ABSTRACT

Thesis Defense Title: **ALTERATIONS IN THE MYOGENIC CAPACITY OF SATELLITE CELLS IN A MOUSE MODEL OF ALS**

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Degree: Masters of Arts, 2012

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Amyotrophic lateral sclerosis (ALS), also known as Lou Gehrig's disease, is a devastating neurodegenerative disease that results in pervasive muscle wasting, paralysis, and ultimately death. Recent research efforts have been made to characterize skeletal muscle in the disease, with some evidence suggesting that the tissue may contribute to ALS pathogenesis. Therefore this study was undertaken to continue to describe ALS skeletal muscle, specifically a population of skeletal muscle-specific stem cells known as *satellite cells* that play a role in regeneration following injury. Satellite cells were isolated and cultured from mutant mice (SOD1 G93A) that recapitulate the disease, assessed for the capacity to differentiate and proliferate, and compared to age-matched control cultures. SOD1 G93A cultures exhibited decreased expression of transcription factors associated with differentiation (i.e. MyoD and myogenin) compared to control cultures, as well as a reduced ability to proliferate *in vitro*. These results indicate that the satellite cell population in a mouse model of ALS displays dysfunctional myogenic capacity *in vitro*, and thus may contribute to the atrophic pathology seen in the disease.

ALTERATIONS IN THE MYOGENIC CAPACITY OF SATELLITE CELLS IN A MOUSE MODEL OF ALS

By

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Thesis submitted to the Faculty of the GraduateSchool of the
University of Maryland, College Park, in partial fulfillment
of the requirements for the degree of
Master of Arts
2012

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Acknowledgements

It was only with the support of many family, friends, and colleagues that this thesis work was possible. I am particularly thankful to my advisor, Dr. Eva Chin, without whom I would have never embarked down the road of graduate study. I owe additional gratitude to my fellow lab members, Davi Mázala and Dapeng Chen, for their technical assistance and thoughtful contributions to the development of this project.

I would also like to thank the rest of my advisory committee, Drs. Espen Spangenburg and Steve Roth, for helping guide both this project and my career as a master's student. Further I am grateful for the generous use of lab space and equipment offered by Dr. Spangenburg and his lab members. I am particularly thankful to Dr. Lindsay Wohlers for her assistance in plate reading and imaging tutorials.

Lastly, I would like to thank my parents for their unyielding support of my academic pursuits. It is only with the support of all who helped with this project that this work was possible.

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List of Abbreviations

AchR – Aceytlcholine Receptor

ALS – Amyotrophic Lateral Sclerosis

BAK – Bcl-2 homologous antagonist killer

BAX – Bcl-2 Associated X Protein

bHLH – basic Helix-Loop-Helix

BrdU – 5-bromo-2-deoxyuridine

Ca^{2+} – Calcium

CARF – Central Animal Resources Facility

Cdk5 – Cyclin Dependent Kinase 5

ChIP – Chromatin Immunoprecipitation

CHOP – C/EBP Homology Protein

CSA – Cross Sectional Area

CSF – Cerebrospinal Fluid

DMEM – Dulbecco's Modified Eagle Medium

DNA – Deoxyribose Nucleic Acid

ECM – Extracellular Matrix

EMG – Electromyography

ER – Endoplasmic Reticulum

fALS – Familial Amyotrophic Lateral Sclerosis

FDB – Flexor Digitorum Brevis

FGF – Fibroblast Growth Factor

FTD – Fronto-temporal Dementia

GPx4 – Glutathione Peroxidase

HGF – Hepatocyte Growth Factor

HSP – Heat Shock Protein

IACUC - Institutional Animal Care and Use Committee

IGF-1 – Insulin-like Growth Factor-1

IL-4; IL-4Rα – Interleukin-4

IL-4-Rα – Interleukin-4 receptor alpha

L-NAME – N^G-nitro-L-arginine methyl ester

MEF-2 – Myocyte Enhancer Factor-2

MEM – Minimal Essential Medium

MHC – Myosin Heavy Chain

MRF – Myogenic Regulatory Factor

MRI – Magnetic Resonance Imaging

OMM – Outer Mitochondrial Membrane

mRNA – messenger Ribonucleic Acid

mTOR – Mammalian Target of Rapamycin

NFL – Neurofilament Light Chain

NMJ – Neuromuscular Junction

NO – Nitric Oxide

NOS – Nitric Oxide Synthase

PTP – Permeability Transition pore

PUT-CAT – Putricine-modifed Catalase

RNA – Ribonucleic Acid

sALS – Sporadic Amyotrophic Lateral Sclerosis

SDF-1α – Stromal derived Factor 1-alpha

Sema3a – Semaphorin 3a

SOD – Superoxide Dismutase

SPSS – Statistical Package for the Social Sciences

TDP-43 – Tar-Binding Protein 43

TGF-β – Transforming Growth Factor-beta

UCP-1 – Uncoupling Protein 1

UPR – Unfolded Protein Response

VDAC1 – Voltage Dependent Anion Channel 1

Chapter 1: *Introduction*

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is a progressive neurodegenerative disease that invariably leads to death in those it affects (Patel & Hamadeh, 2008). Nearly 6000 people are diagnosed with ALS per year in the United States alone, typically surviving from 2-5 years after the onset of the disease (Martin et al. 2009). The pathology of ALS principally results in motor neuron death, muscle weakness, atrophy, paralysis, and ultimately respiratory failure. Therefore the pervasive nature of the disease is manifest as loss of independence, decline in functionality, and overall decrease in the quality of life in ALS patients.

Though they present identical symptomologies, ALS can be broadly categorized into familial and sporadic subtypes (Turner & Talbot, 2008). The familial form of ALS comprises between 10-15% of the total diagnosed cases of the disease, whereby a genetic component accounts for the inheritance of the disorder. A larger proportion of the clinical ALS population – 85-90% of those affected – are diagnosed with sporadic ALS; these cases are characterized by presentation of symptoms with no family history of the disease. The etiology of the sporadic form of the disorder is not currently understood. These circumstances have thus contributed to the current treatment avenue for ALS patients: the majority of diagnoses are made without warning, leaving those affected with no curative option. The drug therapy approved for ALS, Riluzole, only prolongs life by 2-3 months (Boilee et al. 2006). Thus significant efforts must be made on the part of researchers to further understand the hallmarks of the disease prior to overt symptom presentation, and eventually make headway for effective therapeutic solutions for ALS.

Research during the past two decades has significantly advanced our current understanding of ALS. The identification of genetic mutations in the antioxidant enzyme Superoxide Dismutase (SOD_1), common to 20% of familial ALS cases, has allowed for immeasurable gains to current knowledge of the disease (Rosen et al. 1993). Exploitation of this finding only one year later yielded the discovery of a mutant mouse model that effectively mimics the human form of the disease (Gurney et al. 1994). This model has provided researchers a unique platform for studying ALS. Gurney et al. (1994) were the first to produce mice that have been genetically engineered to express a mutant form of SOD_1 with one of the most common mutations, a glycine to alanine substitution at the 93^{rd} codon (SOD1 G93A). These SOD1 G93A mutant mice develop motor neuron degeneration and exhibit the same drastic motor defects seen in the human disease. Therefore this progress has allowed for subsequent examination of pathophysiology and underlying disease mechanisms that was never before possible in ALS research.

Research using this animal model has provided a newfound understanding of how various molecular, cellular, and tissue-specific mechanisms converge and result in the dysfunction seen in ALS. The broad range of work concerning these varying levels of study have yielded numerous hypotheses intended to both describe the disease, from a basic research perspective, and to contribute to applied research efforts concerned with new therapeutic strategies. Much of these efforts have focused on developing a hypothetical working model that describes intracellular motor neuron dysfunction, subsequent motor neuron death, and eventually symptoms. In particular, mitochondrial abnormalities within the motor nerve have been suggested to be a proximal cause of ALS (Vielhaber et al. 2000**;** Dupuis et al. 2010). Indeed, observations consistent with this hypothesis have been made in

both humans and the SOD1 G93Amouse model (Martin et al. 1999**;** Higgins et al. 2003**;** Israelson et al. 2010), but therapeutics targeting oxidative stress associated with these defects have been generally unsuccessful (Turner and Talbot, 2008). Another well established hypothesis regarding motor nerve dysfunction and resultant ALS symptomology concerns glutamate excitotoxicity in the nerve cell. The excitotoxicity hypothesis and targeting of Na^+ channels lead to development of the Na^+ channel antagonist, Riluzole, the only clinically accepted pharmacological treatment for the disease (Doble, 1996). Nevertheless, use of this drug has yielded disappointing results, and has questioned the role of motoneuron hyperexcitability and glutamate toxicity in ALS pathology.

Despite localization of the primary defect at the motor neuron, there has been some indication that influence from non-neuronal cell types is critical in the progression of ALS. Microglial cells of the central nervous system have been shown to be reactive before motor neuron loss (Henkel et al. 2006). Pharmacological treatment of this dysfunction slows disease progression in ALS mice (Kriz et al. 2002). Moreover it has been asserted that astrocytes, auxiliary neural cells responsible for maintenance of neurotransmitter and ion concentration in the synapse, exhibit pathological characteristics consistent with disease progression and thus may contribute to motor neuron dysfunction (Rothstein et al. 1995; Deforges et al. 2009).

Interestingly, a number of recent publications indicate an emerging role for skeletal muscle as a critical target (of both toxicity and therapeutic focuses) in ALS. One such study in support of this hypothesis showed that the G93A SOD1 mouse model of ALS, engineered with concomitant expression of potent growth factor insulin growth facor-1 (IGF-1) exclusively in skeletal muscle, improved muscle-associated symptoms of the disease (Dobrowolny et al. 2005). These double transgenic mice also showed a significant reduction

in the rate of motor neuron death, suggesting that the growth factor released by muscle can impart a significant neurotrophic effect on the nerve. Beyond demonstrating the potential of IGF-1 as a potential therapeutic for ALS, this novel experiment suggests that treatment at the level of skeletal muscle can provide beneficial effects for the motor nerve and overall ALS pathology. Perhaps even more strikingly, experiments utilizing selective tissue-specific expression of mutant SOD_1 have further implicated skeletal muscle as the primary tissue of concern in ALS. Studies of mice with skeletal muscle-specific mutant SOD1 expression have demonstrated the stimulus is sufficient to induce toxicity in muscle (Dobrowolny et al. 2008) and motor neuron degeneration (Martin et al. 2010). These findings highlight the importance of exploring the pathology of skeletal muscle in ALS to further elucidate critical mechanisms in the disease and provide potential targets for therapeutic development.

Recently however, overexpression of transcriptional co-activator PGC-1α exclusively in skeletal muscle of SOD1 G37R ALS-mutants led to improved muscle function and delays in muscle atrophybut no increase in lifespan,suggesting a rescue of the skeletal muscle phenotype alone may not be sufficient to alter disease progression (Da Cruz et al. 2012). PGC-1α activation may prove to be controversial in treating ALS, however, as its targeted overexpression significantly increased mitochondrial biogenesis in skeletal muscle. Given the suspected role of mitochondrial dysfunction possibly initiating critical pathology in the disease (Dupuis et al. 2009; Zhou et al. 2010; Yi et al. 2011), the unintended consequences of PGC-1α overexpression may in fact worsen the skeletal muscle phenotype in ALS.

The overt skeletal muscle phenotype most apparent in the SOD1 G93A mouse is indiscriminant atrophy. Muscle fibers show decreased cross sectional area and progressive reduction in hind limb muscle volume as early as 8 weeks of age compared to wild type

control mice (Marcuzzo et al. 2011; Brooks et al. 2004). In addition to an atrophic response, skeletal muscle fibers also respond with phenotypic derangement to the neurodegeneration in the ALS model. Hegedus et al. described a phenotypic shift in atrophying SOD1 G93A skeletal muscle toward the slow twitch phenotype, attributed to the selective vulnerability of the type IIB motor neuron (2008). These authors concluded that the parallel denervation of fast-fatigable (i.e., IIB) motor units and reinnervation of fibers by fast oxidative glycolytic and slow oxidative motor neurons accounts for an observable decrease in force output per motor unit (Hegedus et al. 2008). Thus, skeletal muscle atrophy and motor neuron defects in the SOD1 G93A mouse both contribute to the functional deficit and ultimate demise in cases of ALS.

Further characterization of skeletal muscle dysfunction in the SOD1 G93A mouse has elucidated other mechanisms possibly underlying ALS pathogenesis. Investigations of skeletal muscle mitochondria have described morphological aberrations and reduced oxidative capacity (Krasniaski et al. 2005), membrane depolarization (Zhou et al. 2010), and impaired calcium handling(Yi et al. 2011). The importance of this aspect of skeletal muscle dysfunction in ALS is highlighted by studies that have localized this dysfunction near the neuromuscular junction (Zhou et al 2010; Yi et al 2011). This suggests a link between skeletal muscle dysfunction and neurodegeneration (Dupuis et al. 2009), and is consistent with the observation that motor neurons "die-back" from their interface with skeletal muscle (Fischer et al. 2004).

Recent characterization of the gene expression profile in human ALS skeletal muscle found alterations in genes related to contraction and development, as well as lipid metabolism (Pradat et al. 2011*a*). mRNA expression of myogenic regulatory factors, MyoD and

myogenin, has been reported to be upregulated in the skeletal muscle of symptomatic SOD1 G93A mice (Manzano et al. 2011). Other evidence suggesting an ongoing remodeling of skeletal muscle includes reports of centrally located nuclei (Marcuzzo et al. 2011), angulated fibers (Fischer et al. 2004) and increased Pax-7 protein levels (Dobrowolny et al. 2005) at terminal stages of disease progression.

Despite the indications of ongoing remodeling of skeletal muscle architecture, there exists minimal characterization of the myogenic capacity of satellite cells in ALS. Satellite cells are skeletal muscle specific stem cells that reside wedged between the basal lamina and sarcolemma of muscle fibers (Mauro, 1961). These cells remain quiescent during adulthood, until activation of the population by muscle injury (Zammit et al. 2011). Activation of this population allows for withdrawal from quiescence and entrance to the cell cycle, prompting the initiation of a tightly regulated myogenic program whereby the cells proliferate, differentiate, and fuse to either existing muscle fibers or other muscle progenitors (Tedesco et al. 2010; see Figure 1 below).

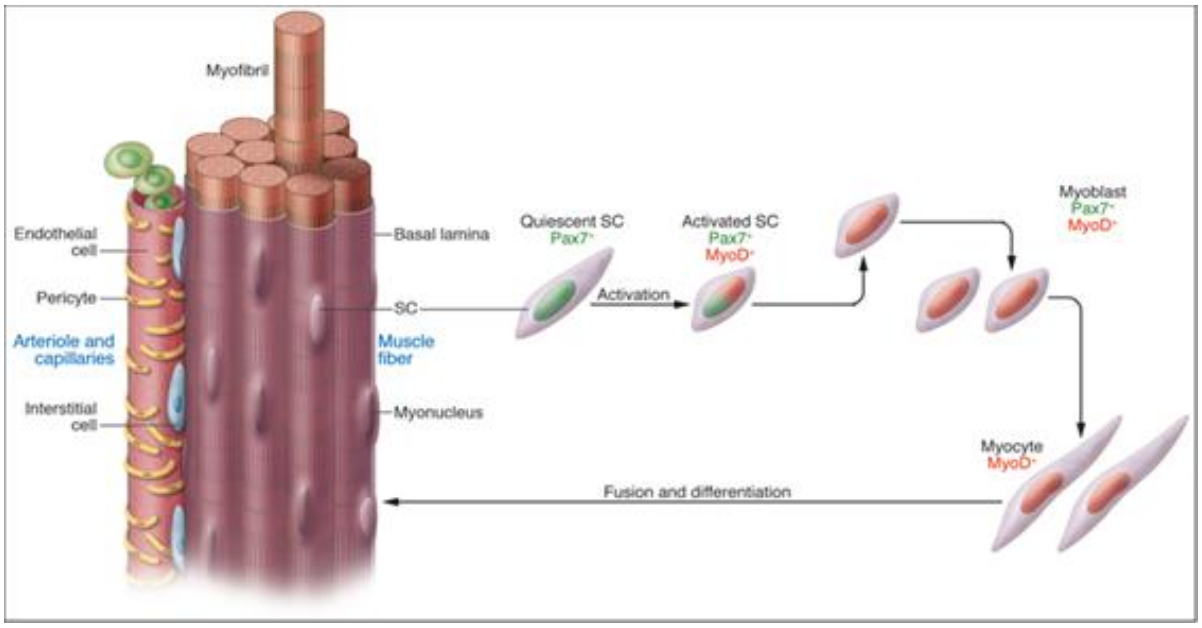


Figure 1.The myogenic program. Quiescent satellite cells reside within a distinct compartment and express Pax-7. Following activation, the cells upregulate MyoD and are committed to myogenic lineage as myoblasts. Terminal differentiation and fusion occurs with concomitant expression of myogenin (not pictured). Adapted and modified from *Tedesco et al*. 2010.

The myogenic process coincides with a distinct temporal expression pattern of a number of myogenic regulatory factors (MRFs) (Cosgrove et al. 2008). The expression of Pax-7 is considered the hallmark of satellite cell identification and is present during quiescence (Olguin & Pisconti, 2011). Asymmetric divisions of Pax-7^{+} precursors yield a population of committed myoblasts that express the transcription factor MyoD (Kuang et al. 2008). Terminal differentiation into myocytes requires the expression of myogenin, ultimately yielding a fusion-competent population of cells (Cosgrove et al. 2008).

The timing and execution of MRF expression (Londe & Davie, 2011) is critical to maintenance of the myogenic program, as evidenced by the dysfunction of conditional knock-outs of one of these factors (Sambasivan et al. 2011; Yablonka-Reuveni et al. 1999; Hasty et al. 2011). Interestingly it has been shown that MyoD and myogenin are regulated by electrical

activity (Eftimie et al. 1993; Gunderson, 2011; Manzano et al. 2011), and given the irregular neuronal stimulation pattern in ALS, their expression in the satellite cell population of diseased skeletal muscle is of particular relevance. Thus it is reasonable to speculate that the myogenic regulatory program may be dysregulated and could contribute to the pervasive atrophy seen in the SOD1 G93A mouse.

Other notable disorders with a distinct skeletal muscle phenotype have shown a dysfunctional satellite cell population. Satellite cell cultures derived from dystrophic mouse muscle (the *mdx* mouse) display aberrant kinetics of the myogenic program, with premature expression of MyoD and myogenin (Yablonka-Reuveni & Anderson, 2006). These findings have provided the basis for recent therapeutic efforts targeting the satellite cell population in muscular dystrophy, highlighting the potential importance of characterization of these cells in other diseases.

Only one study has evaluated ALS satellite cell behavior *in vitro*, and found abnormal myotube morphology and decreased MHC expression compared to controls (Pradat et al. 2011*b*). Unfortunately the *in vitro* analysis used failed to characterize changes in MRF protein or temporal evaluation of MRF expression in the satellite cell population. Moreover the authors reported difficulty in obtaining muscle samples and maintaining cells in culture, frequently observing excessive infiltration of non-muscle cells. Thus only observations were made for three human subjects. Therefore a more robust evaluation of the regulatory program in satellite cell cultures with high myogenicity should be conducted to effectively characterize the population of these cells in ALS.

Preliminary findings from our lab showed that cells from 13 week old SOD1 G93A mice were deficient in their ability to form new myotubes in culture. There was a reduction in the number of nuclei per myotube, a reduction in the number of total nuclei that were found in myotubes and a reduction in myotube diameter in SOD1 G93A compared to control satellite cells, suggesting that there was a reduced myogenic capacity of these cells *in vitro* (English et al. 2012). The purpose of the present study was to more comprehensively characterize the satellite cell population in ALS. Specifically of interest was the temporal expression pattern of the MRFs MyoD and myogenin, two regulatory factors critical for the repair of skeletal muscle. Using an *in vitro* approach, satellite cell cultures derived from SOD1 G93A mice were compared to cultures of control animals. A previously developed culture timeline was used to evaluate the specific aims of the current study:

Specific Aim 1: to determine if the SOD1 G93A cultured satellite cells are MRF positive (i.e., MyoD positive) during early commitment.

