-IGF-1. Control AAV1-GFP-treated mice showed no expression of the human IGF-1 transcript.

No IGF-1 was detected in the serum of AAV-IGF-1-treated animals, indicating that the IGF-1 delivery was not systemic. Little to no IGF-1 was detected in green fluorescent protein (GFP)-treated animals, with background levels detected <20 ng/mg (data not shown).

To determine whether IGF-1 detected at locations distal to the site of injection resulted at least in part from retrograde transport of the AAV vectors, IGF-1 mRNA levels were measured in different regions of the brain using reverse transcriptase-PCR. Our results demonstrated significant transport and expression of the IGF-1 transcript in all regions of the spinal cord including sites distal to the injection site, such as the lumbar and sacral regions of the spinal cord (Fig. 4.4b). No IGF-1 mRNA was found either in the AAV1-GFP-treated (Fig. 4.4b) or AAV2-GFP-treated animals (data not shown), or in the reverse transcriptase–minus controls. These results demonstrate that both the AAV1-IGF-1 and AAV2-IGF-1 vectors underwent retrograde transport to all regions of the spinal cord after DCN injection.

IGF-1-expressing AAV vectors reduced ALS-associated neuropathology in SOD1<sup>G93A</sup> mice. We next evaluated the ability of this therapy to attenuate the
neuropathological features characteristic of ALS disease. Activated microglia and astrocytes contribute to the propagation of the disease process in ALS [169]. Widespread gliosis is readily apparent in the brain stem and spinal cord of both human ALS patients and mouse models of the disease [70,185]. In addition, biochemical assays and gene expression profiling studies showed that inflammatory cascades are activated before and during motor neuron degeneration [186,187]. Microglial activation, astrogliosis, NO synthase expression, and peroxynitrite levels were assessed in mice treated with the AAV-IGF-1 and AAV-GFP vectors. MetaMorph analysis of our results showed that delivering AAV-IGF-1 to the DCN led to a reduction in gliosis both within the brain stem and throughout the spinal cord at 110 days of age compared with AAV-GFP-treated animals. Markers of microglial activation (F4/80 staining) and astrogliosis (glial fibrillary acidic protein staining) were diminished throughout the brain stem, including the motor trigeminal, hypoglossal, and facial nuclei (Fig. 4.5a and c and Fig. 4.6a and c).
Figure 4.5. AAV1-IGF-1 attenuated microglial activation in amyotrophic lateral sclerosis (ALS) mice. Microglial activation (F4/80 staining) in the (a) brain stem (7 = facial nucleus, 12 = hypoglossal nucleus, and Mo5 = motor trigeminal nucleus) and (b) spinal cord in 110-day-old superoxide dismutase-1 mice that were treated with either AAV-GFP or AAV-IGF-1 at 90 days of age. (c) MetaMorph analysis of F4/80-stained brain stem
and spinal cord sections taken from ALS mice treated with either AAV-IGF-1 or AAV-GFP and wild-type (WT) controls. AAV, adeno-associated virus; GFP, green fluorescent protein; IGF, insulin-like growth factor.
Figure 4.6. AAV-IGF-1 significantly attenuated astrogliosis in amyotrophic lateral sclerosis (ALS) mice. Astrogliosis [glial fibrillary acidic protein (GFAP) staining] in the (a) brain stem (7 = facial nucleus, 12 = hypoglossal nucleus, and Mo5 = motor trigeminal nucleus) and (b) spinal cord in 110 day old superoxide dismutase-1 mice that were
treated with either AAV-GFP or AAV-IGF-1 at 90 days of age. (c) MetaMorph analysis of GFAP-stained brain stem and spinal cord sections taken from ALS mice treated with either AAV-IGF-1 or AAV-GFP ($P < 0.05$) and wild-type (WT) controls. AAV, adeno-associated virus; GFP, green fluorescent protein; IGF, insulin-like growth factor.

Throughout the entire length of the spinal cord, microglial activation and astrogliosis were also dramatically reduced (Fig. 4.5b and c and Fig. 4.6b and c).

NO has been implicated as a contributing factor to the pathogenesis of ALS [188]. Upregulation of NO has been shown to be involved in initiating Fas-triggered cell death, a programmed cell death pathway that appears to be restricted to motor neurons [189]. Elevated NO has also been linked to the generation of peroxynitrite, formed by the reaction of NO with superoxide anions, resulting in the nitration of tyrosine residues in neurofilaments. This, in turn, causes irreversible inhibition of the mitochondrial respiratory chain, and inhibition of glutamate transporter activity [190]. Moreover, increased 3-nitrotyrosine immunoreactivity (a marker of peroxynitrite) has been reported in the spinal cord of both sporadic and familial ALS patients [191]. Similar elevations in 3-nitrotyrosine have also been observed in the CNS of ALS mouse models [192,193]. MetaMorph analysis of our results showed that delivery of IGF-1 resulted in reductions in the levels of both NO synthase (Fig. 4.7a and c) and 3-nitrotyrosine (Fig. 4.7b and c) throughout the spinal cord.
Figure 4.7. AAV-IGF-1 treatment reduced disease-induced elevations in nitric oxide synthase (NOS) activity and peroxynitrite formation in amyotrophic lateral sclerosis (ALS) mice. (a) NOS and (b) 3-nitrotyrosine staining (peroxynitrite marker) in the spinal cord of 110-day-old superoxide dismutase-1 mice treated with either AAV-GFP or AAV-IGF-1 at 90 days of age. (c) MetaMorph analysis of NOS and 3-nitrotyrosinestained spinal cord sections taken from ALS mice treated with either AAV-IGF-1 or AAV-GFP.
(P < 0.05) and wild-type (WT) controls. AAV, adeno-associated virus; GFP, green fluorescent protein; IGF, insulin-like growth factor.

Recent studies using embryonic stem cell–derived motor neurons have demonstrated that in vitro models of ALS could be developed that mimic the motor neuron death seen in animal models of the disease [83,84]. We developed similar models using embryonic stem cell–derived motor neurons containing the Hb9-GFP reporter that were transduced with a lentivirus containing the $SOD1^{G93A}$ gene or control $SOD1^{WT}$. As previously shown, the mutation had minimal effects when expressed only in motor neurons [83,84]. However, when motor neurons with or without the $SOD1^{G93A}$ gene were cocultured with astrocytes containing the $SOD1^{G93A}$, motor neurons exhibited shorter axon lengths, increased cell death, and apoptosis shown by caspase-9 activation, which demonstrates and confirms that astrocytes expressing the mutant SOD1 are toxic to motor neurons (Fig. 4.8a and b).
**Figure 4.8.** Insulin-like growth factor-1 (IGF-1) rescues amyotrophic lateral sclerosis (ALS) motor neuron toxicity and attenuates glial activation and toxicity in an *in vitro* coculture model of ALS. (a) SOD1-G93A motor neurons cultured with SOD1-G93A astrocytes in the presence of IGF-1 extend axons comparable to wild-type (WT) motor neurons cocultured with WT astrocytes. (b) SOD1-G93A motor neurons in a coculture with SOD1-G93A astrocytes survive longer with IGF-1 assessed by HB9-GFP counts and cleaved (cl.) caspase-9 + cells per embryoid body (EB) and compared to SOD1-WT motor neurons cultured with WT astrocytes. (c) SOD1-G93A microglia produce increased amounts of tumor necrosis factor-α (TNF-α) and nitric oxide and IGF-1 attenuates the release of these factors. (d) Coculture of WT or SOD1-G93A motor neurons with WT or SOD1-G93A containing astrocytes in the presence of IGF-1-conditioned media and/or astrocytes expressing dominant negative (dN) AKT or constitutively active (cA) AKT demonstrates IGF-1’s neuroprotective ability and its actions on both motor neurons and astrocytes to protect motor neuron survival. (e) IGF-1 serves dual roles as an antiapoptotic factor and to block microglial activation and astrogliosis for motor neuron protection in ALS via activation of AKT. (*P < 0.05). GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; LPS, lipopolysaccharide; SOD1, superoxide dismutase-1.
Figure 4.8. Insulin-like growth factor-1 (IGF-1) rescues motor neuron toxicity and attenuates glial activation.
To test whether IGF-1 could rescue the motor neuron toxicity, we replaced the medium from the SOD1\textsuperscript{G93A}-expressing cocultures with either IGF-1-conditioned medium or mock-transfected control-conditioned medium and compared the results with wild-type cocultures. We observed significant rescue and neuroprotective effects of IGF-1 in coculture experiments with motor neurons and astrocytes both containing the SOD1\textsuperscript{G93A} mutation, which was comparable to control wild-type cultures. IGF-1 treatment resulted in extensive preservation of neuritic extensions along with decreased caspase-9 activation indicating that IGF-1 was potently neuroprotective in this model (Fig. 4.8a and b). We next sought to determine whether IGF-1 may be acting to delay glial cell activation, because earlier studies have demonstrated that glial cells containing the SOD1\textsuperscript{G93A} mutation were a major contributor to disease progression and motor neuron death. We obtained the BV2 microglial cell line and transduced the cells using a lentivirus expressing wild-type SOD1 or SOD1\textsuperscript{G93A}. Upon lipopolysaccharide induction, these cells produce high levels of TNF-α and NO. SOD1\textsuperscript{G93A} microglia expressed higher levels of both TNF-α and NO compared with wild-type SOD1 microglia as previously demonstrated, suggesting that SOD1\textsuperscript{G93A} mutation modestly activated these microglia [86,194]. Consistent with our earlier in vivo experiments, when BV2 microglial cells were cultured with IGF-1-conditioned media before lipopolysaccharide-mediated activation, IGF-1 significantly reduced TNF-α levels and completely suppressed the NO release to baseline levels of nonstimulated microglia, suggesting that IGF-1 directly attenuates microglial cell activation (Figure 4.8c).
We next tested the specific action of IGF-1 in our ALS-motor neuron/astrocyte coculture system using wild-type motor neurons or SOD1<sup>G93A</sup> motor neurons cocultured with SOD1<sup>G93A</sup> astrocytes. Because IGF-1 is a secreted molecule, it is difficult to test the effects of IGF-1 protein solely on motor neurons or astrocytes; hence, we utilized a signaling pathway of IGF-1 to test our hypothesis that IGF-1 was acting both on motor neurons and astrocytes for neuroprotection. IGF-1 is one of the most potent natural activators of the AKT signaling pathway. We confirmed that IGF-1 could activate AKT in astrocytes (data not shown) and therefore used an adenovirus encoding a constitutively activated AKT that was restricted to expression solely in astrocytes to mimic IGF-1 signaling. A dominant negative AKT adenovirus expressed only in astrocytes was also used as a negative control and as a control to inhibit IGF-1 signaling through AKT activation in astrocytes. As demonstrated in our earlier study, motor neurons with or without SOD1<sup>G93A</sup> perished when cultured on SOD1<sup>G93A</sup> astrocytes. IGF-1 added to the cultures significantly rescued the motor neurons from this toxicity. Interestingly, when motor neurons were cultured on top of SOD1<sup>G93A</sup> astrocytes expressing the constitutively activated AKT, there was significant protection of motor neurons compared with untreated (Figure 4.8d) or dominant negative AKT only expressing astrocytes (data not shown). To test whether IGF-1 signaling was required in astrocytes for motor neuron protection, a dominant negative AKT was expressed in astrocytes and IGF-1-conditioned media were added to the coculture. Blocking AKT signaling in astrocytes significantly reduced motor neuron survival, but did not completely abolish the neuroprotective effects of IGF-1, indicating that IGF-1 signaling to activate AKT acts on both motor neurons and astrocytes. These results suggest that IGF-1 signaling via AKT activation in astrocytes is
sufficient in part to provide protection to motor neurons from astrocyte-derived toxicity and that there are additive effects of motor neuron protection by IGF-1 when both motor neurons and astrocytes are exposed to IGF-1.

4.4 Discussion

Trophic factors such as IGF-1 have shown promise for the treatment of ALS [49,168,169]. In this study, we report that CNS-restricted delivery of IGF-1 is sufficient to modify disease progression in symptomatic ALS mice. Specifically, we showed that injecting a recombinant AAV vector encoding IGF-1 within the DCN of SOD1\(^{G93A}\) mice resulted in axonal transport of vector and/or expressed IGF-1 protein to the brain stem and all segments of the spinal cord. This, in turn, led to improved muscle function and a significant extension of life span. Furthermore, IGF-1 also attenuated astrogliosis, microglial activation, peroxynitrite formation, and glial cell–mediated release of TNF-\(\alpha\) and NO.

