GENE THERAPY DEMONSTRATES THAT MUSCLE IS NOT A PRIMARY TARGET FOR NON-CELL AUTONOMOUS TOXICITY IN FAMILIAL ALS

A Dissertation

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2006

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2006
Amyotrophic lateral sclerosis (ALS) is a fatal, progressive neuromuscular disease arising from premature death of motor neurons in the brainstem and spinal cord. Approximately 20% of familial cases of ALS are genetically linked to mutant Cu/Zn superoxide dismutase-1 (SOD1). While the hypothesis that gain of toxicity of mutant SOD1 may cause the disease is generally accepted, the ubiquitous expression of mutant SOD1 makes it hard to delineate its primary target. Previously, neither neuron specific expression nor astrocyte-specific expression provoked motor neuron degeneration. It suggested a non-cell autonomous toxicity, and the implication of multiple cell types in pathogenesis. Recent study showed that the toxicity of mutant SOD1 within motor neuron is crucial to onset and early phase of disease while mutant SOD1 within microglia accelerates disease progression. Given that deterioration of neuromuscular junction is one of earlier symptoms and the depletion or insufficient supply of various neurotrophic factors from degenerated muscle may initiate or promote motor neuron disease, mutant SOD1 damage within skeletal muscle is also a likely primary source for toxicity. This hypothesis was tested with gene therapeutic and genetic approaches. Even though viral delivered siRNA (short interfering RNA) against mutant SOD1 into
various muscle groups significantly diminished mutant SOD1 expression exclusively in muscles, it was insufficient to maintain grip strength. In contrast, the reduction of mutant SOD1 both in motor neuron and muscle is sufficient to preserve motor function. The conditional deletion of mutant SOD1 from muscle did not affect onset or survival. Alternatively, a viral vector encoding Follistatin, a muscle enhancing gene, was administrated to delay disease progress by rescuing muscle atrophy. Follistatin produced sustained increase of whole muscle mass, but the motor function was transiently improved. Indeed, survival was not extended. Therefore, mutant SOD1 within muscle is not a primary contributor to non-cell autonomous pathogenesis of ALS, and the enhancement of muscle mass provides no benefits in slowing disease onset or progression.
Dedicated to my parents
I wish to thank my adviser, Brian K. Kaspar. His scientific guidance, patience, commitment, and contagious goodwill allowed me to complete my Ph D. study. Also, I’d like to thank Hannah Arnson for helping the analysis of muscle pathology and animal behavior study.

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<td>adeno-associated virus</td>
</tr>
<tr>
<td>Ad</td>
<td>adenovirus</td>
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<td>ALS</td>
<td>amyotrophic lateral sclerosis</td>
</tr>
<tr>
<td>Arg</td>
<td>arginine</td>
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<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
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<tr>
<td>BIM1</td>
<td>Bcl2 interacting mediator of cell death</td>
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<tr>
<td>BMP</td>
<td>bone morphogenetic protein</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
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<tr>
<td>CMV</td>
<td>cytomegalovirus</td>
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<tr>
<td>CNS</td>
<td>central nervous system</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>EIAV</td>
<td>equine infectious anemia virus</td>
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<tr>
<td>fALS</td>
<td>familial amyotrophic lateral sclerosis</td>
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<tr>
<td>FGFR1</td>
<td>fibroblast growth factor receptor-1</td>
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<td>FS</td>
<td>follistatin</td>
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<tr>
<td>GDF8</td>
<td>growth and differentiation factor-8</td>
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<tr>
<td>GFP</td>
<td>green fluorescence protein</td>
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<tr>
<td>Gly</td>
<td>glycine</td>
</tr>
<tr>
<td>HEK</td>
<td>human embryonic kidney</td>
</tr>
<tr>
<td>IGF-1</td>
<td>insulin growth factor-1</td>
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<tr>
<td>ITR</td>
<td>inverted terminal repeat</td>
</tr>
<tr>
<td>LTR</td>
<td>long terminal repeat</td>
</tr>
<tr>
<td>miRNA</td>
<td>micro ribonucleic acid</td>
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<tr>
<td>MTT</td>
<td>(3-[4,5-dimthetylthiazol-2-yl]-2,5-diphenyl tetrazolium tetrazolium bromide)</td>
</tr>
<tr>
<td>NSE</td>
<td>neuron specific enolase</td>
</tr>
<tr>
<td>RFP</td>
<td>red fluorescence protein</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNAi</td>
<td>ribonucleic acid interference</td>
</tr>
<tr>
<td>RISC</td>
<td>RNA-induced silencing complex</td>
</tr>
<tr>
<td>shRNA</td>
<td>short hairpin ribonucleic acid</td>
</tr>
<tr>
<td>siRNA</td>
<td>small interference ribonucleic acid</td>
</tr>
<tr>
<td>SOD1</td>
<td>superoxide dismutase 1</td>
</tr>
<tr>
<td>TGFβ</td>
<td>transforming growth factor beta</td>
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ALS background

In this present study, we attempted understand the contribution of the muscle to Amyotrophic Lateral Sclerosis (ALS) pathogenesis. ALS is a progressive neuromuscular disease. The median age of onset is 55 years, and the median survival is three years\(^1\). The lifetime risk is approximately 1 in 2000. 30,000 patients in the United States and 5,000 in the United Kingdom are affected by ALS. Clinical signs involve the dysfunction and death of both lower and upper motor neurons\(^2\), which lead to spasticity, hyperreflexia, generalized weakness, muscle atrophy and paralysis. Symptoms of weakness typically begin asymmetrically and distally in one limb, and then spread within the neuroaxis that involves contiguous groups of motor neurons\(^3\). The symptoms can begin either in the bulbar or limb muscles. Most patients die from the failure of the respiratory muscle within one to five years of onset. In addition to a significant loss of motor neurons, reactive gliosis\(^4\), intracytoplasmic neurofilament abnormalities\(^5-8\), and axonal spheroids\(^5\) are obvious (Table 1). Numerous studies ascribe motor neuron toxicity to multiple factors. The potential pathological mechanisms include...
excessive excitation of motor neuron, free radical-mediated oxidative cytotoxicity, mitochondrial dysfunction, cytoskeletal abnormalities, and trophic factor deprivation. However, the pathological mechanism of ALS is still obscure.

**The Gain of toxicity of mutant SOD-1 in ALS mice models**

Approximately 90% of ALS are sporadic, and the remaining 10% are familial. Approximately 20% of familial cases of ALS are genetically linked to mutant Cu/Zn superoxide dismutase (SOD1). SOD1 is a homodimer of a ubiquitously expressed 153 amino acid polypeptide (Figure 1). As the main function of SOD1 is to convert superoxide, a toxic by-product of mitochondrial oxidative phosphorylation, to water or hydrogen peroxide, SOD1 plays a critical role in the regulation of oxidative stress and in the protection against oxygen-radical induced cell damage (Figure 2). Even though the hypothesis that the active copper and zinc sites of the misfolded mutant SOD1 may cause the toxicity is generally accepted, the mechanism by which mutant SOD1 triggers motor neuron degeneration still remains to be elucidated. Neither the null mutation of SOD1 nor the chronic increase in the levels of wild type SOD1 does cause motor neuron disease. Taken together, these levels of SOD1 activity do not closely correlate with disease in mice or humans. In addition the most popular ALS mouse model SOD1 developed progressive motor neuron degeneration despite the elevated level of SOD1 activity.
Other transgenic mice with different SOD1 mutations, such as SOD1\(^{G37R18}\) and SOD1\(^{G86R19}\), developed disease even though they had elevated or unchanged enzyme activity. Thus, both disease onset and disease progression is independent of the level of SOD1 activity, which supports the gain of toxicity hypothesis.

One fundamental question is whether mutant SOD1 directly damages motor neurons or indirectly targets other adjacent cell populations that might contribute to motor neuron death. Since motor neurons specifically die in ALS, it seems reasonable to hypothesize the toxicity might be originated from motor neurons. However, as SOD1 expression is ubiquitous, it is possible that mutant SOD1 in the adjacent nonneuronal cells might wholly or in part trigger the toxic cascade. Because transgenic mice expressing mutant SOD1 recapitulate human ALS pathologies, they have been used to enlighten the mechanism of ALS. Although more than 100 mutations of human SOD1 have been reported (Figure 3), G93A, G37R and G86R have been the most intensively characterized (Table 2). In 1994, Gurney \textit{et al.} first succeeded in highly and ubiquitously expressing human SOD1 that contains a substitution of glycine to alanine at position 93\(^{17}\). These transgenic mice still had a normal SOD1 enzyme activity at an equivalent level. However, they developed motor neuron disease leading to paralysis in one or more limbs and died by 5 to 6 months of age. They displayed typical pathological features of ALS, including severe loss of large and myelinated axons where SOD1 expression was abundant from the ventral motor roots. Severe loss of motor neurons in intramuscular nerves was usually accompanied by a
denervated muscle and extensive muscle atrophy. Fiber-type grouping and small-angulated (atrophied) fibers are obvious in ALS mice while wild type muscle usually showed randomly distributed muscle fiber types. In the late stages of disease, the muscles were more severely deteriorated, displaying denervated endplate and grouped atrophy. Similar pathological features developed in the other mutant SOD1\textsuperscript{A37R} or mutant SOD1\textsuperscript{G85R} transgenic mice\textsuperscript{18, 19}. Interestingly, mutant G85R showed many inclusion bodies in astrocytes where mutant SOD1 expression is dominant and an increase in the population of aggregated astrocyte. These findings suggest that astrocytes may be the primary targets of mutant SOD1 mediated damage\textsuperscript{19}.

The primary target of mutant SOD1

Although prior studies demonstrated that mutant SOD1 causes inherited ALS, the mechanism underlying disease initiation and progression still remains unclear. To elucidate the primary cellular target of mutant SOD1, tissue specific promoters have been used to modify the ubiquitous expression of mutant SOD1. Although mutant SOD1 accumulates in many and possibly all tissues and cells, motor neurons primarily perish. Therefore, this suggests that mutant SOD1 accumulated in motor neurons may cause the disease. To test whether mutant SOD1 exclusively expressed within motor neurons is sufficient to develop the disease, the neurofilament light chain promoter (NFL) was used to generate transgenic mice carrying neuron-specific expressed mutant SOD1\textsuperscript{G37R}\textsuperscript{20}. The
level of G37R mutant SOD1 expression was high enough to lead to ALS pathology that be expressed constitutively. However, neuron-specific expression of mutant SOD1 \( ^{G37R} \) failed to develop any apparent motor neuron dysfunction. Since the NFL promoter is slightly down regulated later in life, the G93A or G85R mutant SOD1 was expressed in the postnatal motor neuron highly and constitutively under control of Thy1 promoter. The level of SOD1 in postnatal motor neuron was sufficient to develop the disease. However, no pathology of ALS was detected\(^{21}\). Therefore, even highly accumulated mutant SOD1 in postnatal motor neurons may not be sufficient to provoke or accelerate motor neuron disease in ALS. Alternatively, the accumulation of mutant SOD1 in non-neuronal cells may contribute to the selective vulnerability of motor neurons. Motor neurons can be damaged by excitotoxicity derived from the environment. Astrocyte plays a critical role in maintaining the homeostasis of synaptic glutamate level. Aberrant synaptic glutamate should be rapidly removed. Otherwise, glutamate-mediated excitotoxicity can cause repetitive firing and/or the elevation of intracellular calcium leading to motor neuron death\(^{22, 23}\). So far, five subtypes of transporter have been identified. Among them, EAAT2, a glial glutamate transporter, clears 90% of synaptic glutamate\(^{24}\). The reduction of EAAT2 expression is one of the characteristics in the familial ALS (FALS) mouse model\(^{25}\). Consistently, a highly increased level of glutamate in the cerebrospinal fluid of ALS has been reported\(^{26}\). Down-regulated EAAT2 levels with an antisense oligonucleotide induced neuronal death\(^{27}\). So far, reduced glutamate-mediated toxicity only showed therapeutic benefits. To evaluate the potential role
of astrocyte in the etiology of ALS, G86R mutant SOD1 was expressed in astrocyte under GFAP promoter control\textsuperscript{28}. Even though transgenic mice exhibited astrocytosis, they did not develop any motor neuron disease. Thus, even though damage to astrocytes may be involved in the pathogenesis\textsuperscript{29}, motor neuron disease is unlikely to be initiated in astrocytes. Previous studies showed that neither neuron specific expression nor astrocyte-specific expression provoked motor neuron pathology. This suggests that multiple cell types may be implicated in pathogenesis of ALS.