Hypothesis 1: SOD1 G93A cultures would show decreased MyoD positive nuclei during the early commitment phase compared to control cultures.

Specific Aim 2: to determine if the SOD1 G93A cultured satellite cells have decreased MRF positive nuclei during terminal differentiation (i.e., myogenin positive).

Hypothesis 2: SOD1 G93A cultures would show decreased myogenin positive nuclei during terminal differentiation compared to control cultures.

Specific Aim 3: to determine if the proliferative capacity of SOD1 G93A cultured satellite cells are reduced when compared to cultures of control satellite cells.

Hypothesis 3: SOD1 G93A derived satellite cell cultures would display an impaired capacity to proliferate *in vitro* when compared to controls.

Chapter 2: *Review of Literature*

It is the purpose of this literature review to summarize the pathological mechanisms of disease in Amyotrophic Lateral Sclerosis (ALS), and to explore the potential role of skeletal muscle in contributing to the pathogenesis of the disease.

Amyotrophic Lateral Sclerosis: Epidemiology and Clinical Presentation

Amyotrophic Lateral Sclerosis (ALS), also known as Lou Gehrig's disease, is a progressive and ultimately fatal neurodegenerative disorder. Approximately 2 in every 100,000 adults are diagnosed per year, with prevalence of ALS being about 4 in 100,000 worldwide (Boilee et al. 2006). Incidence of the disease is slightly higher in males than females (1.3:1, respectively; McCombe & Henderson, 2010). ALS in an adult-onset disorder such that those diagnosed are 45-55 years of age on average (Kiernan et al. 2011), with life expectancy following diagnosis about 3-5 years (Chio et al. 2010). The neurodegeneration characteristic of the disease exclusively affects motor neurons, disrupting transmission of neural impulses to muscle and eventually resulting in paralysis. Clinically, presentation of hyper-reflexia, wasting of the limbs, and electrophysiological aberrations detected by electromyography (EMG) are classic diagnostic criteria that may indicate the presence of ALS (Carvalho et al. 2008). Following diagnosis, progressive muscle atrophy and weakness contribute to a decreased ability of the patient population to remain functionally independent. A significant decline in the quality of life precedes the invariably fatal consequence of the disease, typically a result of respiratory failure (Rothstein, 2009).

Beginnings and Recent Research History

ALS was first described in 1868 by French neurologist Jean-Martin Charcot in a group of patients with progressive muscular atrophy (Charcot, 1868). Nearly 150 years later a singular cause of the disease is still unknown, though it has been determined that about 10 percent of diagnosed cases are familial in nature (i.e., attributable to an inherited genetic component, known as fALS); the other 90 percent of cases are deemed sporadic (sALS), though they share the same pathology as the familial variant (Patel & Hamedah, 2008). Of the cases diagnosed as fALS, approximately 20 percent share a common mutation of the Cu/Zn superoxide dismutase (SOD1) gene (Rosen at al. 1993). The crucial discovery of this commonality in these patients led to the generation of a transgenic mouse model (the SOD1 G93A mouse) that recapitulates the hallmarks of ALS and allows for study of the disease (Gurney et al. 1994). Variations of the SOD1 mutant have been subsequently developed (e.g., the G37R, G85R, and G86R transgenics); these models differ in dismutase activity and protein content but develop the same motor neuron degeneration characteristic of ALS (Turner & Talbot, 2008).

In the two decades since the discovery of the mutation in the SOD1 gene, an exhaustive effort to investigate ALS has broadened our understanding of the disease. Epidemiological studies have identified discrete subtypes of ALS that share the neurodegenerative phenotype but differ in age of onset, progressivity, and have unique mutation loci on various genes (Gros-Louis et al. 2006). To date, at least eight variations of the disease have been identified (ALS*1*-ALS *8*), suggesting the complexity of the pathogenesis that underlies motor neuron degeneration. Determination of these distinct disease subtypes has led to the development of novel hypotheses that attempt to identify

pathogenic cellular mechanisms of interest in ALS. Implication of the TAR-DNA binding protein, TDP-43, has been suggested by reports of mutations in the TARDBP gene common to sporadic and fALS cases, indicating a role for dysfunctional RNA-processing in neurodegeneration (Mackenzie et al. 2007; Sreedharan et al. 2008). Similarly a causative mutation in the gene encoding the fused in sarcoma/translated in liposarcoma (FUS/TLS) RNA-processing protein has been reported in fALS and sALS cases absent the SOD1 mutation, highlighting the potential importance of this disease mechanism in the greater ALS population (Liscic & Breljak, 2010).

Another notable finding describes an expansion of a hexanucleotide repeat in a non-coding sequence on chromosome region 9p21 in both sporadic and familial ALS cases (DeJesus-Hernandez et al. 2011; Renton et al. 2011). Interestingly this mutation is also seen in patients with frontotemporal dementia (FTD), another condition characterized by neurodegeneration. Given the non-coding nature of the mutation location, it is hypothesized that this discovery lends further credence to the possibility that dysfunctional dynamics of transcription and RNA translation may mechanistically link these neurodegenerative phenotypes (Orr, 2011).

Other research has described mutations in the genes encoding optineurin (Maruyama et al. 2011), angiogenin (Greenway et al. 2007), and the vesicle trafficking protein VAPB (Nishimura et al. 2004) suggesting the implication of various cell signaling pathways in neurodegeneration. Yet despite our growing knowledge of the rich variety of genetic mutations in the human ALS population, transgenic animal lines targeting the SOD1 enzyme remain the most widely studied models of the disease. The most frequently studied of these mutants is the SOD1 G93A mouse; these mice express human copies of the SOD1 enzyme

bearing a Glycine to Alanine substitution at the 93rd codon in the gene coding for the protein – a mutation sufficient to induce the hallmark neurodegeneration, denervation and muscle atrophy seen in canonical ALS (Gurney et al. 1994). The SOD1 G93A mutant mice develop normally, beginning to show tremors and hindlimb weakness at 12 weeks; overt motor performance deficits continue progressively, ultimately leading to paralysis and death at approximately 19 weeks (Turner & Talbot, 2008).

Proposed mechanisms underlying ALS pathogenesis

Oxidative stress

It was initially thought that a loss of dismutase function in the SOD1 G93A mouse led to the development of the disease phenotype, but it is now established that the mutation results in a toxic gain of function (Turner & Talbot, 2008). The SOD1 G93A transgenics express multiple copies of human genomic fragments encoding the mutant protein in addition to endogenous SOD1, consistent with the gain of function hypothesis (Gurney et al. 1994). Moreover the SOD1 knockout mouse does not develop the ALS-like phenotype (Reaume et al. 1996).

In normally functioning redox states, the SOD1 enzyme functions to scavenge (i.e., dismutate) superoxide radicals ($O_2^{\bullet -}$) into H_2O_2, which subsequently reacts to either spontaneously form hydroxyl radicals ($OH^{\bullet}$) or is reduced to water (H_2O). SOD1 also acts to prevent nitric oxide (NO) from reacting with superoxide to form the powerful oxidant peroxynitrite ($ONOO^-$), further protecting the cell from oxidative damage (Fukai & Ushio-Fukai, 2011; see Figure 2).

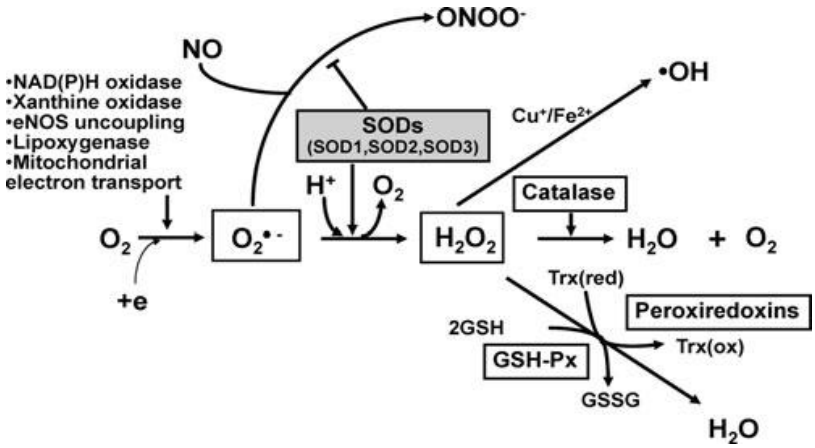


Figure 2.Antioxidant functions of superoxide dismutase (SOD1). Various sources of free radicals increase intracellular superoxide (O_2^-) production. SOD1 dismutates O_2^- into hydrogen peroxide (H_2O_2), which is typically reduced to water (H_2O) though it may spontaneously form hydroxyl radicals ($OH^{\cdot}$). SOD1 also prevents the formation of the oxidant peroxynitrite ($ONOO^-$). Adapted from *Fukai & Ushio-Fukai*, 2011.

The toxic gain of function mutation in the SOD1 G93A mouse is thought to overwhelm the intracellular redox capacity of this system, generating excessive hydroxyl radicals and other reactive species (Patel & Hamadeh, 2008). Such an imbalance imparts oxidative stresses within the cell and causes damage to lipids and DNA, and modifies proteins (Bruijn, Miller, & Cleveland 2004). Thus one of the dominant hypotheses regarding the causative mechanisms underlying ALS pathology is that of oxidative stress. Early experiments cemented the importance of this hypothesis, reporting an accumulation of $OH^{\bullet}$ and concomitant lipid peroxidation in the spinal cords of SOD1 G93A mice (Hall et al. 1998). Oxidatively modified proteins have also been found in the spinal cords of SOD1 G93A mice, a phenomenon also seen in the human form of the disease (Andrus et al. 1998). SOD1 itself was described as heavily modified, a finding that has been confirmed by subsequent investigations (Barber & Shaw, 2010).

Other targets of interest have been determined by using a proteomics approach, specifically identifying those proteins that have been subject to oxidation. One study found, in addition to SOD1, proteins involved in ubiquitin-dependent proteolysis, calcium (Ca^{2+})

binding and protein folding were subject to significant oxidation (Poon et al. 2005). Another publication using a proteomics approach described modifications to proteins involved in energy metabolism, neural development and protein folding, using the presence of reactive aldehyde species (4-hydroxy-2-nonenal, HNE) as a marker of oxidative stress (Perluigi et al. 2005). These reports suggest that oxidative protein modification is the direct link between oxidative stresses engendered by mutant SOD1 and dysfunction within affected cells. This approach offers powerful insight into the indiscriminate oxidative damage imparted by the SOD1 mutant and offers strong evidence as to the involvement of various cell signaling pathways that may be defunct in ALS.

In addition to oxidative protein modification, it is hypothesized an imbalanced redox environment caused by the SOD1 mutation exacerbates the susceptibility of motor neuron to oxidative stress and cell death. Nitrotyrosine levels, an indicator of oxidative stress, were elevated in SOD1 G93A motor neurons and coincided with cleavage and activation of cell death mediator Caspase-12 (Wootz et al. 2004). *In vitro* experiments studying the NSC-34 motor neuron-like cell line transfected with mutant SOD1 have described mitochondrial dysfunction and comparatively higher rates of cell death when subjected to oxidative stress and compared to wild type controls (Rizzardini et al. 2005). Additional investigations have found that mutant cultures of this cell line show oxidative-stress induced mitochondrial dysfunction and cytochrome *c* release, suggesting that redox imbalances may signal cell death through the mitochondria (Liu et al. 2002).

Unfortunately attempts to stem oxidative damage and ameliorate the pathology of the SOD1 G93A mutant have been disappointing. Vitamin E (α–tocopherol) was initially thought of as an ideal antioxidant therapeutic, given its wide availability and protective role against

lipid peroxidation (Ascherio et al. 2005). Treatment studies, however, have shown no effect on survival in mice receiving vitamin E supplementation (Patel &Hamadeh, 2008). Catalase, an enzyme that catalyzes the reduction of the oxidant H_20_2 into H_20 (See Fig. 1), has been delivered (as modified putrescine-modified catalase, PUT-CAT) to augment the antioxidant capacity of the SOD1 G93A mutant. Though this trial delayed the onset of symptoms in a group of SOD1 G93A mice, the treatment had no effect on survival or lifespan (Reinholz et al. 1999). Similarly administration of a litany of antioxidants (e.g., NOS inhibitors, SOD1 mimetics, green tea, red wine) were shown to have only a mild effect in delaying symptom onset or extending survival, despite administration of the treatment across the whole lifespan (Turner & Talbot, 2008). This study design presents significant limitations to clinical interpretation, given the sporadic, adult-onset nature of most human ALS cases (Scott et al. 2008). However one of the few promising antioxidant agents, manganese-porphyrin, was administered at disease onset and extended lifespan compared to vehicle-treated mice (Benetar, 2007). Nevertheless, the varied attempts to mitigate oxidative stress in the SOD1 G93A mouse with few beneficial outcomes have been a frustration to the ALS research community and suggest other significant mechanisms of pathogenesis are at play in the disease.

Mitochondrial dysfunction

One potential target not yet fully understood is the mitochondria. Functions of the mitochondria including apoptotic regulation, reactive oxygen species generation, and metabolic operation may all be implicated in the development of disease features in ALS (Manfredi and Xu, 2005). These operations are inherently linked to the function of the SOD1 enzyme – indeed, the mitochondria is the principal producer of the superoxide substrate that

wild-type SOD1 normally catalyzes. In this respect, the crossroads of mutant SOD1 with the mitochondrial function in ALS patients is of particular interest.

Observations of mitochondrial dysfunction in the clinical population have highlighted the importance of this aspect of the disease in ALS. sALS patients show drastic morphological aberrations in neuronal mitochondria. The organelles show vacuolization (Xu et al. 2004), budding, fission, swelling (Martin et al. 2009), and inclusions (Sasaki et al. 2007). Reduced mitochondrial enzyme activity and damage to mitochondrial DNA was detected in another cohort of sALS subjects (Vielhaber et al 2000). Reports of mitochondrial dysfunction in the SOD1 G93A mouseare largely consistent with these clinical presentations (Martin, 2011).Interestingly, observations made in the SOD1 mutant mouse have been madeduring the presymptomatic phase of the disease, thereby indicating a potential underlying cause of ALS pathogenesis.

The mitochondria play a number of critical roles within the cell, primarily regarding energy production and cytosolic regulation of various metabolites. Byproducts of these mitochondrial operations, including $O_2^{\bullet -}$, H_2O_2, $ONOO^-$ and $OH^{\bullet}$, contribute to oxidative stress and potentially cell death in ALS (Martin et al. 1999). Therefore the impaired redox capacityofthe SOD1 G93A mouse is inherently linked with the function of the mitochondria in affected cells. Thus the mitochondria have become an important target in fully understanding the redox imbalance seen in the SOD1 G93A mutant.

The mitochondria in ALS seem to have a functional defect as well, as indicated by respiratory chain dysfunction and elevated cytoplasmic calcium levels in motor neurons with mutant SOD1 (Carri et al. 1997). Observations of similar calcium related dysfunction were repeated in a more recent study, where comparison of intracellular calcium measurements

with mitochondrial calcium stores showed a disparity between the wild type and SOD1 G93A expressing neuroblastoma cells (SH-SY5Y), an established culture model for studying motor neurons *in vitro* (Jaiswal et al. 2009).

The mechanisms by which SOD1 mutants and other pathological features of ALS interact with the mitochondria are not clearly understood, and may provide a crucial piece in deciphering the causes of motor neuron death in the disorder. Investigations using immunohistochemistry have described SOD1 localization in and around the outer mitochondrial membrane suggesting physical interaction with the organelle may contribute to its dysfunction (Higgins et al. 2002). A more recent study that investigated this phenomenon further was published by *Israelson et al*. (2010). This group was able to show direct interaction between the mitochondria and mutant SOD1 via the voltage-dependent anion channel (VDAC1), a conductance channel located on the outer membrane of the mitochondria. By analyzing voltage across the transport channel, the authors were able to determine that mutant SOD1 binding to VDAC1 reduced conductance of the porin, contributing to mitochondrial dysfunction (Israelson et al. 2010). Another transmembrane channel on the mitochondria that has been shown to be implicated in ALS is the permeability transition pore(PTP). In the motor neurons of the SOD1 G93A mouse, mitochondrial PTP exhibit peculiar trafficking tendencies and may lead to the formation of large, dysfunctional mitochondria (Martin et al. 2009).

It has been suggested that the mitochondrial damage sustained in ALS may directly confer cell death signals and result in motor neuron degeneration. The mitochondria-dependent apoptotic signaling cascade involves cytochrome *c* release into the cytosol and subsequent activation of the caspase family of apoptosis mediators; indeed these cell death

signals are present in the spinal cords of SOD1 G93A mice consistent with disease progression (Guegan et al. 2002). Consistent observations have been recapitulated in a motor neuron-like cell line that described cytochrome*c* release in cells transfected with mutant SOD1 (Liu et al. 2002).

Based on knowledge of mitochondrial defects, efforts have been made to target mitochondrial dysfunction in SOD1 mutants and to determine whether thismitigates disease progression. Treatment with minocycline, an inhibitor of cytochrome *c* release, delayed symptom onset and increased lifespan in a cohort of SOD1 G93A mutants (Zhu et al. 2002). Targeting mitochondrial free radical generation via overexpression of mitochondrial-resident antioxidant enzymes superoxide dismutase 2 (SOD2) and glutathione peroxidase (GPx4) reduced lipid peroxidation, cytochrome *c* release, and cell death in an *in vitro*study of motor neuron-like SOD1 mutant cultures (Liu et al. 2002). In a similar attempt to counteract cell death signaling through the mitochondria, a rather sophisticated investigation crossed mutant mice with deletions of the genes encoding the mitochondrial BAX and BAK pro-apoptotic signaling proteins with SOD1 G93A mice (Reyes et al. 2009). SOD1 mutant mice that lacked BAX and BAK as a result of the genetic deletiondemonstrated improved motor performance and increased lifespan compared to those who still expressed the pro-apoptotic proteins. Consistent with previous therapeutic attempts to target the mitochondrial dysfunction, these results further argue the important role that cell death signaling through the mitochondria plays in ALS disease progression.

Protein aggregation

In addition to a toxic gain of function and the induction of mitochondrial defects, the SOD1 G93A mutation engenders particular susceptibility to protein aggregation and its

associated consequences. As seen in a number of neurodegenerative diseases (e.g. Parkinson's disease, Alzheimer's disease, and FTD), protein aggregation is an axis of cellular dysfunction with the capacity to induce devastating damage in neural cells (Cleveland et al. 2011). Thus observations of protein aggregation in cases of ALS have raised the specter of its role in the pathology of the disease. Destabilization and demetallation of the SOD1 dimer has been shown to promote self-aggregation of SOD1 monomers into insoluble proteinaceous inclusions (Valentine et al. 2005). These inclusions rich in the SOD1 apoenzyme have been observed in human ALS patients (Bruijn, Miller, & Cleveland 2004) and SOD1 G93A mutant mice alike (Watanabe et al. 2001). Studies of the oligomeric SOD1 aggregates have determined a propensity to form donut-shaped and tubular structures that retain but show modified enzymatic activity (Shaw & Valentine, 2007). Evidence that these aggregates complex in an age-dependent fashion point to this defect as a potential source of toxicity in ALS (Turner et al. 2003).

The unique capacity of mutant SOD1 to self-aggregate has also led to the novel hypothesis that ALS is a prion-like disease - misfolded SOD1 proteins form oligomeric inclusions, acting as templates that mediate dysfunctional changes in conformation of various proteins (Polymenidou & Cleveland, 2011). These aggregations can be secreted and may spread from cell to cell, transmitting mutant proteins throughout the motor neuron population. Consistent with this hypothesis, TDP-43 and FUS/TLS have prion-like domains (Gilter & Shorter, 2011), and cell cultured seeded with mutant SOD1 form tubular arrays reminiscent of inclusions seen in the disease (Chia et al. 2010).

