Effects of SMN Deficiency on Muscle Development and Maintenance in Spinal Muscular Atrophy

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Abstract of Dissertation

Effects of SMN Deficiency on Muscle Development and Maintenance in Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disease caused by mutations in the survival of motor neuron 1 (*SMN1*) gene, resulting in reduced expression of the ubiquitously expressed SMN protein. SMA is characterized by motor neuron loss and severe muscle atrophy. It is unclear to what extent SMN deficiency in peripheral tissues may contribute to the pathophysiology of SMA. However, there is growing evidence from human tissue and model organisms that SMN deficiency can lead to intrinsic muscle defects. Here we investigate the role of SMN in muscle development and maintenance using pharmacological and cell biological approaches. We first show that atrophy markers are induced in SMA model mice and SMA patient muscle in association with increased myogenin and histone deacetylase-4 (HDAC4) expression. These results indicate that myogenin-dependent induction of pro-atrophy genes is a mechanism for atrophy in SMN-deficient muscle. The activation of this atrophy pathway is suppressed by treatment with histone deacetylase inhibitors, suggesting that muscle atrophy in SMA is amenable to pharmacological intervention.

SMA model mice have small, weak muscles before induction of atrophy, indicating that SMN may have an intrinsic role in muscle development. Using muscle cell lines we derived from wild-type and SMN-deficient mice, we found that the SMN-deficient muscle cells had reduced capacity to fuse into multinucleate myotubes

compared to the wild-type cells. Expression of key muscle development factors such as myogenin and MyoD was increased and induction upon differentiation was reduced in SMN-deficient compared to wild-type cells. In addition, SMN-deficient muscle cells had impaired cell migration and altered organization of focal adhesions and the actin cytoskeleton. Restoring SMN increased expression of muscle differentiation markers and improved myoblast fusion. These data are consistent with a role for SMN in myotube maturation through changes in gene expression and effects on focal adhesions and the actin cytoskeleton. Overall, this dissertation shows a role for SMN in the formation of myotubes and delineates a pathway for muscle atrophy in SMN-deficient muscle.

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List of Abbreviations

AchR	acetylcholine receptors
Akt/PKB	protein kinase B
ALS	amyotrophic lateral sclerosis
BrdU	bromodeoxyuridine
ChIP	chromatin immunoprecipitation
CNS	central nervous system
Dex	dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	dimethyl sulfoxide
EDL	extensor digitorum longus
FGF	fibroblast growth factor
GFP	green fluorescent protein
H2K	H-2Kb-tsA58 transgene
HDAC	histone deacetylase
HEK	human embryonic kidney
hnRNP-R	heterogeneous nuclear ribonucleoprotein R
htt	huntingtin
IGF-1	insulin-like growth factor 1
MND	motor neuron disease
MuRF1	muscle ring finger 1
MHC	myosin heavy chain

Olig2	oligodendrocyte lineage transcription factor 2
NF-κB	nuclear factor of kappa light polypeptide gene enhancer in B-cells
NMJ	neuromuscular junction
PBS	phosphate buffered saline
PDL	poly-D-lysine
PI3K	Phosphatidylinositide 3 kinase
PLS3	plastin3
PVDF	polyvinylidene fluoride
RNAi	RNA interference
RT-PCR	reverse transcriptase polymerase chain reaction
SBMA	spinal and bulbar muscular atrophy
SMA	spinal muscular atrophy
SMN	survival of motor neuron
SOD1	superoxide dismutase 1
snRNP	small nucleolar ribonucleoprotein
TA	tibialis anterior
TBST	tris buffered saline with Triton X
TNF-α	tumor necrosis factor alpha
TSA	Trichostatin A

Chapter 1: Introduction

Adapted from: Bricceno KV, Fischbeck KF, Burnett BG. Neurogenic and Myogenic Contributions to Hereditary Motor Neuron Disease. Neurodegener Dis. 2012; 9:199-209.

Motor neuron diseases (MNDs) are a group of neurological diseases characterized by progressive loss of motor neurons in the cerebral cortex, brainstem, and spinal cord, leading to muscle weakness and wasting. Spinal muscular atrophy (SMA) is characterized by lower motor neuron loss and skeletal muscle weakness. SMA is caused by deficiency of survival of motor neuron (SMN) protein, with alterations in spliceosome assembly, snRNP biogenesis, and axonal transport of mRNAs reviewed in (Boyer et al., 2010).

This autosomal recessive disease presents in infancy or early childhood. Patients have symmetric muscle weakness, with proximal muscles more affected than distal. The facial muscles are relatively spared, and in severe forms of SMA respiration may also be impaired due to weakness of the intercostal muscles while the diaphragm is relatively spared. The clinical presentations of SMA have been recently reviewed in (Wee et al., 2010).

SMA is caused by a mutation in the survival of motor neuron 1 gene (*SMN1*) that is widely expressed. Our understanding of the effects of SMN has increased greatly over the years, yet the selective vulnerability of motor neurons remains unexplained. One area of active investigation considers the contributions of cellular structures beyond the motor neuron cell body to the disease mechanism. These studies have been guided by observations in animal models and insights from other MNDs such as spinal bulbar muscular atrophy and amyotrophic lateral sclerosis. Three alternate sources of pathology

have emerged: (i) peripheral motor axons, (ii) the neuromuscular junction (NMJ), and (iii) muscle (summarized in Figure 1.1).

DISEASE MECHANISMS

The SMN gene is normally present in multiple copies. SMA is caused by mutation (usually deletion) of *SMN1*, which encodes most of the mRNA for the SMN protein (Lefebvre et al., 1995). SMA is caused by deficiency of the SMN protein and modified by the *SMN2* gene, a near copy of *SMN1* (Feldkotter et al., 2002; Lefebvre et al., 1997). Although motor neuron degeneration and consequent muscle denervation are the primary manifestations of the disease, the role of other tissues cannot be excluded. SMN is expressed in all mammalian tissues (Liu et al., 2010) and is present in both the nucleus and the cytosol of cells (Liu and Dreyfuss, 1996). Genetically modified mice with reduced SMN in motor neurons have a milder phenotype than those with ubiquitously low levels of SMN, a finding which suggests that other cell types contribute to the disease manifestations (Park et al., 2010b). Several functions including snRNP biogenesis and spliceosome assembly have been attributed to SMN (reviewed in (Boyer et al., 2010; Burghes and Beattie, 2009)), but its best characterized role is in snRNP biogenesis. In the cytoplasm, SMN associates with Gemin proteins to form a complex that facilitates the assembly of pre-snRNAs with a (heteroheptameric) ring of Sm proteins to form the snRNP core complex (Paushkin et al., 2002; Pellizzoni et al., 1998). Protein interacting regions of SMN are diagramed in Figure 1.2. Sm core assembly is reduced in cell extracts from SMA patients, and snRNPs have cell-dependent alterations in an SMA mouse model (Workman et al., 2009; Zhang et al., 2008). Presumably, these altered snRNPs

Figure 1.1. Sources of pathology in SMA. Contributions of axonal transport, NMJ, and muscle to the disease mechanisms (as discussed in the text). MN = Motor neuron.

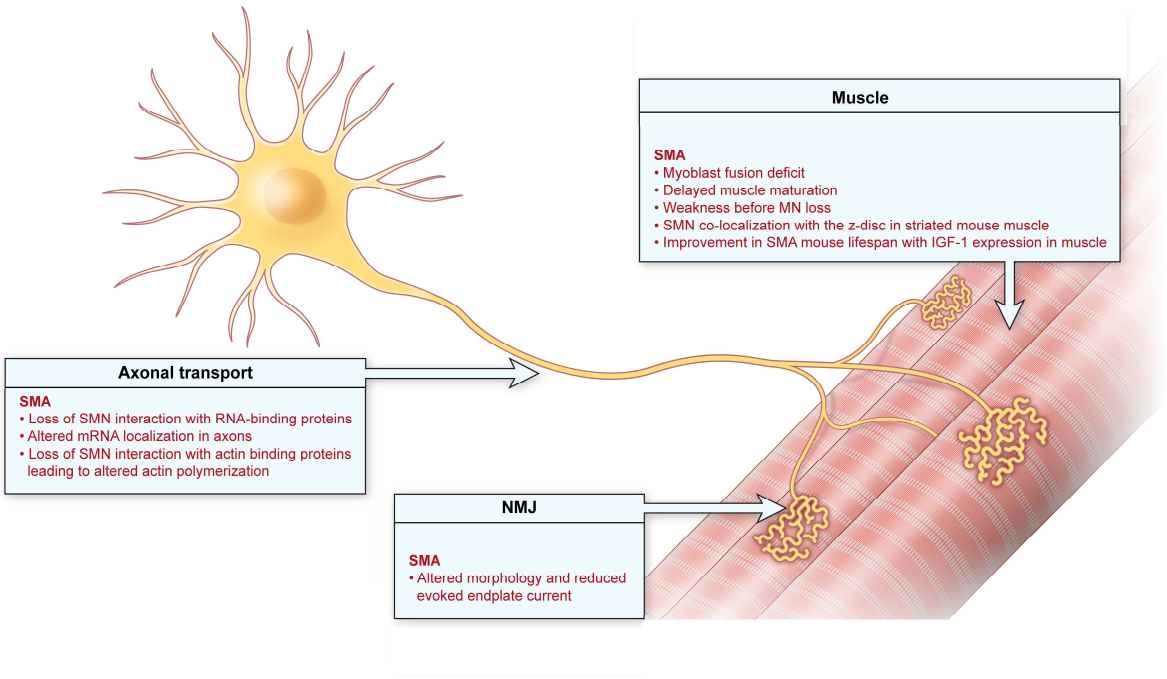
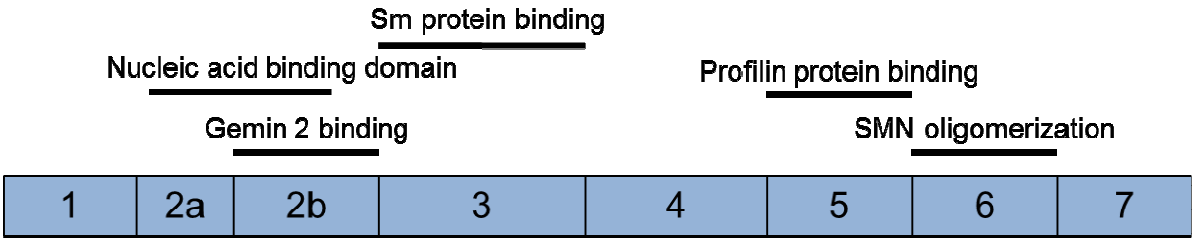