Results obtained using mouse models of motor neuron disease have demonstrated that trophic factors (e.g., IGF-1, BDNF, CNTF, and GDNF) have potent effects on motor neuron survival. However, systemic administration of some of these recombinant trophic factors into subjects with ALS showed only very modest clinical benefit [125,126,195,196]. Studies in ALS mice suggested that inadequate delivery of these trophic growth factors to the CNS may have been responsible for the poor response. Only systemic administration of vascular endothelial growth factor has been reported to be effective in treating SOD1\(^{G93A}\) mice [197]. Intrathecal administration of purified IGF-1
to the same mouse model was also efficacious [119]. However, in both cases, positive effects were reported only when treatment was initiated in presymptomatic animals. In contrast to the results observed with systemic or intrathecal delivery of purified trophic factors, intramuscular injections of viral vectors encoding these factors demonstrated significant therapeutic benefit in the SOD1G93A mice, even when administered after the onset of overt disease symptoms [113,117]. The results of this study indicate that trophic factor delivery to the CNS is sufficient to modify disease progression in symptomatic ALS mice. An advantage of this delivery strategy over existing approaches is that it permits the targeting of multiple areas that undergo neurodegeneration in ALS with a single injection site and obviates the need for injecting directly into the spinal cord where neurodegeneration is taking place. Comparison of survival benefits achieved with DCN versus intramuscular delivery of AAV-IGF-1 is difficult, given that a “death event” in an ALS mouse is artificially determined (i.e., occurs when the mouse can no longer right itself within 30 seconds). In the mouse model, testing a therapeutic is limited to measuring the ability to offer protection to motor neurons predominantly residing in the lumbar division of the spinal cord, which is responsible for the righting reflex. Indeed direct intraspinal injections of AAV-IGF-1 led to significant increases in survival [120]. However, given that respiratory failure is the primary cause of death in ALS patients, we believe that delivering AAV-IGF-1 to the DCN may offer an advantage in that it permits targeting regions of the CNS that control respiration. This, in turn, may lead to a level of efficacy in ALS patients beyond what is observed in ALS mice.

Cellular mechanisms that modulate disease progression in ALS have not been known until just recently. While disease onset is initiated by motor neurons in ALS, it
appears that glial cells modulate disease progression [71,72]. The benefit provided by IGF-1 has mainly been thought to be attributable to activation of anti-apoptotic pathways (e.g., AKT and Bcl-2) within motor neurons of the spinal cord [198]. However, efficacy is still observed when treatment is initiated during disease onset suggesting that the actions of IGF-1 may also be mediated through additional mechanisms, including muscle enhancement. While IGF-1 has potent effects on muscles, it has been recently demonstrated that muscle is not a direct target for mutant SOD1-mediated toxicity [82]. Our in vivo studies here showed that IGF-1 may also attenuate a number of pathological features that have been linked to motor neuron cell death including increased NO activity, elevated peroxynitrite expression, astrogliosis, and microglial activation. Using a newly developed in vitro model of ALS, we corroborate our in vivo findings and also confirm earlier published results demonstrating that motor neurons containing the SOD1 mutation required coculture with astrocytes containing the SOD1 mutation for motor neuron death [83,84,194]. To our knowledge, no study to date has compared the efficacy of a potential therapeutic such as IGF-1 in this in vitro ALS models system with efficacy results obtained using ALS mice. We show here that IGF-1 is potently neuroprotective when present in the coculture system. We observed a delay in neuritic atrophy, cell death, and caspase-9 activation in motor neurons treated with IGF-1. To decipher whether IGF-1 had effects on non-neuronal cells, we performed experiments using microglial cells that contained the mutant SOD1, because these cells have been directly linked to disease progression. Surprisingly, IGF-1 significantly lowered TNF-α and NO production when these cells were activated with lipopolysaccharide. Furthermore, we show here that the neuroprotective effects of IGF-1 are not exclusively limited to motor neurons in the
coculture system, rather IGF-1 exerts strong inhibitory effects on SOD1\textsuperscript{G93A}-mediated toxicity in astrocytes. These results strongly implicate that pleiotropic effects for IGF-1 in multiple non-neuronal subtypes of ALS suggested by the activation of AKT (Figure 8e) could be a potent neuroprotective factor for motor neurons along with the ability to attenuate aberrant glial cell activation and subsequent products produced by astrocytes and microglia, which have been implicated in ALS, such as glutamate, peroxynitrite, TNF-\(\alpha\), and NO. While not all of the signaling pathways of IGF-1 were evaluated in this coculture study, AKT activation appears to play a significant role in protecting motor neurons, in part by its actions for neuroprotection in motor neurons themselves, as well as suppressing the toxicity derived from SOD1\textsuperscript{G93A}-containing astrocytes. This is of interest because it has recently been demonstrated that activated phosphorylated AKT is absent in motor neurons of both sporadic and familial ALS patients, and that motor neurons from mutant SOD1 mice lose activated AKT early in the disease [199]. IGF-1 signaling through other signaling pathways may also be responsible in part for the effects on motor neuron protection.

In summary, these results highlight a novel approach to deliver IGF-1 to multiple regions of the CNS by a single injection site and show for the first time that CNS-restricted delivery of IGF is sufficient to modify disease progression in ALS mice. We also show that in addition to providing motor neuron protection, IGF-1 also modulates pathological events mediated by glial cells in ALS. These findings support the development of therapies that are designed to treat ALS by targeting motor neurons and their cellular environment. Furthermore, the data support the strength of developing
therapeutic screens in the ALS coculture system and that potential future therapies may exploit the activation of AKT pathways in the CNS to curtail ALS progression.
CHAPTER 5

ASTROCYTES FROM SPORADIC ALS PATIENTS ARE TOXIC TO MOTOR NEURONS

5.1 Introduction

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder affecting ~30,000 in the US. This disease is caused by the degeneration of motor neurons leading to progressive paralysis and eventual respiratory failure within 2-5 years of diagnosis. About 10% of ALS cases are dominantly-inherited familial ALS (fALS) while the other 90% of cases are sporadic ALS (sALS). The cause is unknown in the majority of sporadic and familial cases; however, mutations in superoxide dismutase 1 (SOD1) cause 20% of fALS (~2% of total ALS). Mouse models of ALS have been created by transgenically overexpressing the mutated human SOD1 and these mice develop progressive motor neuron disease which closely recapitulates human ALS [65].

Using these mouse models of ALS, it has been shown that glial cells expressing mutant SOD1 can affect motor neurons via a non-cell autonomous mechanism. Reducing expression of mutant SOD1 specifically in astrocytes or microglia in the ALS mouse model slowed disease progression suggesting that mutant SOD1 within glia causes these cells to adopt a more neurotoxic phenotype [72,79]. Several groups have tested this
hypothesis *in vitro* by co-culturing astrocytes derived from the ALS mouse model with motor neurons [83-85]. The mutant SOD1-expressing astrocytes were shown to cause death of motor neurons *in vitro* and one group demonstrated that conditioned media from mutant astrocytes could confer neuronal death [83]. In addition, another study showed that human primary astrocytes expressing mutant SOD1 were toxic to human motor neurons *in vitro* [89]. Together, these studies demonstrate that in SOD1-mediated familial cases of ALS, astrocytes cause neurotoxicity through a non-cell autonomous mechanism.

These reports revolutionized the ALS field spurring numerous studies to investigate the mechanisms underlying this astrocyte-derived toxicity and develop therapies to correct these aberrant glial cells. However, it is still unknown whether this astrocyte-mediated toxicity occurs in sALS which accounts for 90% of human ALS cases. Currently, there are no models to study sALS since the cause is still unidentified. It is disconcerting that the vast majority of ALS research has been conducted on familial SOD1-mediated ALS which only accounts for 2% of all ALS. While the hope is that similar mechanisms lead to motor neuron death between fALS and sALS, the common pathways are still a mystery.

The goal of our study was to test whether astrocytes derived from sALS patients were toxic to motor neurons as has been shown with fALS. We isolated post-mortem neural progenitor cells from both fALS and sALS humans to differentiate patient-specific astrocytes. Using *in vitro* co-culture models of astrocytes and motor neurons, we present the first report that shows astrocytes derived from sALS patients are similarly toxic as familial SOD1 mutant ALS astrocytes. Importantly, our data highlights that astrocytes from both familial and sporadic ALS patients are toxic to motor neurons through a non-
cell autonomous mechanism. We also introduce the first model system to investigate molecular disease mechanisms and evaluate therapies for sporadic ALS.

5.2 Materials and Methods

**Human tissue samples.** Post-mortem spinal cord and skin samples were obtained from the National Disease Research Interchange (NDRI, Philadelphia, PA), and informed consents were obtained from all subjects before sample collections by the NDRI. Receipt of human tissues was granted through Nationwide Children's Hospital Institutional Review Board (IRB08-00402, Investigating the Role of Glia in Amyotrophic Lateral Sclerosis), and use of all human samples is in accordance with their approved protocols. Tissues samples were processed in approximately 24 to 72 hours post-mortem.

**Neural progenitor cell isolation and maintenance.** All procedures were performed in sterile fashion in a class II biosafety cabinet. A representative portion (2.5 cm) of three regions (thoracic, cervical and lumbar) of the spinal cord was used for Neural Progenitor Cell (NPC) isolation as described previously [200]. Briefly, tissue was diced and a single cell suspension was obtained by enzymatic dissociation of the tissue at 37 °C for approximately 30-40 minutes with 2.5 U/ml papain (Worthington Biochem, Lakewood, NJ), 250 U/ml of DNase I (Worthington Biochem), and 1 U/ml neutral protease (Roche, Indianapolis, IN). After dissociation, the cell suspension was mixed with DMEM/F12 (Invitrogen, CarlsBad, CA) with 10% fetal bovine serum (FBS, Gibco, CarlsBad, CA), passed through a 70 µM filter, and pelleted. The cell pellet was resuspended in
DMEM/F12 with 10% FBS and combined 1:1 with percoll (GE Healthcare, Piscataway, NJ). The cell/percoll mixture was centrifuged at 20,000 g for 30 min at room temperature and the low buoyancy fraction (10 ml) above the red blood cell layer was collected. Cells were washed and resuspended in NPC medium containing DMEM/F12 (Invitrogen) supplemented with 10% FBS (Gibco), 10% BIT9500 (Stem Cell Technologies, Vancouver, BC), 1% N2 supplement (Invitrogen), 20 ng/ml of FGF-2 (Peprotech, Rocky Hill, NJ), 20 ng/ml of EGF (Peprotech), 20 ng/ml of PDGF-AB (Peprotech). NPCs were cultured on fibronectin (Chemicon, Billerica, MA) coated plates and after 24 hours, the media was replaced with serum-free NPC medium. Half of the medium was subsequently replaced every 2 days. Cells were passed when 60-70% confluence was reached in about 3-4 weeks.

**Neural progenitor cell differentiation.** Neurons, astrocytes and oligodendrocytes were obtained from NPC cultures by removal of growth factors from the media and supplementation with differentiation factors. To differentiate NPCs into neurons, media was supplemented with 2 uM all-trans retinoic acid (Sigma, St. Louis, MO) and 5 uM of forskolin (Sigma). To induce astrocytic differentiation, NPCs were supplemented with 10% FBS (Gibco). Oligodendrocyte differentiation was induced by culturing cells in 200 ng/ml of IGF-1. In all conditions, cells were allowed to differentiate for 1 week.

**Astrocyte culture and maintenance.** Once NPC cultures from ALS patients and controls were established, astrocytes were generated by media supplementation with 10% FBS. Astrocytes were cultured in laminin coated plates and media was changed every
three days. Astrocytes were passaged when 80% confluent. Fetal human NPCs, isolated from the frontal brain cortex of a 28 week term fetus, (SCP-27, P1) were obtained from the National Human Neural Stem Cell Resource (NHNSCR, Orange, CA). Human fetal astrocyte cell line (1800) isolated from cerebral cortex was obtained from ScienCell research laboratories (Carlsbad, CA).

**Skin fibroblast isolation and maintenance.** Human skin fibroblasts were isolated and cultured according to a previously defined protocol [201]. In brief, a 2 cm² skin sample was rinsed twice in PBS and then placed in a 10cm² dish with the epidermal side facing down. The subcutaneous layer was then scraped off using a surgical scalpel and cut into 0.5 cm² strips. Skin tissue was then allowed to incubate with 25 ml of 0.05% trypsin/EDTA (Invitrogen, Carlsbad, CA) in a 50 ml conical tube at 37 ºC for 45 minutes with gentle agitation every 15 minutes. After the incubation period, 20 mls of DMEM media containing 10% FBS was added to inactivate the trypsin and cells were pelleted at 350g for 4 minutes. Cells were then resuspended in DMEM media containing 10% FBS and plated to a 6 well dish, and within two weeks fibroblasts were usually confluent on a 10cm² tissue culture dish.