Other aggregate species have been detected in ALS, broadening the implication of protein aggregation beyond SOD1 mutants exclusively. Ubiquitinated cytoplasmic inclusions

of TDP-43 aggregates have been reported in sALS motor neurons (Neumann et al. 2006). Further, more recent investigation of a TDP-43 mutation seen in fALS found the mutant protein unusually stable and, as a result, more frequently bound to the ALS-implicated RNA-processing protein FUS/TLD (Ling et al 2010). This important finding suggests a commonality in disease pathogenesis among SOD1 mutation-linked fALS cases and those with other inherited mutations, underscoring neuronal protein aggregation as a critical mechanism in eliciting the neurodegenerative phenotype.

Aberrant protein-protein interaction and aggregation is thought to initiate a series of intracellular responses that may ultimately result in cell death. Detection of mutant SOD1 aggregates in endoplasmic reticulum (ER) compartments isolated from the spinal cords of SOD1 G93A mice indicates these aggregates may activate the ER stress response in ALS (Kikuchi et al. 2006). Accordingly the ER stress signaling apparatus, including protein chaperone BiP and ER stress apoptotic-mediator CHOP, are upregulated in the spinal cords of ALS humans and SOD1 G93A mice (Ito et al. 2009). Additional evidence that suggests mutant SOD1 aggregates inhibit the proteasome, a protein degradation system within the cell, indicates further that protein aggregation disrupts intracellular homeostasis (Ilievia et al. 2009).

Therefore therapeutic approaches have been designed to mitigate the protein aggregation arm of ALS pathology. Pursuit of this end has shown some promise: several trials administering the heat shock protein (HSP) agonist arimoclomol to boost protein folding and chaperoning have increased lifespan when delivered to SOD1 G93A mice at symptom onset (Kieran et al. 2004; Kalmar et al. 2008). Additional attention has recently been given to novel arylsulfanyl pyrazolone compounds (ASPs) that may stem mutant SOD1 aggregation (Chen et

al. 2011; Chen et al 2012), though treatment with this agent has yet to be evaluated in either mice or humans. These promising results imply the relative importance of protein aggregation in ALS pathogenesis and warrants further investigation of this treatment approach.

Glutamatemishandling

Neurotoxicity resulting from the mishandling of the amino acid glutamate is yet another prominent disease mechanism proposed to explain ALS pathology. Normally functioning motor neurons mediate firing patterns and prevent excitoxicity by way of regulated glutamate clearance from the synaptic cleft (Rothstein et al. 2009). Without coordinated removal of glutamate, the motor neuron is subject to overstimulation and hyperexcitability that triggers toxic signaling cascades within affected neural cells. Continual stimulation with glutamate results in excessive calcium influx, increased oxidative stress, and ultimately cell death (Kiernan et al. 2011).

The first evidence of elevated glutamate levels in the cerebrospinal fluid (CSF) of human ALS patients predates the SOD1 G93A mouse and led to the development of this hypothesis (Rothstein et al. 1990). Subsequent validation in this phenomenon in a large cohort (n=377) of sALS patients suggests the broad importance of this disease hallmark in neurodegeneration of varying etiologies (Spreux-Varoquaux et al. 2002).

Much of the research concerning glutamate abnormalities in ALS has focused on the glutamate transporter EAAT2, an astrocytic transport protein that is primarily responsible for the clearance of neurotransmitters from the synapse (Sasabe & Aiso, 2010). EAAT2 protein expression in spinal cords from ALS patients is reduced (Lin et al. 1998) and nearly absent in late-stage symptomatic SOD1 G93A mutants (Howland et al. 2002), suggesting a mechanism

whereby glutamate is mishandled in the disease. Interestingly the knockout of glutamate transporters in mice results in neuronal death (Rothstein et al. 1996), whereas overexpression of the EAAT2 protein in SOD1 G93A mutants is neuroprotective (Guo et al. 2002). Treatment of SOD1 G93A mice with a β-lactam antibiotic that increases EAAT2 expression also beneficially affects disease progression, specifically delaying the onset of symptoms and increasing lifespan in the mutants (Rothstein et al. 2005).

It is under the auspices of this line of evidence that the anti-glutaminergic drug Riluzole was designed. Riluzole, a Na^+ channel antagonist, is the only FDA-approved drug for the treatment of ALS and functions to decrease glutamate toxicity (Bruijn, Miller, & Cleveland 2004). Unfortunately clinical prescription and administration following symptom onset only extends lifespan a few months on average (Turner & Talbot, 2008).

Summary of Neuronal Disease Mechanisms in ALS

Tremendous research effort in the previous two decades has elucidated a number of mechanisms that underlie the motor neuron-specific neurodegenerative pathology in ALS (see Figure 3). The seminal discovery that a subset of fALS patients share a mutation of the SOD1 enzyme (Rosen et al. 1993) and subsequent generation of a mouse model that recapitulates the disease (Gurney et al. 1994) led to the still-prominent hypothesis that oxidative stress is critically involved in disease pathogenesis (Yim et al. 1996). The mitochondrion, given its capacity to generate free radicals and apoptotic signals, has also become the object of much attention concerning ALS pathology (Martin, 2011). Observations of protein aggregates in motor neurons in both human cases and animal models of ALS indicate another source of

toxicity to the neuron in the disease (Polymenidou & Cleveland, 2011). Excitotoxicity as a result of glutamate mishandling has proved perhaps the most clinically relevant discovery, as it has led to the development of Riluzole, the only FDA-approved prescriptive treatment for ALS (Bruijn, Miller, & Cleveland 2004). Attempts to target other causes of dysfunction in the disease have shown some promise, though few have demonstrated an ability to translate to humans (Turner & Talbot, 2008). Thus the search for druggable targets and alternative disease mechanisms in ALS continues. Indeed novel hypotheses have recently arisen, as focus on the motor nerve as the primary target of ALS pathology gives way to non-neuronal cells as a major source of toxicity and potentially causative in disease pathogenesis.

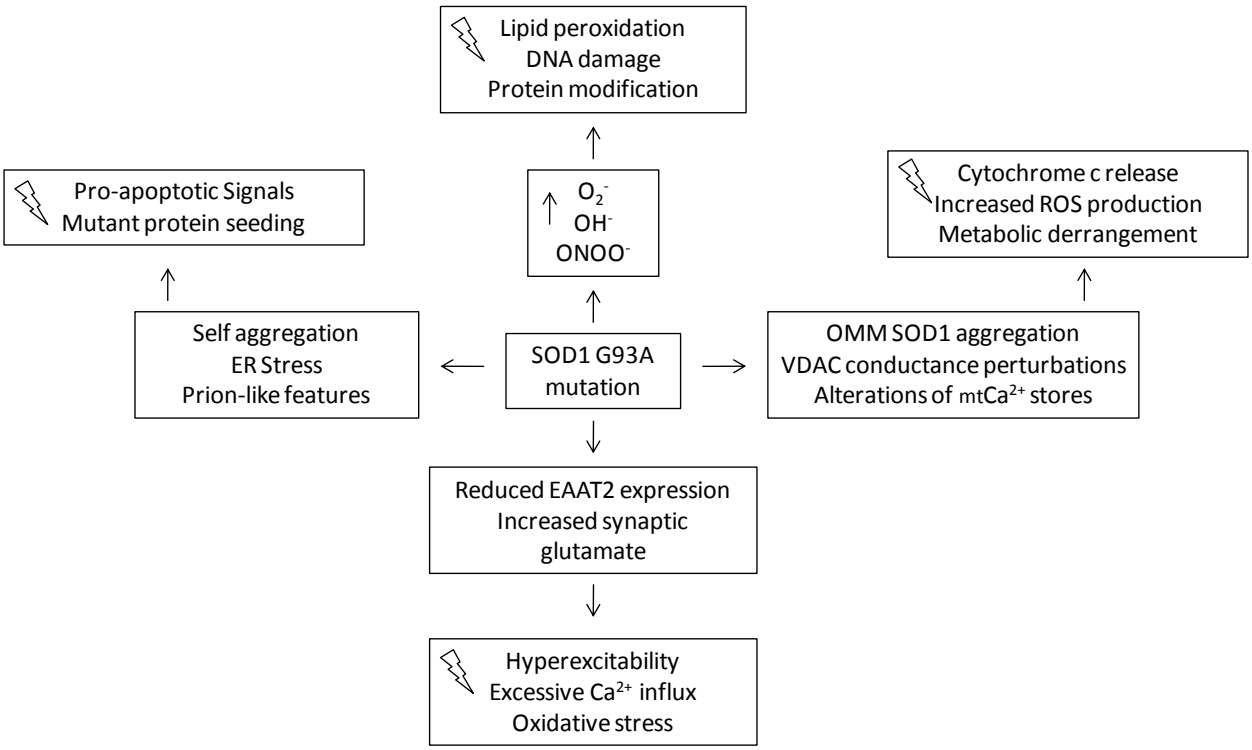


Figure 3.Summary of primary neuronal defects inALS. Sources of dysfunction proposed to contribute to neurodegeneration include oxidative stressors (Top), mitochondrial aberrations (Right), cytotoxic misfolded proteins (Left), and excitotoxicity (Bottom).

Non-neuronal Cell Injury

Neural Cells

Accumulating evidence has begun to illuminate the role of non-neuronal cell populations in contributing to the pathogenesis of ALS. A number of transgenic mice have been generated that selectively express mutant SOD1 in a tissue-specific manner in order to investigate the effect of the mutant enzyme in cell-types other than the motor neuron. This approach has been justified by evidence of dysfunction in neural cells other than motor neurons (e.g., astrocytes and microglia) seen in both human ALS and mutant mice (Gong et al. 2000). Thus the first study to suggest that SOD1 mutant toxicity restricted to motor neurons was not sufficient to cause ALS used a transgenic mouse with mutant SOD1 G37R expression driven by the neurofilament light chain (NFL) promoter (Pramatarova et al. 2001); indeed these mice develop no neurodegeneration despite high expression levels of the mutant protein in motor neurons. Subsequent confirmation of this finding saw no neurodegenerative pathology in transgenic mice expressing mutant SOD1 G93A or SOD1 G85R exclusively in postnatal neurons (Lino et al. 2002). Both of these results support the hypothesis that pathological contribution of multiple cell types is necessary to develop ALS.

These findings are not without controversy, however. A more recent examination of this experimental paradigm was employed to ensure sufficient mutant SOD1 protein was present in motor neurons of the original mutant SOD1-NFL transgenics, citing the waning expression of NFL across mouse lifespan (Jaarsma et al. 2008). The expression of mutant SOD1 in these mice was driven by the Thy 1.2 cassette designed to induce more robust mutant protein content in adult motor neurons (Caroni, 1997). Indeed these mice had 2-5x

more mutant SOD1 protein in adult motor neurons than the original study by *Pramatarova et al.*(2001), and developed motor neuron disease similar to the SOD1 G93A transgenic (Jaarsma et al. 2008). However these mice were absent certain pathologies found in the original G93A (e.g., abnormal mitochondrial morphology and vacuolization), had a longer lifespan, and expressed mutant SOD1 in some other spinal neurons. Taken together, the results of this study and its predecessors indicate the non cell-autonomous nature and progression of the motor neuron degeneration in human ALS and mouse models of the disease.

The diverse cellular constituency of the nervous system has implicated a number of cell types in ALS pathology. Astrocytes are specialized cells that populate the nervous system and facilitate neuronal health by performing antioxidant functions, storing metabolites, and clearing neurotransmitters from the synaptic cleft (Vargas & Johnson, 2010). Consistent with impaired glutamate handling observations suggesting astrocytic dysfunction in ALS (Rothstein et al. 1990), damage to astrocytes has been observed in human ALS postmortem tissue and SOD1 mutant mice (Boilee et al. 2006). Astrocytes exhibit reactive gliosis (Schiffer et al. 1996) and show aberrant protein aggregates similar to those in motor neurons (Steiber et al. 2000). In fact this damage precedes motor neuron degeneration and becomes more marked with disease progression, suggesting astrocytic dysfunction may contribute to ALS pathogenesis (Bruijn et al. 1997). However, mice that express mutant SOD1 solely in astrocytes fail to develop motor neuron degeneration (Gong et al. 2000), refuting the hypothesis that astrocyte-mediated dysfunction may be causative in ALS. Nevertheless, elimination of mutant SOD1 expression in the astrocytes of SOD1 G93A mutants extends

lifespan and delays symptom onset (Wang et al. 2011), arguing for the importance of astrocytic dysfunction in disease progression and therapeutic development.

Microglia, thought of as the primary immune cells of the nervous system, have also been implicated in ALS pathogenesis. Under normal physiological conditions, microglia roam the nervous system and function to clear debris and recruit the inflammatory response in case of infection (Lasiene & Yamanaka, 2011). However in the neurodegenerative circumstance of ALS, it is hypothesized that encountering abnormal protein aggregations and degenerative neuronal cells stimulates excessive microglia activation and stimulation of neuroinflammation. Detection of inflammatory factors associated with microglial activation in spinal cords of SOD1 G93A mice as early as 40 days of age posits the contribution of gliosis to motor neuron degeneration (Alexianu et al. 2001). Additional studies of inflammatory factor expression in the spinal cords of SOD1 G93A mice have shown increased cytokine mRNA levels that correlate with the temporal expression pattern of apoptotic-related genes (Yoshihara et al. 2002; Hensley et al. 2002). Though the causative link between microglial activation and neurodegeneration is difficult to establish, mutant SOD1 G93A mice that lacked native microglia and were infused with donor wild-type cells demonstrated slowed motor neuron loss, indicating a potential role for microglia as a therapeutic target in ALS (Beers et al. 2006).

Peripheral myelin generating cells (called Schwann cells) have a close physical relationship with motor neurons and thus are also of interest in ALS. Consistent with this supposition and the evidence of peripheral axonopathy preceding neurodegeneration in ALS (Fischer et al. 2004), myelin dissolution is apparent along distal portions of motor neurons in presymptomatic SOD1 G93A mutants (Niebroj-Dobosz et al. 2007). A recent investigative

attempt to establish a causal role for Schwann cell dysfunction in ALS expressed mutant SOD1 G93A protein exclusively in these cells, though these mice failed to develop neuropathology or motor deficits (Turner et al. 2010).

These studies and others using chimeric mice (Clement et al. 2003; Yamakana et al. 2008) with varying expression levels of mutant SOD1 protein in diverse combinations of neural cell populations suggest a complex convergence of defective systems leads to ALS-like pathology. Continued efforts in this respect create invaluable knowledge in understanding the contributions of neural cell dysfunction to ALS pathogenesis.

Neuromuscular Junctionuncoupling is a key pathogenic event in ALS

Examination of disease progression in the SOD1 G93A mouse has demonstrated that terminal axons at the neuromuscular junction (NMJ) begin to retract well before symptom onset, with denervation of 40% of the motor end plates before 8 weeks of age and prior to apparent motor neuron death (Fischer et al. 2004). Other longitudinal observations of the NMJ in the SOD1 G93A mouse report damage as early as 40 (~6 weeks) of age (Frey et al. 2000). The occurrence of this event early in disease progression independent of motor neuron death has led to the characterization of ALS as a *distal axonopathy*, whereby the NMJ is the site critical for disease initiation (Dadon-Nachum et al. 2011).

Characterization of aberrations specific to the NMJ in ALS have thus become of interest to those studying the disease. In particular, the signaling axis required for coordinated regeneration and remodeling of the NMJ has been shown to be defective in ALS. Investigations of the neurite outgrowth inhibitor Nogo-A, which functions to inhibit axonal growth following neural injury, show the protein is upregulated prior to motor symptoms in

the SOD1 G86R ALS mouse. Accordingly, crossing SOD1 G85R mutants with Nogo-A knockout mice significantly increased lifespan and delayed denervation in the crossbred SOD1 G86R/Nogo-$A^{(-/-)}$ double mutants (Jokic et al. 2005). The critical role Nogo-A plays in NMJ integrity was confirmed by observations of abnormally small, fragmented NMJs in mice overexpressing the protein (Jokic et al. 2005). However this finding was contrasted by more recent study that found a protective effect of the Nogo/Reticulon family, where deletion of the Nogo-A,-B gene accelerates disease onset and progression (Yang et al. 2009).

Cyclin dependent kinase 5 (cdk5), responsible for acetylcholine receptor (AchR) clustering near the muscle-nerve interface, is another factor critical for NMJ maintenance that has been implicated in ALS pathology. Presymptomatic, 27 day old SOD1 G39A mice showed decreased cdk5 activity and coimmunoprecipitation of the protein with mutant SOD1 (Park & Vincent, 2008). The strikingly early report of this aberration well before symptom onset indicates the significance that the neuromuscular region plays in disease pathogenesis. Especially important is the suggestion of defective AchR clustering, given the role that functional AchRs play in transmission of neural input to muscle (Ferraro et al. 2012). Nevertheless a recent investigation using a novel patch-clamp technique determined the AchRs derived from human ALS muscle and microtransplanted into *Xenopus* oocytes show no functional defect compared to healthy, denervated control protein evaluated with the same technique (Palma et al. 2011).

Semaphorin3a (Sema3a) is a chemorepellant that plays an important role in coordinating axonal growth, and thus may also have ramifications with regard to ALS pathogenesis. Specifically, Sema3a expression has an inhibitory effect on growing neurites, and thus has been posited to act as a brake on premature axonal sprouting immediately

following acute injury, whereas subsequent coordinated downregulation allows for the NMJ remodeling process to continue (Tatsumi et al. 2009). Intriguingly, an upregulation of Sema3a has been reported in SOD1 G93A mice, upon disease onset, exclusively at the NMJ of type IIb muscle fibers (De Winter et al 2006). This striking parallel to the pattern of enhanced susceptibility of type IIb motor neurons to degeneration and type IIb muscle fibers to atrophy in ALS (Hegedus et al. 2008) may signal a novel role for Sema3a in disease pathology. Though these findings were made in the terminal Schwann cells located at the NMJ, other cell types in the region have been shown to express and secrete Sema3a, including satellite cells (Tatsumi et al. 2009*a*).

Various pathological mechanisms have been suggested to induce distal insult to the motor nerve and thus manifest as NMJ damage and uncoupling. Alterations in axonal transport imparts enough damage to the motor neuron to induce denervation (LaMonte et al. 2002), and consistent observations of slowed axonal transport along motor neurons have been made in presymptomatic SOD1 G93A mice (Bilsland et al. 2010). The significance of this aspect of the disease pathology has been questioned however, by evidence that the axonal transport defect is not necessary for axonal degeneration in G85R mice (Marinkovic et al. 2012).

Given the distinct mitochondrial phenotype observed in both the neuronal cells and muscle of SOD1 mutants, defective mitochondria in the vicinity of the end plate may also contribute to degenerative processes at the NMJ. A line of transgenic mice that overexpress uncoupling protein-1 (UCP-1) specifically in skeletal muscle were designed to mimic the hypermetabolic phenotype seen in the SOD1 G93A mice; interestingly these mice develop NMJ uncoupling and motor neuron degeneration, with disease course similar to that seen in

ALS (Dupuis et al. 2009). Accordingly, a study of SOD1 G93A skeletal muscle mitochondria Ca^{2+}-handling dysfunction localized the mitochondrial defect to regions of the skeletal muscle fiber associated with the NMJ (Zhou et al. 2010). The observation of this defect at 37 days, concurrent with early NMJ decoupling events (Fischer et al 2004; Frey et al. 2000), underscores the contribution of defective mitochondria in this region to initiation of degeneration of the NMJ. Furthermore, it evokes speculation that dysfunction native to skeletal muscle may induce deleterious effects to the neighboring motor nerve.

The role of skeletal muscle in ALS pathogenesis

The past two decades of research regarding ALS pathogenesis have largely made headway towards a greater understanding of the neuronal basis underlying the disease, and done so fittingly – indeed ALS is classically recognized as a neurodegenerative disease. However, the accumulation of recent evidence has begun to beg the question: is the motor neuron the primary site of pathogenic toxicity in ALS? Experiments exploring the role of non-neuronal cells in the central and peripheral nervous systems have suggested the motor neuron-autonomous mechanism of disease hypothesis is tenuous (Bruijn et al. 1997; Alexianu et al. 2001). Moreover the experimental evidence from motor neuron-restricted expression of the SOD1 G93A mutation remains equivocal (Lino et al. 2002; Pramatarova et al. 2001; Jaarsma et al. 2008), further suggesting the importance of non-neuronal cell populations in the pathogenic processes of ALS.