The Cleveland group performed an elegant study using chimeric mice to answer several fundamental questions about the potential contribution of non-neuronal cells to disease onset and progression\textsuperscript{30}. Chimera mice were generated by mixing wild-type SOD1 expressing cells and SOD1 mutant expressing ones. The level of mutant SOD1 was sufficient to cause fatal motor neuron disease. Interestingly, non-neuronal cells expressing wild-type SOD1 delayed motor neuron degeneration. They significantly extended the survival of motor neuron that expressed mutant SOD1. These mice survived much longer compared to parental mice that expressed mutant SOD1 ubiquitously. Even within the same animal, the survival period of two sides of spinal motor neuron was biased, and closely correlated to the proportions of adjacent wild-type nonneuronal cells. In a reverse fashion, normal motor neurons that were surrounded by mutant-expressing nonneuronal cells acquired pathology. This indicates that the nonneuronal neighboring cells could be nurturing or detrimental to motor neurons. Additionally, a genetic study using the Cre-LoxP system was used to diminish the
accumulated mutant SOD in motor neurons or microglia. Suppressed mutant SOD1 within motor neurons substantially delayed disease onset and an early phase of disease progression, while reduced mutant SOD1 in microglia dramatically slowed disease progression after onset. Therefore, it is likely that multiple cell types determine the pathogenesis of ALS. Presumably, surrounding non-neuronal cell types including microglia, astrocyte or muscle can contribute to motor neuron death if the toxicity arises from a distance in a paracrine manner rather than from neurons in an autocrine manner (Figure 4). Previously, the protection of the muscle and motor neurons by Insulin-like growth factor 1 (IGF-I) elongated the life span of ALS mice significantly. This suggests that the muscle may be implicated in pathogenesis.

The role of muscle and the spinal cord in ALS

Notably, the expression of mutant SOD1 in a single cell type failed to produce detectable signs of pathology or motor neuron disease. This suggests that damaged multiple types of adjacent cells could act in concert to initiate disease. In this case, other untested cell types could be a primary target in ALS-associated neurodegeneration. Indeed, the previous analysis of chimeric mice supported non-neuronal autonomous toxicity. A selective and progressive loss of muscle innervation is an early and reliable indicator of advancing neuromuscular pathology in ALS (Figure 5). Further, adult muscle fibers are a source of signals that influence neuron survival, axonal growth, and
maintenance of synaptic connections\textsuperscript{34, 35}. Therefore, it is conceivable that denervation may result from the toxicity of mutant SOD1 within the skeletal muscle that may provoke muscle atrophy and motor neuron death. When myostatin, a negative regulator of muscle growth, was inhibited by a neutralizing antibody, it produced a transient increase in muscle mass and strength in SOD1\textsuperscript{G93A} mice. However, it is still unclear whether this limited benefit may result from the relatively short half-life of this antibody. IGF-1 has been known to be implicated in anabolism of muscle and nerve tissues. It induces muscle hypertrophy and promotes neuronal survival\textsuperscript{36}. Injection of adeno associated virus (AAV) carrying an IGF1 gene into ALS mice significantly prolonged life and delayed disease progression\textsuperscript{31}. In this study, AAV-IGF1 that was transduced both into motor neuron and into the muscle via retrograde transport achieved significant therapeutic effects. But, LV-IGF1 that was locally transduced into the muscle displayed restricted beneficial effects. Thus, motor neuron targeting may be required for maximal therapeutic response. Alternatively, the transduction efficiency of LV-IGF1 may be not sufficient to ameliorate muscle atrophy. Supporting the second possibility, transgenic mice expressing skeletal muscle specific IGF-1 in the SOD1\textsuperscript{G93A} background counteracted the symptoms of ALS. They exhibited not only muscle hypertrophy but also concomitant stimulation of muscle satellite cell proliferation. The increased population of centrally nucleated muscle fiber indicates regeneration\textsuperscript{37}. Also, the muscle-specific expression of local IGF-1 (mIGF-1) isoform stabilized neuromuscular junctions, reduced inflammation in the spinal cord, and enhanced motor neuronal survival in
SOD1<sup>G93A</sup> mice. This study strongly suggested that the muscle may be involved in ALS pathogenesis. In addition, a number of studies have shown that exercise is beneficial to ALS transgenic mice due to the synergic effect of exercise and IGF-1. Combined treatment of exercise and IGF-1 resulted in an extended lifespan. Significantly elevated oxidative stress and compensatory antioxidant enzyme upregulation in the skeletal muscles of SOD1 G93A mice was observed. Thus, mutant SOD1 mediated oxidative stress from the muscle may contribute to ALS pathogenesis<sup>38</sup>. These studies raise the possibility that skeletal muscle may be a target for the dominant action of inherited SOD1 mutation, and/or that muscle fibers may serve an important role in neuron survival. Based on previous studies, there are the two possibilities. First, muscle may be an initiation site of disease. Second, muscle may facilitate the disease progression after onset regardless of the involvement of the muscle in disease initiation.

A muscle enhancing gene: its function and a gene therapy approach to muscle atrophy

Growing evidence has shown that ALS may be a "multi-systemic" disease in which several cell types are involved to develop and exacerbate the disease. Given the numerous studies supporting the involvement of muscle in ALS pathogenesis wholly or partially, the rescue of muscle atrophy would be beneficial. Previously, myostatin inhibition has significantly boosted muscle mass. It shows a great promise for the survival and regeneration of compromised
muscle tissue (Figure 5). A novel members of the transforming growth factor-β superfamily, myostatin (previously called growth and differentiation factor-8 (GDF-8)), is a negative regulator of muscle mass\(^{39}\). Myostatin begins to be expressed in the myotome compartment of developing somites at E9.5. It is continuously expressed throughout adulthood, predominantly in skeletal muscles and in adipose tissue\(^{40}\). The myostatin precursor protein consists of a N-terminal propeptide domain that harbors the signal sequence and a C-terminal domain that forms a disulfide-linked dimer. After cleavage of the propeptide, a large fraction of myostatin non-covalently bound to its propeptide needs to be released from the propeptide to attain its biological activity\(^{41}\). The released C-terminal domain of myostatin binds the Activin Receptor Type IIB, leading to intracellular phosphorylation of Smad3. The complex of phosphorylated Smad3 and other Smad proteins translocates into the nucleus where they regulate the transcription of target genes such as p21\(^{42}\). Myostatin activates TGF-β signaling and upregulates p21, which inhibits cyclin-E-Cdk2 activity. Therefore, the proliferation of the myoblast is arrested\(^{43}\). Animals with mutations or deletions in the myostatin have shown over a two-fold increase in the mass of individual muscle groups\(^{44,45}\). This double-muscled phenotype is the result of hyperplasia and hypertrophy of the muscle with less fat and less connective tissue. Therefore, the manipulation of the myostatin pathway is a potential approach to treat a muscle disorder. One promising antagonist of myostatin is follistatin, a secreted glycoprotein that has been shown to antagonize numerous members of the TGF- β superfamily\(^{46-48}\). Human Follistatin mRNAs are alternatively spliced to produce either a short or
long form. The short isoform is composed of 288 amino acids (FS-288). It is eight to ten times more biologically active than the product of the long isoform (FS-315). Both follistatin and myostatin are expressed in or near muscle during development. Over-expression of follistatin resulted in muscle enlargement, whereas the follistatin-/- KO mouse displayed muscle deficiency. In the presence of follistatin, myostatin fails to bind its receptor preventing myostatin-induced muscle loss. Since follistatin is capable of not only blocking myostatin but also modulating other inhibitors of muscle development, such as BMPs and Activin, Follistatin might be a very potent molecule to prevent muscle loss in muscle-degenerating diseases.

**Gene therapy**

The delivery of trophic factors such as IGF-I, glial cell derived neurotrophic factor (GDNF), and brain derived neurotrophic factor (BDNF) produced protective effects on perishing CNS cell populations, including motor neurons, suggesting a promising therapeutic potential for ALS. However, the administration of recombinant trophic factors showed limited beneficial effects owing to the relatively short half-life and poor access to motor neurons. Alternatively, gene transfer of therapeutic genes to targeted cell populations has solved some of these problems, since the exogeneous gene could be expressed constitutively with cell type specificity. Either non-viral gene delivery systems or viral gene delivery systems can be utilized to introduce therapeutic genes. Synthetic gene
delivery systems rely on direct delivery of either naked DNA or a mixture of DNA with cationic lipids (liposomes). Even though synthetic gene delivery exhibits low toxicity, this approach is generally inefficient and often transient\textsuperscript{57}. Viral delivery systems use genetically manipulated viruses to express therapeutic genes. For the proper gene therapy, the viral vector should not elicit any pathogenic or adverse effects. These redesigned viruses are simply carriers, since the most dispensable components of the wild-type genome are replaced with the gene of interest. The components for replication and virion production are separately supplied \textit{in trans}. Viral vectors are roughly categorized into the RNA virus, an integrating virus that can only infect dividing cells, and the DNA virus, a non-integrating virus that is maintained as an episome in the infected cell.

AAV is a nonpathogenic and single stranded DNA virus whose genome is only 4.7 kb\textsuperscript{58}. The genome of AAV comprises 2 viral ORFs, rep and cap, which are flanked by ITRs. The ITRs are essential for AAV life cycle including replication, packaging, integration and circularization. AAV2, the most well studied serotype, uses heparan sulfate proteoglycan as the primary receptor, and integrin $\alpha v \beta 5$ and/or FGFR1 (fibroblast growth factor receptor 1) as co-receptors\textsuperscript{59, 60}(Figure 6). AAV2 enters the host cell by endocytosis, and traffics through the endosomal compartment. The escaped virus from the endosome translocates into the nucleus. In the nucleus, the uncoated virus converts its genome from a single strand to a double strand to express encoded genes. AAV, a dependovirus, requires a helper virus such as adenovirus (Ad) or Herpes Simplex virus (HSV) to propagate its replication\textsuperscript{61}. However, the helper virus
causes contamination, which triggers undesirable immune responses. To overcome this problem, Ad virus free packaging system was developed. In this Ad free packaging system, a helper plasmid provides three essential helper functions of an Ad, such as E2a, E4, and VA RNA. In this system, packaging cells such as HEK 293 cells provide E1a and E1b, which are essential for AAV replication \(^{62,63}\). AAV can efficiently deliver genes into nondividing cells such as hepatocytes, muscle, and neurons since AAV transduction can occur in non-dividing cells \(^{64-66}\). AAV has been recognized as an ideal gene therapeutic virus since it fulfills several requirements including no related etiology, a broad host range, an efficient and easy high titer production, and long-term gene expression. But, the limited packaging capacity hinders its ability to deliver large genes. In addition, the other unsolved problem is a strong humoral immune response against the viral capsid during the re-administration of vector. Indeed, circulating neutralizing antibodies are highly prevalent in the majority of population who were naturally infected. Thus, the modification of capsid protein or the use of the cap protein from a different serotype may overcome those problems \(^{67}\).