**Immunocytochemistry.** Cells in culture were fixed with 4% PFA for 15 minutes, followed by three rinses of TBS for 5 minutes, and then blocked for 1 hour with 10% donkey serum with 0.1% Triton X-100. Spinal cord tissue sections were blocked similarly prior to antibody addition. All primary antibodies were diluted in blocking solution and tissue sections or cells were incubated overnight at 4°C. Primary antibodies
and dilutions utilized are as follows: GFAP (Advanced Immunochemical, Santa Clara, California, 1:1000), SMI32 (Millipore, Billireca, MA, 1:1000), NESTIN (Millipore, Billireca, MA 1:500), TUJ1 (Covance, Princeton, New Jersey, 1:1000), 04 (Millipore, Billireca, MA 1:50), RIP (Developmental Studies Hybridoma Bank, Iowa City, Iowa, 1:100). Nuclei were counterstained with DAPI. All images were captured using a Zeiss LSM510-META confocal laser-scanning microscope (Zeiss, Thornwood, NY).

**DNA sequencing of ALS patient samples.** The *SOD1* and the *TARDBP* genes were sequenced in all ALS samples. For *SOD1* sequencing, primers were designed that flanked all 5 exons of the *SOD1* within intronic regions. High fidelity accuprime polymerase (Invitrogen, Carlsbad CA) was utilized to amplify exonic regions and was subsequently sequenced at Eurofins MWG Operon (Huntsville, Al) utilizing standard Sanger sequencing protocols. In all ALS cases *SOD1* coding regions were wild-type, except for one familial ALS case containing a C>T transition 14 nucleotides downstream of the start adenine nucleotide rendering an alanine to a valine amino acid substitution at position 4 in the protein (*SOD1A4V*). For *TARDBP* DNA sequencing, genomic DNA samples from all ALS cases were sent to Ambry Genetics (Aliso Viejo, CA) for analysis. Similar to *SOD1* DNA sequencing analysis, all 6 exons were amplified and sequenced on an ABI3730xl capillary sequencer utilizing Sanger sequencing techniques. All ALS cases were devoid of mutations within the *TARDBP* coding region; however, samples 64089 and 09050 showed a previously described intronic nucleotide deletion (c.714 +70delG (het))[202].
**RNA isolation and quantitative RT-PCR.** RNA was harvested from proliferating NPCs (P2) and astrocytes (P3) with the RT² q-PCR grade RNA isolation kit (SABiosciences, Frederick, MD). Total RNA was reverse transcribed with RT² First Strand Kit (SABiosciences) according to the manufacturer’s instructions. Real-time quantitative PCR reactions were performed using RT² Real-Time SYBR Green/Rox PCR Master Mix (SABiosciences, Frederick, MD) and run on a customized RT² Profiler PCR Array for astrocyte-enriched genes (SABiosciences, Frederick, MD).

**Virus production.** SOD1 expression in astrocytes was knocked down by lentiviral transduction expressing siRNA sequences previously described [78]. In addition, lentiviruses were used to overexpress either human wild-type SOD1, SOD1 G93A or SOD1 A4V by the CMV promoter in astrocytes. Viruses were produced by transient transfection into HEK293 cells using calcium phosphate, followed by supernatant viral purification by ultracentrifugation [203].

**ES motor neuron differentiation.** Mouse embryonic stem cells that express GFP driven by the Hb9 promoter (HBG3 cells, kind gift from Tom Jessell) were cultured on primary mouse embryonic fibroblasts (Millipore, Billireca, MA,) and differentiated to motor neurons (MNs) in the presence of 2 µM retinoic acid (Sigma) and 2 µM purmorphamine (Calbiochem) as previously described[179]. After 5 days of differentiation, the embryoid bodies were dissociated [204] and sorted for GFP on a BD FACS Vantage/DiVa sorter.
Co-culture of motor neurons and astrocytes. Human astrocytes were plated in 96 well plates coated with laminin (5 ug/ml, Invitrogen) at a density of 10,000 per well. The following day, GFP positive motor neurons (MN) were sorted by FACS and cultured on top of the astrocytes at a density of 10,000 per well in MN media [DMEM:F12 (Invitrogen), 5% horse serum, 2% N2 (Invitrogen), 2% B27 (Invitrogen) + GDNF (10 ng/ml, Invitrogen), BDNF (10 ng/ml, Invitrogen), CNTF (10 ng/ml, Invitrogen)]. After 24 hrs, cytosine arabinose (1 mM) was added for 48 hrs in order to eliminate any remaining dividing NPCs or embryonic stem cells. Media was changed every other day subsequently. For the conditioned medium experiments, media was collected from the co-culture every 2 days and added to MNs that were cultured on laminin coated plates in conditioned media supplemented with fresh GDNF, CNTF, and BDNF.

Western Blot. Cell lysates were obtained using Tissue Protein Extracting Reagent (TPER, Pierce, Rockford, IL) and 20 ug of protein was loaded onto a NuPAGE 4-12% Bis-Tris gel (Invitrogen). The proteins were run at 150 V for 1 hr. then transferred to an Invitrolon PVDF membrane (Invitrogen). The membrane was blocked in 5% non-fat milk, 0.1% Tween-20 in TBS for 1 hr. and then incubated overnight in primary antibody to human SOD1 (#2770, Cell Signaling, 1:1000). Bound primary antibody was detected by horseradish peroxidase conjugated secondary antibody (Jackson) followed by chemiluminescence (ECL™ Western Blotting Detection Reagents, Amersham Biosciences). The blots were then stripped and re-probed with a ß-actin antibody (#A5060, Sigma-Aldrich, 1:1000) to control for protein loading.
**GABAergic neuron differentiation.** Mouse fetal neural progenitors were cultured on laminin-coated plates (5 ug/ml, Invitrogen) in Neural Stem Cell Media [DMEM:F12 (Invitrogen) and 5% N2 (Invitrogen)]. GABAergic neurons were differentiated from mouse fetal neural progenitors in DMEM:F12 (Invitrogen) with 0.1% FBS (Invitrogen), retinoic acid (1 µM, Sigma), and forskolin (5 µM, Sigma) for 7 days.

**Statistical analyses.** All statistical tests were performed by multiway analysis of variance followed by a Bonferroni post hoc analysis of mean differences between groups (GraphPad Prizm Software). Experiments were performed in triplicate.

**5.3 Results**

To test whether sALS astrocytes are similarly neurotoxic as fALS mutant SOD1 astrocytes, we sought to co-culture sALS astrocytes with motor neurons. We have previously shown that neural progenitors isolated from ALS mice can be differentiated to astrocytes *in vitro* and confer toxicity to motor neurons in a co-culture. Therefore, we hypothesized that neural progenitors isolated from post-mortem ALS spinal cord could be differentiated to astrocytes *in vitro* and cause motor neuron death.

We obtained fresh post-mortem spinal cord tissue from three non-ALS controls, four sALS patients, and one fALS patient with a SOD1A4V mutation for comparison (Table 5.1).
Table 5.1. Patient-derived cells.

<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Age</th>
<th>Gender</th>
<th>Diagnosis</th>
<th>Time from Diagnosis to Death</th>
</tr>
</thead>
<tbody>
<tr>
<td>1800</td>
<td>&lt;1</td>
<td>Unk.</td>
<td>Normal</td>
<td>N/A</td>
</tr>
<tr>
<td>SCP-27</td>
<td>&lt;1</td>
<td>Female</td>
<td>Normal</td>
<td>N/A</td>
</tr>
<tr>
<td>63358</td>
<td>87</td>
<td>Male</td>
<td>Normal</td>
<td>N/A</td>
</tr>
<tr>
<td>09050</td>
<td>73</td>
<td>Male</td>
<td>sALS</td>
<td>Unk.</td>
</tr>
<tr>
<td>090322</td>
<td>70</td>
<td>Male</td>
<td>sALS</td>
<td>1 yr, 8 mo.</td>
</tr>
<tr>
<td>64089</td>
<td>67</td>
<td>Male</td>
<td>sALS</td>
<td>9 mo.</td>
</tr>
<tr>
<td>090364</td>
<td>67</td>
<td>Male</td>
<td>sALS</td>
<td>36 days</td>
</tr>
<tr>
<td>4944MA</td>
<td>57</td>
<td>Male</td>
<td>fALS (SOD1A4V)</td>
<td>8 mo.</td>
</tr>
</tbody>
</table>

We verified by sequencing that the fALS patient indeed had a SOD1<sup>A4V</sup> mutation and also established that none of the sALS patients carried SOD1 or TDP43 mutations in the coding regions of these genes (Fig. 5.1).
**Figure 5.1.** fALS patient 4944MA indeed carries a C>T mutation causing an alanine to valine amino acid switch in SOD1.

The patient spinal cord tissue was received within 72 hours of death and we isolated neural progenitor cells (NPCs) using a percoll gradient density separation method as described previously [180,200] (Fig. 5.2).
Figure 5.2. Neural progenitor cells (NPCs) isolated from human post-mortem ALS patients are tripotent in vitro. (a) Schematic of NPC isolation from post-mortem spinal cord tissue. (b) Phase contrast of human NPCs isolated from a sALS patient. (c) NPCs are positive for the NPC marker nestin. (d) NPCs differentiated in retinoic acid (RA) and forskolin (Fsk) stain positive for the neuronal marker TUJ1. (e) NPCs differentiated in IGF-1 form oligodendrocytes characterized by O4 and RIP expression. (f) NPCs cultured in media containing 10% FBS differentiate into GFAP+ astrocytes.
NPCs isolated from human ALS patients were nestin-positive and self-renewing in vitro. We were also able to differentiate these NPCs into neurons, astrocytes, and oligodendrocytes, verifying their tripotency (Fig. 5.2). Since our goal was to derive astrocytes for a co-culture with motor neurons, we further characterized astrocytes differentiated from the human NPCs. Specifically, we analyzed the maturity of our astrocyte cultures and investigated for contaminating microglia (Fig. 5.3).
Figure 5.3. Human NPC-derived astrocytes are free of microglia and express a similar gene profile as primary astrocytes. (a) NPC-derived astrocytes were stained for the astrocytic marker, GFAP, and microglial marker, CD11b. Human primary microglia are shown as a positive control for CD11b staining. (b) Gene expression profile analysis of NPC-derived astrocytes. Comparisons were made to a human sample of mesenchymal stem cells (MSCs), undifferentiated NPCs, and primary astrocytes derived from human
spinal cord. (c) Comparison of CD11b expression levels by quantitative RT-PCR in NPC-derived astrocyte cultures from control and ALS patients. Mesenchymal stem cells (MSCs) and human microglia were used as negative and positive controls respectively.

The NPC-derived astrocytes stain positive for the astrocytic marker, GFAP, and display a mature gene expression profile comparable to that of primary spinal cord astrocytes (Fig. 5.3a and b). Additionally, these astrocyte cultures lack microglia as determined by immunofluorescence and real-time PCR analysis (Fig. 5.3a and c).

To test whether the NPC-derived astrocytes from sALS patients were toxic to motor neurons, we co-cultured mouse embryonic stem (ES) cell-derived motor neurons with these astrocytes. To derive motor neurons, we used an ES cell line that contains an Hb9-GFP reporter to allow for visualization of these motor neurons in co-culture [179]. To enrich for Hb9-GFP+ motor neurons, we differentiated ES cells and then FACS sorted these cells for GFP fluorescence (Fig. 5.4).
Figure 5.4. FACS sorting for GFP fluorescence yields an enriched population of Hb9-GFP+ motor neurons. (a) Forward and side scatter for a representative sort of differentiated Hb9-GFP+ motor neurons. (b) A negative control of 293 cells was used to set the gate for GFP fluorescence. (c) A representative sort shows ~23% of cells were positive for Hb9-GFP. (d) Post-sort analysis verifies that sorted cells are positive for GFP.

The Hb9-GFP positive motor neurons were plated on top of astrocytes derived from non-ALS controls or ALS patients. No differences in motor neuron survival were noted after co-culture for 24 hours. However, after 120 hours, there were fewer motor neurons
surviving in co-culture with all fALS and sALS patient astrocytes compared to all non-ALS controls (Fig. 5.5a and b).
**Figure 5.5.** Astrocytes derived from sALS and fALS patients are toxic to motor neurons.

(a) Representative fields of Hb9-GFP+ motor neurons in co-culture with human astrocytes after 24 or 120 hours. Original magnification, 40X. (b) Counts of Hb9-GFP+ motor neurons per well after 120 hours of co-culture with astrocytes. An siRNA was used to knockdown SOD1 expression in some experiments. (c) Representative fields of Hb9-GFP+ motor neurons treated with astrocyte-conditioned media for 24 or 120 hours. Original magnification, 40X. (d) Counts of Hb9-GFP+ motor neurons per well after 120 hours of treatment with astrocyte-conditioned media. *p<0.05 compared to 63358, #p<0.05 compared to 4944MA + siRNA.
To verify that the fALS astrocytes were causing motor neuron death as a result of mutant SOD1\textsuperscript{A4V} expression, we knocked down SOD1 expression in the astrocytes using a lentivirus encoding an siRNA against SOD1 (Fig. 5.6).