Accordingly, more recent research has examined skeletal muscle as a potential source of toxicity in the disease. Early indications that skeletal muscle is sensitive to the SOD1 G93A mutation independent of neurodegeneration arose from examination of a skeletal

muscle-specific mutant driven by the myosin light chain (MLC) promoter (Dobrowolny et al. 2008). These mice develop atrophy, metabolic dysfunction and decline in contractile function of the skeletal muscle in the absence of motor neuron loss. Notably however, expression of the mutant SOD1 protein was significantly lower in slow muscles, where the MLC promoter is less predominant, when compared than the conventional whole-body SOD1 mutant (Dobrowolny et al. 2008).

A more recent finding offering strong evidence that cytotoxicity within skeletal muscle can initiate ALS pathology used an α-actin promoter to drive more robust mutant SOD1 expression in all skeletal muscles (Wong & Martin, 2010). Strikingly, these mice develop motor neuron degeneration, exhibit caspase-3 activation in the spinal cord, and progressively show decreased motor performance and muscle atrophy. Though these mice have a later disease onset and longer lifespan (8-10 months and >12 months , respectively) than the average SOD1 G93A mouse (~ 3 months and 4-5 months), the characteristics of disease progression in the skeletal muscle SOD1 mutant are more comparable to the human phenotype (Wong & Martin, 2010).

The suggestion that skeletal muscle toxicity may causally contribute to ALS pathogenesis is supported by the patterning of observed pathological hallmarks across the time course of disease progression. Indications that skeletal muscle dysfunction precedes neurodegeneration in the SOD1 G93A mutant support the notion that myotoxicity may contribute to NMJ uncoupling and neurodegeneration in ALS. Longitudinal Magnetic Resonance Imaging (MRI) measures of muscle volume across the lifespan of the SOD1 G93A mouse show muscle atrophy at 8 weeks of age, prior to symptom onset (Marcuzzo et al. 2011). Consistent with this, changes in muscle fiber characteristics occur as early as 60 days

of age in the SOD1 G93A mouse: presymptomatic mutant muscles have fibers reduced in cross-sectional area (CSA) and an exaggeration in the slow-twitch phenotype compared to their wild type counterparts (Atkin et al. 2005; Hegedus et al. 2008). Interestingly these studies describe a preferential loss of the large, fast fatigable type IIb muscle fibers in the SOD1 G93A mouse, confirming an earlier report describing the same phenomenon at 50 days of age (Frey et al. 2000).

Other pathological hallmarks of SOD1 G93A skeletal muscle occur prior to motor neuron degeneration, and thus may contribute to NMJ instability. Abnormalities of skeletal muscle mitochondria are well documented in human ALS (Vielhaber et al. 2000; Krasniaski et al. 2005), SOD1 G93A mutants (Zhou et al. 2010; Yi et al. 2011) and skeletal muscle restricted SOD1 G93A transgenics (Dobrowolny et al 2008; Wong and Martin 2010). These studies have described excessive ROS production (Wei et al. 2012), depolarized mitochondria near the NMJ (Zhou et al. 2010), and defective Ca^{2+}- handling (Zhou et al. 2010; Yi et al. 2011) – defects that parallel dysfunction seen in neuronal mitochondria (Rothstein, 2009). Evaluation of ROS production across the lifespan of SOD1 G93A mutant mice indicates ROS production increases (~2-fold) by 50 days of age, and progressively increases (up to ~10-fold) by terminal stages of the disease (Muller et al. 2006). Moreover, the increase in ROS as a result of muscle-specific SOD1 G93A expression has been shown to contribute to atrophy by inhibition of the protein synthesis signals Akt, mTOR, and p70s6k (Dobrowolny et al. 2011). Further the recent generation of a muscle-specific UCP-1 overexpressing mouse that uncouples mitochondrial electron transport and ATP synthesis induces a similar hypermetabolic phenotype seen in the various SOD1 mutants. These mice show progressive dismantling of the NMJ, providing evidence that mitochondrially-derived myotoxicity can

contribute to neurodegeneration (Dupuis et al. 2009). In addition, the impaired ability of the mitochondria to buffer Ca^{2+} transients during E-C coupling (Yi et al. 2011) may contribute to skeletal muscle atrophy in ALS, in light of the intimate involvement of the organelle with Ca^{2+}-dependent cell death signals (Giorgi et al. 2012).

Efforts on part of the skeletal muscle to resist the atrophic signals appear defective. Insulin-like growth factor-1 (IGF-1) is an important anabolic signaling molecule expressed in skeletal muscle that provides a trophic stimulus to both muscle fibers and motor nerves. Expression of both IGF-1 and IGF-1 binding proteins 3, 4, and 5 are all decreased in ALS muscle (Lunetta et al. 2012). The importance of this defect has been highlighted by previous studies that have targeted the IGF-1 signal for therapeutic purposes (Kaspar et al. 2003; Dobrowolny et al. 2005). Viral delivery of a recombinant form of IGF-1 increased survival and delayed symptom onset in SOD1 G93A mice when injected directly into the respiratory and limb muscles (Kaspar et al. 2003). A subsequent study of skeletal muscle-specific overexpression of IGF-1 in SOD1 G93A mice also proved beneficial, also extending survival and symptom onset (Dobrowolny et al. 2005). Both studies also reported a preservation of motor neuron number and size and delayed astrocytosis, indicating that therapies targeting the muscle can improve the health of the motor nerve in ALS.

The pervasive atrophy of skeletal muscle in ALS may also indicate an impaired ability of the tissue to regenerate in the face of denervation. There are limited investigations exploring this possible defect in ALS muscle, however. The few studies that have evaluated the factors associated with myogenesis present conflicting results. One recent report comparing SOD1 G93A skeletal muscle gene expression across the lifespan with a denervated cohort describes a divergent expression pattern of Pax7, an important factor in myogenesis

(Calvo et al. 2012). Interestingly the expression of Pax7 in skeletal muscle increased following surgical denervation, whereas a progressive decrease in expression occurred throughout disease progression in SOD1 G93A mice. Therefore suppressed Pax7 expression may be an indicator of impaired myogenesis in response to the neurodegenerative pathology specific to ALS. Additionally, the gene expression of other factors involved in myogenesis (i.e., myogenic regulatory factors; MRFs), has been reported across the lifespan in comparison to wild type controls. These observations describe the upregulation of MyoD and myogenin mRNA, two factors critical for myogenesis, concomitant with disease progression (Manzano et al. 2011). In contrast, there are no significant increases in MyoD or myogenin protein expression throughout the course of the disease that corresponds to the upregulation of their transcripts. This discordance may indicate an inability of the skeletal muscle in the SOD1 G93A to fully execute the myogenic program possible due to defects in protein translation.

A key component of the myogenic program and regeneration is the action of *satellite cells* - a population of skeletal muscle-specific stem cells that become activated following injury and fuse with existing fibers to repair the tissue (Ciciliot & Schiafinno, 2010). Given their role in muscle maintenance and the pervasive muscle wasting and dysfunction seen in ALS, characterization of this population of cells is of particular interest. Nonetheless, the topic remains largely unexplored by the ALS research community.

Satellite Cells: Role and Regulation in Health and Disease

Electron microscopy first revealed a population of mononuclear cells within adult frog muscle with a distinct anatomical location: situated between the basal lamina and plasma

membrane of skeletal muscle fibers (Mauro, 1961). Referred to as ‘satellite’ cells because of their position around the outside of the fiber, they account for 3-6% of all nuclei associated with a given skeletal muscle cell (Gopinath and Rando, 2008). The hallmarks of satellite cell morphology include a high nuclear to cytoplasmic ratio, few organelles, and small nuclear size relative to myonuclei (Hawke & Garry, 2001). Functionally, the satellite cell population is maintained in a quiescent (i.e., mitotically inactive) state by various cues received from the surrounding microenvironment, referred to as the niche (Cosgrove et al. 2008).Upon disruption of homeostasis of the niche (e.g., from local injury or denervation), a variety of signals activate the population to proliferate in number, differentiate into myogenic precursors, and ultimately fuse with existing damaged fibers for repair (Ciliciot & Schiafinno, 2010). In particular the satellite cell niche is composed of signals from a distinct set of sources, including the muscle fiber itself. Expression of M-cadherins on both the muscle fiber plasma membrane and satellite cell surface allows protein-protein interaction for adhesion and promotes transmission of mechanical cues from the sarcolemma to the satellite cell (Cosgrove et al. 2008). The muscle fiber can also secrete Stromal cell-derived factor (SDF-1α), which interacts with surface receptor CXCR4 on the satellite cell membrane and is required to facilitate proper migratory and fusion responses (Pavlath et al. 2010).

Where the apical surface is in direct contact with the muscle fiber membrane, the basal surface of the satellite cell is bathed in a gelatinous, extracellular matrix (ECM) composed mainly of collagens and laminins that function both as a biophysical support network and as ligands for membrane receptors (Sanes, 2003). These interactions, especially with laminin, encourage proliferation and migration upon satellite cell activation (Foster et al. 1987). Satellite cells are also exposed to a diverse milieu of systemic factors that function to regulate

their activity. Particularly critical is the action of various growth factors, including Fibroblast Growth Factor (FGF), IGF-1, and Hepatocyte Growth Factor (HGF) (Sheehan & Allen 1998).

Of these factors it is thought that HGF is perhaps of most proximal importance for satellite cell activation, as experiments inhibiting the HGF signal nearly abolished proliferation (Tatsumi et al. 1998). In addition to circulating HGF, satellite cells are also subject to local HGF signals, as the factor is expressed in skeletal muscle and released upon injury (Tatsumi et al. 1998). Subsequent experiments have determined that local HGF signaling occurs in NO-dependent fashion, as treatment of muscle-fiber preparations with NO synthase (NOS) inhibitor N^G-nitro-L-arginine methyl ester hydrochloride (L-NAME) prevented stretch-induced HGF release and satellite cell activation (Tatsumi et al. 2006). This evidence supports earlier findings that NOS inhibition delayed satellite cell activation following *in vivo* crush injury (Anderson, 2000). More recent study of this pathway has determined further that an activated Ca^{2+}-calmodulin complex is required for NOS activity, and thus satellite cell activation appears Ca^{2+}-dependent (Tatsumi et al. 2009*b*).

Following activation, satellite cells divide and give rise to progeny of distinct phenotypes. Rounds of asymmetric divisions allow the satellite cell pool to provide regenerating fibers with committed progenitors and simultaneously renew the pool with undifferentiated cells (Wang & Rudnicki, 2011). This phenomenon was reported first in satellite cells that differentially expressed Numb, an inhibitor of Notch signaling, immediately following cell division (Conboy & Rando, 2002). A later study similarly found evidence of asymmetric division of $Myf5^-$ cells giving rise to $Myf5^+$ daughters using time lapse image analysis following cardiotoxin-induced injury (Kuang et al. 2007). Thus it is now the widely

held view that satellite cells use both symmetric and asymmetric modes of division to yield a diverse population of progeny.

The broader expression pattern of surface markers within the satellite cell pool is heterogeneous and includes distinct combinations of CD34, Myf5, α7-/β1-integrins, and CXCR4 (Beauchamp et al. 2000), though all quiescent cells express the paired-box transcription factor Pax7 (Tedesco et al. 2010). This is supported by the finding that satellite cells are noticeably absent in the skeletal muscle of Pax7 null mice (Seale et al. 2000). Pax7 is thought to actively preserve quiescence in the satellite cell population by inhibition of the transcription of various factors known to drive myogenic commitment and differentiation (Olguin & Olwin, 2004). Pax7 functions to repress premature differentiation specifically through transcriptional activation of Id3, an inhibitor of MyoD, (Kumar et al. 2009).

Ultimately Pax7 expression is suppressed as activated satellite cells (i.e., myoblasts) differentiate, possibly through a proteasome-degradation dependent pathway (Olguin & Pisconti, 2011). The process of differentiation that follows is coordinated by the expression of a number of factors which mediate the regeneration process. Temporally the process is regulated by the two key transcription factors, MyoD and myogenin. Early commitment and differentiation is associated with an upregulation of MyoD, thought to be the master transcriptional activator of myogenesis (Edmondson & Olson, 1989). MyoD belongs to a family of basic-helix-loop-helix (bHLH) transcription factors, and upregulates the transcription of muscle-specific contractile and cytoskeletal genes as well as genes associated with more terminal phases of myogenesis, such as myocyte enhancer factor 2 (MEF2) and myogenin (Cao et al. 2011). The role of MyoD in initiating a feed-forward transcriptional signal necessary for differentiation is exemplified by the findings from numerous studies of

MyoD null mice ($MyoD^{(-/-)}$). Satellite cells from these mice exhibit reduced ability to differentiate *in vitro*(Sabourin et al. 1999; Yablonka-Reuveni et al. 1999; Cornelison et al. 2000), underscoring the importance of MyoD in the successful execution of the myogenic program.

More critical for the terminal phase of differentiation is the bHLH transcription factor myogenin, itself a target of MyoD transcriptional activation (Aziz et al 2011). The importance of myogenin in coordinating myogenesis was demonstrated with the development of a mouse expressing a nonfunctional myogenin mutant (Hasty et al. 1993). Mice with the mutation are stillborn at birth, kyphotic, and have many fewer skeletal muscle fibers than their wild type counterparts (Hasty et al. 1993). Recent time course experiments using chromatin immunoprecipitation (ChIP) assays in differentiating C2C12 cells have confirmed that myogenin transcriptional activity predominates during terminal differentiation (day 6), whereas MyoD expression is upregulated upon differentiation (day 1) and subsequently downregulated (day 4) (Davie et al. 2011). In particular, myogenin occupied the promoters of genes whose expression is associated with late myogenesis, including muscle creatine kinase and desmin (Davie et al. 2011). The role of myogenin to induce *and* maintain terminal differentiation was recently shown by the results of knockdown experiments that used an siRNA construct to reduce endogenous myogenin expression (Mastroyiannopoulos et al. 2012). Treatment of terminally differentiated C2C12 myotubes with the siRNA construct induced cleavage in the multinucleate cells and reversion to mono-nuclear myoblast-like cells (Mastroyiannopoulos et al. 2012). These results suggest a more critical role for myogenin in executing the myogenic program than previously thought, and highlight the potential for deleterious consequences in the event of its dysregulation.

Impaired coordination of the myogenic program and consequential satellite cell dysfunction has significant implications for the health of skeletal muscle and contributes to the disease state in various muscle disorders. Muscular dystrophies are a diverse group of muscle-wasting conditions characterized by an enhanced susceptibility to contraction-induced muscle injury, progressive weakness, shortened lifespan, and cardiac dysfunction (Emery, 2002). Because of the dramatic muscle wasting phenotype seen in muscular dystrophy, it has been suggested that the inability of the satellite cell pool to repair repeatedly damaged, dystrophic fibers may contribute to the disease. Indeed both single fiber preparations and cultured satellite cells derived from a transgenic mouse developed to recapitulate the disease (the *mdx* mouse) display premature differentiation *in vitro*, coinciding with accelerated MyoD and myogenin expression (Anderson, 2006). Additional suggestions that repeated rounds of fiber damage lead to exhaustive recruitment of satellite cells early in life and depletion of this pool later in life (Morgan & Zammit, 2010) support the accepted view that satellite cell dysfunction contributes to disease progression in muscular dystrophies.

There are also indications that satellite cell dysfunction is associated with the age-related decline in muscle mass and function. Studies of aged murine skeletal muscle have shown decreased numbers of $Pax7^{+}$ cells compared to younger animals (Brack et al. 2005; Collins et al. 2007). Moreover, cultured satellite cells isolated from aged skeletal muscle display a decreased ability to differentiate *in vitro* (Lorenzen et al. 2004), reduced myosin and muscle creatine kinase protein levels (Lees et al. 2005), and diminished capacity to replenish the pool via self-renewal (Day et al. 2010). Interestingly though, the myogenic potential of aged satellite cells can be restored upon treatment with media supplemented with the serum from young mice, suggesting an alteration in the aged niche is driving the defect in these cells

(Conboy et al. 2005). This finding further suggests these progenitors can retain myogenic capacity into old age, and systemic factors present in young circulation serve as promising targets for developing therapeutic strategies to treat this aspect of muscle dysfunction. Consistently, satellite cell cultures derived from aged murine skeletal muscle subject to 10 days of immobilization-induced atrophy showed decreased proliferative capacity *in vitro*, the effect of which is reversed with infusion of IGF-1 (Chakravarthy et al. 2001). More recent experiments with aged mice treated with an antagonist of TGF-β signaling, the angiotensin II receptor inhibitor Losartan, showed improved regeneration and satellite cell myogenic capacity following cardiotoxin-induced muscle injury (Burks et al. 2011). These results suggest the potential rescue of satellite cell function can restore skeletal muscle health in the context of muscle wasting, indicating the value of understanding the potential role these cells play in a broad range of pathologies. Therefore the focus of this thesis is on the defects in satellite cell function as a possible contributor to the muscle atrophy observed in ALS.

Chapter 3: *Thesis Research Findings*

Introduction

ALS is a devastating neurodegenerative disease that the clinical and biomedical research communities have known to exist for almost 150 years, yet no efficacious treatments are currently available for those afflicted. Our understanding of the disease remains dubious, though the past twenty years have seen significant advancement of our insight into the elusive cellular and molecular mechanisms that ultimately cause ALS. In particular, dysfunction of the motor nerve has been thoroughly characterized in an effort to identify the critical source of pathology that could guide future research to the successful development of a pharmacological strategy that can mitigate, or even reverse the destructive effects of ALS.

Although efforts to delineate both the genetic underpinnings and the neuronal defect in the disease have recently made substantial headway, a new focus has emerged on exploring the possibility that dysfunctional skeletal muscle plays a pathogenic role in ALS. The findings of investigations into this possibility, coupled with our burgeoning knowledge of neuronal disease mechanisms, could prove beneficial in developing therapeutic strategies for the disease population. Therefore the purpose of the current study was to evaluate the satellite cell population in ALS, a critical cellular constituent of skeletal muscle that is not fully characterized in this disease. Using an *in vitro* experimental approach, my thesis research investigated whether satellite cells in the SOD1 G93A ALS mouse model display aberrations in execution of the myogenic program and proliferative capacity. Findings from this research contribute to our growing understanding of the skeletal muscle phenotype in ALS, and may lead to a new therapeutic strategy for disease intervention.

Methods

Animals

SOD1 G93Amice and wild typelittermate controls were obtained from the colony established by the Chin lab at the Central Animal Resources Facility (CARF) at the University of Maryland College Park. All mice were genotyped as either SOD1 G93Amutants (ALS) or wild typecontrols (CON) in accordance with genotyping protocols described previously (Rosen et al. 1993; Gurney et al. 1994) and housed with littermates of the same sex following weaning at 21 days of age. The animals were ear-punched for purposes of identification and housed with *ad libitum* access to water and standard feeding chow. All housing protocols, handling procedures, and experimenters were previously approved for these practices by the Institutional Animal Care and Use Committee (IACUC) at the University of Maryland College Park.