Besides the DNA virus, the RNA viruses have received much attention because they can stably integrate into host genome. Among them, lentivirus is categorized as a Retrovirus, a large family of enveloped RNA viruses that contains two copies of a single-stranded RNA genome. The viral RNA genome contains three essential genes: gag, which encodes capsid protein, matrix, and nucleoprotein complex; pol, which encodes the viral protease, reverse transcriptase, and integrase; and env, which encodes the envelope
glycoproteins. These three essential viral proteins are flanked by long terminal repeats (LTR). Lentiviruses, which are complex retroviruses, encode three to six additional regulatory genes for viral replication and persistence of infection. Two of these regulatory proteins, tat and rev, are commonly present in all lentiviruses. They mediate transactivation of viral transcription and nuclear export of unspliced viral RNA, respectively. For biosafety reasons, other dispensable HIV-1 accessory genes, including vif, vpr, nef, and vpu, were deleted to prevent homologous recombination and replication.

Lentivirus can infect a broad spectrum of cells from prokaryotes to many eukaryotic cells. Also, their ability to integrate into the host genome allows them to achieve a stable lifelong gene expression. The major advantage of lentivirus over other retroviruses is that lentivirus can infect nondividing, terminally differentiated mammalian cells. Lentivirus enters the host cell through the nuclear pore even without the disassembly of the nuclear membrane. In contrast, other retroviruses can enter into the host nucleus only when the nucleus membranes are disassembled during mitosis. The receptor of lentivirus mediates membrane fusion and initiates the endocytosis. In the host cell, the viral RNA is reverse transcribed into a double-stranded proviral DNA, which is associated with viral proteins like nucleocapsid, reverse transcriptase and integrase (preintegration complex). The nuclear localized newly synthesized proviral DNA is integrated into the host cell genome by integrase. The integrated viral DNA mimics a cellular gene and utilizes host-cell machinery to express their genes. Because they depend on receptor-mediated endocytosis, the infection of lentivirus is
strictly restricted to cells that express CD4 and the coreceptor. However, 
lentivirus can incorporate env glycoproteins originated from related as well as 
unrelated viruses allowing them to alter tropism. Pseudotyping with the 
vesicular stomatitis virus glycoproteins (VSV-G) has broadened its host range\textsuperscript{72,73}, while rabies-G pseudotyped equine infectious anaemia virus (EIAV) based 
vectors are able to conduct retrograde axonal transport to the nervous system\textsuperscript{74}.

In CNS-related neurodegenerative disease such as ALS, the relative 
inaccessibility of the CNS and the broad anatomical distribution of neurons make it difficult to target affected neurons. To rescue the perishing cell population efficiently and specifically, cell type specific promoters can be utilized. The neuron specific enolase promoter (NSE) has shown neuron specific transgene expression in the brain and spinal cord\textsuperscript{75-77}. Alternatively, retrogradely transported viruses can be harnessed to transduce genes within motor neuron populations\textsuperscript{78-80}. In addition, the wide distribution of the virus is another obstacle. Since the injected virus is usually present locally, multiple injections are required to disseminate the virus beyond the injection site. Multiple injections may cause trauma or paralysis. Alternatively, the retrogradely transported viruses for example, AdV, HSV and the pseudo rabies virus, show promising results\textsuperscript{78-80}. These viruses express transgenes in entire neuronal projection pathways. Additionally, instead of a difficult intraventricular injection, retrograde transport viruses can be intramuscularly injected. Interestingly, despite the denervation and impairment of axonal transport in mouse models of ALS, the axonal retrograde transport of intramuscular injected AdV is dramatically improved\textsuperscript{81,82}. 
Although those retrogradely targeted virus showed very encouraging results, the robust immune response against AdV render translation difficult. Since AAV is associated with no etiology or pathology, it is a potentially attractive viral vector. Non toxic AAV virions labeled with fluorescent molecules were rapidly taken up exclusively in neurons indicating that AAV was suitable for transgene expression in motoneurons of the spinal cord without robust immune response. Furthermore, gene therapy utilizing AAV has shown pre-clinical successes for a number for neurodegenerative disease, including Parkinson’s disease, Alzheimer’s disease, and spinal cord injury. Finally, therapeutic gene expression using AAV was successful in the brains of primates.

siRNA approaches

Besides the viral delivery of therapeutic genes, small interference RNA (siRNA) has been recently considered a promising therapy to silence detrimental mutant SOD1 expression in neurodegenerative disease where the accumulation of a toxic protein drives neuropathogenesis. Accumulating biochemical and genetic evidence has begun to elucidate a mechanism for RNAi mediated gene silencing in mammalian cells. Depending on the organism, RNAi is triggered by various types of molecules, including long dsRNAs, plasmid-based short hairpin RNAs (shRNAs) or endogenous hairpin micro RNAs (miRNAs). The ribonuclease-III activity of the evolutionarily conserved Dicer enzyme processes those molecules to generate 21–22-nt siRNAs. Sequentially, newly processed
siRNAs are incorporated into a protein complex, RNA-induced silencing complex (RISC), where the duplex siRNA is unwound in an ATP-dependent manner. The antisense strand of the duplex siRNA guides the RISC to the homologous mRNA where the RISC-associated endoribonuclease cleaves the target mRNA and silences the target gene.

However, the siRNA gene therapeutic approach has two major hurdles to overcome. The first hurdle is the achievement of sustained synthesis of small RNA species. Plasmids expressing siRNA can be utilized to overcome this obstacle. RNA polymerase III (pol III) U6 and H1 snRNA promoters, tRNA promoters, and RNA-pol-II-based CMV (cytomegalovirus) promoters have shown constitutive expression of plasmid-based shRNAs successfully suppressing target genes. Among them, the pol III promoter is widely used because it is active in broad cell types and efficiently directs the synthesis of transcripts. The second challenge is the efficient delivery of the siRNA into target cells. In particular, the transfection of siRNA into freshly isolated primary cells, neural cells, and stem cells has experienced technical difficulties. Viral delivery of siRNA is one way to overcome these shortcomings. For example, shRNA-expression cassettes have been efficiently and stably integrated into the host genome by using the Moloney murine leukaemia virus or lentiviral vector systems. By contrast, the adenovirus vector system that does not integrate into the host is more suitable for RNAi-mediated human gene therapy. Lentivirus-mediated delivery has added advantages over others. This virus can transfect
non-dividing cells, such as terminally differentiated cells. Transgene carried by lentiviral vectors are resistant to silencing. Previously, lentivirus-delivered siRNA successfully suppressed GFP\textsuperscript{102}, the pro-apoptotic BIM1 (Bcl2-interacting mediator of cell death) gene\textsuperscript{103} and the gene encoding the IL2 receptor (CD25)\textsuperscript{103}.

In previous studies, performed siRNA viral delivery studies have generated tremendous excitement owing to their potential therapeutic application for amyotrophic lateral sclerosis (ALS). Rabies-G-pseudotyped EIAV-mediated silencing of mutant SOD1 expression in both vulnerable motor neurons and muscle increased motor neuron survival and improved motor performance\textsuperscript{104}. Kaspar and his colleagues showed that the intramuscular injected AAV2 siRNAs against mutant SOD1 were retrogradely transported into motor neurons\textsuperscript{105}. Virally delivered siRNA efficiently delayed muscle deterioration and extended their survival in the mice model of ALS. Additionally, intraspinal injected lentiviral vector only affected the spinal cell population, including motor neuron and glial cells\textsuperscript{106}. These lentiviral delivered siRNA successfully rescued motor neuron death, delayed disease onset and reduced muscular atrophy. Taken together, those studies demonstrated that the silencing of mutant SOD1 in muscle and/or spinal cells is beneficial to ameliorate ALS pathology.

Growing evidence has highlighted the importance of cellular environments in the initiation of disease. Previously, chimera mice that express wild-type motor neurons and mutant non-neuronal cells substantially compromised motor neuron
degeneration and extended life span\textsuperscript{30}. In contrast, mutant adjacent non-neuronal cells triggered the demise of wild-type motor neurons. This study suggests the involvement of non-neuronal cells in the pathogenesis\textsuperscript{30}. However, the ubiquitous expression of mutant SOD1 in the transgenic mouse model makes it harder to delineate the cellular target of mutant SOD1. Interestingly, the Cre-Lox mediated silencing of the toxic mutant SOD1 in the motor neuron significantly delayed disease onset as well as extended the life span. In contrast, the reduction of mutant SOD1 within microglia dramatically delayed disease progress at the later phase, but it did not have any effect on disease onset\textsuperscript{107}. Normally, microglia have a critical role in the mediation of inflammatory responses. However, neuronal damage activates them to release oxygen radicals, nitric oxide, and cytokines, which affect the neighboring cells, such as neurons and astrocytes. Noticeably, activated microglia were observed surrounding the perishing motor neurons in ALS at the late stage of disease\textsuperscript{108}. These studies support the hypothesis of non-cell autonomous toxicity of mutant SOD1, in which the damaged motor neuron and/or other cell types may initiate the disease and microglia may ameliorate it. Even though the target of mutant SOD1 is still unclear, a recent study suggested that the disease may progress distally and the deterioration of neuromuscular junctions may be involved in the development of the disease. Quantitative pathological analysis performed at multiple ages showed that the denervation of neuromuscular junctions preceded motor neuron loss\textsuperscript{109}. At 47 days, 40% of end plates were deteriorated whereas any ventral root or cell body loss was not detected yet. Sixty percent of ventral root axons
were lost at the age of 80 days, and motor neuron loss started at 100 days. Microglial and astrocytic activation around motor neurons followed the onset of distal axon degeneration, supporting the hypothesis that motor neuron pathology may begin at the level of the neuromuscular junctions and proceed from distal to proximal\textsuperscript{109}. As the neuromuscular junction has been considered a potential pathogenesis site, skeletal muscle may be a potential target for the dominant action of inherited SOD1 mutation. Since muscles provide various neurotrophic factors to the motor neuron, the depletion/insufficient supply of neurotrophic factors from deteriorated muscles can initiate or facilitate motor neuron disease. Moreover, the preservation of motor neurons and muscle by the delivery of siRNA against mutant SOD1\textsuperscript{105, 106} or neurotrophic factor, such as IGF-1\textsuperscript{31}, improved the motor function substantially and extended the life span. These studies raised the possibility that the muscle may be a target of toxic mutant SOD1. When IGF-1 was transduced exclusively into skeletal muscle by using VSVG pseudotyped lentivirus, the survival rate was only slightly extended. However, it is hard to exclude the possibility that the low transduction efficiency may limit the efficacy. The transgenic mice expressing IGF-1 solely within skeletal muscle impressively reversed the pathology of ALS. This highlights the contribution of the muscle to ALS etiology\textsuperscript{37}, even though IGF-1 might protect the adjacent motor neurons in a paracrine manner at the neuromuscular junction. More intensive efforts have been made to rescue muscle atrophy using neutralizing antibody. The anti-myostatin antibody preserved limb muscle transiently and improved diaphragm morphology and function through the late
stage. However, disease onset was not delayed and survival was not extended. The observed restricted preservation of muscles may be from the relatively short half life of the antibody. While these studies implicate the muscle in ALS, the exclusive role of skeletal muscle has not been intensively evaluated yet. Delineating the target of mutant SOD1 is important to evaluate the therapeutic significance of blocking damage within target sites in the course of disease progress. Especially since the skeletal muscle is an attractive target for virus-mediated gene therapeutic approaches in ALS, it is important to determine whether an initiating toxic insult or a determinant of disease progress after onset is developed within the muscle. In this study, we investigated the hypotheses that skeletal muscle may be the initiation site of disease and/or the preservation of skeletal muscles may be therapeutically beneficial. To test these hypotheses, we utilized three different approaches (Figure 8). First, siRNA was targeted to various muscle groups to evaluate whether silencing mutant SOD1 toxicity solely within the muscles can block disease onset. Alternatively, mutant SOD1 within skeletal muscle was exclusively excised by the Cre-LoxP system. Finally, the muscle-enhancing gene, follistatin, was administrated using a virus to estimate whether disease progression can be delayed by the rescue of muscle atrophy.
<table>
<thead>
<tr>
<th>Early Symptoms</th>
<th>Late Symptoms</th>
</tr>
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<tbody>
<tr>
<td>Twitching</td>
<td>Shortage of breath</td>
</tr>
<tr>
<td>Loss of motor control in the hands and arms</td>
<td>Difficulty breathing</td>
</tr>
<tr>
<td>Weakness and fatigue</td>
<td>Difficulty swallowing</td>
</tr>
<tr>
<td>Tripping and falling</td>
<td>Paralysis</td>
</tr>
<tr>
<td>Dropping things</td>
<td>Death by respiratory involvement</td>
</tr>
<tr>
<td>Uncontrollable periods of laughing or crying</td>
<td>Slurred speech and difficulty in projecting the voice</td>
</tr>
</tbody>
</table>

Table 1 The pathological symptoms of ALS
Nature Reviews Neuroscience, 2001, Cleveland et al.