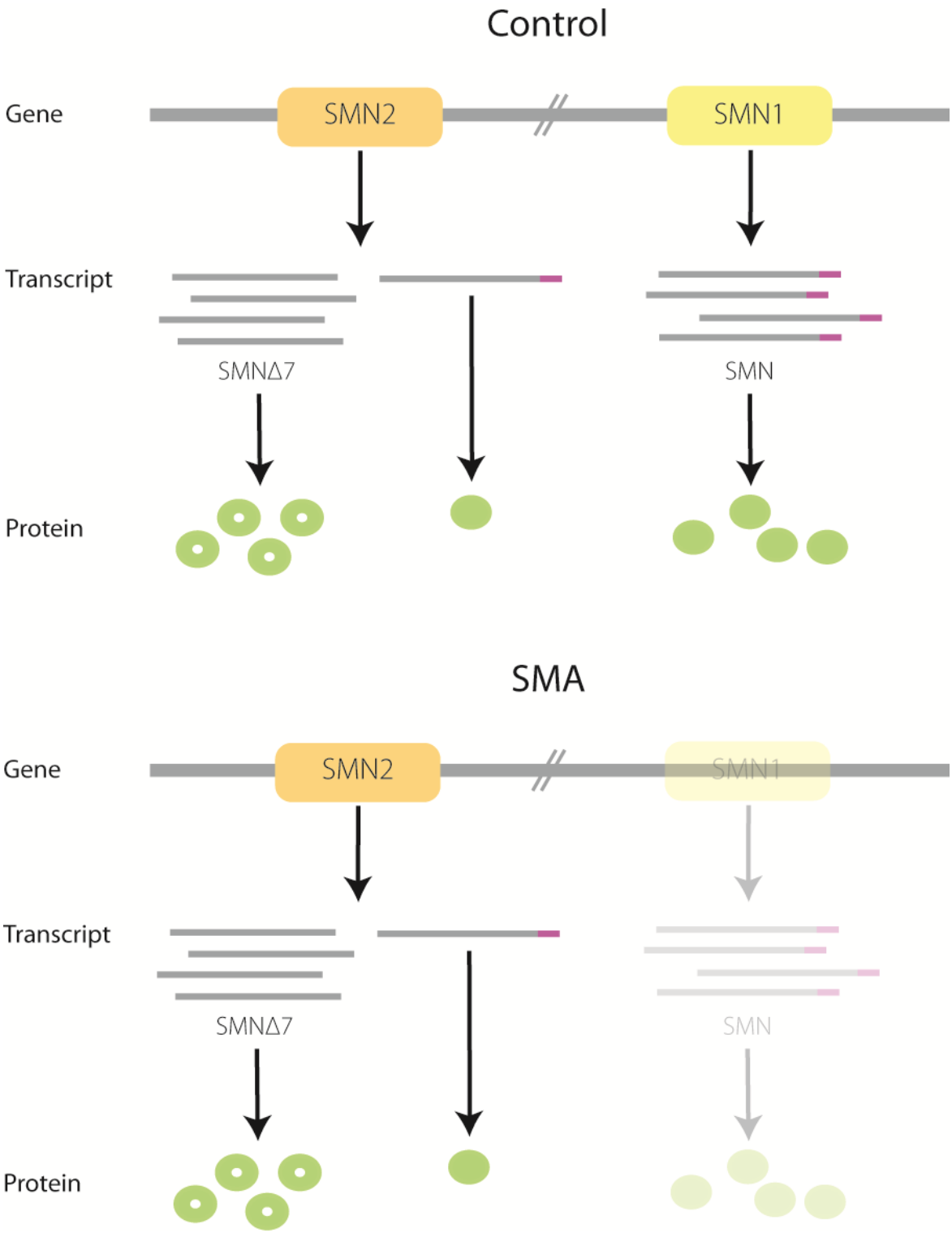
Figure 1.2. SMN full-length transcript and protein interacting regions.



result in the mis-splicing of transcripts that are important for motor neuron survival, but less crucial for other cells. This mis-splicing of key transcripts may contribute to the motor neuron loss in SMA. Studies using exon arrays have not produced a well-defined subset of transcripts with altered splicing in the spinal cord of SMA mice (Baumer et al., 2009; Zhang et al., 2008). SMN's role in snRNP assembly, a critical cellular function, supports the possibility that motor neurons are not uniquely affected by SMN. Loss of the *SMN* gene in several animal model systems is early embryonic lethal, demonstrating the need for a minimum level of SMN protein in prenatal development (Miguel-Aliaga et al., 1999; Paushkin et al., 2000; Schrank et al., 1997). Specifically, mice with complete loss of the *Smn* gene have massive cell death at 90-104 hours post coitus (Schrank et al., 1997).

All patients with SMA have at least one copy of the *SMN2* gene, which is similar to *SMN1* but encodes mostly a transcript lacking exon 7. The resulting truncated protein is quickly degraded (Figure 1.3). Two copies of the human *SMN2* gene rescue embryonic lethality in mice lacking the endogenous mouse *Smn* gene, although the mice live only 5 days on average (Le et al., 2005). However, *Smn*-/- mice expressing 8 copies of *SMN2* do not develop an SMA phenotype (Monani et al., 2000). These mouse models demonstrate that *SMN2* is a disease modifier; ameliorating disease severity and preventing disease manifestations if present in sufficient quantity. In humans, individuals with the mildest form of SMA have higher copy numbers of the *SMN2* gene (Feldkotter et al., 2002) while individuals with at least 5 copies of *SMN2* may be phenotypically normal (Prior et al., 2004). It is unclear how much SMN protein is needed to prevent SMA.

Figure 1.3. Genetics of SMA. The loss of the SMN1 gene and therefore its protein by mutation or deletion causes SMA. Image courtesy of Deborah Kwon.



MOLECULAR MECHANISMS

The genetic cause of SMA is known, but the underlying molecular mechanisms of the disease remain elusive. It is possible that the disease mechanisms are not completely cell-autonomous, i.e., that the primary effects of the disease causing mutations are not only in motor neurons, but also in other cells and tissues.

Disruption of axonal transport and cytoskeletal dynamics

Motor neurons, with their very long and highly branched axons, may be particularly vulnerable to axonal transport defects. Critical proteins and mRNAs as well as organelles are synthesized in the neuronal cell body and transported along the axon toward the nerve terminals; this retrograde transport is important for recycling cytoskeletal and synaptic vesicle-associated proteins (Shea, 2000). Retrograde axonal transport defects have been reported in motor neurons of amyotrophic lateral sclerosis (ALS) model mice (Kieran et al., 2005; Williamson and Cleveland, 1999). In SMA, compromised anterograde and retograde axonal transport has been proposed to (partially) account for the selective vulnerability of motor neurons. The work of Zhang and colleagues indicates that SMN protein must be full length for proper cytoplasmic localization, and that SMN protein is transported along the cytoskeleton in neurons (Zhang et al., 2003).

In motor axons and growth cones, SMN co-localizes with the alpha subunit of the vesicle coat protein complex (α-COP) (Peter et al., 2011). The entire complex has 7 subunits and is involved in vesicle trafficking within the Golgi and between the Golgi and the endoplasmic reticulum. α-COP is transported along axons in primary neurons and associates with β-actin mRNA in HEK 293T cells. In SH-SY5Y human neuroblastoma

cells, SMN has α-COP-dependent co-localization actin at the cell perimeter. In the absence of α-COP, SMN is reduced and filamentous actin is increased at the cell perimeter. Collectively, these data suggest SMN is involved in α-COP–mediated axonal transport of β-actin mRNA and actin cytoskeleton re-organization (Peter et al., 2011). The involvement of SMN in β-actin mRNA transport is notable because it is consistent with earlier work by Rossoll and colleagues, who showed that SMN co-localizes with the RNA-binding protein, hnRNP-R (Rossoll et al., 2002), a protein that mediates β-actin mRNA accumulation in the growth cones (Rossoll et al., 2003). SMN also interacts directly with β-actin mRNA (Todd et al., 2010). Thus, through interactions with α-COP and hnRNP-R, SMN may facilitate β-actin mRNA transport to the growth cones, potentially affecting cellular polarity and motility (Kislauskis et al., 1997). This function may be especially important in long, highly branched motor axons. Reduction in SMN may compromise these cellular processes; consistent with this, path-finding defects in motor axons are observed when SMN is knocked down in zebrafish (McWhorter et al., 2003). Injection of zebrafish embryos at the one- to four-cell stage with constructs for full-length SMN rescued the axonal defect in 2 day old zebrafish, suggesting a function for SMN in the axon distinct from its role in snRNP biogenesis. Injection of SMN with a patient-derived mutation that is still able to bind Sm proteins, critical snRNP components, did not rescue the defect. (Carrel et al., 2006).