Study Design

The present study examined the temporal expression of MRFs MyoD and myogenin *in vitro* during early and terminal differentiation (Aims 1 and 2), and evaluated proliferative

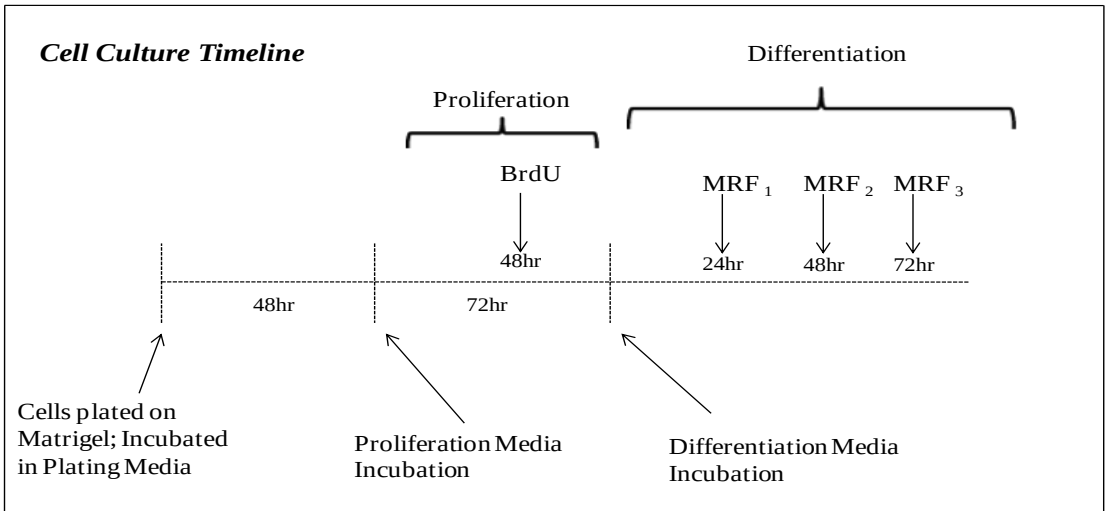


Figure 4.Satellite Cell Culture Timeline. Satellite cells were plated and maintained in culture for evaluation of myogenic factors during differentiation and proliferative capacity. Three time points during the differentiation period were used to assess the expression pattern of MyoD and myogenin(24, 48 and 72 hrs, shown as MRFs $_{1\text{-}3}$ respectively). Proliferative capacity was assessed using BrdUat 96hr.

capacity (Aim 3) during the proliferative phase of culture (see Figure 4).

For Aims 1 and 2, SOD1 G93A and control mice aged 13 weeks were used (n=12 each) and divided into three groups, such that four mice were evaluated per MRF time point (n=4 at 24, 48 and 72 hrs; see Fig. 4, $MRFs_{1-3}$). For each animal, there were six wells of cultured satellite cells: three wells of culture were used to evaluate MyoD expression, and three wells were used to evaluate myogenin expression. Thus a total of twelve cultured wells per timepoint for each of MyoD and myogenin were evaluated (see *Satellite Cell Culture Stain,Immunofluorescence and Imaging* section for details). Animals of the same sex were used at each time point (i.e., all males were used at MRF time points 24hr and 72hr; all females were used for MRF 48hr). Although this was not the ideal study design, it was necessary for the optimum use of all mice in the colony. The number of animals and cultures used were based on historical data from the lab and are consistent with the numbers used in experiments published by other labs (Yablonka-Reuveni & Anderson, 2006).

An additional subset of animals (n=4 females of each condition) were used to harvest and establish satellite cell cultures for proliferative capacity assessment (Fig. 4, BrdU). The use of four animals of each condition thusly yielded 24 wells of cultured satellite cells for each of the SOD1 G93Aand control mice. This number of mice and replicates of satellite cell cultured is greater than those used in published data from other labs (Tatsumi et al. 2009).

Single Fiber Harvest and Satellite Cell Culture

At thirteen weeks of age, animals were sacrificed by cervical dislocation and the paws from both hind limbs were removed above the heel and dissected of their flexor digitorum brevis (FDB) muscles were dissected as previously described (Shefer & Yablonka-Reuveni,

2005). The FDB was cut into strips and digested in 0.2% Collagenase + MEM solution (MEM + 10%FBS + 1% PenStrep) in a tissue culture incubator at 37°C for 4 hours. After 4 hrs, FDB muscle strips were triturated to separate single muscle fibers. Following dissociation, single fibers were incubated (37°C, 95% O_2, 5% CO_2) in MEM solution overnight. Single fibers were plated the following day on Matrigel-coated 24-well culture plates and supplemented with 0.5mL Plating Media (DMEM + 10% Horse Serum + 0.5% Chick Embryo Extract + 1% Penstrep). Single fibers from two (2) FDB muscles of one mouse were sufficient for plating six wells, and thus one six-well row of cultures was maintained per animal. Fibers werekept in Plating Media for 48 hours, with media replaced at 24 hours to account for depletion of substrates in the media. During this period the satellite cells migrate off of the single fibers and settle onto the Matrigel-coated plates. After 48 hrs in Plating Media, the cultures were shifted to Proliferation Media (DMEM + 20% Fetal Bovine Serum + 10% Horse Serum + 2% Chick Embryo Extract + 1% PenStrep). Proliferation was induced for three days with media replaced every 24 hours. For the series of satellite cells evaluated for MRF expression, differentiation was induced by shifting the cells into a low serum media (DMEM + 2% Fetal Bovine Serum + 1% PenStrep) for 24, 48, or72 hours for each MRF evaluation (Fig. 4; separate 24 well plate for MRF_1, MRF_2, andMRF_3 respectively). As with the earlier phases, low serum media was replaced every 24 hours to maintain culture conditions. The cultures assessed for proliferative capacity were evaluated at 48 hours following proliferation initiation.

Immunofluorescent Staining and Imaging of Cultured Satellite Cells

In order to evaluate MyoD and myogenin expression at 24, 48, and 72 hours in the differentiation phase of culture, immunofluorescence microscopy was performed on both SOD1 G93Aand control satellite cell cultures. In order to gain insight of the concomitant expression of both MRFs in cultures derived from the same animal, both MyoD and myogenin expression were evaluated in each animal (see Figure 5). For detection of MRF expression, cells were fixed with 10% neutral buffered formalin, blocked with a Fab-fragment to minimize non-specific binding, stained with primary antibodies specific for either MyoD or myogenin, and incubated with anti-mouse FITC conjugated secondary antibody for subsequent fluorescence analysis. All cells were additionally stained with DAPI to confirm nuclei location.

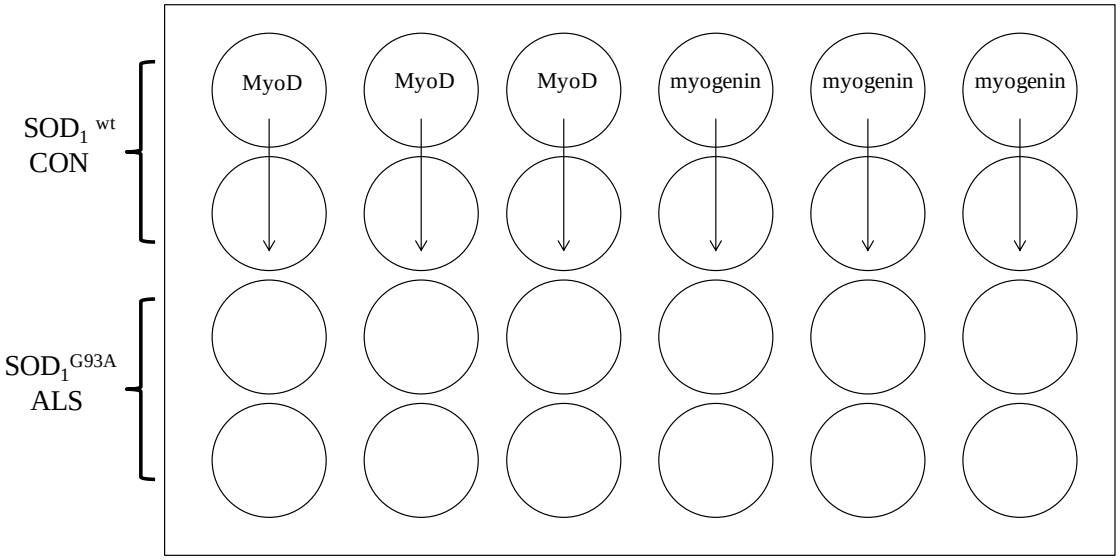


Figure 5.Satellite cell plating schematic. 24-well plates were used to culture satellite cells from SOD1 G93Aand controls mice, with each row of wells corresponding to one animal. Evaluation of MRF expression was carried out in parallel, such that three (3) wells from each row were stained for MyoD, three (3) were stained for myogenin.

Immunofluorescence was detected and images were obtained using a Nikon TiU microscope and Nikon Elements Imaging software. Five fields were selected randomly and

imaged from each well. Both the total number of nuclei per field (DAPI stained) and nuclei staining positive for MRF expression were counted from each field. The ratios of MRF expressing nuclei were determined by normalizing the total number of MRF positive nuclei divided to the total number of nuclei present (DAPI positive). This index will be referred to as the MRF Index (i.e., Total MRF (+) / Total DAPI). The MRF Index values were calculated per field and then an average value obtained for each well (n=12) and for each mouse (n=4) for both control and SOD1 G93A genotypes.

In order to assess the relative abilities of satellite cell cultures derived from SOD1 G93Amutants and control animals to proliferate *in vitro*, BrdU labeling experiments were conducted as described previously (Tatsumi et al. 1998). Cultures were pulse labeled with 10µL 5-bromo-2-deoxyuridine (BrdU) during the 46-48th hours of the 72 hour proliferation period. Incorporation of BrdU occurs in satellite cells undergoing DNA synthesis, and therefore subsequent detection of BrdU was used as an index of proliferative capacity. BrdU positive cells were detected following fixation using an anti-BrdU monoclonal antibody and a horseradish peroxidase conjugated anti-mouse secondary antibody. Following BrdU antigen retrieval and staining, each well was stained with DAPI to index the total number of nuclei present per well. Detection of the BrdU-positive, colorimetric stain was made using live Nikon TiU microscope bright field quantification. The total number of BrdU-positive nuclei was counted per well and averaged across all wells for each condition. These raw numbers were then normalized to total nuclei present per well by measuring total DAPI fluorescence using a fluorescence plate reader (Synergy H1 Hybrid Reader;Biotek). These DAPI fluorescence measures were further used to compare nuclei density at 48hr following induced proliferation in each of the SOD1 G93A and control conditions. In a separate set of

experiments using C2C12 cells, the linearity of the DAPI fluorescence stain was validated (see Appendix A). The indices of proliferation (both raw and normalized BrdU counts) were averaged for each well (total of 12 wells) and each animal (4 wells per animal) for both control and SOD1 G93A genotypes.

Statistical Analysis

Differences in the MRF index values for both MyoD and myogenin were compared between control and SOD1 G93A mice using a two-tailed unpaired t-test with SPSS. Comparisons of indices of proliferation (both Raw and normalized BrdU counts) were also compared between control and SOD1 G93A mice using the two-tailed unpaired t-test (SPSS). Differences were deemed significant at $p < 0.05$.

Results

MRF Experiments

Our lab previously described an impaired ability of cultured satellite cells derived from the SOD1 G93A mouse to form myotubes *in vitro* compared to age-matched wild-type control cells. To investigate if this impairment coincides with dysfunctional execution of the myogenic regulatory program, this study examined the temporal protein expression pattern of the two key myogenic regulatory factors (MRFs) MyoD and myogenin, critical for early and terminal differentiation respectively. MyoD expression in differentiating satellite cell cultures derived from 13-week old SOD1 G93A mice and age-matched wild-type controls at 24, 48, and 72hr following serum starvation is shown in Figure 6**.**At 24hr following induced-differentiation, MyoD MRF Index for SOD1 G93A mouse-derived satellite cell cultures was reduced to 53.3% of the MyoD MRF Index of control satellite cells (24.2 ± 3.8% *vs.* 12.9%,

CON *vs*. ALS, $p < 0.05$; mean ± SE). The reduced MyoD MRF Index in satellite cells from SOD1 G93A mice at 24 hrs indicates a reduced capacity to initiate the events necessary for early differentiation. Further, SOD1 G93A cultures failed to show an increase in MyoD MRF Index at subsequent 48hr and 72hr time points (12.9 ± 2.1% and 11.1 ± 1.8%, 48, and 72hr respectively) suggesting that there was no delayed increase in MyoD protein expression after 24 hrs. In satellite cells from control mice, the MyoD MRF Index was subsequently reduced (15.3 ± 2.1% at 24hr and 8.9 ± 1.5% at 48hr) as cells continued to differentiate, consistent with previous reports of the acute temporal increase in MyoD expression during early-phase differentiation (Cao et al. 2011; Davie et al. 2011).

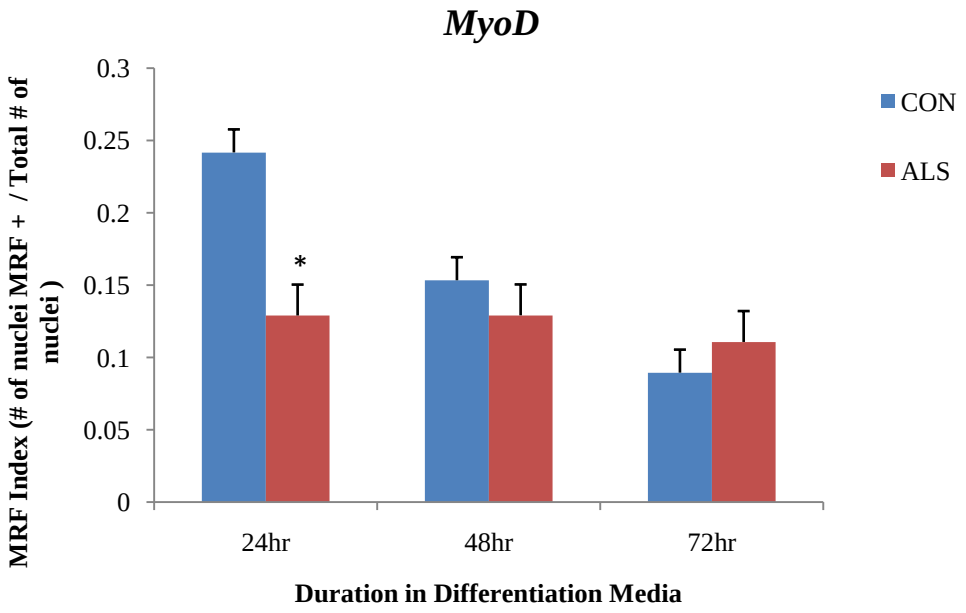


Figure 6. MyoD MRF Index during differentiation. Ratio is significantly reduced in ALS animals at 24hr, but show no differences at 48 or 72 hr. n = 4 animals of each condition at each time point. Data represented as means ± SE. Significance indicated as * = p <0.05.

The myogenin MRF Index in differentiating satellite cell cultures from 13-week old SOD1 G93A mice and age-matched wild-type controls are shown in Figure 7. Myogenin

MRF Index at 24hr was significantly lower in SOD1 G93A animals compared to controls (16.25 ± 2.8% *vs.* 8.7 ± 1.2%; $p<0.05$), but was not different at either 48 or 72 hr (8.1 ± 1.4% *vs.* 9.2 ± 1.6%, CON *vs.* ALS at 48hr; 5.9 ± .85% *vs.* 9.0 ± 1.4%, CON *vs.* ALS at 72hr).

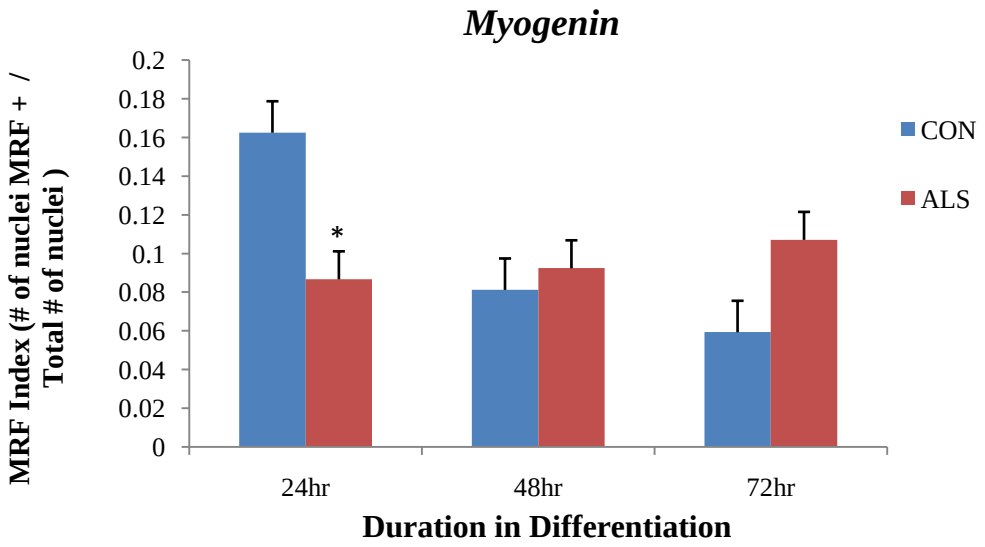


Figure 7. Myogenin MRF Index during differentiation. Myogenin MRF Index is reduced at 24hr in the ALS group compared to the CON group, but no significant changes were seen in MRF Index at 48hr or 72 hr. n = 4 animals of each condition at each time point. Data represented as means ± SE. Significance indicated as * = p <0.05.

In order to address the within vs. between animal variability in the behavior of cultured satellite cells, additional analyses were carried out to examine the MRF Indices at the 24hr time point. Distribution of the MRF Index values per animal for both control and SOD1 G93A genotypes are shown in Figure 8. Qualitative evaluation of the distribution of average MyoD MRF Index per animal(Figure 8A) indicates a small range associated with the SOD1 G93A at 24hr (0.067 - 0.155) compared to the control mice(0.124-0.512). Similarly, the MRF Index for myogenin (Figure 8B) in the SOD1 G93A cohort had a smaller range of values (between 0.062 - 0.111) relative to the control values (0.024-0.247).

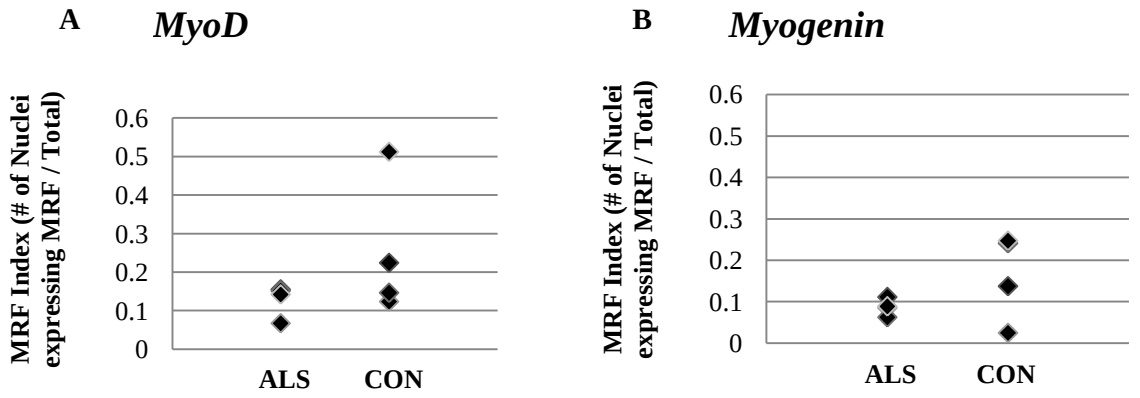


Figure 8 (A-B).Myogenic Regulatory Factor (MRF) Index for MyoD (A) and Myogenin (B) per animal following 24hr of differentiation. n = 4 in both the ALS and CON conditions. Each data point (♦) represents the MRF Index for a given animal, averaged across 15 fields imaged from 3 wells of culture.

In order to gain further insight into the spread of the MRF Index values per field of imaged satellite cells for both genotypes, the distribution of the MRF Indices are represented graphically for the 24hr time point (Figure 9; see also Appendix B). The distribution of MRF Index values per field for both MyoD and myogenin are also shown in Table form (Appendix B). The distribution of MRF Index values for SOD1 G93A satellite cell cultures show no fields with a MRF (+) / Total DAPI (+) ratio greater than 0.5 (i.e., fields of view with 50% or more nuclei positive for MRF) in either the MyoD or myogenin stained wells. In contrast, control cultures more frequently revealed fields with an MRF Index greater than 0.5 (20% of MyoD-stained and 8.3% of myogenin-stained fields with 50% or more cells staining positive for the MRF). Additional per well and per field distributions can be viewed in the Appendix D.