Figure 1 The structure of SOD1 (Superoxide dismutase 1)
ALS linked mutations are scattered throughout the SOD1 protein. The positions of mutations in the three dimensional structure of crystallized human SOD1 are indicated. Rasmol program was used to get the structure (R. Sayle, Biomolecular Structure Group, GlaxoWellcome Research & Development).
Superoxide dismutase catalyzes the conversion of superoxide to peroxide and water via two asymmetric steps. Essential copper atom is oxidized or reduced by superoxide, alternatively.
More than 100 mutations of SOD1 have been reported in familial ALS. Mutations are distributed among all five exons and alter the various regions of SOD1 protein.
<table>
<thead>
<tr>
<th>Transgenic Mice</th>
<th>Human/mouse SOD1 ratio</th>
<th>Onset of Disease</th>
<th>Pathology</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>hSOD1</td>
<td>1-10</td>
<td>No disease</td>
<td></td>
<td>Epstein et al., 1987, Ceballospicot et al., 1991</td>
</tr>
<tr>
<td>hSOD1 null</td>
<td>No SOD1</td>
<td>No disease</td>
<td></td>
<td>Reaume et al., 1990</td>
</tr>
<tr>
<td>hG37R</td>
<td>10-12</td>
<td>3.5-4 months</td>
<td>Vacuoles in motor neuron, muscle atrophy</td>
<td>Wong et al., 1995</td>
</tr>
<tr>
<td>hG37R</td>
<td>/4-5</td>
<td>/6-8 months</td>
<td></td>
<td>Pramatarova et al., 2001</td>
</tr>
<tr>
<td>hG85R</td>
<td>1</td>
<td>8-10 months</td>
<td>Astrocytic inclusions, aggregates in motor neurons, clinical signs (hindlimb, paralysis &amp; muscle atrophy), late onset, rapid progression</td>
<td>Bruijin et al., 1997</td>
</tr>
</tbody>
</table>

Table 2 Summary of previously published SOD1 transgenic mice
Table 2 (Continued)

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<table>
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<tr>
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</thead>
<tbody>
<tr>
<td>hG85R</td>
<td>0.2</td>
<td>12-14 months</td>
<td></td>
<td>Bruijin et al., 1997</td>
</tr>
<tr>
<td>mG86R</td>
<td>1</td>
<td>3-4 months</td>
<td>Motor neuron degeneration</td>
<td>Ripps et al., 1995</td>
</tr>
<tr>
<td>mG86R</td>
<td></td>
<td></td>
<td>Restricted to astrocytes</td>
<td>Gong et al., 2000</td>
</tr>
<tr>
<td>hG93A</td>
<td>4</td>
<td>4-6 months</td>
<td>Cytoskeleton abnormality, NF aggregation, astrocytosis</td>
<td>Gurney et al., 1994</td>
</tr>
</tbody>
</table>

Table 2 Summary of previously published SOD1 transgenic mice
Figure 4 The potential targets of mutant SOD1 toxicity
Figure 4 The potential targets of mutant SOD1 toxicity

Mutant SOD1 is expressed ubiquitously. Numerous studies support multiple cell types are involved in pathogenesis. Mutant SOD1 expression in single cell type, such as motor neuron or astrocyte, was not sufficient to develop motor neuron pathology. Severe muscle atrophy suggests the contribution of skeletal muscle to ALS pathogenesis.
Figure 5 Inhibition of Myostatin by Follistatin
Myostatin induces p21 to arrest the proliferation of muscle. Follistatin, an antagonist of Myostatin, releases cell cycle arrest promoting muscle hypertrophy and hyperplasia.
Viral binding to a receptor/co-receptor on the surface of cells is followed by endocytosis. Virus is trafficked through the endosomal compartment. Escaped virus from endosome is translocated into nucleus. In the nucleus, uncoated virus converts its genome from a single strand to a double strand to express an encoded gene.
Figure 7 Graphic representation of the behavioral and neuropathological progression of G93A transgenic mice
Figure 8 Graphic representation of various studies performed
Figure 8 Graphic representation of various studies performed

Virus expressing siRNA or Follistatin was injected into hindlimb muscles prior to disease onset. At the end stage of disease, Follistatin level in serum was monitored and muscle pathology was analyzed.
CHAPTER 2

MATERIALS AND METHODS

Construction of AAV1, AAV2 and lentivirus Vectors

Human Follistatin-344 was a kind gift from Dr. Shumazaki (University of California-San Diego, La Jolla, CA) and was subcloned into the EcoR I site of Bluescript containing a novel 5’ Sfi site and 3’ Pme I site (Figure 9). The cDNA was then subcloned directionally into these sites of AAV1-ITR containing the vector under the control of the human cytomegalovirus (CMV) promoter. Short interfering RNA (siRNA) directed against SOD1 was designed as described earlier. Sequences were determined to be unique to the human SOD1 gene by BLAST searching of the GeneBank database. Oligonucleotide primers of the sequences 5’-GGCCTGCATGGATTCCATG-3’ for the siRNA and 5’-GGCTTGCATGGATTACATG-3’ for the siRNA mismatch were annealed and cloned into the pSuper vector. Sequentially, this cDNA was subcloned into recombinant AAV2-ITR (Figure 10) or recombinant lentivirus downstream of the H1RNA promoter (Figure 11). This vector also carries an enhanced green florescent protein (EGFP; ClonTech, Palo Alto, CA) driven by the cytomegalovirus promoter. All constructs were verified by automated sequencing.
**Viral vector production**

Human T293G cells were grown in Dulbecco’s minimum essential medium supplemented with 10% fetal bovine serum and 50 ug/ml pen/strep (Invitrogen, La Jolla, CA). Transfections with pSuper vectors were conducted by using Effectene (Qiagen, Valencia, CA). Recombinant AAV1-FS, AAV2-siRNA, or lentivirus-siRNA was produced by triple transfection using calcium phosphate in human embryonic kidney carcinoma HEK293 cells as described previously\(^\text{111}\). For production of recombinant AAV1-FS, the AAV Helper-Free System was used to produce an infectious recombinant adeno-associated virus-2 virions without the use of a helper virus. A plasmid containing the Rep from serotype 2 and capsid form serotype 1\(^\text{112}\) was provided along with a helper adenoviral plasmid that includes most of the adenovirus gene products E2A, E4, and VA RNA genes required for AAV replication (Stratagene, Palo Alto, CA). HEK 293 cells stably supplied the remaining adenoviral gene, E1a and E1b genes in trans. Vector plasmid, pAAV/Ad8, and pXX6 helper plasmid were used for recombinant AAV2-siRNA production\(^\text{62}\). The HEK 293 cells were plated to 3 X10\(^6\) cells 48 hours prior to transfection. The calcium phosphate-based triple transfection was performed at 70-80% confluency. One ml of 0.3 M CaCl\(_2\) was added to the mixture of the three plasmids to be co-transfected, the recombinant pAAV expression plasmid, pAAV/Ad8, and Pxx6 helper, and mixed well. Sequentially, the mixture of DNA/CaCl\(_2\) was added to 2 X HBS (280 mM NaCl, 1.5 mM NaHPO\(_4\), 50 mM HEPES, pH 7.1) in a dropwise fashion. Immediately after
several gentle inversions or pipetting, the DNA/CaCl₂/HBS suspension was applied to the plate in a dropwise fashion and swirled gently to distribute the DNA suspension evenly in the medium. After 6 hours of incubation at 37 °C, the medium was removed from the plates and replaced with the medium containing 10% serum. The cells were incubated for 66-72 hours at 37 °C. The virus was collected 72 hours post-transfection from both the growth medium and intact cells. The transfected cells plus the DMEM growth medium was transferred to a 15ml conical tube. The collected cell suspension was subjected to four rounds of freeze/thaw by alternating the tubes between the dry ice-ethanol bath and the 37°C water bath, vortexing briefly after each thaw. Each freeze and each thaw took 10 minutes' incubation time. Cellular debris was removed after centrifugation at 10,000 X g for 10 minutes at room temperature. Collected viruses were treated with benzonase at a final concentration of 35 U/ml for 30 minutes at 37° C to remove the cellular DNA contamination before purification. Clarified viruses were processed on cesium chloride density gradients. The lysate was subjected to centrifugation at 41,000 rpm of Beckman rotor in 1.37g/ml cesium chloride density gradient to separate the packaged and unpackaged AAV particles. The packaged AAV was collected and a buffer exchange was followed. To purify the AAV2 serotype, the viruses were passed through heparin affinity column chromatography (Amersham Biotech) using vector services from Virapur, LLC (San Diego, CA) (Figure 12). The titer was determined by Q-PCR (quantitative PCR) techniques. Q-PCR was carried out
using the PRISM/700 Sequence Detector (PE Applied Biosystems). The primers were designed to amplify the CMV (cytomegalovirus) sequence of the AAV genome. The forward and reverse primers were 5’ TGG AAA TCC CCG TGA GTC AA 3’ and 5’ CAT GGT GAT GCG GTT TTG G 3’, respectively. The reaction was performed according to the instructions of the manufacturer using the SYBR Green PCR Core Reagents Kit (PE Biosystems). Initially, since the SYBR Green I dye binds to the double-stranded DNA template, they are fluorescent. During the PCR reaction, double-strand DNA is denatured and the SYBR Green I Dye is released, reducing the fluorescence drastically. The primers anneal and the PCR product is extended. When polymerization is complete, the SYBR Green I Dye is completely bound, producing a net increase in fluorescence. A DNA plasmid standard curve was constructed using $10^3$ to $10^7$ AAV genome equivalents. The virus DNA samples were prepared by digesting DNase-resistant virus with proteinase K in 1 X PCR buffer at 50 °C for 1 hour, followed by boiling for 20 minutes.Titers were $3 \times 10^{12}$ DNase Resistant Particles/ml. Silver staining was used to examine the purity (Figure 13).

Lentivirus pseudotyped with VSVG was produced by quadruple transfection in HEK293 cells as previously described\textsuperscript{113}. Briefly, the lentiviral vectors based on the pRRL vector was used to transfect HEK 293 cells along with three packaging plasmids, gag/pol, rev, and the VSV-G envelope, to supply helper functions and viral proteins \textit{in trans}. The HEK 293 cells were transfected approximately at 90-95% confluency. Before transfection, the cultured media was
removed from the 293 cells and replaced with fresh medium containing serum. 2.7ml of 2.5M CaCl₂ was added to the quadruple mixture of the lentivirus vector and the packaging, and vortexed. Sterile water was added up to 14 ml, and then poured into the same amount of 2 X BBS (50mM BES, 280mM NaCl, and 1.5Mm Na₂HPO₄) placed in a 50 ml Corning tube. The virus containing supernatants was harvested at 48-72 hours post-transfection by removal of the medium when the HEK 293 cells started to detach from the plates. The collected virus plus media was centrifuged at 3,000 rpm for 15 minutes at 4 °C to pellet cell debris or filtered to remove cell debris (Figure 14). Titers were determined to be 5 x 10⁸ transducing units/ml based on GFP expression on serial dilution infections of HEK 293 cells¹¹⁴. The lentivirus was aliquoted and stored at -80°C.