**Figure 5.6.** Transduction of patient astrocytes with a lentivirus (LV) encoding an siRNA against SOD1 reduces levels of SOD1 protein by western blot.

When the mutant SOD1\textsuperscript{A4V} expression was reduced by siRNA, motor neuron toxicity was completely rescued (Fig. 5.5b). Similarly, we tested whether viral-mediated overexpression of mutant SOD1 in non-ALS control astrocytes would cause MN toxicity.
Indeed, overexpression of either \textit{SOD1}^{G93A} or \textit{SOD1}^{A4V} in control astrocytes led to a decrease of MNs in co-culture by 120 hours compared to controls. Astrocytes expressing \textit{SOD1}^{A4V} caused a greater level of toxicity towards MNs in comparison to \textit{SOD1}^{G93A} expressing astrocytes which is consistent with the observation that \textit{SOD1}^{A4V} mutations are known to cause a rapidly progressing form of human ALS [205] (Fig. 5.7).
**Figure 5.7.** Non-ALS control astrocytes expressing mutant SOD1 are toxic to motor neurons. (a) Counts of Hb9-GFP+ motor neurons after 120 hours in co-culture with non-ALS control astrocytes infected with a lentivirus (LV) expressing mutant SOD1.
*p<0.05. (b) Counts of Hb9-GFP+ motor neurons after 120 hours of treatment with astrocyte-conditioned media. *p<0.05. (c) Western blot analysis verifies astrocytes infected with lentivirus express mutant SOD1. Spinal cord homogenate from transgenic SOD1<sup>G93A</sup> mice is shown for SOD1 expression comparison.

To establish that the motor neuron death was specifically due to astrocyte-derived factors, we co-cultured motor neurons with ALS patient-derived fibroblasts and saw no neuronal death compared to controls (Fig. 5.8).

**Figure 5.8.** ALS patient-derived fibroblasts do not confer motor neuron toxicity. (a) ALS patient-derived fibroblasts do not cause motor neuron death in co-culture. p>0.05 between all groups. (b) Conditioned media from ALS patient-derived fibroblasts is not toxic to motor neurons. p>0.05 between all groups.
Recent work has demonstrated that astrocytes isolated from the ALS SOD1 mouse model can cause motor neuron death \textit{in vitro} via an unknown secreted factor [83]. To determine whether our patient-derived sALS astrocytes also secrete neurotoxic molecules, we treated motor neurons with astrocyte-conditioned media and measured motor neuron survival. Motor neurons cultured in sALS astrocyte-conditioned media or fALS astrocyte-conditioned media died ~50% faster than motor neurons cultured in conditioned media from non-ALS control astrocytes (Fig. 5.5c and d). We again infected the fALS (SOD1^{A4V}) astrocytes with a lentivirus expressing a SOD1 siRNA and then collected conditioned media. Suppressing SOD1 expression in these astrocytes eliminated the toxic properties of the conditioned media and completely rescued motor neuron death (Fig. 5.5d). Likewise, when non-ALS control astrocytes were infected with a lentivirus overexpressing mutant SOD1^{G93A} or SOD1^{A4V}, the conditioned media now conferred motor neuron toxicity (Fig. 5.7b). Motor neurons cultured in ALS-patient fibroblast conditioned media survived well over time, demonstrating the specificity of the astrocyte-derived toxicity (Fig. 5.8b).

In human ALS, neuronal death is surprisingly selective to motor neurons with most other neuronal types relatively spared [49]. To determine whether human sALS astrocytes selectively kill motor neurons, we cultured GABAergic (gamma aminobutyric acid) neurons with the patient-derived astrocytes or with conditioned media prepared from these astrocytes. Overall, the GABAergic neurons survived well on both non-ALS control astrocytes as well as sALS and fALS astrocytes, suggesting the ALS astrocyte toxicity is specific toward motor neurons (Fig. 5.9).
Figure 5.9. Toxicity of patient-derived ALS astrocytes is specific to motor neurons. (a) Neither fALS nor sALS astrocytes cause death of GABAergic neurons in co-culture. p>0.05 between all groups. (b) Conditioned media collected from fALS or sALS patient-derived astrocytes is not toxic toward GABAergic neurons. p>0.05 between all groups. GAD67= Glutamate decarboxylase 67.

5.4 Discussion

Together, our results demonstrate for the first time that sALS astrocytes are toxic to motor neurons through a non-cell autonomous mechanism, similar to fALS astrocytes expressing a SOD1 mutation. Additionally, this astrocyte-derived toxicity is caused by either a toxic factor secreted into the media or the reduction of neuroprotective factors released into the media. The ALS astrocyte toxicity is specific for motor neurons since
another neuronal type, GABAergic neurons, were unaffected by co-culture with these astrocytes or treatment with the conditioned media. These data highlight a common pathway to motor neuron death between fALS and sALS. Furthermore, we introduce the first model system for the study of sALS.

One criticism of the co-culture studies using primary astrocytes isolated from the SOD1\textsuperscript{G93A} mouse model is the potential for contaminating microglia in these astrocyte cultures. Therefore, we extensively characterized our astrocyte cultures for the presence of microglia. Our results show that pure cultures of ALS astrocytes can cause motor neuron death and microglia are not required for this toxicity.

However, we did notice an interesting requirement when preparing the ALS patient astrocyte conditioned media. In order for the conditioned media to confer neuronal toxicity, the astrocytes had to first be co-cultured with motor neurons. We hypothesize that motor neuron death triggers activation of pathways in astrocytes which cause these cells to adopt a more neurotoxic phenotype. Since we used mouse motor neurons in co-culture with human astrocytes, the pathways leading to astrocyte toxicity are likely conserved across species. Although we did not address how the conditioned media causes motor neuron death, one could imagine future experiments analyzing these ALS astrocytes for loss of glutamate transporters, aberrant inflammatory gene expression, or conducting a complete proteomics analysis for toxic factors secreted by these astrocytes. Additional studies may also help identify which specific signals are relayed from the motor neuron to the astrocyte leading to the adoption of a neurotoxic phenotype. Strategies devised to block these signals could prevent the reactive astrogliosis thought to be harmful in the context of ALS.
It is worthy to note that not all of the ALS patient-derived astrocytes caused the same level of motor neuron death. One might speculate that ALS patients with prominent neuroinflammation and immune cell infiltration would have a faster progressing disease course and astrocytes isolated from these patients would be more activated and neurotoxic. We did investigate whether the level of motor neuron toxicity correlated with the speed of the patient’s disease progression. Overall, we did not see a correlation between time from diagnosis to death and the astrocytes’ propensity for killing motor neurons \textit{in vitro}. However, it is difficult to make these comparisons because there are individual variations between when a patient first notices symptoms and the time when they seek medical attention as well as how long it takes to obtain a correct diagnosis. Therefore, the rate of disease progression may still play a role in the level of neurotoxicity from the ALS patient-derived astrocytes. In support, familial ALS caused by an A4V mutation in SOD1 is known to be one of the fastest progressing types of ALS [205]. Our results demonstrate that astrocytes expressing the \textit{SOD1}^{A4V} mutation kill motor neurons over twice as fast as astrocytes expressing the \textit{SOD1}^{G93A} mutation.

The discovery that glial cells could cause motor neuron death via a non-cell autonomous mechanism in the SOD1 mouse model of ALS transformed the field. Numerous groups are in the process of developing therapies to target the astrocyte and microglial-derived toxicity in ALS. However, prior to this study, it was unknown whether these glial-targeted therapies would apply to sALS, which accounts for 90% of ALS cases. Our study shows for the first time that sALS astrocytes are similarly toxic to motor neurons through a non-cell autonomous mechanism and suggests that therapies targeting aberrant astrocytes may be effective for sALS as well. Additionally, we introduce a
system in line with the growing field of personalized medicine. One could envision using
this model system to assess therapies for different types of ALS in order to evaluate
effectiveness prior to patient administration.

To date, the vast majority of ALS research has been conducted on fALS with the
hope that similar pathways lead to motor neuron death between fALS and sALS. Here we
demonstrate a common mechanism leading to motor neuron death between familial and
sporadic ALS through astrocyte-derived toxicity. We also present the first model system
for the study of sALS. One could forsee the addition of motor neurons reprogrammed
from sALS patient fibroblasts into these co-cultures using induced pluripotent stem cell
(iPS) technology. Future experiments will aim to identify common pathways activated in
motor neurons undergoing death by fALS and sALS astrocytes. Discovery of these
shared mechanisms may lead to greater understanding of mechanisms central to ALS
pathogenesis.
CHAPTER 6

ACTIVATION OF THE NF-KB PATHWAY IN ASTROCYTES ISOLATED FROM AN ALS MOUSE MODEL

6.1 Introduction

Amyotrophic Lateral Sclerosis (ALS) is a fatal neurodegenerative disorder affecting ~30,000 in the US characterized by progressive paralysis and death usually within 5 years of diagnosis. ALS is caused by the degeneration of motor neurons and accompanied by glial cell activation and proliferation. While the primary insult is thought to be motor neuron death, recent studies have highlighted the involvement of neuroinflammatory processes in disease progression. Activated astrocytes and microglia are found in areas of neurodegeneration in human ALS as well as in ALS mouse models. Using the SOD1 mouse model of familial ALS, a number of studies have shown that mutant SOD1 within glial cells can affect neurons through a non-cell autonomous mechanism. First, mice that express mutant SOD1 solely in neurons do not develop motor neuron disease, suggesting a need for expression in glial cells [73,74]. Secondly, in chimeric animals generated from mixtures of wild-type and mutant SOD1-expressing cells, motor neurons are healthier if they are surrounded by wild-type glia [71]. Finally, reducing expression of mutant SOD1 specifically in astrocytes or microglia dramatically
slows disease progression [72,79,80]. Together, these studies imply that the mutant SOD1 protein within glia causes these cells to adopt a neurotoxic phenotype. Additionally, these transgenic mouse studies suggest that correcting the aberrant phenotype of glial cells may be an effective strategy to slow ALS disease progression.

Not surprisingly, astrocytes and microglia derived from the mutant SOD1 mouse are more neurotoxic in vitro than wild-type glia [83,84,86]. While several groups have modeled this glial-derived neurotoxicity, there is no clear mechanism for how these mutant SOD1-expressing glial cells kill motor neurons. Interestingly, mutant SOD1-expressing microglia show increased activation of specific genes involved in the inflammatory response including inducible nitric oxide synthase (iNOS), NADPH oxidase (NOX2), TNF-α, and IL-1 [91,96,102-107]. In addition, astrocytes isolated from the ALS mouse model also demonstrate elevated levels of iNOS, prostaglandin E₂ (PGE₂), COX-II, TNF-α, and IL-1 [97,103]. Surprisingly, genetic approaches to eliminate specific inflammatory genes in the ALS mouse model have mostly failed. Crossing ALS mice to knockout mice lacking TNF-α[110], IL-1β[104], COX1[111], or iNOS[112] resulted in no extension in survival. One reason may be that a variety of inflammatory factors contribute to motor neuron toxicity and that targeting only one gene may not be sufficient to slow disease.

Because various inflammatory genes are likely playing a role in ALS disease progression, we hypothesized that central mediators of inflammation are activated in ALS glial cells. Specifically, we investigated whether the transcription factor nuclear factor-κB (NF-κB) signaling pathway is activated in mutant SOD1-expressing astrocytes since NF-κB is well known for its role in inflammation and the immune response. First, we
performed an array to identify inflammatory genes that are differentially expressed in mutant SOD1<sup>G93A</sup> astrocytes. We found 30 genes upregulated in the mutant SOD1-expressing astrocytes compared to wild-type astrocytes, including a variety of inflammatory cytokines and chemokines which are NF-κB target genes. In support, we found significant activation of the NF-κB signaling pathway by western blot analysis in astrocytes derived from ALS animals at the time of disease onset. Inhibiting NF-κB signaling <i>in vitro</i> by expressing a dominant negative mutant of IκBα reduced the expression of inflammatory genes upregulated in mutant astrocytes. To test this approach in the ALS mouse model, we delivered the dominant negative IκBα gene to glial cells using an adeno-associated viral (AAV) vector of serotype 9. Preliminary data demonstrates that inhibiting the NF-κB pathway specifically in glia of the ALS mouse model can improve functional performance and extend survival. These findings suggest that NF-κB signaling contributes to the aberrant inflammatory gene expression in mutant SOD1 astrocytes and we offer a central target for reducing inflammation and slowing ALS disease progression.