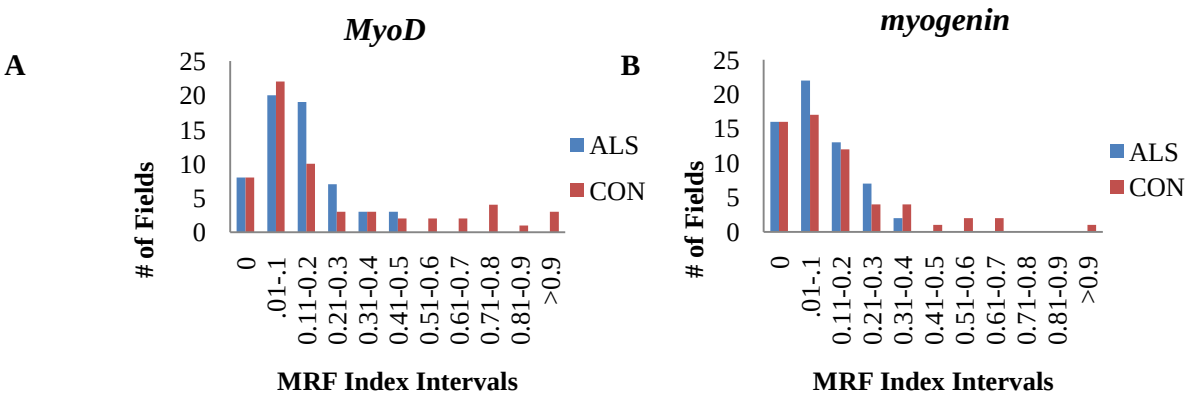


Figure 9.Frequency distribution of MRF Index ratios per field at 24hr for MyoD (A) and myogenin (B). Both MyoD and Myogenin distributions indicate numerous CON fields with MRF Indices above 0.5, whereas no ALS Indices were found > 0.5. Data represented as total number of observances of fields with MRF Index values within the intervals shown.

BrdU Experiments

In order to investigate if SOD1 G93A satellite cells show a reduced capacity to proliferate *in vitro*, BrdU labeling experiments were carried out 48hrs following induced proliferation in culture. Nuclei positive for BrdU incorporation were counted per well and averages of these raw counts are indicated in Figure 10. Wells containing SOD1 G93A-derived satellite cell cultures had significantly fewer BrdU-positive nuclei compared to control cultures (230.2 ± 46.9 *vs.* 58.3 ± 11.9; CON *vs.* ALS; $p < 0.001$; means ± SE).

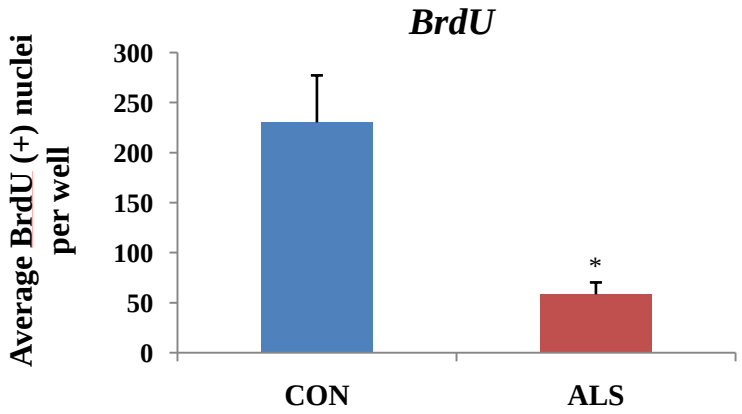


Figure 10.Average BrdU-positive nuclei per well. Average BrdU-positive nuclei in the ALS wells exhibit a 75% decrease relative to CON cells. Data represented as means ± SE. n = 4 animals per condition.

Because total BrdU positive cell counts per well may be biased by a different number of satellite cells seeded per well, nuclei were counterstained with DAPI and resultant fluorescence measured to indicate total nuclei present in each well. Averages for SOD1 G93A and control BrdU positive nuclei normalized to the DAPI fluorescence value are displayed in Figure 11. The ratio calculated for SOD1 G93A cultures was significantly reduced compared to control cultures (1.72 ± 0.389*vs.*0.363 ± 0.103; CON *vs.*ALS). Thus, the relative reduction in BrdU positive nuclei in SOD1 G93A vs. control satellite cells is 25% and 21% (raw values and normalized, respectively) indicating a significant reduction in proliferative capacity in the cells from SOD1 G93A mice.

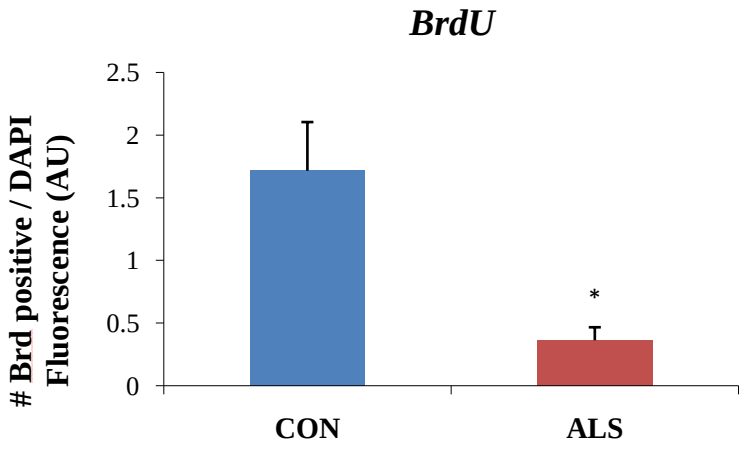


Figure 11. Total BrdU (+) nuclei normalized to per well DAPI fluorescence. The 21% reduction in ALS culture BrdU(+) cells normalized to DAPI fluorescence indicates reduced proliferative capacity at 96hr compared to CON cultures. n = 4 animals of each condition, * indicates p <0.01. Data represented as means (arbitrary units) ± SE.

The DAPI fluorescence values also provide some insight into differences in total number of cells that migrated and adhered to the matrigel coated plates. There was a significant difference in the total DAPI fluorescence between control amd SOD1 G93A cultured satellite cells (178.9 ± 19.1 *vs.* 104.8 ± 8.8, respectively; p <0.01) (Figure 12). This

42% reduction in DAPI fluorescence confirms our previous findings of fewer nuclei per well of cultured satellite cells.

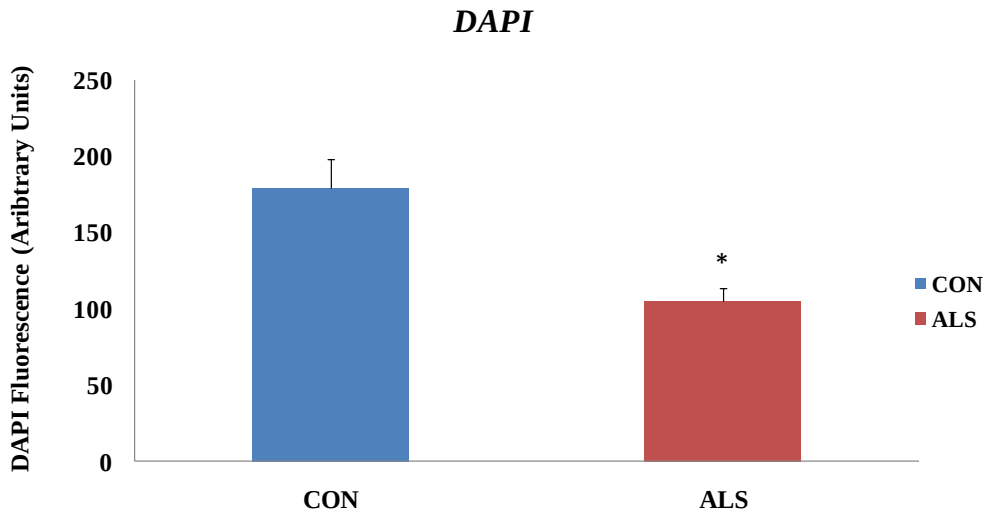


Figure 12.Average DAPI fluorescence across CON and ALS wells at 96hr. Significantly decreased signal in ALS wells indicates smaller number of nuclei compared to CON. n = 4 animals of each condition, * indicates p <0.01. Data represented as means (arbitrary units) ± SE.

Discussion

Our lab has previously detected alterations of the ability of satellite cells derived from presymptomatic SOD1 G93A mice to form myotubes*in vitro* (English et al. 2012). The results from this study demonstrate, for the first time, that the impaired fusion capacity of these cells may be due to the dysregulated expression of myogenic regulatory factors, reduced proliferative capacity, or a combination of the two. SOD1 G93A satellite cell cultures induced to differentiate showed significantly reduced MyoD positive cells 24 hours following serum-starvation when compared to control cells subjected to the same differentiation stimulus. However no differences in MyoD expression were detected at 48 or 72 hours. Similarly at 24 hours, frequency of myogenin (+) nuclei was significantly reduced in SOD1 G93A cultures compared to controls, though average myogenin (+) nuclei was not different at the more

terminal 48 and 72 hour time points. Additional experiments labeling actively proliferating satellite cells revealed a significant reduction (~75% decrease) in the number of SOD1 G93A-derived cells that had incorporated BrdU, a marker of DNA synthesis, compared to control cells. These results suggest that the cellular and molecular mechanisms that regulate both differentiation and proliferation in the satellite cell population of SOD1 G93A mice are defective and may contribute to the impaired capacity of these cells to successfully form new myotubes *in vitro*.

MyoD expression is dysregulated early in differentiation

The results of the current study indicate a failed ability of SOD1 G93A satellite cells to substantially upregulate MyoD at any point during the first 72 hours following induced differentiation, with a notable decrement number of MyoD-(+) nuclei at 24 hours compared to control nuclei. MyoD belongs to a family of basic helix-loop-helix transcription factors required for myogenesis, called myogenic regulatory factors (MRFs), that facilitate the commitment of myogenic precursors to the muscle lineage and the concomitant expression of genes required for myogenic development (Edmonson & Olson, 1989). Specifically the temporal expression pattern of MyoD is crucial, where its expression is upregulated early in differentiation and subsequently downregulated (Cao et al. 2011, Davie et al. 2011). Without upregulation of MyoD in early phases of myogenesis, differentiation is delayed and regeneration is deficient (Sabourin et al. 1999; Yablonka-Reuveni et al. 1999; Cornelison et al. 2000). Thus the observed decrease of MyoD expression 24 hours following induced differentiation may have significant implications for successful progression of SOD1 G93A satellite cells through myogenesis. The sustained suppression of MyoD expression in the SOD1 G93A cultures at 48 and 72 hours suggests that the failure to initiate myogenesis

extends beyond early phases of differentiation and persists, yielding few fusion-competent myoblasts for terminal differentiation.

A previous report of satellite cell behavior from human skeletal muscle of ALS patients also indicates an impaired ability to differentiate (Pradat et al. 2011*b*). In this study, satellite cell cultures from patient muscle biopsies formed thin myotubes with reduced MHC expression compared to the cultures of healthy control subjects. These authors also reported nuclei within ALS muscle sections staining positive for neonatal myosin, MyoD, and myogenin, whereas control sections showed no such staining, suggesting the regeneration process is active in symptomatic patients (Pradat et al. 2011). Further comparison to our study is limited, however, as their tissue samples were derived from symptomatic human muscle.

In seeming contrast to the findings of this study, recent reports have described increased levels of MyoD mRNA in the skeletal muscle of presymptomatic SOD1 G93A mice (Galbiati et al. 2012; Manzano et al. 2011) and C_2C_{12} cells transfected with mutant SOD1 (Galbiati et al. 2012). It should be noted, however, that the MRF mRNA changes reported by *Manzano et al.* (2011) failed to correspond to the changes observed in the MRF protein data from the same muscle samples. Moreover these changes were observed in whole muscle extracts, and given the distinct roles of these factors in muscle (reviewed in Gunderson, 2011), may reflect changes within the muscle fiber itself and not the satellite cell population.

The mutant C_2C_{12} line utilized by *Galbiati et al.* (2012) may represent a more relevant model for studying the possible myogenic defect in the SOD1 G93A mouse, though the absence of protein measures and discordance of MRF mRNA and protein levels reported elsewhere (Manzano et al. 2011) suggest cause for skepticism. Furthermore the C_2C_{12} cells

were transfected merely 48 hours prior to harvest, which fails to recapitulate the chronic pathological phenotype characteristic of the SOD1 G93A mouse.

A recent publication suggesting a possible link between mutant SOD1-related pathology and impaired differentiation shows evidence that the Unfolded Protein Response (UPR) induced C/EBP homology protein (CHOP) directly suppresses MyoD expression by binding to its upstream regulatory sequences and reducing its transcription (Alter & Bengal, 2011). Interestingly, data from our lab indicates an upregulation of CHOP in the skeletal muscle of presymptomatic SOD1 G93A mice, possibly a result of misfolded mutant SOD1 protein accumulation in the endoplasmic reticulum (D. Chen & E. Chin, unpublished observations). Though this observation is not specific to the satellite cell population, it is plausible to speculate that an accumulation of misfolded SOD1 protein and other protein aggregates within the satellite cell results in an upregulation of CHOP expression following UPR induction, and consequentially repressing MyoD transcription and translation (see Figure 13).

Myogenin expression is suppressed early in differentiation

Myogenin expression was significantly reduced in SOD1 G93A cultures compared to controls at the early 24 hour time point following induced differentiation. Typically myogenin expression is upregulated terminally in the differentiation process subsequent to early commitment defined by MyoD expression. However the exact timing of its upregulation during differentiation induction is not precisely known, because published reports vary in satellite cell isolation techniques and culture methods (Tatsumi et al. 2009*b*; Yablonka-Reuveni et al. 2006; Anderson, 2000). It should also be noted that the early increase in

myogenin expression occurred in a small proportion of the total cultured population (16.25% *vs.* 8.7% in CON *vs.* ALS satellite cells, respectively), which is possibly in a subset of prematurely differentiating, contact-inhibited cells.

Moreover, our technique for obtaining satellite cells may activate the quiescent population prior to experimentally induced proliferation and differentiation, giving rise to a small population of precocious cells. Specifically, it is possible that the trauma induced by the dissection technique is activating some of the satellite cells prior to experimentally induced proliferation and differentiation. This would result in two distinct populations of cells: (1) a population that migrates off the fibers but is not activated by mechanical dissection, and (2) the satellite cells that were sufficiently stimulated by the trauma of dissection and proceed through culture on a trajectory of premature proliferation and differentiation. Thus it is possible that the difference observed at 24 hours may be an artifact engendered by our cell-isolation technique and culture methods. Alternatively, it is worthwhile to note that parallel cultures from the same mice exhibited a similar disparity in MyoD expression. Because MyoD expression is critical for myogenin upregulation, it is reasonable to hypothesize that reduced myogenin expression in cultures from the same mice is predicated by the observed concomitant decrease in MyoD.

The absence of robust myogenin upregulation at more terminal (i.e., 48 and 72 hr) phases of satellite cell culture in both SOD1 G93A and control cells may also be due to an insufficient duration allowed for complete execution of the myogenic program. Some reports show myogeninupregulation at 96 hours (Tatsumi et al. 2009*b*) or even as late as eight days (Davie et al. 2011; Yablonka-Reuveni et al. 1999). Investigations beyond 72 hours in low-

serum media may provide a more appropriate model for examination of terminal differentiation in the cell isolation and culture methods used in the present study.

Per animal and per field data offer further insight into MRF dysregulation

Upon closer inspection of the 24 hour time point, MRF Index averages per animal (shown in Figure 8) indicate minimal variability within the SOD1 G93A cohort, whereas there was some spread in the MyoD and myogenin MRF index in the control group. It was expected that there might be greater variability in satellite cell culture response between SOD1 G93A mice given the possibility that each mouse could have a different transgene copy number. However, the reduced variability in the SOD1 G93A averages at 24 hours mitigates the concern that mutant SOD1 gene copy number within this group could result in an inconsistent phenotype, (Mead et al. 2011), at least with respect to satellite cell behavior *in vitro*. Interestingly a wide range of averages was seen in the MRF Index values of the control mice, despite controlling for sex and other factors. These data suggests heterogeneity of the *in vitro* satellite cell behavior within animals of the non-mutant population that is not seen in the SOD1 G93A animals.

Examination of the recorded MRF Index values per field reveals additional information about the myogenic performance of the cultured satellite cells from each group. Though both the SOD1 G93A and control cultures had similar numbers of fields with MRF Index measures of 0.0, 0.0-0.1, and 0.11-0.2, MRF values greater than 0.5 (i.e., more the 50% cells within a given field expressing MyoD or myogenin) were only observed in the cell cultures derived from the control animals. It is well accepted that successful execution of the

myogenic program is in part dependent on the secretion of local factors that act in an autocrine fashion to promote proliferation and fusion. Myoblasts concurrently secrete chemokines and express their complementary receptors, exemplified by studies of IL-4 and its receptor IL-4Rα in satellite cell cultures (Horsley et al. 2003). Various other receptor-ligand pairs have recently been identified, and exhibit temporal expression patterns, suggesting the critical role this signaling mechanisms plays in regulating myogenesis (Griffin et al. 2010). Accordingly, the expression of chemokine receptor CXCR4 has been shown to be critical for differentiation *in vitro*, where loss of the protein significantly reduced the expression of MyoD and myogenin and differentiation into myotubes in cultured C_2C_{12} cells (Ödemis et al. 2007). Satellite cells also express and secrete TGF-β family member myostatin, an inhibitor of satellite cell proliferation and differentiation (Joulia et al. 2003). Upregulation of these autocrine signaling pathways might lead to the spatial dysfunction suggested by the per field measures, Though this hypothesis remains undeveloped, the important role this signaling mechanism plays in regulating myogenesis warrants characterization in the SOD1 G93A satellite cell population.

BrdU labeling is reduced during proliferation

BrdU labeling is a commonly used methodology to measure proliferation in satellite cell cultures (Tatsumi et al. 1998; Anderson, 2000). Results from utilizing this technique in the current study demonstrate reduced proliferative capacity in the satellite cell cultures isolated from SOD1 G93A mice compared to cultures isolated from control mice. Reduced proliferative capacity in these cells was reflected in the comparatively lower total number of nuclei that had incorporated BrdU following its supplementation in the culture media during proliferation *in vitro*. Quantified BrdU positive cells were subsequently normalized for total

DAPI fluorescence per well to confirm the observed phenomenon was not simply due to more total cells present in wild-type cultures. The potential consequences of reduced proliferative capacity in this cell type include an impaired ability to replenish the satellite cell pool (i.e., self-renewal) and/or the diminished capability to mount a sufficient response to injury (e.g., denervation).Therefore one can postulate that satellite cell dysfunction may contribute to the atrophic phenotype seen in ALS.

Interestingly, SOD1 G93A and control cultures also differed in terms of total cells present at 48hr following proliferation, as indicated by reduced DAPI fluorescence in the mutant cultures (Figure 12).This result suggests that in addition to an impaired ability to proliferate compared to controls, the satellite cell population from SOD1 G93A mutants may have a lesser capacity to migrate off of fibers at earlier points in culture, reduced ability to adhere to the Matrigel-coated culture dishes, decreased proliferative function prior to the 48hr time point, or significant rates of apoptosis that suppress increases in cell number. These hypotheses require further investigation, however, to characterize more fully the behavior of these cells *in vitro*.

The observation of reduced proliferative capacity in these cells may offer additional insight into plausible inter- or intracellular signaling defects resulting from the presence of mutant SOD1. Activation of satellite cells is mediated by the presentation of hepatocyte growth factor (HGF) to the c-met receptor following local trauma. An elegant series of studies by *Tatsumi et al.* provide evidence that the activation process occurs in an autocrine manner, whereby HGF tethered to the extracellular matrix (ECM) surrounding the satellite cell is released, following initiation of a Ca^{2+}/calmodulin-, nitric oxide- (NO) dependent signaling cascade (Tatsumi et al. 2005; Tatsumi et al. 2009*a*, Hara et al. 2012). Although this pathway

likely contains intermediaries yet to be described, it is reasonable to speculate that defects in the components of this layered signaling axis may contribute to reduced proliferation in the SOD1 G93A satellite cell. Because this process is facilitated by local HGF, and given the aberrant skeletal muscle phenotype in the SOD1 mutant, a decrement in endogenous HGF may reduce proliferation. No reports to date, however, have addressed this possibility.

c-met receptor dysfunction is another promising target for potential contribution to disruption of the satellite cell activation signaling cascade. An *in vitro*study of renal epithelial cells that readily express c-met found that oxidative stress negatively regulates its transcription and consequent protein levels (Zhang et al. 2010). A similar model could be proposed to describe the SOD1 G93A satellite cell population whereby mutant SOD1 disrupts the intracellular redox balance and results in decreased c-met expression, reducing the capacity of the cell to become activated and proliferate. Equally possible is dysfunctional signal transduction downstream of c-met-dependent activation, or defective cellular mechanisms that normally allow for successful proliferation (e.g., protein synthesis, cell cycle regulation, cytokinesis). Thus for a comprehensive understanding of defects that contribute to reduced proliferation in the SOD1 G93A satellite cell population, further study of the matter is required.