SMN has been shown to interact with another RNA-binding protein, HuD in both cultured cells and rodent spinal cord (Akten et al., 2011; Fallini et al., 2011; Hubers et al., 2011). The SMN-HuD interaction has implications for mRNA localization in motor neurons. SMN is needed for the recruitment of HuD to RNA granules in motor axons,

and these granules are decreased when SMN is reduced (Fallini et al., 2011; Hubers et al., 2011). In addition, mRNAs known to interact with SMA and HuD and involved in neuronal process and neuromuscular synapse formation are decreased when SMN is reduced (Akten et al., 2011; Hubers et al., 2011). Increasing these interacting mRNAs improves the phenotype of a SMA zebrafish model (Akten et al., 2011). In addition, increasing HuD also improves the phenotype of MN-1 cells with shRNA to SMN (Hubers et al., 2011). These data support a role for SMA in mRNA localization in motor neurons and suggest that the interactions between SMA, RNA-binding proteins and RNAs may have an effect on the SMA phenotype.

In addition to its putative role in intracellular trafficking, SMN also interacts with proteins involved in cytoskeletal dynamics, including the actin-binding proteins profilin 1 and 2 (Giesemann et al., 1999; Sharma et al., 2005). Protein interacting regions of SMN are diagramed in Figure 1.2. Profilin regulates the polymerization of globular actin into filamentous actin by catalyzing the exchange of ADP- and ATP-bound actin (Kwiatkowski and Bruns, 1988) and by sequestering globular actin monomers (Witke, 2004). Mutant huntingtin (htt) protein expression enhances degradation of profilin, suggesting a link between Huntington disease pathogenesis and actin dynamics (Burnett et al., 2008). Recent research has linked mutations in profilin 1 with familial amyotrophic lateral sclerosis (Wu et al., 2012). There is also evidence to suggest that SMN's interaction with profilin has an effect on actin polymerization. Van Bergeijk and colleagues showed that knockdown of SMN increases the amount of polymerized actin in PC12 cells and reduces neurite outgrowth (van Bergeijk et al., 2007). Based on these data, the authors proposed a model in which reduced SMN results in increased unbound

or “free” profilin 2, which in turn promotes actin polymerization, disrupts normal actin turnover, and reduces neurite outgrowth (van Bergeijk et al., 2007). Patient-derived missense mutations and the common deletions of exons 5 and 7 result in the loss of association of SMN with profilin 2, the neuron-specific isoform of profilin (Sharma et al., 2005), further implicating this interaction in the disease pathogenesis.

RhoA signaling, via Rho kinase and profilin 2a, regulates neurite outgrowth (Da Silva et al., 2003). Bowerman et al sought to characterize this signaling pathway in SMA model mice and found that these mice have increased GTP-bound (activated) RhoA, while total RhoA remains unchanged (Bowerman et al., 2010). Activated RhoA inhibits neurite differentiation and outgrowth (Hall, 1998). SMA mice treated with Y-27632, a Rho kinase inhibitor (Uehata et al., 1997), had increased lifespan (death between 14 and 33 weeks of age) and increased NMJ and muscle size compared to vehicle treated mice. There was no observable change in SMN protein level in the spinal cord, leading the authors to conclude that the Y-27632-mediated improvement in the NMJ occurs via an SMN-independent pathway. In addition, the number of motor neurons was unchanged in the treated mice, suggesting that the improvements in the motor unit size could not be explained by slowing motor neuron loss (Bowerman et al., 2010).

Increased expression of the actin-binding protein, plastin3 (PLS3) has been reported to ameliorate the SMA phenotype (Opera et al., 2008). PLS3 bundles actin filaments, a necessary step in axonal outgrowth (Delanote et al., 2005). The expression of PLS3 was abnormally high in the unaffected siblings of seven SMA patients (Opera et al., 2008). These unaffected siblings had the same disease mutation (homozygous deletion of *SMN1*) and *SMN2* copy number as their affected family members, suggesting

that PLS3 is a protective modifier of SMA. Over-expression of PLS3 in zebrafish rescues the defects in motor axon outgrowth that are observed when SMN is reduced. Thus, increased PLS3 may help to mitigate the SMA phenotype by altering actin polymerization (Opera et al., 2008).

Deficits in neuromuscular transmission

In addition to altered actin polymerization in the axon, defects at the nerve terminus have been reported in lower motor neuron diseases (Chai et al., 2008; Nishimura et al., 2004). Abnormalities at the NMJ are among the earliest defects observed in SMA mice present as early as P2 (Kariya et al., 2008; Murray et al., 2008). In response to repetitive stimulation, NMJs of severe SMA mice have reduced end plate current, likely due to decreased synaptic vesicle release (Kong et al., 2009). A separate study did not find fatigue or transmission failure, although SMA mice had approximately 50% the response of control littermates (Lee et al., 2011). In a milder mouse model of SMA, intermittent transmission failures were recorded in response to repetitive stimulation (Kariya et al., 2008). An ongoing observational study in a cohort of SMA patients involves analyzing electrophysiological measures to improve understanding of SMA pathophysiology and to develop biomarkers for future clinical trials (Kaufmann et al., 2011). The morphology of the NMJ is also altered in SMA mouse models, consistent with the electrophysiological changes. At the pre-synaptic nerve terminal, SMA mice have an abnormal accumulation of neurofilament (Kariya et al., 2008; Kong et al., 2009; Murray et al., 2008), while at the post-synaptic endplate the acetylcholine receptors (AchR) remain clustered in plaques rather than transitioning to pretzel-like formations as in age-matched controls. In addition, SMA mice retain the fetal isoform of the AchR

longer than control littermates (Kariya et al., 2008; Kong et al., 2009). These data suggest that impaired transmission hinders maturation of the NMJ and contributes to the SMA phenotype. However, these studies do not establish a causal relationship between NMJ abnormalities and the SMA phenotype. It still remains unclear whether the NMJ phenotype is due solely to SMN deficiency in motor neurons or whether there is a muscle component to this aspect of the pathology.

Further morphological studies have assessed the innervation of skeletal muscles in SMA and suggest that the NMJs of different muscles are affected to varying degrees. Studies in different SMA mouse models analyzing different skeletal muscles give contrasting data regarding the axon myelination and caliber, the number and filling of motor endplates, and the structure of presynaptic active zones and postsynaptic folds. The loss of motor neurons or unoccupied neuromuscular junctions would indicate denervation of the muscle. It is also possible that intact neuromuscular junctions could have functional defects in transmission. Defects in the neuromuscular junction ultrastructure, as viewed by electron microscopy, as well as altered protein levels could also indicate that an intact neuromuscular junction is not functionally normal. These data suggest that the NMJs of different muscles are affected to varying degrees. One consistent finding across mouse models and muscle groups is the absence of a shift in fiber size in SMA mice. Initially, the fiber size of SMA mice is indistinguishable from control littermates. As the mice age, the fiber size of control mice increases, but SMA mice do not show a similar shift in fiber size (Dachs et al., 2011; Lee et al., 2011). Although the NMJs of SMA mice show varying degrees of morphological abnormality, the muscles all have a similar pathology. In addition, muscle weakness and degeneration occur before motor

neuron loss, which suggests that defects intrinsic to the muscle may contribute to the disease manifestations.

Muscle involvement in motor neuron disease

While SMA is primarily a lower motor neuron disease, data from patient biopsies and model mice raise the possibility that skeletal muscle is also involved in the disease mechanism. Research in another MND, spinal and bulbar muscular atrophy (SBMA), supports muscle involvement in the disease mechanism (Yu et al., 2006) and suggests that muscle-directed therapy may have an effect on the disease phenotype.

In both normal (Musaro et al., 2001) and disease (Dobrowolny et al., 2005) conditions, IGF-1 induces muscle growth and regeneration. IGF-1 activates the PI3K/Akt pathway that stimulates muscle satellite cells to proliferate, ultimately increasing myotube size (Rommel et al., 2001). Activation of this pathway also reduces the toxicity of the mutant protein in mouse and cell models of SBMA (Palazzolo et al., 2007; Palazzolo et al., 2009). SBMA mice over-expressing a non-circulating muscle isoform of IGF-1 (mIGF-1) lived longer, performed better in behavioral tests, and had increased motor neuron numbers compared to control SBMA mice (Palazzolo et al., 2009). These data demonstrate that blocking mutant protein toxicity in muscle may improve the disease phenotype, including motor neuron survival, and suggest that the muscle is a potential therapeutic target tissue for SBMA and other lower motor neuron diseases.