### 6.2 Materials and Methods

**Animals.** Transgenic male and female littermate mice that express the mutant SOD1<sup>G93A</sup> transgene at high levels were divided equally among groups. C57Bl/6 mice were used as controls in astrocytes isolation experiments. Animals were housed under light/dark (12:12 hour) cycle and provided with food and water ad libitum. All procedures were performed using a protocol approved by the Columbus Children’s Research Institutional
Animal Care and Use Committee. At 50-60 days of age, mice were injected in the tail vein 1 or 2 x 10^{12} viral particles of AAV-IκBα in 100 ul PBS. Mice were monitored weekly and grip strength was measured from 3 separate trials using a grip strength meter (Columbus Instruments). A “death event” was entered when animals could no longer “right” themselves within 30 seconds after the animal was placed on its back.

**Astrocyte isolation.** Mice were anaesthetized with ketamine/xylazine and cervically dislocated. Brain and spinal cord tissue was rapidly isolated and washed in PBS. The tissue was minced and digested with DNase I (Worthington), Dispase II (Boehringer), and Papain (Worthington) for 15-20 minutes with trituration. The mixture was passed through a 70 μM filter, washed with PBS, and plated into 25 cm^2 flasks in Astrocyte Media [DMEM high glucose (Invitrogen), 10% FBS (Invitrogen), 1% antibiotic-antimycotic (Invitrogen), 1% L-glutamine (Invitrogen), and 0.2% N2 supplement (Invitrogen)]. The media was changed on the day following isolation and every 3 days afterwards until the flasks were confluent (1-2 weeks). Cytosine arabinoside (20 μM, Sigma) was added to the media for 2 days to eliminate proliferating microglia and neural progenitors.

**Western blots.** Cell lysates or spinal cord homogenates were collected using Tissue Protein Extracting Reagent (TPER, Pierce) with protease inhibitor (Roche). Protein concentration was measured using the Bradford assay (Pierce) and 10-20 μg of protein was loaded onto a NuPAGE 4-12% Bis-Tris gel (Invitrogen). The proteins were run at 150 V for 1 hr. then transferred to an Invitrolon PVDF membrane (Invitrogen). The membrane was blocked in 5% non-fat milk, 0.1% Tween-20 in TBS for 1 hr. and then incubated overnight in primary antibody. Bound primary antibody was detected by
horseradish peroxidase conjugated secondary antibody (Jackson) followed by chemiluminescence (ECL™ Western Blotting Detection Reagents, Amersham Biosciences). The blots were then stripped and re-probed with a β-actin antibody to control for protein loading. Primary antibodies used: p100, p52, phospho-p65, p65, iNOS, IκBα, phospho-IκBα (Cell Signaling, 1:1000), actin (Sigma, 1:1000).

**PCR Arrays.** RNA was isolated from primary astrocytes using the Qiagen RNeasy mini kit with optional genomic DNA removal step. First strand cDNA synthesis (SA Biosciences) was performed and both RNA/cDNA qualities were checked using the RT² RNA QC PCR Array (SA Biosciences). Inflammatory gene expression was measured using the Inflammatory Cytokines and Receptors PCR Array (SA Biosciences) ran on an ABI 7000 cycler.

**Viruses.** Adenovirus expressing either GFP or IκBα DN was purchased from Vector Biolabs. For all astrocytes infections, a multiplicity of infection (MOI) of 500 was used. The cDNA for IκBα DN was purchased from Addgene. IκBα DN was cloned into the scAAV vector under the chick β-actin promoter using EcoRV/NotI sites. A contract manufacturing company was used for virus preparation of AAV9-IκBα DN (Merrion).

**6.3 Results**

To investigate mechanisms for how mutant SOD1-expressing astrocytes are toxic to motor neurons, we first sought to identify genes differentially expressed in mutant astrocytes. We specifically chose to investigate inflammatory genes since activation of glial cells has been well-documented in both human and mouse ALS. We performed a
PCR array to examine inflammatory cytokine and receptor expression and found 30 genes upregulated in primary astrocytes derived from the SOD1$^{G93A}$ mouse model at 110 days of age (Fig. 6.1).
Figure 6.1. Thirty inflammatory genes are upregulated in astrocytes derived from SOD1<sup>G93A</sup> mice compared to wild-type mice at 110 days of age.
These genes comprised a variety of cytokines, chemokines, and receptors including several genes with known NF-κB transcription response elements in their promoters such as TNF-α [206].

Since such a large number of inflammatory genes were found to be upregulated, we hypothesized that a central mediator of inflammation such as the transcription factor, NF-κB, was activated in mutant SOD1 astrocytes. We specifically chose to investigate the NF-κB pathway because it is well known for its important role in inflammation. We analyzed the NF-κB pathway by western blot to look for activation of both the classical and alternative NF-κB signaling pathways (Fig. 6.2 and 6.3).
Figure 6.2. Schematic of the classical NF-κB signaling pathway.
The classical NF-κB pathway is activated in SOD1\textsuperscript{G93A} primary astrocytes compared to wild-type astrocytes isolated from mice at 100 days of age. The alternative NF-κB pathway shows no change in activation between wild-type and SOD1\textsuperscript{G93A} astrocytes.

The classical NF-κB signaling pathway is triggered by a variety of stimuli including proinflammatory cytokines [206] (Fig. 6.2). Signaling of this pathway leads to activation of the IκB-kinase (IKK) complex which phosphorylates IκBα, targeting this protein for ubiquitin degradation. The absence of IκBα allows the active NF-κB transcription factor
to translocate into the nucleus and bind to DNA. The active NF-κB transcription factor is made up of subunits such as the classical p50 and p65 which form an active heterodimer. The p65 subunit also gets phosphorylated when active so we used phosphorylation-specific antibodies to probe for the activated form of p65. In primary astrocytes from SOD1<sup>G93A</sup> mice, we saw a dramatic increase in the level of phosphorylated p65 compared to wild-type astrocytes with total p65 levels remaining the same. In contrast, we saw no change in the alternative NF-κB pathway. The alternative NF-κB pathway is stimulated by several cytokines such as CD40 ligand [206]. During activation, p100 is ubiquitinated and cleaved to the active p52 transcription factor. We saw no change in the level of active p52 in SOD1<sup>G93A</sup> astrocytes compared to wild-type astrocytes, indicating that the alternative NF-κB signaling pathway is not changed (Fig. 6.3).

We hypothesized that the classical NF-κB activation was contributing to the increase of inflammatory gene expression in SOD1<sup>G93A</sup> astrocytes. To test this, we sought to inhibit the NF-κB signaling pathway in these mutant astrocytes and then assay for inflammatory gene expression. To inhibit the NF-κB pathway, we used a dominant negative (DN) form of the IκBα protein which contains mutated phosphorylation sites and is resistant to phosphorylation-induced degradation [207] (Fig. 6.4).
Figure 6.4. Schematic depicting how the dominant negative (DN) mutant form of IκBα inhibits NF-κB signaling.
We delivered the IκBα DN mutant using an adenoviral vector to primary astrocytes isolated from wild-type mice or SOD1<sup>G93A</sup> mice and tested whether the classical NF-κB pathway was inhibited. We determined that IκBα DN was resistant to phosphorylation and degradation by western blot (Figure 6.5).

Figure 6.5. Adenoviral delivery of IκBα DN inhibits NF-κB signaling. Astrocytes were infected with an adenovirus expressing IκBα DN (Ad-IκBα-DN) and stimulated with TNF-α to activate the NF-κB pathway. Astrocytes expressing IκBα DN show no phosphorylated IκBα and no degradation of IκBα as expected. Adenovirus expressing GFP was used as a control (Ad-GFP).
Next, we inhibited the NF-κB pathway in both wild-type and SOD1$^{G93A}$ astrocytes with Ad-IκBα DN and then assayed for inflammatory gene expression (Fig. 6.6).
**Figure 6.6.** Inflammatory gene expression in SOD1<sup>G93A</sup> astrocytes is reduced when the NF-κB pathway is inhibited. Ad-GFP was used as a control in both WT Astros and SOD1<sup>G93A</sup> Astros.
When the NF-κB signaling pathway was inhibited by IκBα DN, we saw a drastic reduction in the majority of inflammatory genes upregulated in the SOD1\textsuperscript{G93A} astrocytes. In addition to genes in the array, we also looked at the levels of inducible nitric oxide synthase (iNOS) in wild-type and SOD1\textsuperscript{G93A} astrocytes since this inflammatory gene is known to be upregulated in the ALS mouse model [102]. We analyzed the levels of iNOS by western before and after inhibition of the NF-κB pathway and saw that delivery of IκBα DN could reduce the aberrant increase in iNOS seen in SOD1\textsuperscript{G93A} astrocytes (Fig. 6.7).

\textbf{Figure 6.7.} iNOS is upregulated in astrocytes isolated from SOD1\textsuperscript{G93A} mice and this increase is reduced by expression of IκBα DN.
Together, these results emphasize that mutant SOD1 expressing astrocytes acquire a more proinflammatory phenotype compared to wild-type astrocytes. Importantly, we show that this aberrant inflammatory phenotype can be modified by inhibiting the NF-κB signaling pathway.

We next sought to test our approach to inhibit NF-κB signaling in vivo using the ALS mouse model. Again, we chose to use the dominant negative mutant of IκBα to inhibit NF-κB since we demonstrated this approach could suppress aberrant inflammatory gene expression in SOD1G93A astrocytes in vitro. To deliver the IκBα DN gene to glial cells in vivo, we used an adeno-associated viral (AAV) vector of serotype 9. We have previously shown that when AAV9 is intravenously injected into adult mice, this virus will target predominantly astrocytes as well as some microglia [115]. First, we cloned the IκBα DN gene into an AAV vector under control of the ubiquitous chick β-actin (CBA) promoter. We used a self complementary AAV vector (scAAV) because these vectors eliminate the requirement for host-cell DNA strand synthesis and can therefore achieve higher levels of gene expression faster [208]. We tested this construct in vitro for its ability to inhibit NF-κB signaling. When HEK293 cells were transfected with the IκBα DN construct and stimulated with TNF-α, we saw a reduction in the level of phosphorylated IκBα. Consequently, we also saw less degradation of IκBα as expected, verifying that our construct was acting to inhibit NF-κB signaling (Fig. 6.8).
Figure 6.8. Human embryonic kidney carcinoma 293 (HEK293) cells were transfected with a plasmid expressing either GFP (control) or IκBα DN. Two days later, the 293 cells were stimulated with TNF-α to activate the NF-κB pathway. Western blot analysis shows that IκBα DN expression caused a reduction in the levels of phosphorylated IκBα and subsequently, less degradation of IκBα.

We delivered $1 \times 10^{12}$ viral particles per animal of scAAV9 expressing IκBα DN to SOD1$^{G93A}$ mice at 50-60 days of age. Although less clinically relevant, we chose to inject our virus prior to disease onset to first determine whether the disease-associated inflammation could be prevented. We followed each mouse over time and measured their
hindlimb grip strength as an assessment of function. Although the treated group did show improved grip strength, this group did not survive any longer than controls (Fig. 6.9).

Figure 6.9. SOD1^{G93A} mice were injected between 50-60 days with 1 x 10^{12} viral particles of AAV-IκBα DN or PBS (control). (a) Grip strength is improved in the treated group. (p<0.05) (b) There is no change in survival between treated and control groups. (n=10)

We hypothesized that sufficient levels of the IκBα DN protein were not established in the spinal cord following injection with AAV9-IκBα DN. We looked at total IκBα levels in spinal cord homogenate by western blot and saw no difference between control and AAV9-IκBα DN injected mice (Fig. 6.10).
Figure 6.10. The level of IκBα is unchanged in the spinal cord of treated versus control groups.

In order to increase inhibition of NF-κB, we treated another cohort of SOD1$^{G93A}$ mice with double the dose of virus. Due to limitations on virus available, we only injected 3 SOD1$^{G93A}$ mice with $2 \times 10^{12}$ viral particles of AAV9-IκBα DN. Although preliminary, we saw again an increase in grip strength in the treated group now accompanied by an encouraging 15 day median increase in survival (Figure 6.11).
Figure 6.11. SOD1^{G93A} mice were injected between 50-60 days with 2 x 10^{12} viral particles of AAV-IκBα DN or PBS (control). (a) Grip strength is improved in the treated group. (p<0.05) (b) The treated group (n=3) shows a trend toward prolonged survival compared to the control group (n=10).

While preliminary, these results support that inhibiting the NF-κB pathway in glial cells of the ALS mouse model could slow disease progression. We are currently producing more virus to test a larger cohort of ALS mice with a higher dose of virus.