**In vitro Cell Proliferation Assay**

C2C12 myoblasts were grown in DMEM (Invitrogen, Temecula, CA) containing 10% FBS (Invitrogen, Temecula, CA). Cell proliferation assays were conducted in 96-well Nunc Microwell plates and seeded at 1000 cells per well. After attachment, myostatin (R&D Systems) was added at a concentration of 3 ug/ml in the presence or absence of conditioned media.

Conditioned media was created by infecting a well of 12 well dish with either 10⁹ viral particles of AAV-Red Fluorescence Protein (RFP) or AAV-Follistatin (FS) and collecting the media 48 hours after infection. A MTT (3-[4,5-
dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium tetrazolium bromide) proliferation assay was performed using a commercially available kit (Pierce Technologies). Data was collected in quadruplicate and read on a microplate reader set for absorbance at 570 nM. The results were presented as means and standard errors.

**Injection of mice and behavioral testing**

We used transgenic mice carrying the human SOD1 gene with the G93A mutation (B6SJL-TgN[SOD1-G93A]1Gur; Jackson Laboratories, Bar Harbor, ME) in these studies. The mouse line was maintained as a hemizygote by breeding G93A male with the B6SJL hybrid females. Transgenic progeny were identified by PCR using primers specific for human SOD1. To eliminate the possibility of increased survival being as a consequence of a dropped copy number of the SOD1G93A gene, littermates were served as uninjected controls. All surgical procedures were performed in accordance with the National Institute of Health guidelines and with institutional approval. At age 45 days, 20 randomly assigned transgenic mice with the G93A human SOD1 mutation were injected with 25μl AAV-siRNA-GFP or AAV-misesense-GFP into the right lower hind limb using a 5 μl Hamilton syringe, with a total of 5μl of virus used per each injection. A total of 1X10^{11} AAV were injected into one hindlimb of each SOD1 G93A mice.
A total of 1 \( \times 10^7 \) lentiviral particles were injected per hindlimb for lentiviral siRNA studies. Following injections, we left the needle in place for an additional 5 min before the needle was gently withdrawn.

Mice were observed daily for survival. Testing of motor function using a rotarod device (Columbus Instruments, Columbus, OH) began at 35-40 days of age. A 4-cm-diameter rod was set to rotate at 5 rpm/min, and the mice were placed on a rotating rod. The time they remained before sliding off was recorded (latency). Each weekly session consisted of three trials, and the average of three trials was recorded. The muscle strength of forelimb and hindlimb was examined weekly using a grip strength meter (Columbus Instruments, Columbus, OH). Each weekly session consisted of 4 tests per animal per limb. Four tests were done consecutively on each mouse with no more than 30 seconds between each trial. Grip strength meter testing was performed by allowing the animals to grasp a platform with one hind limb (left or right), followed by pulling the animal until it released the platform; the platform was connected to a tension digital force transducer and the force measurement was recorded in four separate trials. The 4 values were averaged and corrected for the weight of the mouse to produce a kg of tension produced per kg weight of the mouse. To determine mortality in a reliable and humane fashion, we used an artificial end point, defined by the inability of the mice to right themselves in 30 seconds after being placed on their sides. The moribund mice were scored as “dead” and were euthanized, and tissues were collected.
**LoxSOD1\textsuperscript{G37R} Mice**

Transgenic mice carrying heterozygous human SOD1G37R that is flanked by loxP sequence were generated (Figure 22). Briefly, a pair of 34 base loxP sequences was cloned at both sides of human SOD1\textsuperscript{G37R} transgene. Cloned the human SOD1G37R transgene flanked by loxP sequences was digested with two different restriction enzymes, Sal I and Not I. The gel purified gene was microinjected into one-cell stage hybrid (C57BL/6J x C3H/HeJ) F2 mice embryos. Western analysis was performed to check the construction of the transgenic founder as described below. The tail protein was used for the identification of human SOD1. The transgenic founder was crossed with the C57BL/6J mice five times before mating to the Cre transgenic mice. MCK-Cre heterozygous transgenic mice were generated. A 6.5 kb DNA fragment of muscle creatine kinase (MCK) was cloned as described before\textsuperscript{115}. The genomic MCK fragment included the MCK promoter, enhancer 1, the untranslated exon 1, and intron 2 containing enhancer 2. This fragment was subcloned into the pBluescript (Stratagene) that has a 1.1 kb Cre recombinase gene with a nuclear localization site. This 1.1 kb Cre recombinase was a kind gift from Dr. V. Episkopou, MRC Clinical Science Center, Hammersmith Hospital, London, UK. A polyadenylation signal from the human growth hormone gene was added (a kind gift from Dr. R. Palmiter, Howard Hughes Medical hormone Institute and Department of Biochemistry, University of Washington, Seattle, USA). Finally, the cloned gene was purified from agarose gel, and injected into fertilized F1:CBAxC57BL/6 eggs.
Heterozygous LoxSOD1\textsuperscript{G37R} mice were crossed with MCK-Cre mice from a C57BL/6 background to excise mutant SOD1\textsuperscript{G37R} solely within the skeletal muscles of heterozygous LoxSOD1\textsuperscript{G37R} mice. The presence of the mutant SOD1 was examined by PCR using the following primers, 5' CAG CAG TCA CAT TGC CCA AGG TCT CCA ACA T3' and 5'CCA AGA TGC TTA ACT CTT GTA ATC AAT GGC 3'. The Cre sequence was identified using the following primers, 5' CCG GGC TGC CAC GAC CAA3' and 5' GGC GCGGCA ACA CCA TTT TT 3'. The deletion of Lox SOD1\textsuperscript{G37R} was determined by quantitative PCR. The level of lox SOD1G37R was analyzed by Western. For the survival analysis, the littermates that were generated contemporaneously were used as negative control. The disease onset was defined as the time when mice reached peak body weight. After this peak point, they started to lose their body weight, which was closely correlated to the initiation of motor performance dysfunction. Mice usually lost 10% of their maximal body weight at the initial stage of the disease. The end point was defined at the time when the animal could not longer right itself within 30 seconds when placed on its side.

**Quantification of SOD1 transgene content by real-time PCR**

After the excision of mutant SOD, levels of LoxSOD1\textsuperscript{G37R} in skeletal muscle were measured by real time PCR. Genomic DNA was extracted from skeletal muscles using the QIAamp DNA micro kit. 33ng of genomic DNA was
amplified with iQ Supermix (Bio-Rad) and 100 nM of each primer and probe (IDT) in a Bio-Rad iCycler real time PCR machine. The following PCR program was used: 1 cycle 50 °C, 2 minutes; 1 cycle 95 °C, 10 minutes; 40 cycles 95 °C, 15 seconds, 60 °C, 1 minute. Specific primers and probe for the human SOD1 gene were: hSOD1-forward, CAA TGT GAC TGC TGA CAA AG; hSOD1-reverse, GTG CGG CCA ATG ATG CAA T; and the hSOD1 probe, fam-CCG ATG TGT CTA TTG AAG ATT CTG –BHQ. Primers and probe for the normalizer apolipoprotein B (apoB) were: apoB-forward, CAC GTG GGC TCC AGC ATT; apoB-reverse, TCA CCA GTC ATT TCT GCC TTT G; and the apoB probe, Texas Red-CCA ATG GTC GGG CAC TGC TCA A-BHQ2. Genomic DNA from LoxSOD1<sup>G37R</sup> was mixed with comparable genomic DNA from C57/B16 mice in different proportions and used to determine the sensitivity and linearity of the real-time PCR.

**Muscle fiber typing**

Skeletal muscle hypertrophy and myofiber number were investigated. Mice were anesthetized with the mixture of ketamine and xylazine and perfused transcardially with ice cold 1% paraformaldehyde for 1 minute followed by 4% paraformaldehyde for 10 minutes. Various muscle tissues were rapidly removed, embedded in Optimal Cutting Temperature Compound (OCT) and snap frozen in liquid nitrogen cooled isopentane. Transverse 10 µm sections were cut through the middle of the muscle. Sections were stained with hematoxylin/eosin (H & E)
and trichromed stains. Cross-sections from the middle part of 2 different muscles such as Gastocmenius and Quadraseps muscle were considered; the fiber number was calculated as a percentage of all the fibers within an entire section. In order to eliminate any variation from one field to the other, the entire muscle section was considered. In all cases, the measurements were performed on four consecutive serial sections for each sample and an average of the results obtained on these four sections was the value considered. The sections (4 sections for each animal) were photographed on a Zeiss Axi ovet microscope connected to a Zeiss micrometer on a Dell Workstation. The total area of the muscle cross-section was calculated. Individual myofibers were counted and diameters measured. Graphs of total fiber numbers per sections and a percentage of fiber diameters were plotted.

**Immunoblotting and ELISA**

Mice were anesthetized and perfused transcardially as described before. The harvested muscles were weighed. Muscles were immediately lysed in cold TPER buffer (25mM bicine, 150mM sodium chloride pH 7.6, Pierce Chemicals). The ratio of 1g of tissue to 20 ml lysis buffer containing the mixture of protease inhibitors was used. The muscle lysate was homogenized untill it was clear using a motorized homogenization device (Fisher Scientific). The sample was centrifuged for 10 minutes at 4 °C to pellet tissue debris. Colleted supernatant
was used for the Bradford assay to determine the total protein concentration (Bio-rad). Since the acidic dye in the assay solution bound to protein, the color of the dye changed in response to various concentrations of protein, and the absorbance shifted from 465 nm to 595 nm. The absorption at 595 nm was measured with a spectrophotometer. Compared to the standard curve that was drawn using bovine serum albumin (BSA), a relative protein concentration of sample was measured. After a 10 minute denaturation at 70 °C, the protein was resolved on a 4-12% Bis-Tris sodium dodecyl sulfate polyacrylamide gel electrophoresis system (Novex, Invitrogen) with 200 constant voltage. Protein was then transferred onto PVDF (Novex, Invitrogen) membrane that was activated with Methanol for 5 minutes and washed twice with distilled water prior to transfer. The protein transfer was confirmed by Ponceiu-S staining and Comassie-Blue staining. The membrane was rinsed in distilled water until the stain disappeared. The blotted membrane was blocked in freshly prepared 5% nonfat milk/0.1% Tween 20 (Sigma) in TBS (136mM NaCl, 200Mm Tris-HCl, pH 7.6) for 1 hour at room temperature with constant agitation before the primary antibody incubation. The blocked membrane was incubated with the primary antibody, 1:2000 dilution of SOD1 antibody (SC-8637; Santa Cruz Biotechnology, Santa Cruz, CA), 1:100 anti-green fluorescent protein (GFP; Molecular Probes, Eugene, OR) or 1:5000 dilution of β-actin antibody (Sigma, St Louis, MO) in 5% nonfat skim milk/0.1% Tween 20 (Sigma) in TBS overnight at 4 °C with constant agitation. It was followed by incubation with 1:50,000 dilution of horseradish
peroxidase-conjugated anti-rabbit or anti-mouse IgG (Amersham Pharmacia Biosciences, Piscataway, NJ) in the blocking solution 1 to 2 hours at room temperature with constant agitation. The membrane was washed five times for 3 to 5 minutes each with 0.1% Tween 20 in TBS. Before detection, the membrane was briefly rinsed in distilled water. The detection of protein was performed using the enhanced chemiluminescence (ECL, Amersham Biosciences). ELISA assay was performed using a commercially available ELISA kit against human Follistatin (R&D Systems). For the ELISA assay, the serum was collected, allowed to clot for at least 30 minutes at room temperature, and centrifuged at 13,000 g for 10 minutes at room temperature. The supernatant was used for the assay. The follistatin standard was reconstituted in 1:9 ratios, allowed to sit for at least 30 minutes, gently mixed, and used to make a serial dilution. The assay diluent (100 ul) was added into the microplate, and 100 ul of standard or sample was added to each well. The mixture in the microplate was covered and incubated 3 hours at 4°C. After incubation, the microplate was aspirated and washed 4 times. Follistatin conjugate (200 ul) was added to each well, incubated 2 hours at 4°C and washed 4 times. The optical density of each well was determined using the color detection solution. The absorption was measured at either 540nm or 570nm and 450nm. After this measurement, the reading at 540nm or 570nm was subtracted from the values at 450nm to correct the optical imperfections. Data was collected in triplicate for each animal and presented as means with standard error.
**Statistical Analysis**