As with the SBMA mouse model, the lifespan of SMA mice is extended by crosses to mice over-expressing muscle-specific IGF-1 (Bosch-Marce et al., 2011). Although the mechanism is not fully worked out, there is evidence that skeletal muscle may influence the SMA phenotype through indirect effects on motor neurons. Guettier-

Sigrist and colleagues investigated this with an experimental system in which human SMA skeletal muscle cells, collected from biopsy, are innervated by rat embryonic spinal cord explants *in vitro*. When the muscle was derived from moderate to severely affected (type 1-2) SMA patients, the innervated myofibers degenerated after 1-3 weeks (Guettier-Sigrist et al., 1998). This degeneration was not observed with co-cultures using muscle from aged-matched controls or mildly affected (type 3) SMA patients. The symbiotic relationship between muscle and nerve was evident in the finding that embryonic spinal cord explants survived for just 2-3 weeks in the absence of muscle, while normal muscle–nerve co-cultures survived for over a year under the same conditions (Guettier-Sigrist et al., 1998).

The molecular basis for this relationship has been examined in a fly model of SMA (Sen et al., 2011). Mesoderm-specific expression of RNAi for the Drosophila SMN homolog (Smn) results in a lethal phenotype that is exacerbated by decreased expression of the Drosophila homolog of the fibroblast growth factor (FGF) receptor or over-expression of a downstream inhibitor, *sprouty*, of the FGF signaling pathway. Altering the expression of FGF components in muscle influences NMJ morphology and neurotransmission. Disrupting FGF signaling results in reduced evoked potential amplitude without affecting the quantal content (number of synaptic vesicles released) of the neurotransmitter. Over-expression of the FGF receptor homolog rescues the NMJ defect caused by reduced Smn. Reduced Smn expression decreases the staining for this homolog at the NMJ. However, in the reverse experiment reduced expression of the FGF receptor homolog does not result in altered Smn. Overall, these data link Smn to the FGF

pathway in Drosophila. Smn affects FGF signaling components, and modifying this signaling alters the SMA phenotype.

Evidence from human tissue and model organisms indicates that SMN deficiency causes intrinsic muscle defects. Data from muscle fibers from control fetuses show an increase in myotube diameter between weeks 10 and 12, but this increase is delayed until weeks 12 to 15 in muscle from SMA fetuses (Martinez-Hernandez et al., 2009). Furthermore, muscle precursor cells from SMA patient biopsies show reduced fusion to myotubes (Arnold et al., 2004). Reducing SMN in C2C12 myoblasts reduces proliferation and fusion, resulting in malformed myotubes (Shafey et al., 2005). Consistent with an intrinsic defect in SMN-deficient muscle, mice expressing only truncated and unstable SMN lacking the domain encoded by exon 7 have a dystrophic phenotype and muscle paralysis resulting in death. Exons of SMN are diagramed in Figure 1.2. Thus, a minimum level of SMN is essential for muscle maintenance and survival (Cifuentes-Diaz et al., 2001). In addition, severely affected SMA model mice show no apparent signs of muscle fiber regeneration (Avila et al., 2007), which could indicate an intrinsic regenerative defect. Muscle biopsies from severe and intermediate SMA patients rarely show changes in muscle fiber architecture such as degeneration, internal nuclei, and target fibers. Such changes may be seen in mild forms of SMA (Love et al., 2008).

Given that SMN deficiency has been implicated in the muscle maturation and maintenance defects seen in SMA patients' cells, interventions to enhance muscle growth and maintenance have been investigated in SMA model mice. In addition to IGF-1, myostatin and follistatin are factors that regulate muscle growth. Myostatin suppresses

muscle growth, and its inhibition results in increased muscle mass (Grobet et al., 2003; Lee and McPherron, 2001). Follistatin blocks myostatin activity *in vivo* (Lee and McPherron, 2001). As SMA model mice age, myostatin expression is increased and follistatin expression decreased compared to control mice (Sumner et al., 2009). One study in SMA mice found a significant increase in muscle mass and life span with follistatin administration (Rose et al., 2009). In a separate study, SMA mice engineered to over-express follistatin showed no improvement in muscle mass or lifespan (Sumner et al., 2009). The contrasting results of these studies may stem from different experimental methodologies. For example, continuous expression of follistatin may be less beneficial than large doses of follistatin at critical time points such as the onset of NMJ abnormalities at P2 (Kariya et al., 2008; Murray et al., 2008) or the onset of weight loss at P10 (Avila et al., 2007).

Is the motor neuron loss in SMA cell-autonomous, i.e., caused by SMN deficiency in motor neurons alone? Recent work by Park and colleagues showed that depleting SMN only in motor neurons is sufficient to cause an SMA-like phenotype, but with milder manifestations than in mice that are universally deficient in SMN (Park et al., 2010b). Mice deficient in SMN in motor neurons only, referred to as Olig2-Cre SMA mice, have an impaired righting reflex compared to controls, but this subsides by 2 weeks of age, after which there is no observable difference. Olig2-Cre mice have better survival than mice where SMN is reduced in all tissues, with 70% of animals reaching one year of age. Nevertheless, Olig2-Cre SMA mice remain distinguishable from controls based on decreased weight and muscle mass as well as kyphosis. These data indicate that non-neuronal cells may have an impact on disease severity in SMA.

Burghes and colleagues also addressed the question of cell-autonomy by examining the effects of over-expressing SMN in the muscle or nervous system of SMA mice (Gavrilina et al., 2008). Increasing SMN expression in the brain and spinal cord of the mice with a prion promoter extended survival, while increasing expression of SMN only in striated muscle with a skeletal actin promoter did not significantly alter the lifespan. However, the muscle-specific skeletal actin promoter drives expression only in post-mitotic myotubes, not proliferating myoblasts or satellite cells, and expression of the prion promoter is not limited to the CNS. Thus SMN may have a role early in muscle development such that SMN expression in these proliferating cells is needed to rescue the SMA phenotype. Data from mice with muscle-specific deletion of exon 7 of murine Smn supports this possibility. Targeted deletion of exon 7 in muscle precursors resulted in muscle necrosis, motor paralysis, and earlier death than in mice with exon 7 deleted only in fused myotubes (Nicole et al., 2003). In response to muscle damage, regeneration was impaired in mice with exon 7 deleted in muscle precursor cells and fused myotubes. In contrast, regeneration in mice with exon 7 deleted only in myotubes is not significantly different from controls. Although muscle necrosis is not seen in other SMA mouse models, the findings from both models indicate that replacement of SMN early in muscle development results in a less severe phenotype and suggest that increasing levels of SMN early in muscle development may ameliorate muscle pathology.

Interactions of SMN with cytoskeletal proteins could affect muscle as well as nerve. SMN interacts with actin-binding proteins, and drosophila SMN protein (dSmn) interacts with alpha actinin (Rajendra et al., 2007), a component of the muscle sarcomere. Alpha actinin cross-links actin filaments (Clark et al., 2002) and co-localizes with SMN

in mouse hind limb and cardiac myofibrils (Rajendra et al., 2007; Walker et al., 2008). This co-localization may indicate a functional interaction between SMN and structural proteins in skeletal muscle. There are reports of arrhythmia and cardiomyopathy in SMA patients and model mice, suggesting that cardiac muscle may be affected by SMN deficiency and involved in the disease pathology (Bevan et al., 2010; Heier et al., 2010; Shababi et al., 2010). Other disorders that are considered primary myopathies (such as oculopharyngeal muscular dystrophy and myofibrillar myopathy), may have peripheral nerve involvement(Finsterer, 2003; Selcen, 2011) (Finsterer, 2003; Selcen, 2011).

Much effort has gone into gaining an understanding of the normal function of disease proteins such as SMN and learning how disruption of normal function leads to motor neuron disease. A growing body of evidence supports a contribution of muscle to the disease mechanisms, indicating that treatment need not be CNS-directed to be at least partially effective. It has recently been shown that the proteasome inhibitor bortezomib improves the motor function and delays the pathology of SMA mice despite limited CNS penetrance of this drug (Kwon et al., 2011). The studies with muscle-specific IGF-1 over-expression also show that compounds that act peripherally may have beneficial effects on the CNS, e.g., through action at the NMJ or trophic effects. Cytoskeletal dynamics and cellular trafficking are critical in muscle as well as motor neurons, and this may explain why both could be involved in the pathology. In ALS, as in SMA, there is evidence that non-neuronal cells, such as glia, influence the disease phenotype. ALS model mice over-expressing the mutant SOD1 protein only in muscle ($SOD1^{mus}$) have decreased muscle strength and motor function (Wong and Martin, 2010). A similar phenotype was reported in another mouse model of ALS (Dupuis et al., 2009). The pathways leading to the

muscle pathology in these models are not fully understood. Cell death, as measured by caspase 3 activity, and increased atrophy, due to negative regulation of the Akt pathway, have both been proposed, but the mouse data are conflicting; perhaps due to the different ALS models used (Dobrowolny et al., 2011; Dupuis et al., 2009; Wong and Martin, 2010). As with the IGF-1 over-expressing SBMA mice, expression of muscle-specific transgenes affects motor neurons in ALS mouse models. In the case of ALS, the over-expression of disease-causing proteins results in NMJ abnormalities, including decreased occupancy of NMJs and loss of motor neurons (Dupuis et al., 2009; Wong and Martin, 2010). Overall, these data support a role for muscle in ALS and suggest non-autonomous mechanisms for motor neuron degeneration that may explain their selective vulnerability. Better understanding of how these pathways are compromised in lower motor neuron diseases could be very helpful in the identification of new therapeutic targets.