Proposed implications of satellite cell dysfunction in the pathogenesis of ALS

The results from the current study suggest defects in both proliferation and differentiation in SOD1 G93A satellite cell cultures compared to wild-type controls. Figure 12proposes a model suggesting mechanisms that may underlie these observed dysfunctions. The hallmark oxidative stress in the SOD1 G93A mouse (Patel & Hamadeh, 2008) may

present a significant stimulus to repress c-met expression (Zhang et al. 2010). Such impairment would decrease the ability of the SOD1 G93A satellite cell population to receive obligatory activation signals from HGF (Tatsumi et al. 1998). The capacity to effectively initiate the myogenic regulatory program and differentiate may itself be due to the upregulation of ER-stress induced protein CHOP, which directly inhibits MyoD expression (Alter & Bengal, 2011). The ramifications these defects impart on the health of skeletal muscle in ALS may be significant, as the satellite cell population is required for regeneration in adult muscle (Sambasivan et al. 2011).

Recent evidence has also suggested that satellite cells play an active role in coordinating reinnervation at the neuromuscular junction, specifically by secretion of neural chemorepellant Semaphorin3a in early differentiation (Tatsumi et al. 2009a). Interestingly, this protein is selectively overexpressed in the IIb fibers of SOD1 G93A mice (De Winter et al. 2006). Because these fibers show an enhanced susceptibility to denervation in ALS mice (Hegedus et al. 2008), Semaphorin3a is a target apt for further characterization in the disease.

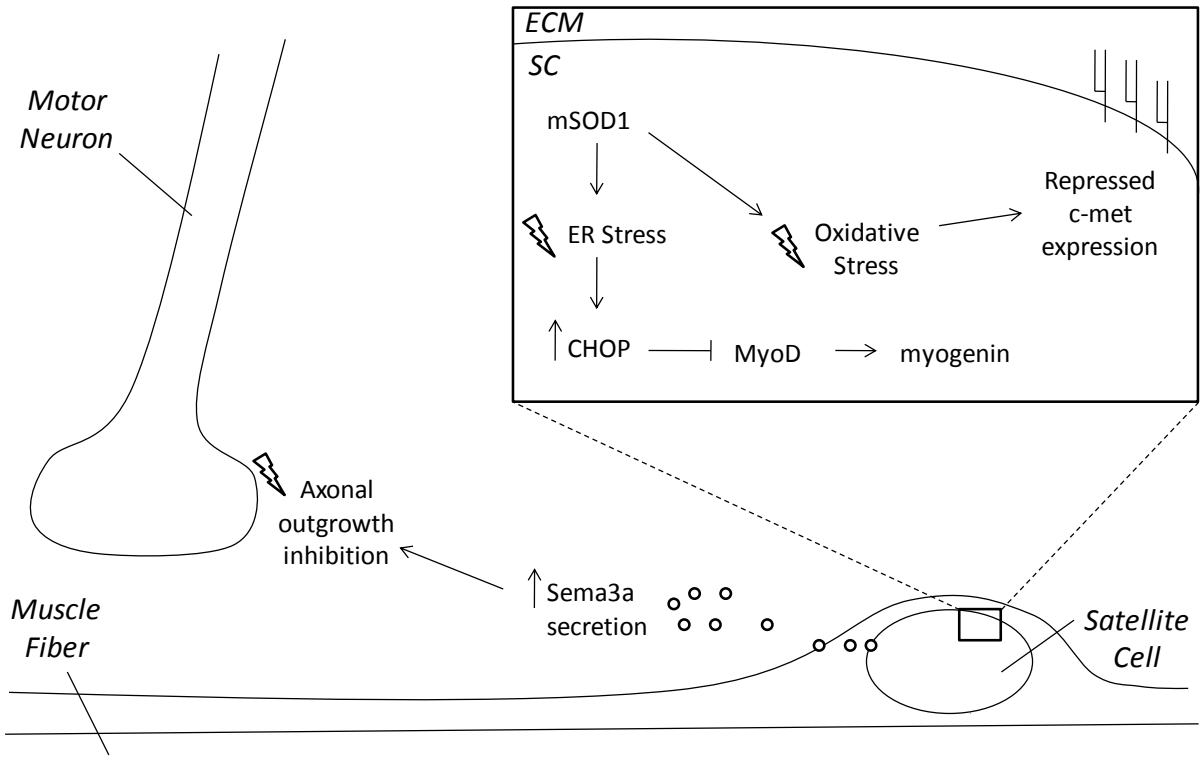


Figure 13.Schematic representation of proposed contribution of satellite cell dysfunction to ALS pathogenesis.The inset graphic (Top Right) summarizes the possible mechanisms underlying the defects in proliferation and differentiation observed in the current study. The larger portion of the cartoon depicts potential interaction of the satellite cell with the motor neuron as suggested by the findings of previously conducted studies (see Tatsumi et al. 2009*b*; De Winter et al. 2006).

Future Directions

Various avenues of future research now appear attractive in light of the findings of this study. Though the immunohistochemistry performed here provides the first evidence of dysfunctional MRF expression and reduced proliferation in the satellite cell population of the SOD1 G93A mouse, these findings would be strengthened if recapitulated in other models of study. Intact single muscle fiber cultures, for example, offer a unique view of satellite cell behavior while contained within their native niche. Using this approach may reveal additional dysfunctional characteristics of differentiation or proliferation otherwise unseen in the culture design used in the current study. Further it would be beneficial to confirm the changes in

immunofluorescently-detected MRF expression with protein levels measured by western blot. Measuring these protein targets from cell scrapings would take the entirety of the wells of culture into account and eliminate concerns of variability within a given well.

The signaling axis responsible for activation of satellite cells may be defunct in the SOD1 G93A mouse, as suggested by the reduced proliferation indicated by BrdU labeling experiments. Thus a logical follow-up to this study would be to assess the viability of the proteins associated with activation. Targets would therefore include HGF and c-met, either at the level of protein content or protein activity.

A question that has not been addressed is whether the SOD1 G93A satellite cell population actively contributes to decoupling of the neuromuscular junction. A recent study has shown that satellite cells contain an axonal growth inhibitor within their secretome, called Semaphorin3a, which is normally secreted following differentiation to coordinate NMJ remodeling (Tatsumi et al. 2009). Aberrant upregulation of this protein has been demonstrated to localize to type IIb skeletal muscle fibers in SOD1 G93A mice (De Winter et al. 2006). Taken together, these pieces of evidence highlight an unexplored and potentially critical pathological process in ALS, warranting further study.

Yet another unexplored aspect of ALS pathology is the capacity of diseased skeletal muscle to respond to injury and regenerate *in vivo*. The notion that regenerative capacity could be impaired supported by the findings of this study, that reduced myogenic capacity resulting inimpairedproliferation and differentiation suggest that SOD1 G93A muscle may be responding to traumatic stimuli other than denervation (i.e., increased myocellular redox stress). Experimentally this could be determined by inducing injury and characterizing the

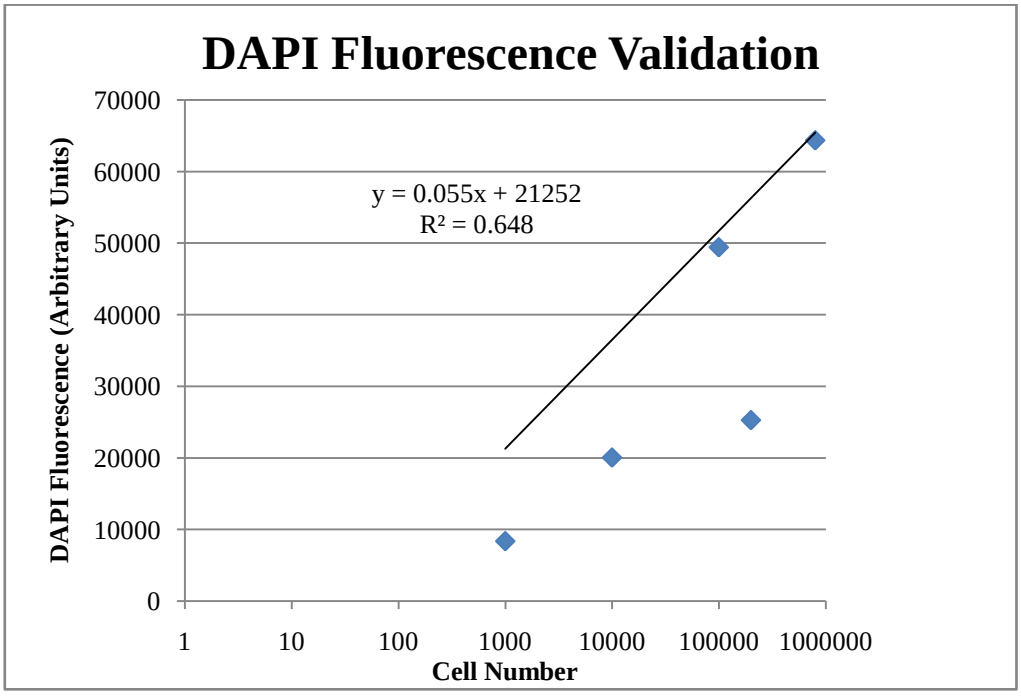
regenerative response in SOD1 mutant mice. One can speculate further that such a response to injury may elicit physiologically distinct consequences to one day be used as a biomarker in clinical ALS diagnoses.

Chapter 4:*Conclusions*

The results from this study demonstrate that the impaired fusion capacity of SOD1 G93A satellite cells may be due to the dysregulated expression of myogenic regulatory factors, reduced proliferative capacity, or a combination of the two. Specifically, as indicated by fewer nuclei positive for either MyoD or myogenin, SOD1 G93A satellite cell cultures appear suppressed in early and terminal differentiation compared to wild type satellite cell cultures. Experiments using BrdU incorporation as an indicator of proliferation show fewer proliferating cells in SOD1 G93A satellite cell cultures when compared to wild type satellite cell cultures. These results suggest that the cellular and molecular mechanisms that regulate both differentiation and proliferation in the satellite cell population of SOD1 G93A mice are defective and may contribute to the impaired capacity of these cells to successfully form new myotubes *in vitro*.

Moreover, these results further contribute to an emerging wealth of evidence that describes the dysfunctional characteristics of skeletal muscle in ALS as significant and distinct. The results from this thesis suggest additional ways whereby satellite cell dysfunction may actively contribute to neuromuscular decoupling.Given the role of the satellite cell population in skeletal muscle and the NMJ regeneration, further investigation should be undertaken to comprehensively evaluate how their dysfunction may contribute to the pathology of the disease.

Appendix A.Linear regression for BrdU Fluorescence and number of cells plated.



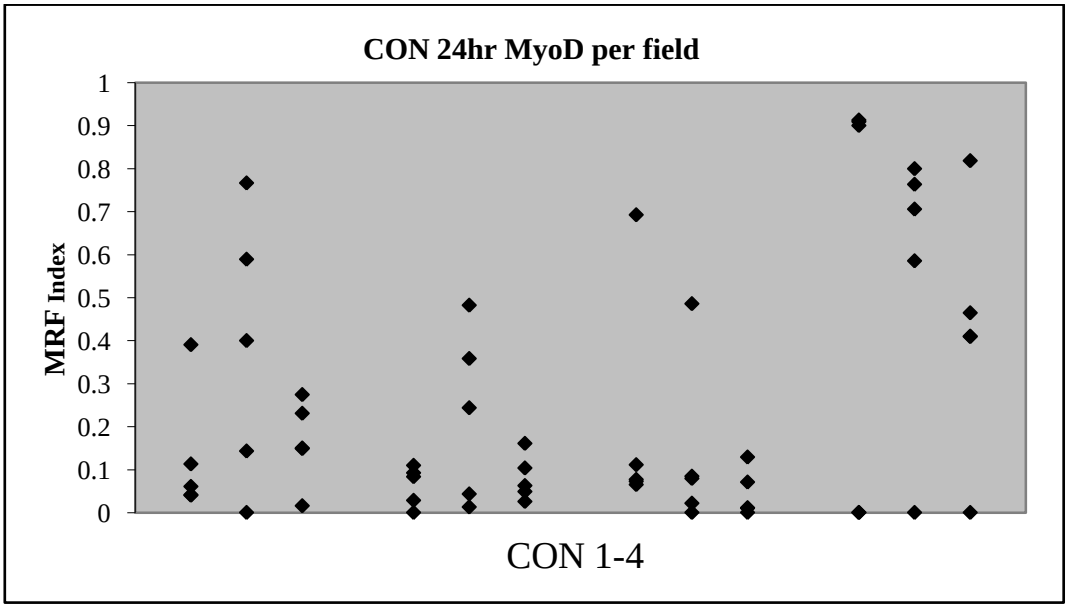
Appendix B.

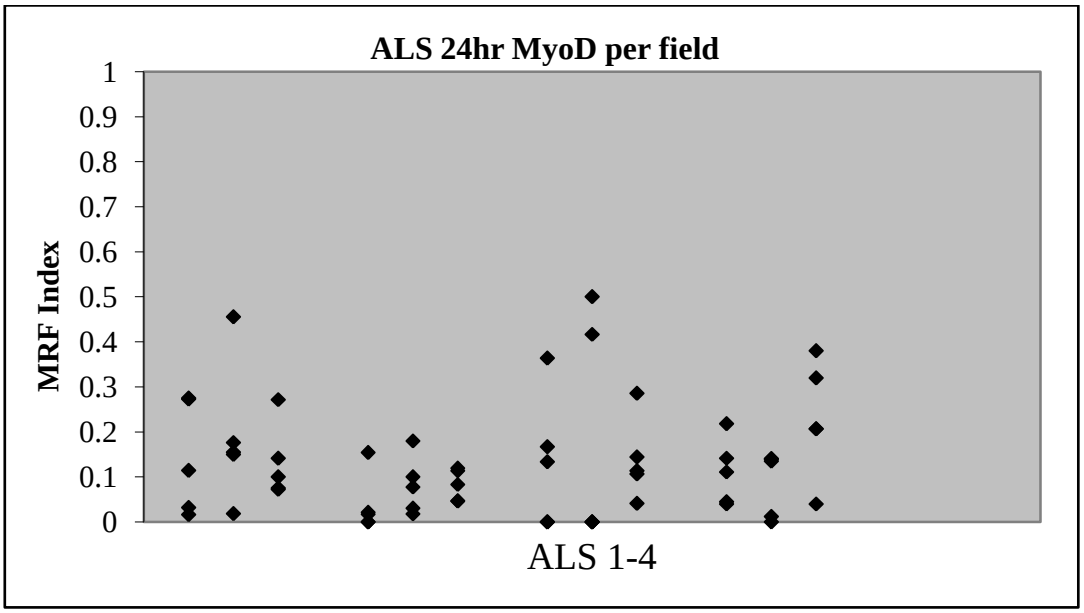
Table 1. Frequency Distribution of MRF Index per field in satellite cell cultures from control and SOD1 G93A muscle at 24hr.

MRF Index (# MRF (+) / Total Dapi (+))	*MyoD*		*Myogenin*	
	ALS	CON	ALS	CON
0	8	8	16	16
0 – 0.1	20	22	22	17
0.11 – 0.2	19	10	13	12
0.21 – 0.3	7	3	7	4
0.31 – 0.4	3	3	2	4
0.41 – 0.5	3	2	0	1
0.51 – 0.6	0	2	0	2
0.61 – 0.7	0	2	0	2
0.71 – 0.8	0	4	0	0
0.81 – 0.9	0	1	0	0
0.9 – 1.0	0	3	0	1

Frequency distribution of MRF Index ratios per field for MyoD and Myogenin expression at 24hr. n = 4 mice percondition. 60 total fields imaged per condition (5 images per well, 3 wells per animal) for both MyoD and Myogenin expression ratios.

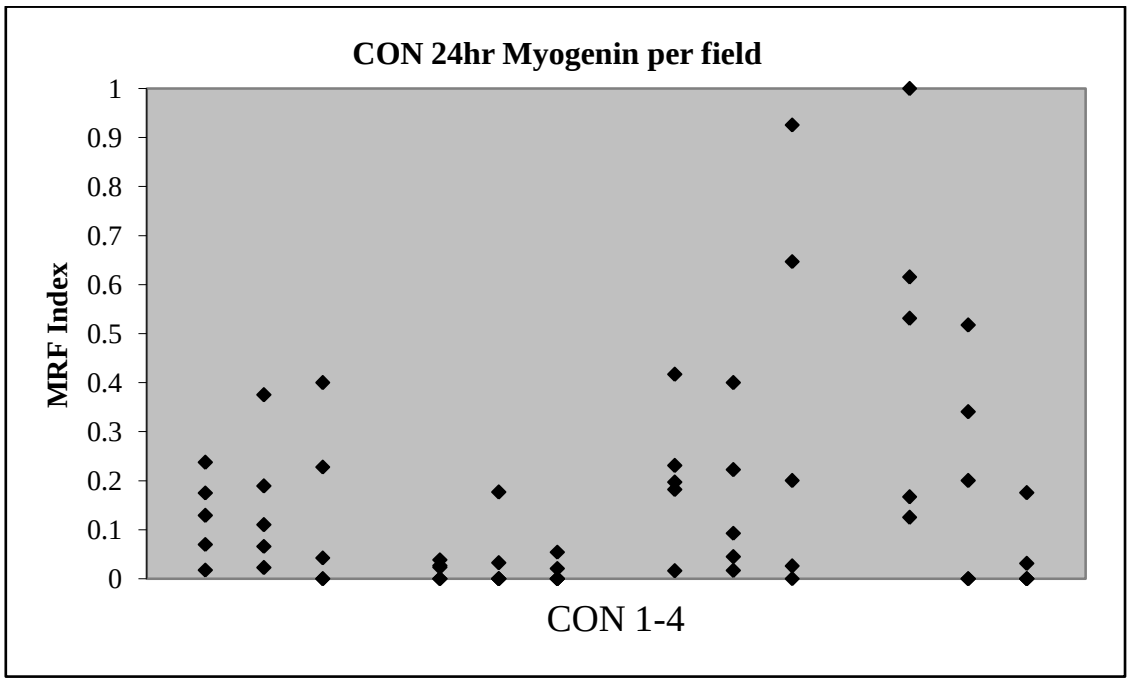
Appendix C. Myogenic Regulatory Factor Index for MyoD at 24hrs.