5.4 Discussion

It is well established that ALS is caused by motor neuron degeneration; however, studies using the familial ALS mouse model have highlighted the non-cell autonomous effects of glial cells on motor neurons. Using in vitro co-culture models, several groups have demonstrated that mutant SOD1-expressing astrocytes and microglia are toxic to
motor neurons although it is still unclear through which exact mechanisms. Since vast inflammation has been documented in the spinal cord in both human and mouse ALS, we investigated neuroinflammatory processes activated in glial cells as one potential mechanism for neurotoxicity.

Using a PCR array to analyze inflammatory gene expression, we demonstrated that various inflammatory genes are differentially expressed in primary astrocytes isolated from the SOD1\(^{G93A}\) mouse model at 110 days of age compared to wild-type mice. Since so many inflammatory genes were upregulated, we hypothesized that a central mediator of inflammation was activated, causing this inflammatory gene expression. We specifically showed the classical NF-κB pathway is activated in mutant SOD1 astrocytes. When we inhibited the NF-κB pathway with a dominant negative mutant of IκBα, the majority of inflammatory gene expression in the SOD1\(^{G93A}\) astrocytes was drastically reduced. These results indicate that activation of the NF-κB pathway is a major cause of aberrant inflammatory gene expression in SOD1\(^{G93A}\) astrocytes.

One question is whether NF-κB activation occurs prior to the increase in inflammatory gene expression or whether NF-κB activation is a consequence of elevated cytokine levels. We saw increased levels of inflammatory gene expression in astrocytes isolated from the SOD1\(^{G93A}\) mice only after 110 days of age. Even at 100 days of age, the time point when NF-κB is activated, we did not find elevated inflammatory gene expression. In contrast, NF-κB activation seems to be an early event occurring around the time of clinical disease onset (100 days). It is likely there are multiple feedback loops
activated where cytokines and NF-κB interact together. However, our studies suggest that NF-κB activation occurs prior to inflammatory gene expression in the ALS mouse model.

Although we specifically looked at the role of NF-κB in astrocytes, these same signaling pathways may play important roles in microglia as well. Our preliminary results suggest that NF-κB is not activated in isolated SOD1\(^{G93A}\) microglia at a 100 day time point compared to wild-type microglia. In support, some studies have implied that astrocytes become reactive just prior to the activation of microglial cells in the ALS mouse model [70,79]. We are currently investigating whether NF-κB becomes activated in microglia at a later time point in disease progression.

To inhibit NF-κB signaling \textit{in vivo}, we delivered the dominant negative IκBα to glial cells of the ALS mouse model using a scAAV9 vector. When 1 x 10\(^{12}\) viral particles were injected, the mice showed an improvement in strength, but no increase in survival. We injected double the dose of virus into 3 additional SOD1\(^{G93A}\) mice and encouragingly, saw an increase in strength as well as prolonged survival. Although these results are preliminary, they demonstrate that inhibiting the NF-κB pathway in glial cells of the ALS model slow disease progression. Future experiments with larger cohorts of animals and higher doses of virus will verify whether this approach could be an effective ALS therapy.

Although AAV9 predominantly targets glial cells in the CNS, the virus will also infect muscle tissue in the periphery. One could argue that inhibition of NF-κB in muscle could protect against the muscle wasting that occurs during ALS, making results difficult to interpret. In fact, activation of the NF-κB pathway has been linked to muscle atrophy in a variety of disease conditions [209]. Therefore, inhibition of NF-κB in ALS muscle
tissue could slow muscle wasting. However, the increase in survival seen with AAV9-IκBα DN treatment argues that inhibition of NF-κB is playing a more important role in the spinal cord. It is crucial to note that muscle wasting is not a primary cause of ALS, but rather a secondary effect of motor neuron denervation. Therapies such as myostatin inhibition which increase muscle mass have a transient effect on strength but no effect on survival in the ALS mouse model [82]. The fact that AAV9-IκBα DN slowed disease progression suggests that inhibition of NF-κB in glial cells is neuroprotective.

In addition to viral-mediated NF-κB inhibition, we are also testing both pharmacologic as well as transgenic approaches to inhibit NF-κB signaling in the ALS mouse model. While transgenic NF-κB inhibition in the ALS mouse model could provide a proof-of-principle for this approach, we believe our viral-mediated strategy is more clinically applicable. In contrast, pharmacologic approaches to inhibit NF-κB are certainly clinically translatable. However, the drawback to a pharmacologic approach is that it is difficult to develop drugs which cross the blood-brain-barrier and target specific cell types such as glial cells in the CNS. This may be important because studies show that the function of NF-κB is cell-type specific [210]. For example, NF-κB can activate anti-apoptotic pathways in neurons that may be protective during motor neuron degeneration and therefore, suppressing NF-κB in these cells may have a detrimental effect [211,212].

Overall, our results demonstrate that mutant SOD1-expressing astrocytes acquire a more proinflammatory phenotype compared to wild-type astrocytes. For unknown reasons, SOD1G93A astrocytes display activation of the NF-κB pathway around the time of clinical disease onset. Subsequently, activation of this pathway leads to aberrant inflammatory gene expression which can be prevented by blocking NF-κB signaling.
Preliminary results suggest that inhibiting the NF-κB pathway *in vivo* may slow ALS disease progression and warrants further investigation of this approach.
CHAPTER 7

GENERAL DISCUSSION

7.1 *Summary of results*

Neuromuscular disorders affect thousands in the US including both patients suffering from these dehabilitating diseases as well as caregivers providing physical, emotional, and financial support. The goal of the studies presented here is to gain a further understanding of the pathogenic disease mechanisms of neuromuscular disorders and develop translational therapies to attempt to treat these diseases.

Many individuals are affected by muscle wasting either as a primary consequence of a muscle disorder or a secondary result of another disease. Inhibition of the myostatin signaling pathway has emerged as a powerful strategy to increase muscle mass and strength. One potent antagonist of myostatin is the glycoprotein, follistatin. The goal of our studies was to design a gene therapy vector for delivery of the follistatin gene to muscle. First, we demonstrated that normal mice injected with an adeno-associated virus expressing follistatin (AAV-FS) display an enlargement of muscle for over 2 years post-injection. We additionally showed that delivery of AAV-FS to a mouse model of muscular dystrophy results in increases in muscle mass and strength, even when treated
after the onset of muscle degeneration. Next, we injected non-human primates with AAV-FS to test the ability of this vector to increase muscle size as well as assess safety. Non-human primates injected with AAV-FS display an increase in quadriceps muscle size and strength compared to the contralateral non-injected leg. We found no adverse effects of AAV-FS treatment in any major organ system suggesting delivery of FS is safe and well-tolerated. Therefore, AAV-FS represents a promising therapy for muscle disorders such as muscular dystrophy and warrants consideration for translation to the clinic.

Amyotrophic Lateral Sclerosis (ALS) is a fatal paralytic disorder caused by the death of motor neurons in the spinal cord, brainstem, and motor cortex. Although the primary insult is motor neuron degeneration, glial cells such as astrocytes and microglia have been found to play an important role in the progression of disease. We sought to model this glial-mediated motor neuron death in vitro to investigate mechanisms of toxicity as well as test potential therapies. First, we showed that astrocytes isolated from the ALS mouse model are toxic to motor neurons in a co-culture system by a non-cell autonomous effect. Motor neuron death in this co-culture can be rescued by the addition of insulin-like growth factor 1 (IGF-1) which is neuroprotective through its actions on both motor neurons and astrocytes in the co-culture. However, the ALS mouse model is based on familial ALS and this inherited form only accounts for 10% of all ALS cases. We investigated whether astrocytes from sporadic ALS patients similarly had a toxic non-cell autonomous effect on motor neurons since sporadic ALS makes up 90% of all ALS cases. Here, we demonstrate that astrocytes derived from sporadic ALS patients also cause motor neuron death in co-culture, highlighting a commonality between the sporadic
and familial forms of ALS. To explain this non-cell autonomous toxicity of ALS astrocytes, we investigated whether inflammatory pathways were activated in these astrocytes. In astrocytes derived from the ALS mouse model, the NF-κB signaling pathway was activated, leading to upregulation of a variety of inflammatory genes. Preliminary data suggests that inhibiting the NF-κB signaling pathway in the ALS mouse model may slow disease progression potentially by modifying the inflammatory profile of astrocytes. Future studies will explore the potential role of inflammatory pathways in both the ALS mouse model as well as in astrocytes isolated from sporadic ALS patients. Ultimately, therapies designed to alter aberrant glial toxicity could slow ALS disease progression resulting in an improved quality of life for these patients.

7.2 Proposed mechanisms of muscle enhancement by follistatin

AAV-mediated delivery of the myostatin antagonist, follistatin, to mice and non-human primates resulted in muscle mass and strength increases [39,40]. We chose to use the FS344 isoform of follistatin instead of the FS317 isoform because evidence suggests that the FS344 isoform is unable to bind to heparan sulfate proteoglycans [140]. Therefore, FS344 represents the soluble, serum-circulating isoform of follistatin and lacks the ability to bind to reproductive tissue [155,156,163]. We believe FS344’s ability to circulate in the serum explains the observed effect of follistatin on remote muscle groups, distant from the injected muscle. Although some muscle diseases affect specific muscles with limited involvement of other muscle groups, the majority of muscular dystrophies target a variety of muscle groups in the body. A therapy such as follistatin that can affect multiple regions may be ideal for treatment of these types of disorders. Not
all muscle fibers would need to be transduced since a muscle fiber secreting follistatin could potentially affect neighboring non-transduced fibers.

While follistatin is recognized for its ability to be secreted from the injection site, the mechanisms for how follistatin acts on the muscle fiber to cause muscle size increase are relatively unknown. Follistatin was first explored in the context of muscle enlargement due to its ability to bind and inhibit myostatin [22]. Myostatin is a negative regulator of muscle growth that signals through activin receptor type IIb on muscle cells [26]. Follistatin can bind myostatin and prevent it from signaling through its receptor. However, it has been shown that follistatin can act to increase muscle size through mechanisms outside of myostatin inhibition alone [37]. Transgenic mice that are null for myostatin display a doubling of muscle mass. Yet, when these myostatin null mice were crossed to mice carrying a follistatin transgene, a quadrupling of muscle mass was observed, indicating that follistatin acts through pathways in addition to myostatin inhibition. Therefore, we believe our strategy to inhibit myostatin by follistatin delivery will prove to be more efficacious than therapies which target myostatin alone.

The additional signaling pathways follistatin utilizes to increases muscle mass are somewhat unknown. Resident muscle stem cells called satellite cells have long been suspected to be involved in the mechanism whereby follistatin induces muscle hypertrophy. In response to various stimuli, satellite cells proliferate and differentiate into myoblasts. These myoblasts can form new myofibers or fuse with existing myotubes to induce fiber hypertrophy. In the case of post-natal myostatin inhibition by follistatin, myofiber hypertrophy has been shown to be the major mechanism in muscle mass increase [30,33,39]. There are several mechanisms that may be contributing to this
myofiber hypertrophy. For example, follistatin is known to be a mediator of myoblast fusion events, supporting its role in muscle regeneration [213]. Indeed, direct expression of follistatin in myoblasts by lentivirus increases their proliferation and differentiation [214]. This mechanism likely plays a role in AAV-mediated gene delivery of follistatin by directing myoblasts to proliferate and fuse with existing myofibers leading to a hypertrophic effect.

However, follistatin’s role in muscle enhancement is not solely due to its actions on satellite cells and myoblasts. When proliferative cells are removed by irradiating the muscle, follistatin is still able to increase muscle size by 20% [215]. This effect was also seen in myostatin null mice, suggesting that follistatin’s role goes beyond myostatin inhibition and its actions on satellite cells. To explore alternative proteins that follistatin may interact with, the activins were investigated since follistatin is known to bind to activins. When follistatin was mutated to remove its activin-binding potential, muscle increase was diminished by about half. In addition, if the activin binding-mutant of follistatin was delivered to myostatin null animals, no increase in muscle size was observed [215]. These results demonstrate that follistatin’s effect on muscle size is likely through a combination of myostatin inhibition, activin inhibition, and satellite cell proliferation and fusion. Further exploration of how follistatin acts in these signaling pathways may lead to even more efficacious strategies to increase muscle mass and strength.

Several observations from our studies also lead us to believe that follistatin plays a role beyond muscle hypertrophy in models of muscle disease. When we delivered follistatin to a mouse model of muscular dystrophy, we saw a decrease in serum creatine
kinase, a marker of muscle damage. In addition, we observed that follistatin could reduce inflammatory cell infiltrates and decrease production of fatty tissue replacement during muscle degeneration in this muscular dystrophy model. One might hypothesize that follistatin caused muscle fiber hypertrophy and these larger muscle fibers were more resistant to disease-related injury. However, follistatin has also been shown to reduce inflammation in a model of endotoxemia [144]. Therefore, in comparison with other strategies that induce muscle hypertrophy, follistatin may be a superior choice for muscle disorders such as the muscular dystrophies characterized by inflammatory muscle damage.