How the reduction of SMN protein results in the specific pathology of SMA remains unknown. Motor neuron degeneration is accepted as the principal disease pathology, but the role of muscle-specific defects cannot be excluded. It remains unclear whether the pathology in SMA is due mainly to the SMN protein deficiency in motor neurons, in muscle and other tissues, or a combination of these tissues. This dissertation addresses the hypothesis that the deficiency of SMN in muscle alters its development and maintenance. This hypothesis is tested with the following specific aims: 1) investigate a mechanism of atrophy in SMA muscle and 2) determine the effect of SMN deficiency on muscle development.

Specific aim 1 (Chapter 2) addresses the cause of atrophy in SMA which is thought to result primarily from denervation. In muscle atrophy, the E3 ligases atrogin-1

and muscle ring finger 1 (MuRF1) increase protein degradation through the ubiquitin proteasome system. These E3 ligases are regulated by a variety of mechanisms. During acute denervation, they are activated by myogenin, which is in turn regulated by histone deacetylases 4 and 5. In chapter 2, we present data showing that atrogin-1 and MuRF1 are induced in SMA model mice and in SMA patient muscle in association with increased myogenin and histone deacetylase-4 (HDAC4) expression. This activation during both acute denervation and SMA disease progression is suppressed by treatment with a histone deacetylaseinhibitor; however, this treatment has no effect when atrogene induction occurs independently of myogenin. These results indicate that myogenin-dependent atrogene induction is amenable to pharmacological intervention with histone deacetylase inhibitors and help to explain the beneficial effects of these agents on SMA and other denervating diseases.

Specific aim 2 (Chapter 3) investigates the role of SMN in muscle development using SMN-deficient muscle cell lines and primary myoblasts. To determine the effect of SMN deficiency on muscle development, we examined expression of known markers of muscle differentiation as well as cell morphology and fusion. We found that SMN-deficient muscle cells have reduced fusion into multinucleate myotubes compared to wild type cells. In addition, we have identified time points at which the expression of differentiation markers between SMN-deficient and control muscle cells diverge. Restoration of SMN improves the fusion and brings the expression of differentiation markers closer to the levels of control muscle cells. SMN-deficient muscle cells have impaired cell migration and altered organization of focal adhesions and the actin cytoskeleton. These data are consistent with a role for SMN in myotube formation

through modulation of focal adhesions and actin dynamics.

Chapter 2: Histone deacetylase inhibition suppresses myogenin-dependent atrogene activation in spinal muscular atrophy

Adapted from: Bricceno KV, Sampognaro PJ, Van Meerbeke JP, Sumner CJ, Fischbeck KF, Burnett BG. Histone deacetylase inhibition suppresses myogenin-dependent atrogene activation in spinal muscular atrophy mice. Hum Mol Genet. 2012; 21(20):4448-59.

INTRODUCTION

Treatment of SMA mice with the histone deacetylase (HDAC) inhibitor trichostatin A (TSA), improves body weight, life span, myofiber number, and myofiber cross-sectional area (Avila et al., 2007). Beneficial effects of TSA on survival, muscle mass, and motor function have also been shown in a mouse model of ALS (Yoo and Ko, 2011). TSA has been shown to modestly increase SMN expression (Avila et al., 2007; Kwon et al., 2011; Narver et al., 2008), but as an HDAC inhibitor it may also inhibit HDAC4 activity, which has been shown to modulate the expression of genes involved in muscle atrophy. We hypothesized that TSA improves SMA muscle pathology, in part, by inhibiting this muscle atrophy pathway.

Muscle atrophy occurs in a variety of denervating diseases (Lecker et al., 1999) and with disuse (Tischler et al., 1990). During atrophy, muscle protein is degraded through the ubiquitin proteasome pathway (Solomon et al., 1998). Expression of genes encoding the skeletal muscle-specific E3 ligases atrogin-1 and muscle ring finger 1 (MuRF1), referred to as atrogenes, is increased in atrophy models, including fasting and glucocorticoid treatment, as well as denervation (Bodine et al., 2001). MuRF1 and atrogin-1 null mice are resistant to loss of muscle mass after denervation (Bodine et al., 2001).

Upregulation of the atrogenes is mediated by multiple pathways, including reduction in activity of the PI3K/Akt signaling cascade, which activates the FoxO1/3 transcription factors (Sandri et al., 2004; Stitt et al., 2004). MuRF1 upregulation in tumor-bearing mice is mediated by the transcription factor NF-κB, which is activated by TNF-α and other pro-inflammatory cytokines (Cai et al., 2004). In atrophy due to acute denervation, atrogin-1 and MuRF1 expression is regulated in part by HDACs and myogenin. Following denervation, HDAC4 is upregulated and suppresses the expression of Dach2, a transcription factor and represssor of the myogenin promoter (Tang and Goldman, 2006; Tang et al., 2009). Myogenin is a muscle-specific transcription factor essential for muscle development (Hasty et al., 1993). Myogenin expression increases following denervation via increased HDAC4 and decreased Dach2 expression (Tang et al., 2009). Myogenin, in turn, positively regulates HDAC4 expression following denervation, creating a positive feedback loop. Atrogin-1 and MuRF1 expression and muscle atrophy following denervation are attenuated in myogenin-null mice (Macpherson et al., 2011; Moresi et al., 2010). While these studies modeled neurogenic atrophy by severing the sciatic nerve, it remains to be determined whether (i) this pathway is conserved in a chronic motor neuron disease such as SMA and (ii) myogenin expression can be pharmacologically modulated to abrogate atrogene induction.

In this study, we demonstrate that the muscle-specific E3 ligases atrogin-1 and MuRF1 are upregulated in a cellular model of muscle atrophy and in the muscle of SMA mice and patients. We provide evidence that myogenin mediates induction of these atrogenes, and that TSA inhibits their upregulation. Thus, it is likely that this HDAC

inhibitor mitigates SMA disease manifestations by inhibition of atrogene upregulation in addition to upregulation of SMN expression.

RESULTS

Atrogin-1 and MuRF1 are upregulated in SMA mouse muscle and reduced by TSA treatment.

We and others have previously shown that myofibers are markedly smaller in size in multiple muscles in SMA mice at disease endstage, postnatal day 13 (P13), but not at P5 (Avila et al., 2007; Kong et al., 2009; Lee et al., 2011). In order to evaluate whether atrogenes are induced in SMA mice and whether their activation can be inhibited by HDAC inhibition, we treated a cohort of mice with either TSA (10 mg/kg) or vehicle (DMSO) and extracted RNA from total hindlimb muscles. The expression of atrogin-1 and MuRF1 was approximately 20-fold and 6-fold higher respectively, in SMA mice compared to phenotypically normal heterozygous littermates at P13 (Fig. 2.1A). As we have also previously shown that a 16% decrease in myofiber size occurs specifically between P9 and P13 in the tibialis anterior muscle of SMA mice (Kong et al., 2009), we further characterized the timing of atrogene induction. The atrogenes were similarly expressed in SMA mice and healthy littermates at P9, however they were significantly upregulated in SMA mice at P11 (Fig. 2.1A). Western blot analysis confirmed that increased atrogene gene expression was accompanied by increased protein expression (Fig. 2.1B). Thus atrogene induction corresponds temporally with the onset of muscle atrophy as well as with the onset of weight loss in these mice (Fig. 2.2) and (Avila et al., 2007).

Figure 2.1. Atrogin-1 and MuRF1 expression are upregulated in muscle of SMA mice. (A) The mice were treated starting at P5 with either vehicle (DMSO) or TSA (10 mg/kg) daily and sacrificed at P9, P11, and P13. Muscle tissue was harvested and RNA isolated for quantitative RT-PCR analysis. (B) Mice were treated starting at P5 with either vehicle (DMSO) or TSA (10 mg/kg) daily and sacrificed at P11. Muscle tissue was harvested and protein isolated for Western Blot analysis. Quantification of Western data is shown in the right panel of B. Values represent the mean ± SEM. (*, P < 0.05; **, P<0.01).