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 multi-way analysis of variance followed by a Bonferroni post hoc analysis of means differences between groups (GraphPad Prizm Software, San Diego, CA).
Figure 9 The Schematic view of Follistatin
It includes both FSK SPARC (secreted protein, acidic and rich in Cys) that modulates cellular interaction with the extracellular matrix by its binding to structural matrix proteins, such as collagen and vitronectin, and KAZAL (Kazal type serine protease inhibitor) that includes the ovomucoid-like inhibitory loop.
AAV2 viral construct was designed to express both siRNA against mutant SOD1 under control of the H1 RNA polymerase promoter and GFP driven by CMV promoter.
Figure 11 Lentivirus SOD1 siRNA construct

siRNA against mutant SOD1 was cloned into lentivirus vector under H1 promoter. The vector also expressed GFP driven by CMV promoter.
Recombinant AAV was produced by triple transfecting using calcium phosphate in HEK 293 cells. Virus was collected at 72 hours post-transfection followed by benzonase treatment to remove plasmid DNA. All serotypes of AAV were purified by cesium chloride gradients. Sequentially, AAV2 serotype isolated by heparin affinity column was eluted with 0.5 M NaCl.
After heparin affinity column purification, the purity of AAV2 was confirmed by silver staining. Capsid protein of AAV2, VP1 (85KDa), VP2 (72KDa), and VP3 (61KDa) were identified.
Figure 14 Lentivirus purification

Lentivirus pseudotyped with VSVG was produced by quadruple transfection in HEK293 cells. Collected virus was filtered and ultra centrifuged to get high titer viral stock.
CHAPTER 3

RESULTS

Reduction of toxicity of mutant SOD1 in muscles does not affect disease

Via retrograde transport to spinal motor neurons\textsuperscript{116}, intramuscularly injected AAV encoding siRNA against SOD1 increased motor neuron survival, elongated life span, and improved motor function in SOD1 G93A mice\textsuperscript{104, 105, 116}. This demonstrates that the silencing of mutant SOD1 in muscle and/or spinal cord cells is beneficial to block disease progress. Furthermore, this suggests that muscle may be an initiation site of disease. siRNA was delivered into the muscle to investigated whether the reduction of mutant SOD1 exclusively within muscle can reverse pathogenesis in motor neurons. Small interfering RNA (siRNA) can promote degradation of specific messenger RNA (mRNA) and further reduce protein expression\textsuperscript{92}. Thus, this procedure has been proposed as a viable therapy for ALS in which the accumulation of mutant SOD1 drives motor neuron death in a dosage dependent manner. A potent 19-nucleotide siRNA was designed to target the region containing nucleotides 270-290 of the human SOD1 coding sequence. To test the efficacy of the siRNA approach, we constructed AAV-2 viral vectors expressing a transcribable siRNA against human SOD1
mRNA, which also encodes GFP in order to monitor viral transduction (Figure 10). To evaluate the specificity and efficiency of siRNA against mutant SOD1, HEK 293 cells were infected with the siRNA vector. The siRNA vector decreased SOD1 protein levels by at least 90%, whereas a 2bp missense siRNA did not\textsuperscript{105}. For in vivo study, we utilized two different virus vectors depending on their trophism. Lentivirus pseudotyped Vesicular Stomatis Virus Glycoprotein (VSV-G) were intramuscularly injected to produce siRNA exclusively within the muscles since they are lack of retrograde transport capacity\textsuperscript{113} (Figure 15). Adeno associated virus (AAV2) was administrated into hindlimb muscles to eliminate mutant SOD1 in both the muscle and the motor neurons of the spinal cord via retrograde transport\textsuperscript{110} (Figure 15). If the toxicity of mutant SOD1 solely comes from the muscle group, both lentivirus mediated siRNA and AAV2 mediated siRNA can reverse the pathology of ALS. If the muscle is not actively involved in pathogenesis, lentivirus delivered siRNA will not significantly protect motor neurons compared to AAV2 mediated siRNA. We injected the lower part of the hindlimb on one side of a cohort of 7-week-old SOD1G93A mutant mice with AAV2-siRNA-GFP or lentivirus-siRNA-GFP. Because only one side of the hindlimb was used for injection, the other side of the hindlimb served as a negative injection control. The behavioral effect of siRNA in G93A SOD1 was investigated following the verification of siRNA delivery. By densitometric scanning relative to the β-actin standard, Western analysis in extracts of gastrocnemius demonstrated approximately 60% reduction in mutant SOD1
expression following injection of either lentivirus-siRNA or AAV2-siRNA (n=10 each) compared to ALS mice at the age of 100 days (Figure 16). This suggests that either AAV2 or lentivirus delivered siRNA produced a long-lasting and substantial reduction of mutant SOD1 protein until the late stage of the disease. Since both viruses also express GFP, GFP expression in injected muscle was used to verify the equivalent delivery of siRNA by the two different viruses. The expression pattern of GFP verified that both viruses were comparably distributed throughout the entire injected muscles. Immunoblotting for GFP demonstrated that the same amount of siRNA was delivered by the two different virus constructs (Figure 17). Taken together, AAV and lentivirus delivered the equivalent siRNA into the muscle and suppressed mutant SOD1 expression at comparable levels. Fifty percent reduction of mutant SOD1 expression successfully rescued motor neuron dysfunction \(^{104}\). In the present study, animals were subjected to a grip strength test to monitor hindlimb muscle strength that would be preserved by siRNA silencing mutant SOD1 (Figure 18). AAV-siRNA treated animals remarkably maintained muscle strength between 75 and 95 days and did not present any sign of unameliorated motor strength compared to the untreated SOD1G93A animals, which lost grip strength (Figure 19). This demonstrated the efficacy of AAV2-siRNA on the delay of disease onset in ALS mutant mice. Despite comparable suppression within the muscle, lentivirus-siRNA treated mice developed progressive physical weakness and dramatically deteriorated grip strength at 70 days even though they had increased muscle
strength transiently between 50 days and 70 days (Figure 19). They behaved just like uninjected SOD1<sup>G93A</sup> littermates, and the therapeutic effects were limited in this group. Consistently, the measurement of the wet weight of injected muscle showed that AAV-siRNA injected animals presented less muscle loss compared to those injected with lentivirus-siRNA at 98 days of age (Figure 20).

In sum, even though mutant SOD1 expression was silenced prior to disease onset, the silencing of mutant SOD1 within the muscle failed to preserve muscle integrity. In contrast, the reduction of mutant SOD1 expression in both the motor neurons and the muscle preserved muscle integrity and maintained motor function. These results suggest that the toxicity of mutant SOD1 may be derived from motor neurons or adjacent non-neuronal cells within spinal cord. Further, both neuromuscular dysfunction and muscle atrophy may be a secondary effect of toxic mutant SOD1 within the spinal cord. This further suggests that the muscle is not the initiation site of ALS pathogenesis.

**Reducing mutant SOD1<sup>G37R</sup> within the muscle by selective mutant gene excision does not affect disease onset or survival**

Although the lentivirus expressing siRNA were efficiently transduced and significantly reduced mutant SOD1 in the muscle, the onset of motor neuron disease was not affected and the motor function was not preserved. Because the hindlimb muscle is the most severely damaged in ALS, we targeted this muscle. However, the toxicity of mutant SOD1 in other muscles might develop the
disease. Indeed, although siRNA efficiently reduced mutant SOD1 expression, it is unlikely that every single muscle cell was transduced by virus. Therefore, to investigate the contribution of mutant SOD1 within all skeletal muscle to disease initiation and progression, we conditionally inactivated mutant SOD1 within the skeletal muscle using Lox and Cre-loxP mediated recombination. Transgenic mice carrying LoxSOD1\textsuperscript{G37R} were made in which the human mutant SOD1\textsuperscript{G37R} gene was flanked at both ends by 34 base pairs of LoxP sequences. To determine the effect of the Lox sequence on transgenic mice carrying mutant SOD1\textsuperscript{G37R}, the pathology of the mice was monitored. As expected, they showed progressive weight loss, motor function deterioration, and eventually paralysis. Overall, the phenotype was indistinguishable from that of the SOD1\textsuperscript{G37R} transgenic mice. To diminish mutant SOD1 within skeletal muscle, heterozygous LoxSOD1\textsuperscript{G37R} mice was mated to heterozygous MCK-Cre mice (Figure 21). To this end, the muscle creatine kinase (MCK) promoter was chosen. MCK is exclusively expressed within skeletal muscle\textsuperscript{117}, and the expression of MCK begins at embryonic day 17 and remains constant throughout the rest of life\textsuperscript{117}. Genomic DNA was extracted from quadriceps femoris and gastrocnemius muscles for quantitative PCR analysis to determine the Cre-dependent reduction of mutant SOD1. The quantitative PCR analysis showed that the mutant SOD1 expression in LoxSOD\textsuperscript{G37R}/MCK-Cre+ mice (n=3-5) was reduced up to 25% compared to that of littermate LoxSOD\textsuperscript{G37R} mice (n=3) that did not express Cre recombinase (Figure 22). A previous study demonstrated that the equivalent
level of inactivation of mutant SOD1 within microglia impressively slowed down the late progression of disease by an average of up to 99 days\textsuperscript{107}. Furthermore, the reduction of mutant SOD1 within motor neurons slowed disease onset by an average delay of 18 days, and the early progression of disease was significantly retarded with a mean extension of 31 days\textsuperscript{107}. In contrast to these impressive delays, the later disease development was modestly slowed with a mean extension of 15 days. In the present study, to investigate the therapeutic benefit of limiting mutant SOD1 damage in skeletal muscle, the disease onset as well as the life span was monitored. For these analyses, the littermates were used as a negative control. In contrast to the results of reduced mutant SOD1 expression within microglia or motor neurons, diminishing mutant SOD1 expression within the muscle did not delay the disease onset (Cre-: 298 ± 20 days, Cre+: 285 ± 16 day, P value 0.12) (Figure 24). For this study, the disease onset was defined as the time when mice reached peak body weight as previously described\textsuperscript{107}. When mice passed this peak point, they started to lose their body weight. This weight loss was closely correlated to the initiation of motor performance dysfunction. Mice usually lost 10\% of maximal body weight at the initial stage of the disease. Consistently, the survival was not affected as well (Cre-: 376 ± 13 days, Cre+: 384 ± 11 days, p value 0.12) (Figure 25). This demonstrates that even though mutant SOD1 was efficiently removed in vivo as early as embryonic stage, the progression of the disease was not changed at any stage.
Finally these results underscore that the toxicity of mutant SOD1 within muscle
does not substantially contribute to the disease development while the damage
of motor neurons and microglia is associated with the disease at different phases.