7.3 Future Directions for Follistatin Gene Delivery

Here we have developed an AAV vector to deliver the follistatin gene and have shown that follistatin expression in muscle tissue results in muscle size and strength increases. Although we demonstrate that follistatin causes muscle enlargement due to myofiber hypertrophy, it is still unknown through which exact mechanisms this occurs. Follistatin is a known inhibitor of the myostatin signaling pathway but its muscle enlarging effects go beyond myostatin inhibition alone [37]. Therefore, one future direction may be to investigate how follistatin acts to cause muscle hypertrophy. One could envision studies using a yeast-2-hybrid system to identify proteins that specifically bind to follistatin. It would be intriguing to discover whether follistatin interacts with proteins involved in the inflammatory process since we saw a decrease in muscle inflammatory infiltrates when we treated a mouse model of muscular dystrophy. To uncover pathways that follistatin may be involved in beyond myostatin inhibition, a
microarray analysis might be beneficial. In this experiment, one cohort of mice would be
treated with a specific inhibitor of myostatin such as a neutralizing antibody and the other
cohort of mice would be treated with follistatin. An experiment such as this would allow
for subtraction in the analysis of genes related to myostatin inhibition and solely focus on
genes specifically changed as a result of follistatin’s additional properties.

In addition to looking further into the molecular mechanisms of follistatin-
mediated hypertrophy, future directions should include more translational studies with
the goal to optimize delivery of the follistatin gene. In our studies to this point, we have
delivered follistatin to muscle via direct intramuscular injection. However, diseases such
as Duchenne muscular dystrophy affect more than one muscle group. Although follistatin
can be secreted from the targeted muscle, all of our results have demonstrated that the
greatest effects are seen at the injection site. We utilized an AAV1 serotype in our
experiments; however, some serotypes of AAV such as AAV8 and AAV9 have recently
been shown to transduce muscle after vascular delivery [11,13]. Future experiments
testing these additional AAV serotypes for their ability to deliver follistatin through the
vasculature may result in more systemic gene delivery and enhanced muscle effects.
These systemic delivery approaches may also be able to target muscles such as the
diaphragm that are more difficult to inject directly, but have a large impact on respiration
in some of the muscular dystrophies.

Lastly, given the robust effects of follistatin in both mice and non-human
primates, an obvious future direction is toward a clinical trial. While we did not observe
any adverse effects from follistatin gene delivery in mice or non-human primates, a
formal toxicology study will be required. Indeed, we have already initiated this study in
mice under good-laboratory-practices (GLP) in preparation for an investigational new
drug application (IND). Since some isoforms of follistatin do play a role in reproductive
physiology, an important part of this toxicology study will be to determine whether gene
delivery of follistatin-344 affects this organ system. Assuming follistatin proves safe in
the toxicology study, a clinical trial could initiate in less than a year. In accordance with
our preclinical studies, follistatin would be delivered by direct intramuscular injection to
human patients. Thus, a patient population with focal muscle atrophy may be an ideal
group to test this therapy. For example, patients with sporadic inclusion body myositis
display specific atrophy of the quadriceps muscle with less severe involvement of other
muscle groups [166]. Targeted delivery of AAV-follistatin to the quadriceps muscle
could potentially increase muscle mass and strength in this muscle which is crucial for
standing and ambulating. As safe systemic delivery approaches become available, one
could foresee delivery of follistatin to muscular dystrophy populations in which multiple
muscle groups need to be targeted.

7.4 Relevance to humans- Follistatin gene delivery

As with most research conducted in vitro or on animal subjects, the ultimate goal
is to translate our findings to improve the health and well being of humans. Our studies
testing gene delivery of follistatin could have obvious impacts on disease if this therapy
makes its way into the clinic. In contrast to many gene therapies, follistatin does not
specifically target a disease mechanism such as replacement of dystrophin for Duchenne
muscular dystrophy. Rather, follistatin can act to increase muscle size and strength in a
variety of contexts. Patients suffering from many diseases, both muscular and
neurological, exhibit muscle atrophy which impacts their daily life. In a mouse model of muscular dystrophy, follistatin was able to increase strength even when administered after the onset of muscle degeneration, making it an ideal therapy for clinical development. Although follistatin may not fix the underlying genetic problem, increasing strength may provide for an improved quality of life. For example, follistatin treatment could combat muscle atrophy so that a patient may not require a wheelchair for another year. Even in diseases such as ALS where muscle wasting is a secondary event resulting from neuronal damage, follistatin treatment was able to increase strength transiently although follistatin could not prolong survival [82]. While this improvement may seem trivial, it has the potential to change the life of an ALS patient who may be searching for ways to maximize the quality of life for their remaining months or years.

In addition to follistatin’s potential impact on human disease, follistatin may also be useful in the context of the meat industry. Gene therapy for livestock may not be the first use of follistatin that comes to mind, but it certainly is an idea already in development within the meat industry. The rapidly growing world population and protein demands have become a significant contribution to global warming. Currently, the United Nations Food and Agriculture Organization estimate that 18% of greenhouse gas emissions come from livestock, which is an even greater share than transportation-related emissions [216]. Some of this impact is due to expansion of grazing land for livestock which now accounts for 26% of the Earth’s surface. However, the majority of these emissions come directly from livestock in the form of methane release or nitric oxide from manure [216]. Currently, the livestock industry is working to develop strategies to increase efficiency in production of meat for the increasingly demanding market. One
potential avenue to address this challenge is to produce larger animals with a greater amount of muscle mass. Targeting pathways that inhibit myostatin such as through delivery of follistatin, may be a feasible strategy to increase muscle mass in livestock. In wild-type mice, follistatin delivery was able to increase body mass by 20% [39]. If each cow could deliver 20% more protein, the potential exists to decrease the number of animals needed by 1/5 to arrive at the same amount of meat for production. Obviously, the industry may need to develop a more cost-effective strategy besides gene therapy to accomplish this goal, but the possibility still exists for a major impact utilizing this pathway.

7.5 Proposed Mechanisms for Glial-Mediated Motor Neuron Death in ALS

Here we have demonstrated that ALS astrocytes can cause the death of motor neurons by a non-cell autonomous effect. Astrocytes isolated from both the SOD1 mouse model of familial ALS as well as from post-mortem spinal cords of sporadic ALS patients display this motor neuron toxicity. However, these studies raise questions regarding discrepancies between in vivo studies conducted in the ALS mouse model and in vitro co-culture studies. In the mouse model of ALS, reducing mutant SOD1 expression specifically in astrocytes by crossing GFAP-Cre expressing mice to SOD1 floxed mice results in a dramatic extension in survival [79]. Conversely, if mutant SOD1 is expressed in wild-type mice only in astrocytes by the GFAP promoter, mice do not develop motor neuron disease [76]. These findings suggest that mutant SOD1 expression is required in both astrocytes as well as neurons. However, if mutant SOD1-expressing astrocytes are co-cultured with wild-type motor neurons, neuronal death is observed in
One may suggest that these in vitro co-culture models do not accurately represent what is occurring in vivo. Yet, reduction of mutant SOD1 in vivo in astrocytes clearly has a motor neuron protective effect, supporting the non-cell autonomous role of these cells in disease.

This discrepancy could be explained by the effect of in vitro conditions on motor neuron cell survival. Motor neurons will die in culture over time even without exposure to mutant-SOD1 astrocytes. Perhaps motor neuron death is a naturally occurring event in these cultures and the death serves as a trigger to initiate toxicity in the mutant SOD1 astrocytes, mimicking the motor neuron death occurring in ALS. In fact, our studies conducted with sALS astrocytes support this hypothesis. We found that conditioned media collected from ALS astrocytes would confer motor neuron toxicity only if the astrocytes were co-cultured with motor neurons prior to conditioned media preparation. Perhaps as motor neurons die, they release factors into the media which cause activation of astrocytes. If the astrocytes contain a SOD1 mutation, they may react to these factors in a more harmful manner toward motor neurons than wild-type astrocytes. This may explain differences between ALS astrocytes and how astrocytes react in other paradigms of injury or disease. It is thought that astrocytes may play a protective role in some models of injury or neurodegeneration [217,218]. These protective mechanisms may also occur in ALS astrocytes, but for unknown reasons, expression of mutant SOD1 causes these astrocytes to adopt a more neurotoxic rather than a neuroprotective phenotype.

Based on these observations, we propose a mechanism for glial-mediated motor neuron death in ALS where motor neurons are the initial trigger for disease onset. Motor neurons likely exhibit early abnormalities, initiated either by a mutated gene, aberrant
RNA splicing, or altered epigenetic gene regulation. Since ALS is a disease of middle to late age, possibly an environmental or age-related insult is needed to trigger the disease. As motor neurons start to die, they release factors which are recognized by neighboring astrocytes. Since these astrocytes also contain an altered genetic profile, they react to the motor neuron-derived factors by adopting a neurotoxic phenotype. Microglia may also respond to factors released by motor neurons and it is debatable whether microglia or astrocytes react first to the motor neuron damage. In some models of injury, microglia react first to neuronal damage followed by astrocyte activation [219]. However, some evidence suggests that astrocytes become reactive prior to microglial activation in ALS mouse models [70]. In either scenario, it is likely that activated astrocytes and microglia crosstalk. As glial cells recognize the motor neuron damage, they may initially attempt to respond in a neuroprotective manner. However, since these cells harbor a SOD1 mutation, the glia are pushed toward a more neurotoxic phenotype. A variety of factors including pro-inflammatory cytokines, chemokines, and reactive oxygen and nitrogen species are released by glia contributing to the spread of motor neuron death. Once this cascade is initiated, disease progresses rather rapidly, leading to severe motor neuron loss and eventual death.

One question that remains is why glia respond in a damaging manner to motor neuron death in ALS. A potential mechanism, although likely not the only explanation, is that the NF-κB signaling pathway is upregulated in the ALS astrocytes. We have demonstrated that astrocytes isolated from the ALS mouse model have increased activation of the NF-κB signaling pathway, causing aberrant inflammatory gene expression. However, the reasons for specific activation of NF-κB are unknown. One
possibility is that mutations in SOD1 cause this protein to adopt a toxic gain of function that allows for its interaction within the NF-κB signaling pathway. However, a more likely scenario is that initial injury within the motor neuron triggers activation of NF-κB in glial cells. Indeed, injury or trauma in the CNS has been shown to cause activation of NF-κB in both astrocytes and microglia [210]. Subsequent increases in inflammatory gene expression probably only perpetuate the NF-κB pathway activation leading to further neuronal damage. While this hypothesis suggests that NF-κB activation may not be specific to damage in ALS, this pathway may still be a suitable target for reducing glial inflammation which is known to play a role in disease progression.

7.6 Future directions for ALS research

We have demonstrated that the NF-κB pathway is specifically activated in astrocytes derived from the ALS mouse model. This NF-κB activation leads to aberrant increases in inflammatory gene expression in these astrocytes and preliminary data suggests inhibition of this pathway in vivo may slow disease progression in ALS mice. Although we used primary astrocytes to show activation of the NF-κB pathway, we have not yet demonstrated NF-κB activation in vivo in the ALS mice. We have tried to use antibodies for immunohistochemistry on the spinal cord of ALS mice, but these NF-κB antibodies work poorly on spinal cord tissue. To accomplish this goal, we have obtained a line of mice which express GFP driven by an NF-κB transcription response element. Crossing these mice with the ALS mouse model will allow for easy visualization of the cell types where NF-κB is activated and at which time during the course of disease. With
this data, we may be able to determine the optimal time for viral-mediated inhibition of NF-κB.

To inhibit NF-κB in the mouse ALS model, we have chosen to first test an AAV expressing a dominant negative mutant of IκBα. However, we were only able to inject 3 mice with a dose of $2 \times 10^{12}$ viral particles. Future experiments with larger cohorts of mice and an increased viral dose will demonstrate whether this strategy can truly slow disease progression. In addition to using a gene therapy approach to inhibit NF-κB, we have also started to work on transgenic approaches to show that inhibition of NF-κB in the ALS mouse model slows disease progression. To accomplish this goal, we have obtained mice which have the IKK gene floxed. In the classical NF-κB pathway, IKK is essential for phosphorylation of IκBα and subsequent degradation of IκBα, allowing NF-κB to translocate. We plan to cross these IKK floxed mice to mice expressing cre driven by the GFAP promoter to specifically remove IKK in astrocytes. Crossing these mice with the ALS mice will allow us to assess for extension in survival and demonstrate proof-of-principle for our NF-κB inhibition approach.