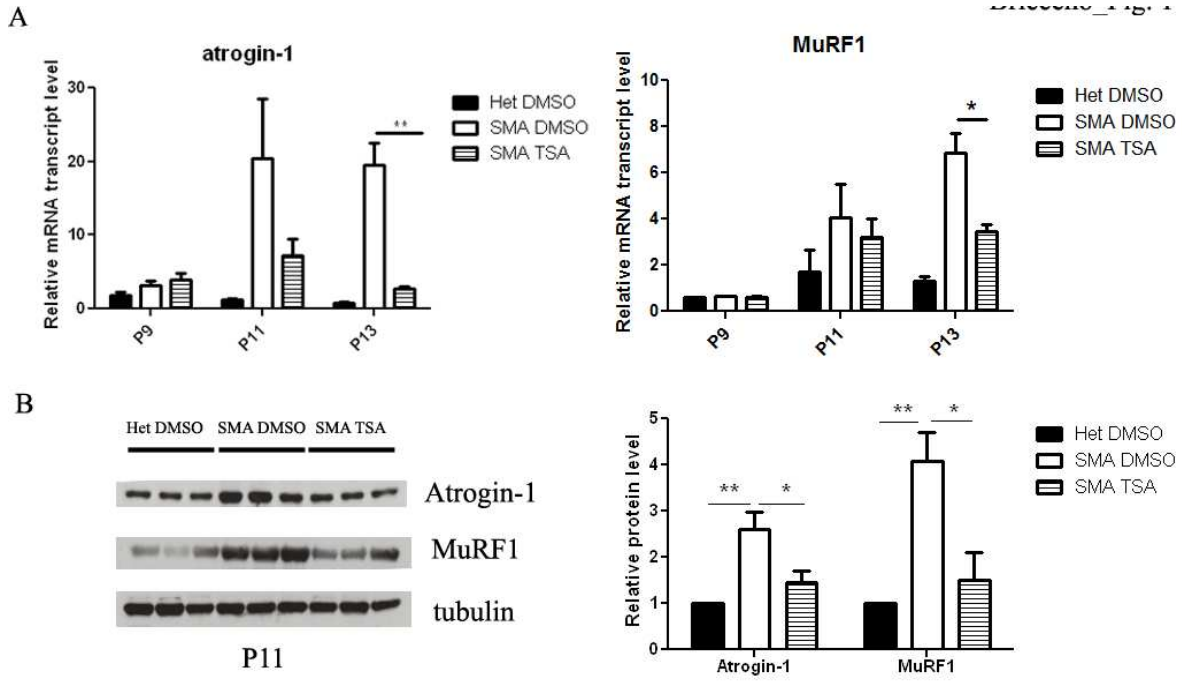
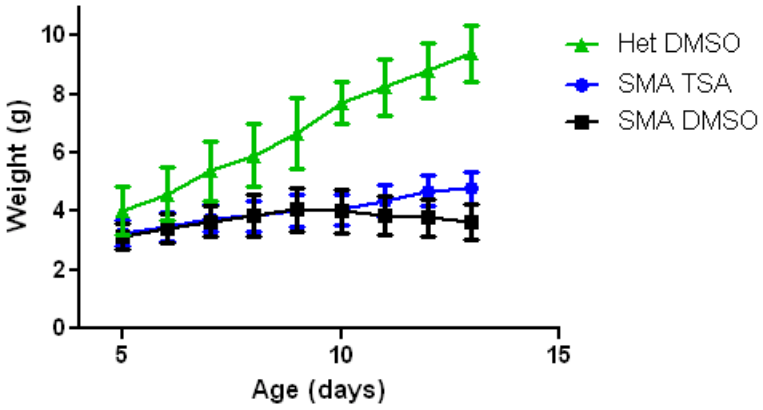


Figure 2.2. Body weight of SMA and heterozygous mice. Mice were weighed daily starting at P5 and were treated either with vehicle (DMSO) or TSA (10mg/kg). Values represent the mean body weight ± SEM.

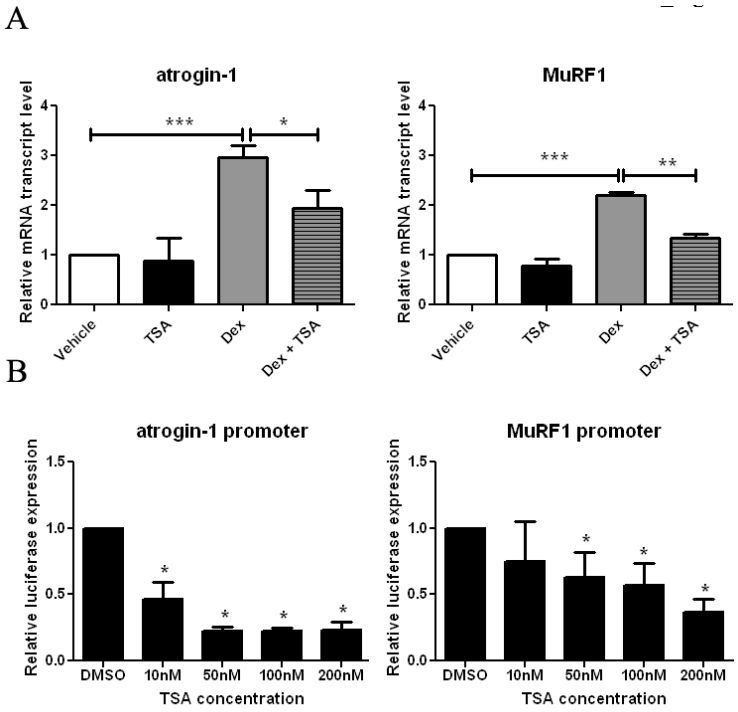


Treatment with TSA significantly inhibited atrogin-1 and MuRF1 activation at both P11 and P13 (Fig. 2.1A & B).

TSA reduces atrogin-1 and MuRF1 expression and inhibits protein breakdown in C2C12 cells.

To examine the molecular mechanisms by which HDAC inhibition may suppress atrogin-1 and MuRF1 expression, we treated C2C12 myotubes with dexamethasone (10 μM), TSA (50 nM), or a combination of TSA and dexamethasone for 24 hours. A synthetic glucocorticoid, dexamethasone promotes protein breakdown and induces atrogin-1 and MuRF1 expression in myotube cultures (Sandri et al., 2004) and adult mouse muscle (Baehr et al., 2011). We found that atrogin-1 and MuRF1 expression in the C2C12 myotubes was increased with dexamethasone treatment and that this effect was blocked by TSA (Fig. 2.3A). To determine whether this was due to decreased transcription at the promoter, we transfected C2C12 myoblasts with reporter constructs containing the atrogin-1 and MuRF1 promoters upstream of luciferase and quantified expression in the presence or absence of TSA. TSA blocked atrogin-1 and MuRF1 promoter activity in a dose dependent manner (Fig. 2.3B). Interestingly, TSA did not affect atrogene promoter activity when the reporters were expressed in a non-muscle cell line (HEK 293T), suggesting that the effect of TSA may be mediated by muscle-specific factors (Fig. 2.4). The structurally unrelated HDAC inhibitor valproic acid also reduced atrogin-1 and MuRF1 promoter reporter expression in C2C12 cells, indicating that atrogene downregulation was likely not due to an off-target effect of TSA (Fig. 2.5).

Figure 2.3. TSA inhibits atrogin-1 and MuRF1 induction and muscle protein breakdown in C2C12 myotubes. (A) Quantitative RT-PCR analysis of atrogin-1 and MuRF1 expression in C2C12 cells after 24 hr treatment with dexamethasone (Dex, 10 µM), and/or TSA (50 nM). Expression level is represented relative to the vehicle control (*n*=4 experiments per treatment). (B) Transfection of C2C12 myotubes with either an atrogin-1 or a MuRF1 luciferase reporter and treatment with DMSO, 10 nM, 50 nM, 100 nM, or 200 nM TSA. Luciferase activity is represented relative to DMSO control (*n*=4 experiments per dose). (C) The rate of protein breakdown in C2C12 myotubes labeled with L-[3,5-3H]tyrosine. Myotubes were treated with vehicle, Dex (10 µM) and/or TSA (50 nM), and release of L-[3,5-3H]tyrosine from cell proteins was used as an index of the breakdown of long-lived myofibrillar proteins. Dex treatment compared to Dex and TSA treatment (*n*=3 experiments per treatment). Values represent mean ± SEM. (*, P < 0.05; **, P<0.01;***, P<0.001).



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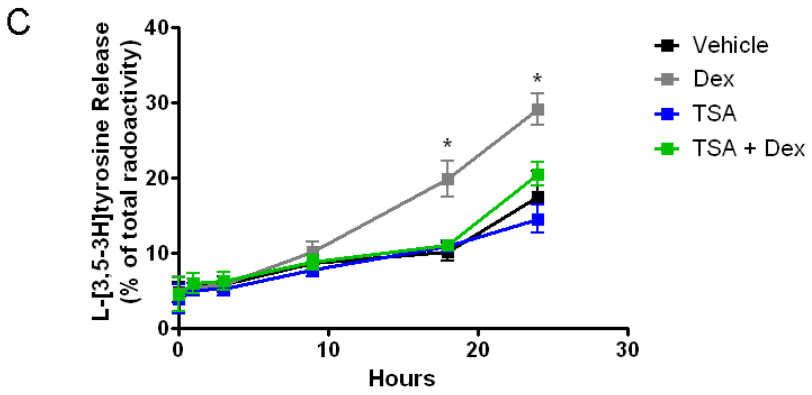


Figure 2.4. HEK 293T cells were transfected with luciferase reporters for either atrogin-1 or MuRF1 and treated with DMSO, 50 nM, 200 nM, or 500 nM TSA. Luciferase activity is represented relative to DMSO control (*n*=3 experiments per dose). Data represent the mean ± SEM.