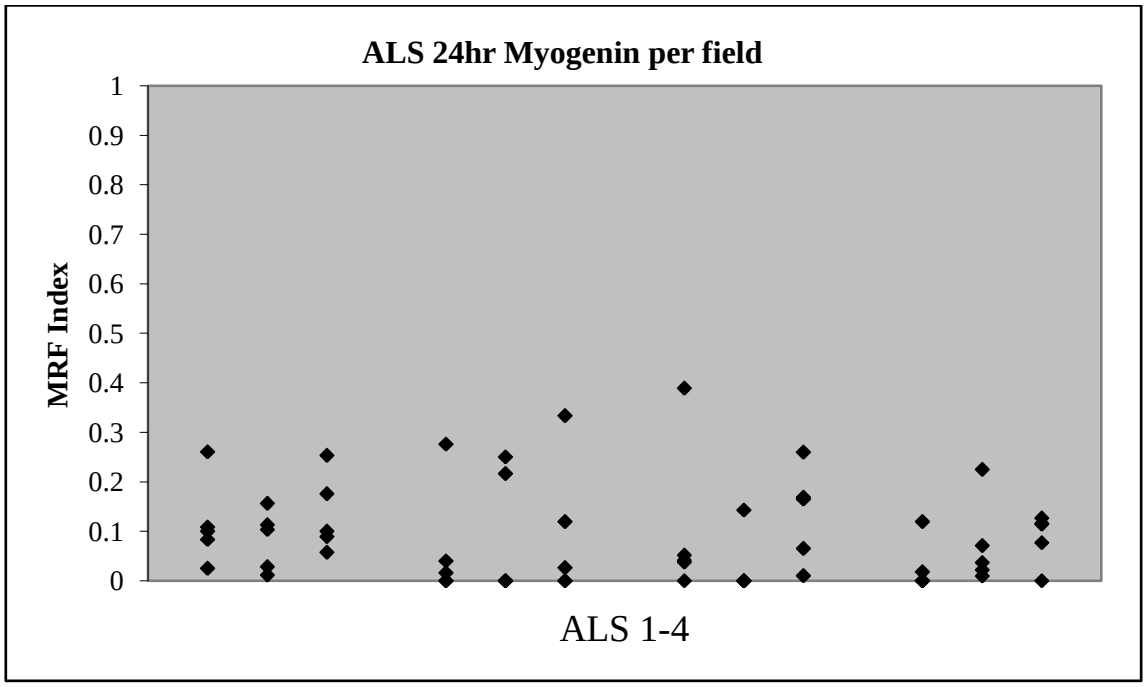




Data represents number of MyoD positive nuclei per total nuclei in each field analyzed.

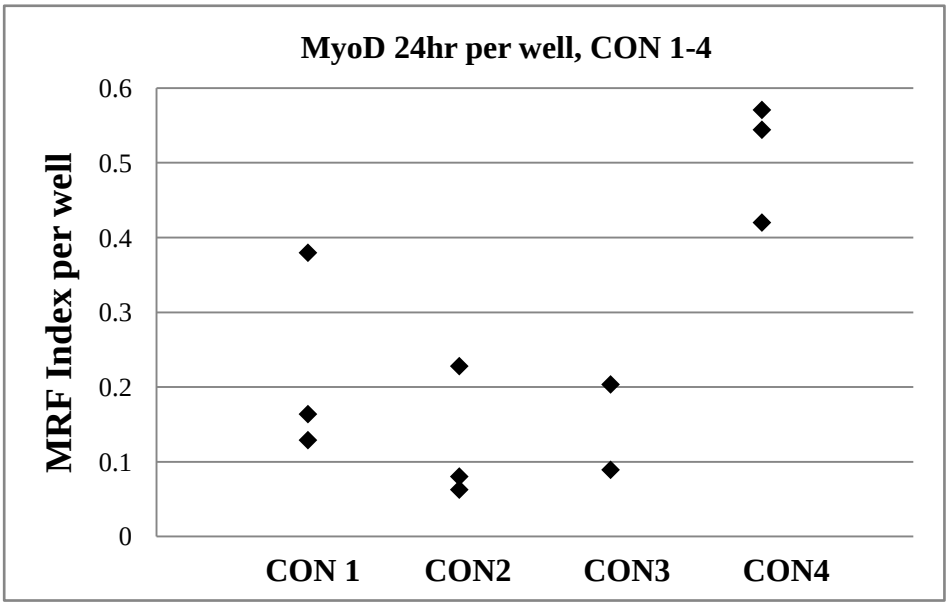
Appendix D. Myogenic Regulatory Factor Index for Myogenin at 24hrs.

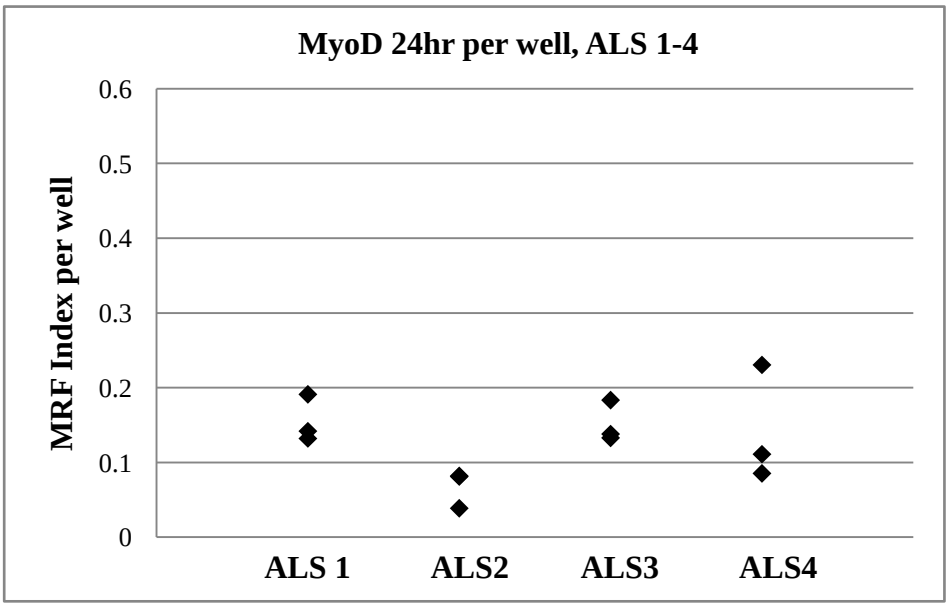




Data represents number of Myogenin positive nuclei per total nuclei in each field analyzed.

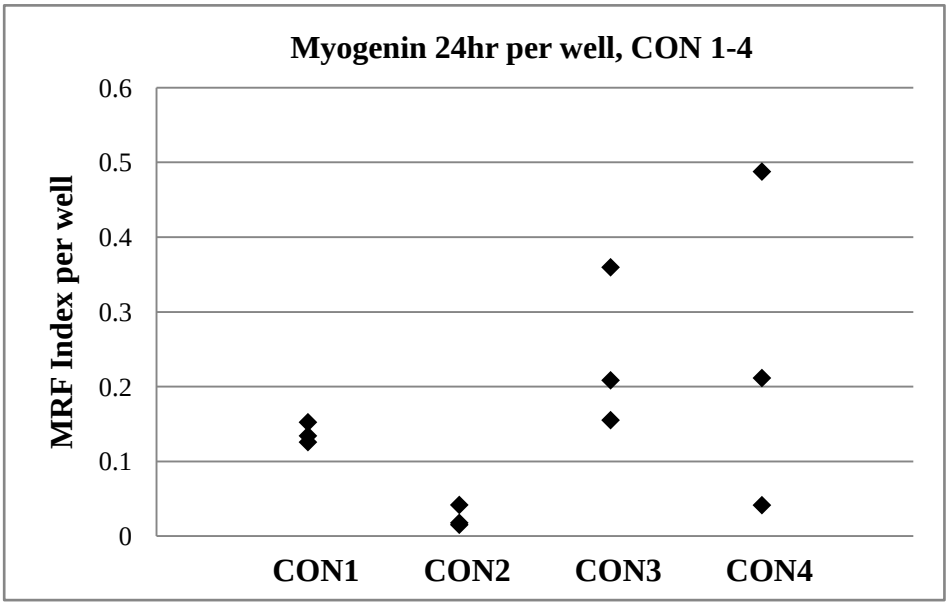
Appendix E. Myogenic Regulatory Factor Index values for MyoD at 24 hrs per well of culture.

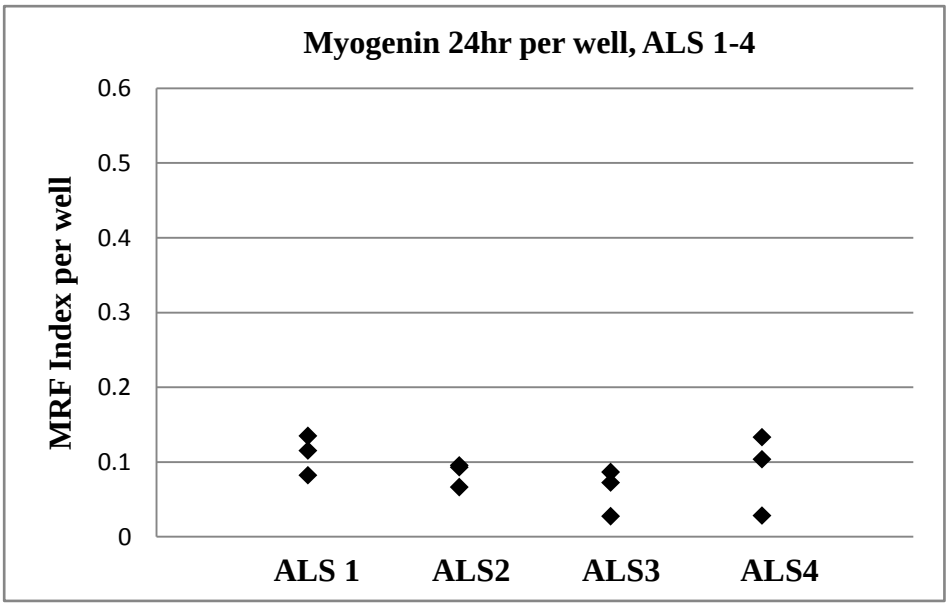




As shown, each data point corresponds to one well of culture. Three wells of culture were maintained for each animal.

AppendixF. Myogenic Regulatory Factor Index values for Myogenin at 24 hrs per well of culture.

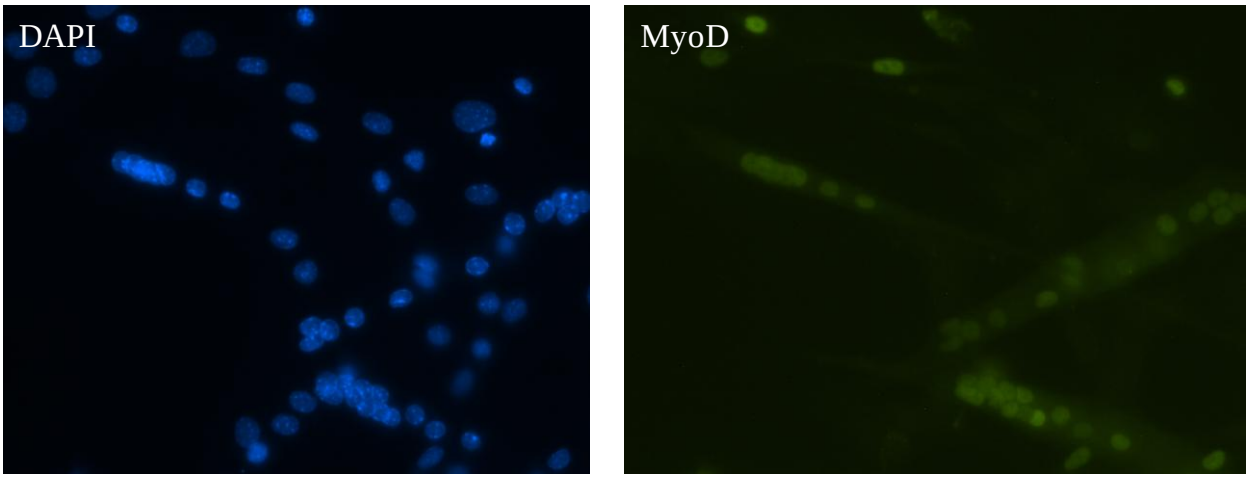




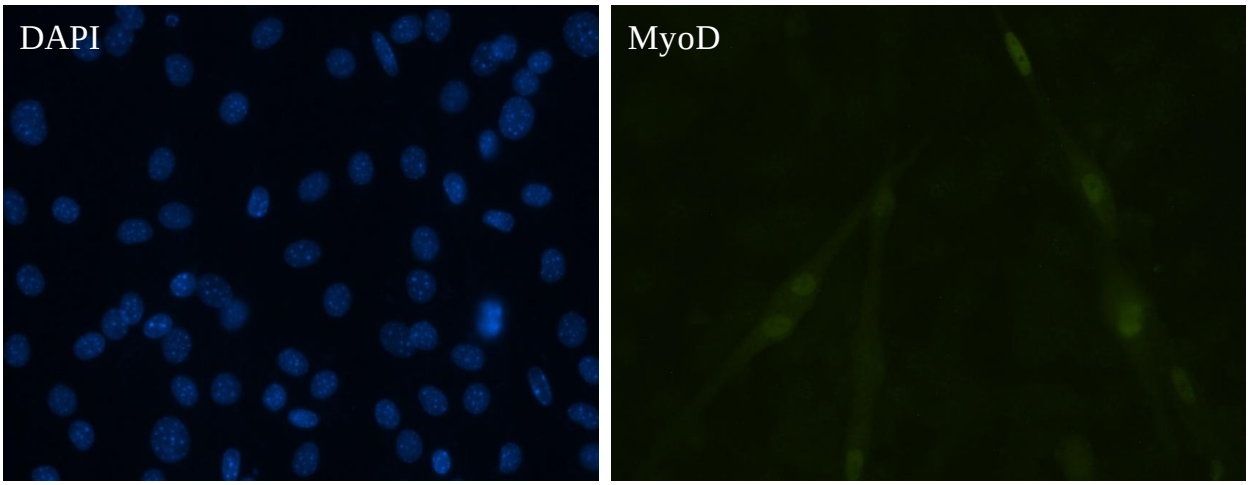
As shown, each data point corresponds to one well of culture. Three wells of culture were maintained for each animal.

Appendix G. Representative Images for MyoD staining at 24hrs.

CON 24hr

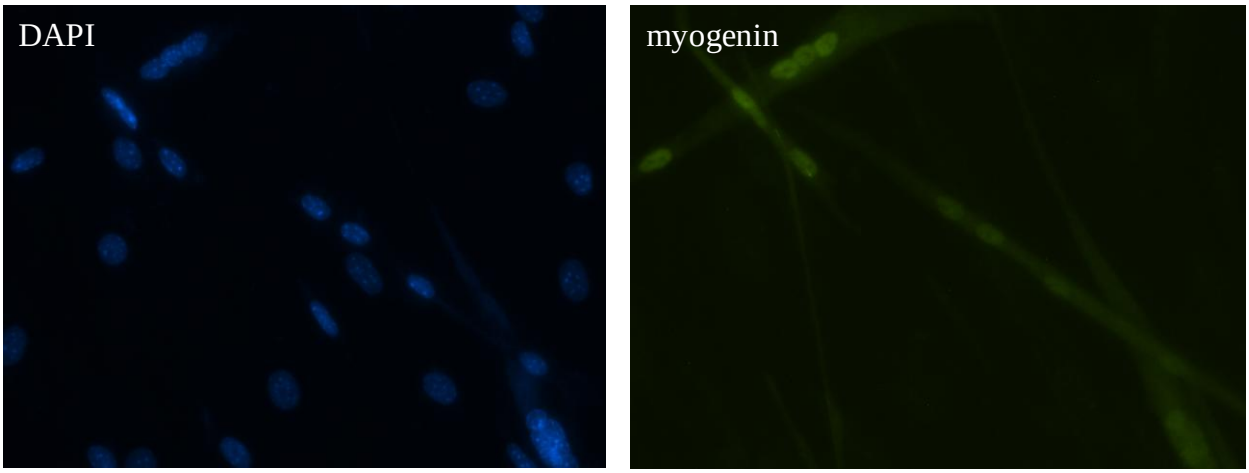


ALS 24hr

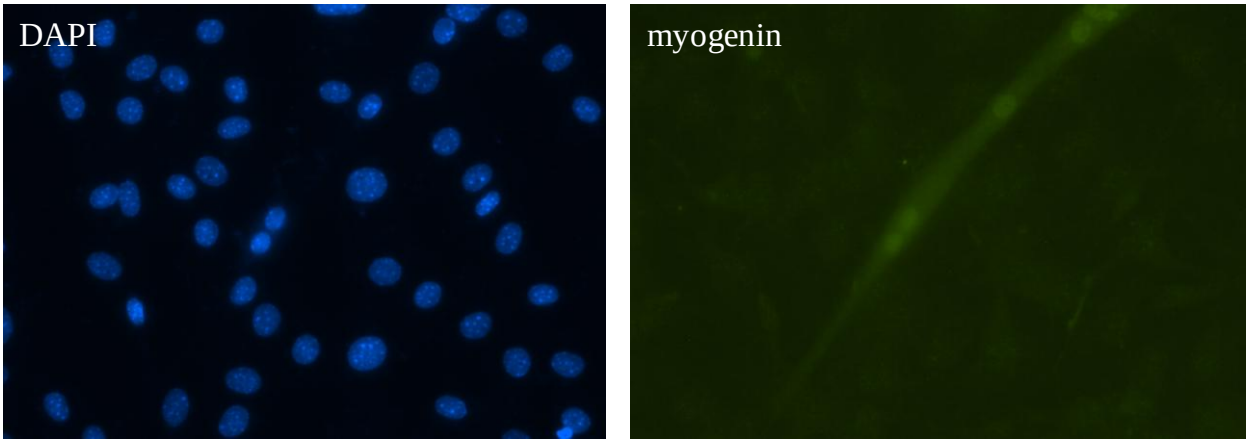


Appendix H. Representative Images for Myogenin staining at 24hrs.

CON 24hr

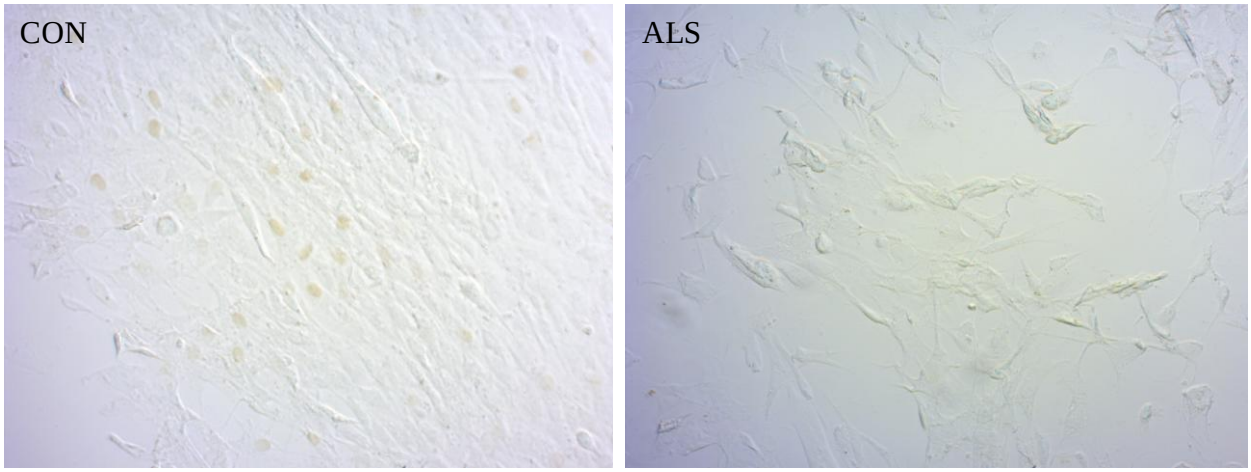


ALS 24hr



AppendixI.Representative Images for BrdU staining during proliferation.

BrdU



Appendix J. Approval for Animal Use Protocol

UNIVERSITY OF MARYLAND
GRADUATE STUDIES AND RESEARCH
Institutional Animal Care & Use Committee

Jim Dietz
IACUC Chair
jmdietz@umd.edu
Phone: (301)405-6949

April 7, 2009

Dr. Eva Chin
Department: Kinesiology
University of Maryland
erchin@umd.edu
Phone: (301)405-2450

Dr. Chin,

This letter is to inform you that on **April 12, 2009**, the members of the Institutional Animal Care & Use Committee (IACUC) reviewed and approved the protocol for:

Analysis of Skeletal Muscle Function in Transgenic Mice and in Mouse and Rat Models of Muscle Wasting

R-09-20

Please note that an approved protocol is valid for three (3) years unless there is a change in the protocol. Thus, this protocol is valid until **April 12, 2012**. Federal laws indicate that protocols must be reviewed yearly. Thus, in order to keep your approved protocol active you **MUST** submit a protocol renewal/update by the first of the month of the anniversary of your approval (April 2010 & April 2011). All work extending beyond the approval date of the protocol must be submitted to the IACUC as a new protocol.

Sincerely,

Jim Dietz
Professor, Biology
Chair, IACUC

CC: Doug Powell, Amanda Underwood

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