Although we demonstrated these ALS astrocytes cause death of motor neurons in a co-culture system, we have not yet shown a mechanism for this killing. Therefore, an important experiment will be to inhibit NF-κB specifically in astrocytes of our co-culture system and assess for motor neuron survival. To accomplish this, we can utilize an adenovirus expressing the dominant negative form of IκBα as we have previously used. The adenovirus can be specifically delivered to astrocytes prior to co-culture with motor neurons. In addition to testing astrocytic NF-κB inhibition in a mouse co-culture system, these same experiments could be carried out using our human sALS patient-derived
astrocytes. Proving that NF-κB is activated in these sALS astrocytes and that inhibition rescues motor neuron death in a co-culture would reveal whether this strategy may be therapeutic for human sALS patients.

Thus far, many of our experiments have focused on the role of astrocytes in ALS disease progression. However, microglia are equally important for directing disease progression as astrocytes. Future experiments will be aimed at adding microglia into our co-culture systems and examining their role in motor neuron death. One could envision co-culture systems with various combinations of wild-type and mutant SOD1-expressing microglia, astrocytes, and motor neurons. These experiments may help to determine the specific contributions of astrocytes and microglia to motor neuron death. We may also be able to determine what effects mutant microglia and mutant astrocytes have on each other and whether these interactions cause the glial cells to become more neurotoxic or neuroprotective. It is currently unknown how microglia and astrocytes crosstalk in the context of ALS and knowledge of these pathways may lead to novel avenues for targeting glial toxicity.

In addition to adding microglia into our co-cultures, another important piece to add to the co-culture system will be human motor neurons. Our experiments thus far have involved co-culturing human ALS patient-derived astrocytes with motor neurons derived from mouse embryonic stem cells. One would expect to obtain similar results using human motor neurons. However, it would be particularly interesting to use human motor neurons derived from ALS patients to investigate whether these motor neurons harbor increased sensitivity to the ALS astrocytes. We have already begun the process of creating human motor neurons derived from ALS patients using stem cell technology.
Motor neurons are most easily differentiated in vitro from embryonic stem cells. In order to create patient-specific lines of embryonic stem cells, we have isolated both ALS patient-derived fibroblasts and neural progenitor cells to induce pluripotency. In the past couple of years, it has been shown that one can induce pluripotency in a terminally differentiated cell type by overexpressing genes which confer pluripotency, essentially “reprogramming” these cells back to an embryonic stem cell-like state [220]. We have infected our ALS patient-derived cells with retroviruses to deliver these pluripotency genes to create induced pluripotent stem (iPS) cells. Future experiments will involve differentiating these patient-specific iPS cells into motor neurons for characterization. It will be intriguing to look for abnormalities in both ALS patient-derived motor neurons cultured alone as well as in a co-culture system with ALS patient-derived astrocytes.

One question posed by these experiments is whether or not reprogramming a cell causes the loss of features leading to ALS. For example, if development of sporadic ALS is caused by epigenetic modifications, reprogramming may eliminate this epigenetic profile leaving essentially a “normal” cell. Therefore, it may be necessary to optimize differentiation of ALS patient-derived neural progenitor cells toward a motor neuron fate to maintain the epigenetic code. This potential hurdle will have to be considered in these experiments.

However, these motor neuron/astrocyte co-culture systems could still be useful to compare different forms of ALS caused by genetic mutations. For example, a co-culture representing SOD1-mediated ALS could be compared with a co-culture using cells with TDP-43 mutations or other mutations linked to ALS. It is our hope that co-culture systems such as these will allow for the dissection of molecular mechanisms involved in
motor neuron death in ALS and help to identify common pathways to motor neuron death shared between fALS and sALS. Discovery of these common pathways may shed insight to the mechanisms central to ALS pathogenesis.

Much of the work presented here involves the development of co-cultures systems as models to study ALS. However, utilization of these models to uncover mechanisms involved in ALS is the primary goal. One example for how these co-culture systems could be used to search for altered cellular pathways is an RNAi modifier screen. In this experiment, we would first deliver an RNAi library to motor neurons using lentiviral vectors. These motor neurons would be co-cultured with mutant SOD1-expressing astrocytes, leading to motor neuron death. Some motor neurons may have enhanced survival in this co-culture environment due to genes being repressed by RNAi. These surviving motor neurons would be collected by FACS sorting for Hb9-driven GFP fluorescence and the DNA would be isolated to identify which siRNA was delivered. The siRNA collected from surviving motor neurons could be cloned back into the lentiviral delivery system to repeat the assay. Repeating this cycle multiple times will help to eliminate false positives and narrow the pool of modifier RNAi to allow for easier analysis (Fig. 7.1). A modifier screen such as this proposed may be able to identify pathways involved in glial-mediated motor neuron death, leading to a greater understanding of ALS pathogenesis.
**Figure 7.1.** Schematic of an RNAi modifier screen performed on motor neurons in a co-culture with mutant SOD1-expressing astrocytes.

### 7.7 Relevance to humans - ALS research

One focus of the work presented here is to gain a greater understanding for the disease mechanisms involved in ALS pathogenesis and develop new therapeutic strategies. Currently, ALS affects 30,000 in the US but its impact is even greater considering the amount of support and care these patients require. Although ALS was first described back in 1869, the cause for over 90% of cases is still unknown. ALS is a
100% fatal disease and there is only one treatment for ALS which extends survival for an average of 3 months.

In particular, we have focused on investigating glial-mediated motor neuron death in ALS. One reason is because there are no predictors of who will develop ALS and by the time of diagnosis, over half of motor neurons may already be degenerating from the spinal cord so motor neurons may not be a feasible therapeutic target. Therefore, our studies have concentrated on slowing the progression of disease rather than preventing the onset. Glial cells such as astrocytes and microglia have been shown to play a role in the progression phase of disease in a familial SOD1-mediated ALS mouse model. We have also demonstrated that astrocytes derived from this ALS mouse model are toxic to motor neurons \textit{in vitro}. However, prior to this point, it was unknown whether these findings applied to sALS, which accounts for 90% of ALS cases. We have shown for the first time that astrocytes derived from sALS patients have a similar non-cell autonomous toxic effect toward motor neurons. These results are important because there are a wide range of therapies currently in development to target glial cells in fALS. Our data suggests that these glial-directed treatments may also be beneficial in the context of sALS. Preceding our study, there were no models for research on sALS. We have now introduced the first model system for the study of sALS which allows for astrocytic comparisons between fALS and sALS. It is our hope that this system will help elucidate common molecular pathways leading to motor neuron death between sALS and fALS. Identification of these common mechanisms may point to the ultimate causes of ALS.

In addition to creating model systems for the study of ALS, we have also strived to develop a new therapy directed toward inhibition of glial-mediated motor neuron death
in ALS. Our data has demonstrated that the NF-κB pathway is activated in ALS astrocytes, leading to the production of pro-inflammatory cytokines and chemokines. Infection of astrocytes with a virus expressing a dominant negative mutant of IκBα DN protein can inhibit the classical NF-κB pathway and cause a decrease in inflammatory gene expression. To target this NF-κB activation in vivo, we designed an AAV vector carrying the dominant negative mutant form of IκBα. We are currently still in the process of testing this therapy in an ALS mouse model, but preliminary data suggests that inhibition of this pathway may result in an extension of survival. In fact, the NF-κB pathway has been targeted by similar approaches in a variety of neurodegenerative conditions with promising results. One group has produced transgenic mice that express the IκBα DN mutant specifically in glial cells driven by the GFAP promoter. These mice demonstrate decreases in inflammation in models of ischemic injury [221], peripheral nerve injury [222], and spinal cord injury [223]. In addition, the GFAP-IκBα DN mice retain more axons following spinal cord injury and show increased neuronal survival after ischemic injury [221,224]. Lastly, these mice exhibit improved functional outcomes in an experimental autoimmune encephalomyelitis model of multiple sclerosis [225]. Obviously, inhibition of the NF-κB pathway has beneficial effects in animal disease models characterized by inflammation. Since glial cells have been shown to be harmful to motor neurons in ALS and since ALS is characterized by inflammation in the spinal cord, we predict that targeting glial NF-κB activation will also benefit a mouse model of ALS. In addition, our AAV-mediated inhibition of NF-κB is more clinically relevant than inhibition of NF-κB using transgenic mouse models. Transgenic GFAP-IκBα DN expression in mice has certainly demonstrated a proof-of-principle concept for inhibition
of NF-κB in a variety of neurodegeneration models. Therefore, our AAV-IκBα DN therapy may be useful for the treatment of other disorders as well as ALS.

7.8 Concluding remarks

Neuromuscular disorders affect thousands in the US each year, leaving patients with pain, disability, and in some cases, little hope for a cure or treatment. The physical, emotional, and financial impact of neuromuscular disorders such as muscular dystrophy and ALS is immense, affecting both patients and their families. The ultimate goal of the studies presented in this thesis is to gain a greater understanding for the mechanisms involved in neuromuscular disease and use this knowledge to develop more effective therapies.

The first sections of this thesis describe the development and testing of a novel gene therapy to increase muscle mass and strength. The delivery of follistatin, a myostatin antagonist, by AAV-mediated gene delivery demonstrated a dramatic ability to enhance muscles in both a muscular dystrophy mouse model as well as in the non-human primate. While the muscular dystrophies certainly represent our main target population, there are many disorders characterized by muscle atrophy that could be impacted by this therapy. In a completely different paradigm, increasing muscle size through the follistatin pathway may be a strategy to maximize efficiency in the livestock industry and combat environmental issues which are currently a global concern.

Since our studies have been translational in nature, some basic biological questions remain unanswered. Although follistatin is known to inhibit myostatin, studies have shown that follistatin also works through additional pathways that are currently
largely unknown. Identification of these mechanisms may lead to the development of even more effective ways to increase muscle mass. In regard to clinical development of AAV-follistatin, safety concerns remain at the top of the list for investigation in future studies. Formal toxicology studies conducted on mice will prove whether or not follistatin gene delivery affects any major organ system including the reproductive system, known to be influenced by certain follistatin isoforms. If follistatin passes safety tests, future translational research will be focused on strategies to deliver follistatin to multiple muscle groups in the body. Since our studies have solely focused on direct intramuscular injection of follistatin, optimizing this gene therapy for systemic delivery will surely be a priority in years to come.

The second part of this thesis describes work focused mainly on modeling pathogenic disease mechanisms occurring in ALS and developing new treatments to combat this devastating disease. It has been known that glial cells such as astrocytes and microglia are involved in ALS disease progression. We have developed novel model systems to study this glial-mediated motor neuron death in vitro. However, our studies have all centered on the contribution of astrocytes to motor neuron death. Future studies will be necessary to articulate the role of microglia and the peripheral immune system in non-cell autonomous motor neuron death in ALS.

One important piece of data described here demonstrates a common pathway to motor neuron death between fALS and sALS. The vast majority of ALS research has been conducted on the familial form of ALS caused by mutations in SOD1 which only accounts for 2% of all human ALS cases. In contrast, we have developed a novel co-culture model to study the non-cell autonomous effects of sALS astrocytes on motor
neurons. We believe this model can be used to investigate disease mechanisms central to motor neuron death in fALS and sALS as well as test therapies. However, this model is not perfect. In order to truly recapitulate the ALS environment, human motor neurons derived from ALS patients need to be generated to replace the mouse motor neurons currently used in the model. Experiments are currently underway to create induced pluripotent stem cells from ALS patients that will have the capacity to differentiate into motor neurons. Using these co-culture systems, unresolved questions have the potential to be finally answered. For example, are human motor neurons derived from ALS patients more susceptible to the astrocyte-mediated toxicity? Are astrocytes necessary to trigger death of ALS patient-derived motor neurons or do these motor neurons display abnormalities without astrocytes in co-culture? What are the molecular mechanisms leading to motor neuron death and are they similar between sALS and fALS? Do motor neurons derived from sALS iPS cells retain an ALS-phenotype or does reprogramming of the epigenetic code clear these aberrations?

While there are many basic mechanistic questions yet to be answered regarding human ALS pathogenesis, we have strived to develop a therapy based on pathways implicated in motor neuron death in the ALS mouse model. Glial-mediated motor neuron toxicity has been described in both the fALS mouse model as well as in \textit{in vitro} co-culture models. Our data offers one potential mechanism for this glial toxicity, through activation of the NF-κB signaling pathway and inflammatory gene expression. Therefore, we designed an AAV vector to deliver a gene to glial cells that inhibits NF-κB activation. Although we have seen preliminary success in the fALS mouse model using this vector, it remains to be seen whether this pathway is similarly involved in sALS. Future
experiments will more rigorously test this therapy in both mice and in the sALS motor neuron-astrocyte co-culture system. If this vector proves to be successful at inhibiting inflammation in ALS, one could envision testing this approach in other neurodegenerative diseases or injury models characterized by destructive inflammation.
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