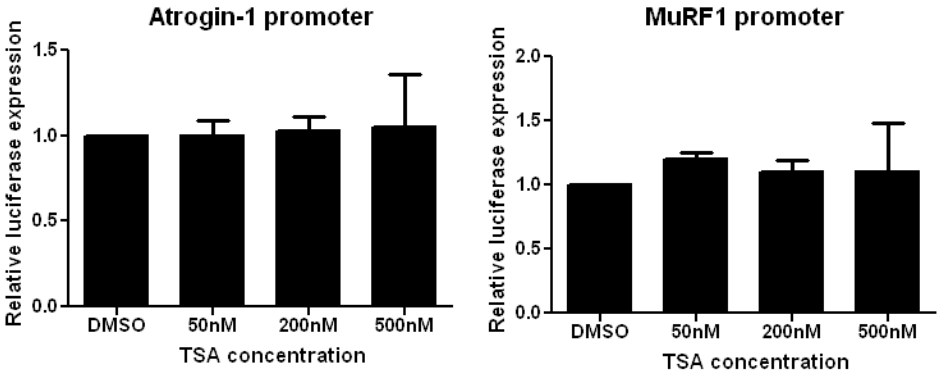
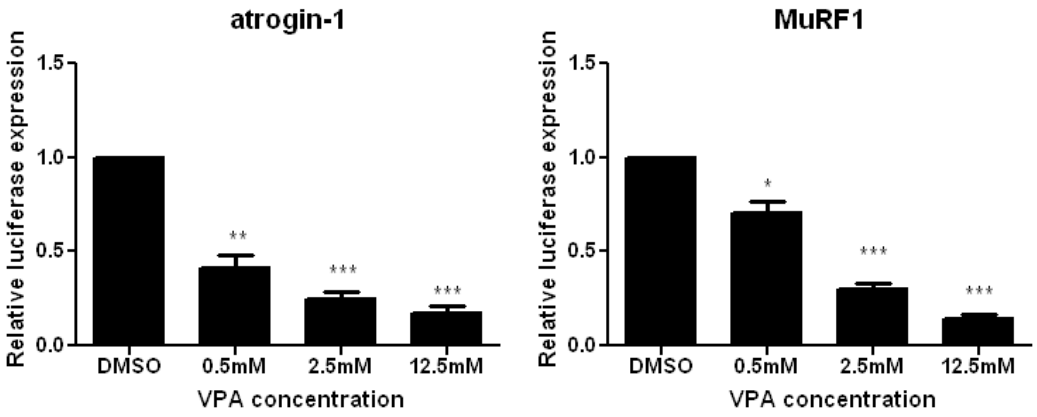


Figure 2.5. C2C12 myotubes were transfected with luciferase reporters for either atrogin-1 or MuRF1 and treated with DMSO, or 0.5 mM, 2.5 mM, 12.5 mM valproic acid (VPA). Luciferase activity is represented relative to DMSO control (n=3 experiments per dose). One sample t-tests were performed using GraphPad Prisim 5 software (GraphPad Software). Data represent the mean ± SEM. (*, P < 0.05; **, P<0.01; ***, P<0.001).



The maintenance of muscle mass is controlled by a balance between protein synthesis and protein degradation, which shifts toward protein degradation during atrophy. Atrogene expression stimulates proteasomal and autophagic/lysosomal proteolysis (Mammucari et al., 2007). We therefore tested whether TSA inhibits muscle protein degradation. After bulk labeling cellular proteins in myotubes with [^{3}H]tyrosine for 20 hours, the rate of degradation of long-lived proteins was measured in C2C12 myotubes after treatment with dexamethasone (10 μM), TSA (50 nM), both TSA and dexamethasone, or vehicle alone. Treatment with dexamethasone stimulated overall proteolysis compared with vehicle-treated myotubes (Fig. 2.3C). However, after 18 hours, TSA inhibited the increase in dexamethasone-induced proteolysis. Together, these data indicate that TSA suppresses the induction of atrogin-1 and MuRF1 and inhibits muscle protein breakdown in cultured myotubes.

TSA modulates atrogenes by reducing myogenin expression

Myogenin is upregulated in skeletal muscle following acute denervation and in turn increases the expression of MuRF1 and atrogin-1 (Macpherson et al., 2011; Moresi et al., 2010). Reducing myogenin in mice abrogates the expression of atrogin-1 and MuRF1 following denervation and slows muscle atrophy. Thus, myogenin plays a dual role as both an inducer of neurogenic atrophy and a regulator of muscle development (reviewed by (Rudnicki et al., 2008). We thus sought to determine whether the decreased expression of atrogin-1 and MuRF1 levels with TSA is associated with reduction of myogenin. We found that myogenin mRNA expression and protein levels were modestly increased in C2C12 myotubes treated with dexamethasome and this was inhibited by co-

administration of TSA (Fig. 2.6A and B). HDAC4 is upregulated in skeletal muscle upon denervation and represses the expression of Dach2, a negative regulator of myogenin (Tang et al., 2009). We examined Dach2 levels in myotubes and found that Dach2 expression is indeed increased in the presence of TSA (Fig. 2.6C). Thus, in C2C12 myotubes, TSA suppresses the expression of myogenin, MuRF1, and atrogin-1 and upregulates the expression of the repressor, Dach2.

HDAC inhibition reduces myogenin levels in SMA mice

Given that TSA repressed myogenin expression in cultured myotubes (Fig. 2.6A) and (Hagiwara et al., 2011), we next investigated whether the same is true in SMA mice. To examine the temporal sequence of events, we examined myogenin expression at P9, P11, and P13. While myogenin levels in untreated SMA mice were similar to heterozygous littermates at P9, TSA-treated SMA mouse muscle had significantly lower myogenin expression (Fig. 2.7A). At P11, when we first detected induction of the atrogenes (Fig. 2.1A), we found that myogenin levels were upregulated in SMA mice, while TSA-treated SMA mice had no significant myogenin induction. Myogenin levels were similar in heterozygous littermates treated with TSA or DMSO (data not shown).

We also examined the expression of the upstream myogenin negative regulator Dach2 at P9 and P11 in SMA muscle. Dach2 levels were unchanged at P9 in vehicle-treated SMA mice compared to heterozygous littermates (Fig. 2.8), but Dach2 levels were approximately 150-fold lower in vehicle-treated SMA mice compared to heterozygous littermates at P11 (Fig. 2.7B). Furthermore, treatment with TSA resulted in

Figure 2.6. TSA treatment modulates myogenin and Dach2 expression in C2C12 myotubes. C2C12 cells were treated with DMSO, dexamethasone (Dex, 10 µM), and/or TSA (50 nM). Quantitative RT-PCR analysis of myogenin (A) and Dach2 (C) expression (*n*=4 experiments per treatment). Western blot analysis of myogenin (B) protein levels (*n*=3 experiments per treatment). Quantification of Western data is shown in the lower panel. Values represent mean ± SEM. (*, P < 0.05; **, P<0.01).

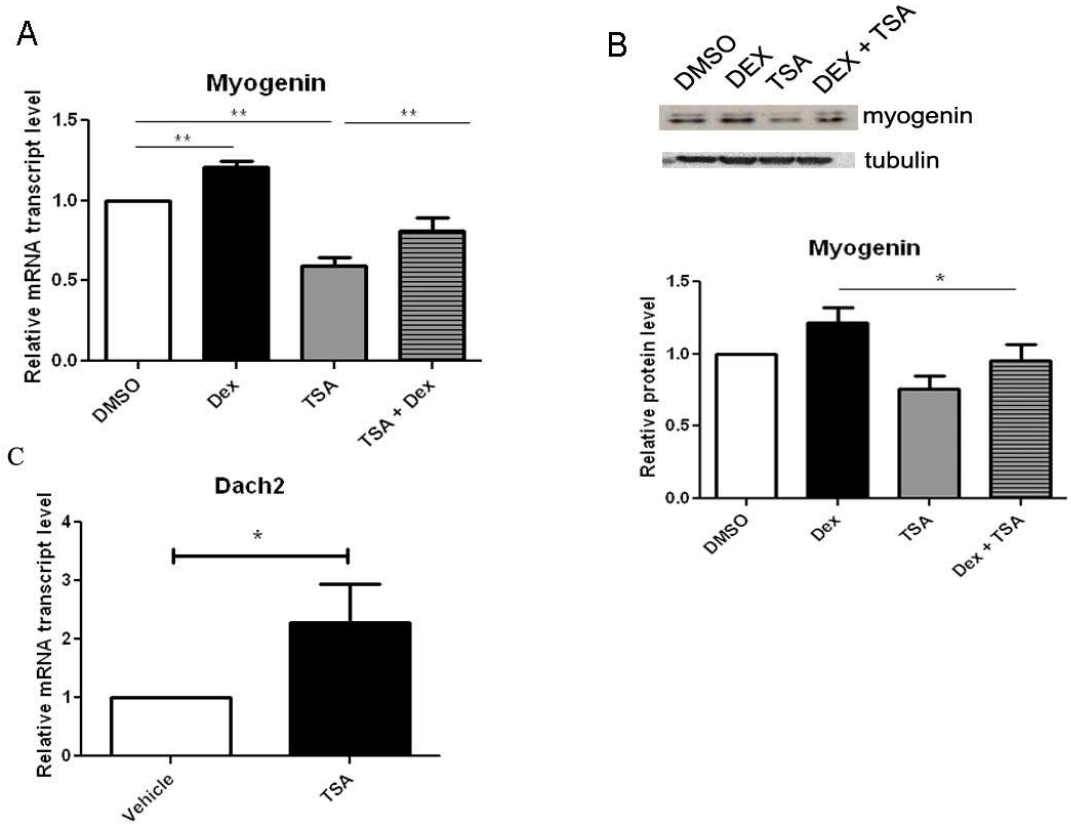
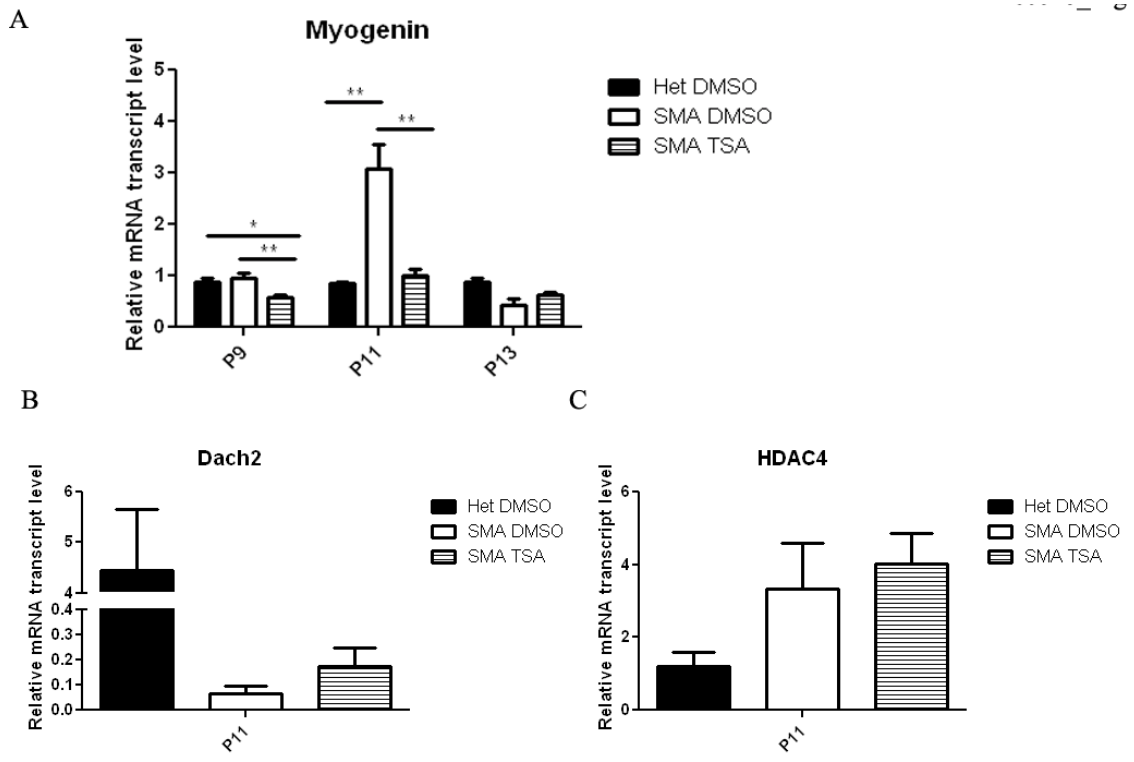