**Follistatin-induced muscle enhancement is not protective in SOD1 mutant-
mediated ALS**

Independently, we evaluated the contribution of the muscle to disease
progress as well as the therapeutic benefits of muscle enhancement. Without
reference to the initiation of mutant SOD1 toxicity within the muscle, if muscle
atrophy substantially promotes the pathological progression of the disease, the
preservation of muscle integrity would halt disease onset and slow disease
development including, the motor functional decline. When we consider the
critical role of the muscle as a supplier of various neurotrophic factors, the
prevention and/or rescue of muscle atrophy may have important implications for
the development of successful therapies. Follistatin, a negative regulator of
myostatin, is not associated with any salient effects on motor neurons while it
enhanced muscle mass and dramatically improved the muscle phenotype
atrophy in mouse models of muscular skeletal disorders including muscular
dystrophy\(^{118, 119}\). Previously, AAV1 was chosen since it showed the capability of
both transducing skeletal muscle fibers efficiently and achieving stable
expression. An AAV serotype I vector expressing human Follistatin-344 under
cytomegalovirus (CMV) promoter was constructed (Figure 26). First, we
investigated the stable and constitutive expression of Follistatin in HEK 293 cells
(Figure 27). Later, the capacity of follistatin to antagonize the myostatin-dependent inhibition of myoblast proliferation was examined. Proliferating C2C12 cells were arrested with myostatin. *In vitro*, we tested the ability of follistatin to reverse the inhibition of muscle cell proliferation. We infected HEK 293 cells with AAV-Red Fluorescence Protein (RFP) or AAV-Follistatin (FS) to produce conditional media. AAV-RFP was used as a negative control. Collected conditional media was added into myostatin-treated C2C12 cells. We tested whether Follistatin (FS) was capable of releasing the arrested proliferation of C2C12 cells following the infection. A MTT assay was performed to measure myoblast proliferation. Briefly, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium tetrazolium bromide) is cleaved by active mitochondrial reductase present in live cells, changing the color from a pale yellow to a dark blue\(^ {120} \). Myostatin was reconstituted in PBS (pH 7.4). PBS, AAV-RFP, or AAV-FS alone did not have any effect on myoblast proliferation. However, myoblasts cultured with myostatin significantly decreased proliferation. Follistatin-conditioned media reversed the inhibition of proliferation by myostatin, whereas control RFP-conditioned media failed to release arrested proliferation. This demonstrates that HEK 293 cells transduced with AAV-FS efficiently produce biologically active Follistatin (Figure 28). Next, to assess our hypothesis that rescued muscle atrophy may delay motor neuron dysfunction and extend lifespan, we utilized AAV1 virus to deliver Follistatin into ALS mice. The ALS transgenic mice developed severely deteriorated motor neurons that were accompanied by the
denervation of neuromuscular junctions and muscle atrophy\textsuperscript{17}. Follistatin has been known as an antagonist of myostatin, which is a negative regulator of muscle mass via TGF-β signaling\textsuperscript{43}. Previous study demonstrated myostatin arrests muscle cell proliferation by binding to the BMP receptor\textsuperscript{43}. However, follistatin can recover this arrest by binding to the BMP receptor or Myostatin \textsuperscript{41}. 

AAV1-Follistatin was injected bilaterally via intramuscular delivery into the hindlimb quadriceps and tibialis anterior muscles of sixteen 40-day-old animals (equal distribution of female and male) at the dose of \(1 \times 10^{11}\) viral genome per injection. Additionally, the AAV1-GFP virus was administrated as a negative control that does not have any effect on muscle mass enhancement. Both AAV1-FS treated mice and AAV1-GFP treated ones reached the similar end stage; 130 days versus 126 days, \(p< 0.05\), Chi Square 3.504. Even though AAV1-Follistatin (AAV1-FS) did not significantly extend mice lifespan compared to AAV1-GFP, gross muscle mass showed a great discrepancy between the two groups (Figure 29). Only mice receiving AAV1-FS showed a gross muscle mass increase compared to the AAV1-GFP treated animals under visual inspection. Consistently, the wet weight of multiple muscles (n=10-15 animal each), including the tibialis anterior, gastrocnemius, medial quadriceps, and triceps muscle (Figure 30) was significantly increased (\(p<0.05\)) after injection of AAV1-FS (Figure 31). Muscle mass increase was not limited to the injection site, but remote muscle also displayed a distinguishable muscle mass increase, suggesting that AAV1-FS affected various muscle groups in an endocrine
manner as well as in an autocrine manner. The ELISA assay specific for human Follistatin was performed to measure the level of circulating Follistatin in the blood stream. For this, whole serum was collected from the injected animals, both AAV-Follistatin-treated animals and the AAV-GFP-treated animal, at 100 days of age. The Elisa assay illustrated that the expression levels of circulating human Follistatin in ALS mice receiving AAV1-FS were 12-15 fold higher (10 ng/ml) than those receiving AAV1-GFP (Figure 32). This assay showed virally delivered Follistatin was efficiently expressed, secreted, and circulated in the whole body leading to muscle mass enhancement. To determine whether the muscle mass increase came from hyperplasia or hypertrophy and/or muscle sparing in the ALS animals, The myofiber number in the injected gastrocnemius muscle group was counted in the serial 4 sections of AAV1-Follistatin- or AAV-GFP- treated animals (n=8 animals) at the end stage, the age of 126-7 days. Photographs from three different areas were taken to determine the average muscle fiber number and the diameter. Compared to GFP-treated animals, which were undistinguishable from ALS mice, The Follistatin-treated animals presented a noticeable increase in muscle fiber number (Figure 33). In addition, they showed a moderate increase in muscle fiber diameter, displaying a slight shift toward larger myofiber diameter. The diameters of muscle fiber from quadriceps and gastrocnemius increased up to 21% and 13%, respectively (Figure 34 & 35). These results indicate that muscle mass increase resulted mainly from hyperplasia. Taken together, Follistatin efficiently prevented muscle atrophy by
inducing muscle mass increase. Next, we tested the effect of muscle enhancement on its function. It is possible that muscle mass increase may not rescue muscle function. To determine this, we conducted grip strength tests of the forelimb and hindlimb and rotarod tests. While wild-type mice normally continued to gain their muscle strength at the age between 50 and 65 days, ALS mice typically failed to increase their muscle strength during the same period, began to decline at the age of 80 days, and died at 131 days.

Overall, Follistatin-treated animals were comparable to wild-type animals during the early stage of disease. They continued to gain strength by 75 days of age. Follistatin-treated animals showed a 40% increase in forelimb strength compared with GFP-treated littermates (Figure 36) and then dramatically declined. Additionally, their hindlimbs started to gain strength from 65 days of age (33%) and continued till the end-stage (Figure 37). The Rotarod test was conducted to check muscle coordination. When the mice are placed on the machine, they need to coordinate different muscle groups such as the forelimb and hindlimb at the same time. Otherwise, they will fall down. Despite the maintenance of muscle strength and increased muscle mass even through the end stage, the latency to fall was not distinguishable between GFP-treated group and follistatin-treated group (Figure 38). This implies that even though follistatin substantially attenuated muscle atrophy even after the onset of the denervation and paralysis stage, muscle function was not completely rescued.
In conclusion, even though until the end-stage virally delivered Follistatin produced sustained increase of whole muscle mass and preserved muscle integrity, the functional improvement was limited and the extension of life span was not statistically significant.
Figure 15 Viral delivery of mutant SOD1 siRNA
Figure 15 Viral delivery of mutant SOD1 siRNA

Genes encoded in adeno-associated virus (AAV) were expressed in muscle and in motor neuron via retrograde transport while genes encoded in lentivirus were expressed only in muscle. Either AAV or lentivirus expressing siRNA against mutant SOD1 was injected into one hindlimb of SOD1G93A mice at 40 days of age. siRNA construct was identical in each case.
Figure 16 Silencing of mutant SOD1
Western blot analysis showed that either AAV or lentivirus delivered siRNA reduced mutant SOD1 expression up to 60% compared to untreated mice. Immunoblot analysis was performed at the end stage of disease.
Because both AAV and lentivirus express GFP, immunoblotting for GFP represents the equivalent amount of siRNA delivery by two different viruses. GFP expression in the injected hindlimbs is comparable between two groups at 100 days of age.
The function of muscles was evaluated by grip strength and rotarod test. Grip strength of forelimb and hindlimb was measured weekly. Animals were allowed to grasp a platform with one hindlimb and pulled till it released the platform. Rotarod test was performed weekly. Mice were placed on the rotarod at 5rpm/min. The time each mouse remained on the rod was registered.
Figure 19 Grip strength test of hindlimb

AAV delivered siRNA enhanced muscle strength of hindlimb in SOD1G93A mice substantially even though the end stage of disease. Despite comparable suppression of mutant SOD1 within muscle, lentivirus delivered siRNA maintained muscle strength only transiently.
Figure 20 Wet weight of injected various muscles at end stage disease (98 days) AAV-siRNA injected animals presented less muscle mass loss compared to lentivirus siRNA injected mice at the end stage disease.
Figure 21 MCK/Cre/LoxP mediated mutant SOD1 excision within skeletal muscles
Figure 21 MCK/Cr/LoxP mediated mutant SOD1 excision within skeletal muscles
Mice heterozygous for mutant SOD1G93R that is flanked at both ends by LoxP site were mated to mice expressing Cre recombinase under MCK promoter. Cre-mediated recombined mouse efficiently excised mutant SOD1 expression exclusively within skeletal muscles.
Figure 22 Q-PCR analysis of systematically downregulated mutant SOD1 expression from skeletal muscle
Quantitative PCR showed that LoxSOD1<sup>G93R</sup> gene level was significantly reduced in quadriceps femoris and gastrocnemius muscle of Lox SOD1<sup>G93R</sup>/MCK-Cre mice (n=3-5, Cre+) compared to Lox SODG<sup>G93R</sup> mice (n=3, Cre-). Bars represent mean and standard deviation. P < 0.05 unpaired t-test. (A) Cre-: 100±37% (n=3), Cre+: 76±17% (n=5), (B) Cre-: 100±27% (n=3), Cre+: 79±20% (n=3)
Western blot analysis showed that LoxSOD1\textsuperscript{G93R} gene expression level in Lox SOD1\textsuperscript{G93R}/MCK-Cre mice was reduced up to 30% compared to that in Lox SODG\textsuperscript{G93R} mice.
Figure 24 The disease onset of LoxSOD1G93A/MCK-Cre mice (Cre+) and littermate LoxSODG93A (Cre-) mice. The reduction of mutated SOD1 expression within skeletal muscle had no effect on disease onset compared to the littermate. Cre+: 298±16 days (p=0.12, unpaired test) Days indicated mean and standard deviation.
Figure 25 Survival graph of LoxSOD1G93A/MCK-Cre mice (Cre+) and littermate LoxSODG93A (Cre-) mice. 
Systemic downregulation of mutant SOD1 from skeletal muscle did not extend the survival compared to the littermates, LoxSOD1G37R/Cre-, Cre-:376 ± 13 days, Cre+:384 ± 11 days, p value 0.12 (unpaired test).
Human Follistatin was cloned into AAV viral vector under CMV promoter control. AAV was packaged into capsid serotype 1 along with the adenoviral helper plasmid.
Figure 27 Western analysis of secreted Follistatin

Immunoblotting analysis presented the secretion of Follistatin from C2C12 cells. C2C12 cells were infected with AAV1 expressing Follistatin under CMV promoter control.
Figure 28 Proliferation assay of myoblasts (C2C12)
Myoblasts were cultured in growth media in the presence or absence of myostatin (3 ug/ml) mixed with conditioned media from AAV transduced cells expressing red fluorescent protein (RFP), Follistatin (FS) or Follistatin related gene (FLRG). (N=4 average ± SE) The proliferation of C2C12 cells was arrested by myostatin. Either FS or FLRG was able to reverse the inhibition but RFP was not. PBS, AAV-RFP or AAV-FS alone didn’t affect on myoblast proliferation.
Figure 29 Gross increase of muscle mass at age 126 days

AAV1 Follistatin was injected bilaterally via intramuscular delivery into the hindlimb quadriceps and tibialis anterior muscles prior to disease onset at the dose of $1 \times 10^{11}$ vp. AAV1-GFP was used for a negative control. Only mice receiving AAV1-FS showed a gross muscle mass increase compared to AAV1-GFP treated animals under visual inspection.
Either AAV-FS or AAV-GFP injected 3 different muscles including Tibialis, Gastrocnemius, Quadriceps, were inspected for histopathological analysis. Additionally, one uninjected muscle, Triceps, was analyzed.

Figure 30 Various muscles used for histopathological analysis
Wet weights of tibialis anterior, gastrocnemius, medial quadriceps, and triceps muscle from AAV-Follistatin and AAV-GFP animals at 126 days of age (N=10-15 per group, average ± SE). Viral delivered Follistatin significantly increased not only injected muscles but also uninjected muscle suggesting AAV-Follistatin affects various muscle groups in endocrine manner as well as in autocrine manner.

Figure 31 Wet weight of various muscles
The expression level of circulating human Follistatin in AAV1-FS injected ALS mice was 12-15 fold higher (10ug/ml) than those in AAV1-GFP injected ALS mice at age 100 days.

Figure 32 ELISA assay for Follistatin at end-stage disease
Myofibers were counted in serial sections of gastrocnemius and quadriceps muscle from AAV-Follistatin and AAV-GFP treated SOD1G93A mice at end stage (126 days). (N=8 per group, average ± SE) Compared to GFP-treated animals, Follistatin treated animals presented a noticeable increase in muscle fiber number indicating that hyperplasia was induced.

Figure 33 The analysis of myofiber number
Figure 34 The fiber diameter of quadriceps

Diameter of the Quadriceps muscle in AAV-Follistatin and AAV-GFP treated SOD1G93A mice at end stage disease (126 days). (N=8 G group, average ± SE). The diameter of muscle fiber increased up to 21% compared to control.
AAV-Follistatin and AAV-GFP treated SOD1G93A mice at end stage of disease (126 days). (N=8 per group, average ± SE) The diameters of muscle fiber from gastrocnemius increased up to 13%.

Figure 35 The fiber diameter of gastrocnemius

Diameters of muscle fibers form gastrocnemius muscle in AAV-Follistatin and AAV-GFP treated SOD1G93A mice at end stage of disease (126 days). (N=8 per group, average ± SE) The diameters of muscle fiber from gastrocnemius increased up to 13%. 
Figure 36 Forelimb Grip strength test
Grip strength of hindlimbs were monitored in AAV-Follistatin and AAV-GFP treated SODG93A mice. Follistatin treated mice started to gain strength form 65 days of age (33%), and continued till the den-stage.
Figure 37 Hindlimb Grip Strength test
Grip strength of hindlimb was recorded in AAV-GFP and AAV-Follistatin-treated SOD1G93A mice. Follistatin treated animals continued to gain muscle strength by 75 days of age, and showed a 40% increase in hindlimb strength compared with GFP-treated littermates.
Figure 38 Rota-rod test
Latency to fall off the rotarod was measured in AAV-follistatin vs. AAV-GFP treated SOD1G93A mice. The latency to fall off was not distinguishable between follistatin treated and GFP treated group suggesting that muscle function was not completely rescued.
Survival analysis of AAV-Follistatin and AAV-GFP treated SOD1G93A mice. AAV-GFP 126 days, AAV-Follistatin 130, p value 0.06. (N=15, litter matched, female=male mice) Time to end-stage was measured using the defined end point of failure to right itself within 30 seconds. The extension of lifespan was not statistically significant suggesting that myostatin inhibition by follistatin could not delay end-stage disease.
CHAPTER 4

DISCUSSION

An understanding of the role of the muscle in ALS has practical implications for developing optimal therapeutics and understanding cellular phenotypic involvement in this devastating disease. Pathologically, motor neurons perish distally. Initially, the lower region of motor neurons starts to die and moves toward upper region, such as the cell body. The denervation of neuromuscular junction precedes motor neuron loss, suggesting that skeletal muscle might have an active role in motor neuron pathogenesis. Supporting this, the delivery of AAV2 mediated IGF-1 preserved neuromuscular junctions and dramatically extended the life span in ALS mouse model via retrograde transport. Because AAV2 was injected intramuscularly, some of virally delivered IGF-1 may have locally resided muscles and protected them. Furthermore, transgenic mice expressing IGF-1 exclusively within the muscle in SOD1G93A background substantially delayed the disease progression and enhanced the survival by attenuating muscle wasting and promoting regenerative pathway. Indeed, IGF-1 maintained the integrity of neuromuscular junction configuration and protected
motor neurons from degeneration. This study suggested that the muscle may have a role in motor neuron disease. However, IGF-1 expressed within muscle could also protect adjacent motor neurons, and IGF-1 has been known to provide neuroprotective benefits. Thus, it is difficult to delineate the role of mutant SOD1 toxicity within the muscle. Besides the neurotrophic factor delivery, siRNA was delivered into motor neurons and the muscle to block the toxicity of mutant SOD1, which is genetically linked to 20% of familial ALS. siRNA approaches have shown great promise in ALS in terms of motor neuron survival, motor neuron function recovery, and extended survival rate\textsuperscript{104,106}. Previous studies using lentiviruses reduced almost 50% of mutant SOD1 expression, which substantially retarded both the onset and the progression rate of the disease\textsuperscript{106}. Since the neurotrophic factor and siRNA delivery showed dual beneficial effects on both muscle and neurons, it is very hard to delineate where the beneficial effects were derived from. In the present study, we evaluated whether reducing the mutant SOD1 expression exclusively within the muscle can delay disease progress. siRNA against mutant SOD1 was delivered solely into muscle or both in the muscle and motor neurons. Both siRNA deliveries decreased mutant SOD1 expression up to 60%. The substantial reduction of mutant SOD1 expression solely within the muscle failed to delay disease progression in contrast to the previous studies of silencing mutant SOD1 within various cell types\textsuperscript{107}. This result suggests that muscle damage is not the primary cause of the disease. However, siRNA was delivered into the hindlimbs. To eliminate mutant
SOD1 within all muscle cells, we utilized the Cre-Lox system. To this end, Cre recombinase was placed under MCK promoter control. The expression of MCK is limited to most skeletal muscles, initiated as early as at the embryonic stage, and maintained throughout the rest of life. The heterozygous MCK-Cre mice were mated to the heterozygous LoxSOD1\textsuperscript{G37R} mice, which express the human mutant SOD1\textsuperscript{G37R} gene flanked at both ends by 34 base pairs of LoxP sequences. Mutant SOD1 expression was efficiently reduced up to 25% compared to the littermates that did not express Cre recombinase. While the previous study showed that the equivalent reduction within microglia substantially extended their survival, the excision of mutant SOD1 within the muscles neither delayed the disease onset nor extended survival. This clearly demonstrates that the toxicity of mutant SOD1 within muscle is not a major contributor to motor neuron pathology, and the muscle may be a secondary target of mutant SOD1. Therefore, the exclusive reduction of mutant SOD1 expression solely in the muscle is not an ideal therapeutic approach. Recently, myostatin, a negative regulator of muscle growth, was inhibited to slow muscle atrophy by administrating a neutralizing antibody into the rodent model of ALS\textsuperscript{121}. Muscle mass increase and functional rescue was limited only to the early stage, however it was not maintained through the late stage disease. Both disease onset and long-term survival were not affected, although the diaphragm was moderately maintained through the end stage of disease. In addition, the short halflife of antibody may result in transient beneficial effects. Therefore, we independently evaluated whether the protection
of the muscle is pathologically significant in ALS. To this end, we harnessed viral
delivery system to express a secreted form of Follistatin, a muscle-enhancing
gene, stably. Follistatin has been known to efficiently inhibit myostatin, enhancing
muscle integrity and improving muscle function \(^{41}\). In the rodent model of inherited
ALS, Follistatin was virally delivered prior to disease onset and secreted into the
blood stream even through the late stage of disease. Circulating Follistatin
significantly enhanced muscle mass in various muscle groups distally through
end stage disease. Even though circulating Follistatin preserved muscle strength
at the early stage of disease (55-80 days after birth), grip strength tests showed
that muscle strength declined later on and the functional coordination of different
muscle groups was only moderately improved at the late stage. Additionally, life
span was not significantly extended. This suggests that muscle mass
enhancement does not delay disease progression even though the preserved
muscle is capable of supplying sufficient trophic factors into motor neurons. This
also implies that the muscles may be deteriorated by toxicity from other cell types
within the spinal cord. In sum, the present study clearly showed that siRNA
against mutant SOD1 delivered exclusively within the muscle did not have any
salient effects on disease onset and progression. It suggests that muscle is not
the primary target of mutant SOD1. Indeed, even though muscle mass was
preserved prior to disease onset,
ALS pathology was alleviated only at the early stage, but not delayed through the late stage. Thus, to prevent muscle atrophy, the toxicity of mutant SOD1 should be diminished within other cell types in spinal cord.

Previously, the therapeutic effects of exercise or IGF-1 expression within skeletal muscles implicated the muscle in motor neuron disease. However, growing evidence suggests that the effects of exercise may be more global not limited to the muscle. The benefits of exercise may be derived from the induction of anti-apoptotic gene expression, recruitment of motor units, increase of neurotrophic factor expression and secretion, and improvement of motor function rather than the increase of muscle mass. Despite all of these benefits, the effects of exercise are still controversial since the adverse effects of strenuous exercise have been also reported before\textsuperscript{122}. Thus, exercise may be a potentially therapeutic approach when the ideal duration and intensity of exercise are determined. Additionally, when we consider that IGF-1 is a crucial neurotrophic factor as well as myotrophic factor, the improved motor function and extended lifespan was more likely to originate from the protection of motor neurons, rather than from the muscle, in IGF-1 treated mice.

Even though the muscle is not a direct target of gene therapy, it is a suitable docking site for retrogradely transported virus to transduce therapeutic genes chronically to motor neurons with high efficiency. Perishing motor neuron population in CNS is difficult to target and the direct injection into the spinal cord may significantly increase the probability of paralysis from the trauma of multiple
injections. Therefore, compared to direct spinal cord injection, the muscle is an accessible and suitable target. Additionally, when several neurotrophic factors were administrated systematically, their therapeutic benefits were very limited because of short bioavailability, poor access to target site, and toxicity. However, when IGF-1 was delivered intramuscularly, it reached motor neurons steadily via retrograde transport, preserved motor neurons, and sustained life in ALS mice. Therefore, this study revealed the exciting possibility that skeletal muscle may be an ideal docking site for gene therapy.

Growing evidence suggests that multiple cell types within the spinal cord may be associated with ALS pathology. For a successful gene therapy, the contribution to damage of mutant SOD1 within a specific cell type should be identified. Transgenic mice carrying mutant SOD1 G37R flanked by LoxP sequences were crossed with the other transgenic mice expressing cell type specific Cre recombinase 107. Diminishing mutant SOD1 expression within motor neuron significantly delayed disease onset and the early progression of disease, while lowering mutant SOD1 expression within microglia substantially extended survival during the late phase. This implies that different mechanisms of toxic mutant SOD1 may control disease initiation and disease progression. Thus, the contribution of each cell type to the disease may be different. The initiation of the disease may be ascribed to damage to motor neurons and possibly to other cell types.
The inflammatory response including microglia and astrocyte activation may exacerbate the symptoms during the late phase after onset. Therefore, the individual cell type should be differentially targeted depending on the phase of ALS progression.

Finally, this study demonstrated that the primary toxicity of mutant SOD1 is not localized within the muscle, but developed within the spinal cord. Neither diminishing mutant SOD1 expression in muscle nor enhancing muscle mass was sufficient to attenuate the disease progression even though gene transfer was initiated prior to disease onset and lasted through the late stage of disease. However, when the inaccessibility of CNS is considered, skeletal muscle is an ideal site, in which a retrogradely transporting viral vector can be safely administrated.
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