Figure 2.7. Expression of myogenin is increased in SMA mice, and this effect is abrogated by TSA. (A) Mice were treated starting at P5 with either vehicle (DMSO) or TSA (10 mg/kg) daily and sacrificed at P9, P11 or P13. Muscle tissue was harvested and RNA isolated for quantitative RT-PCR analysis of myogenin levels. (B and C) Mice were treated starting at P5 with either vehicle (DMSO) or TSA (10 mg/kg) daily and sacrificed at P11. Muscle tissue was harvested and RNA isolated for quantitative RT-PCR analysis of Dach2 and HDAC4 levels. (D) Mice were treated starting at P5 with either vehicle (DMSO) or TSA (10 mg/kg) daily and sacrificed at P11. Muscle tissue was harvested and chromatin was isolated for ChIP analysis of myogenin association with atrogin-1 and MuRF1 promoters. Values represent the mean ± SEM. (*, P < 0.05; **, P<0.01).



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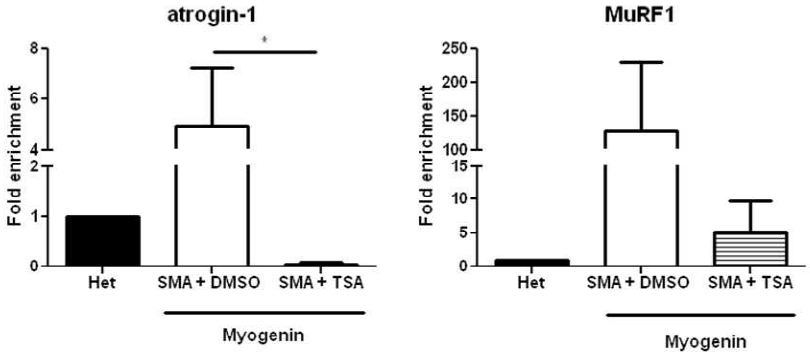
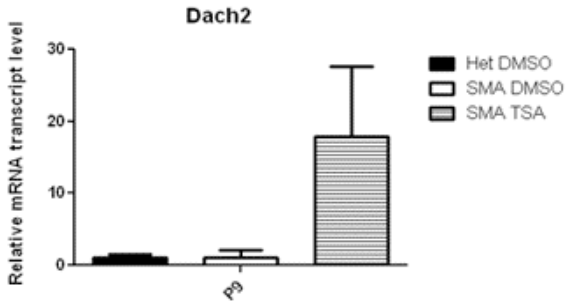


Figure 2.8. Expression of the myogenin repressor Dach2 in SMA mice treated with TSA. Mice were treated starting at P5 with either vehicle (DMSO) or TSA (10 mg/kg) daily and sacrificed at P9. Muscle tissue was harvested and RNA isolated for quantitative RT-PCR analysis of Dach2 levels. Values represent the mean ± SEM.



approximately 12-fold and 2-fold increases in Dach2 expression at P9 and P11, respectively (Fig. 2.8 and Fig. 2.7B). HDAC4 expression was increased in SMA mice compared to heterozygous littermates at P11 (Fig. 2.7C). TSA treatment did not decrease HDAC4 transcript levels, suggesting that TSA results in increased Dach2 expression by inhibiting HDAC4 activity rather than its expression.

We next examined whether myogenin was directly interacting with the promoters of the atrogenes to induce their expression in SMA mice. E boxes (CANNTG) that mediate myogenin binding are located in the promoters for *FBXO32* (atrogin-1) (-79 bp) and *TRIM63* (MuRF1) (-143bp, -66bp, -44bp) (Moresi et al., 2010). We examined the binding of myogenin to the endogenous MuRF1 and atrogin-1 promoters by performing chromatin immunoprecipitation assays using chromatin extracts from P11 hindlimb muscles. Chromatin was immunoprecipitated with antibodies against either myogenin or immunoglobulin G as a control. We found increased association of myogenin with the endogenous atrogin-1 and MuRF1 promoters in chromatin extracts from vehicle-treated SMA mice compared to heterozygous littermates (Fig. 2.7D); however, little association was observed in SMA mice treated with TSA. Together, these data suggest that myogenin directly activates atrogene expression in SMA muscle via transactivation of the atrogene promoters. Furthermore, myogenin activation in SMA possibly occurs as a consequence of increased HDAC4 and reduced Dach2 expression and this can be inhibited by HDAC inhibitors.

HDAC inhibition suppresses myogenin-dependent, but not myogenin-independent atrogene induction

Because the atrogenes can be induced by fasting, and SMA mice likely have inadequate nutrition at disease endstage (Bowerman et al., 2012; Butchbach et al., 2010; Narver et al., 2008), we examined whether TSA is capable of repressing atrogene induction that occurs during fasting. Wild-type mice were treated with 10 mg/kg of TSA daily for three days, and then food was removed and the animals were sacrificed 18 h later. Atrogin-1 and MuRF1 expression were markedly increased in the fasting mice, but this effect was not blocked by TSA treatment (Fig. 2.9A). Importantly, myogenin expression was also not induced in fasting mice, indicating that during starvation atrogene activation occurs independently of myogenin (Fig. 2.9B). We next examined the effects of TSA in mice with acute denervation atrophy as in this context atrogene induction is myogenin and HDAC4/5 dependent (Macpherson et al., 2011; Moresi et al., 2010; Tang and Goldman, 2006; Tang et al., 2009). Wild-type mice were treated with 10 mg/kg of TSA daily for three days before sciatic nerve transaction and 3 days after denervation. The sciatic nerve of the conralateral leg was left intact and used as a control. Increased myogenin, atrogin-1 and MuRF1 expression in the denervated muscle was almost completely inhibited by TSA treatment (Fig. 2.9C and D). Together these data highlight that atrogene induction can occur via distinct upstream mechanisms, and that TSA specifically inhibits the myogenin-dependent pathway.

The atrogenes and myogenin are induced in human SMA muscle

To verify that myogenin-dependent atrogene activation is relevant to SMA in humans, we examined the expression of the atrogenes in the iliopsoas, a severely affected muscle, and the diaphragm, a relatively spared muscle, in type I SMA patients and age-

Figure 2.9. The effect of TSA on atrogene expression is myogenin-dependent. (A and B) Starved wild-type mice were treated by intraperitoneal injection with vehicle (DMSO) or 10 mg/kg of TSA daily for three days and sacrificed 18 hours after removal of food. Muscle tissue was harvested from fed mice and DMSO and TSA-treated starved mice and RNA isolated for quantitative RT-PCR analysis of myogenin, atrogin-1 and MuRF1 levels. (C and D) Wild-type mice were treated by intraperitoneal injection with vehicle (DMSO) or 10 mg/kg of TSA daily for three days before and three days after denervation by sciatic nerve cut. The contralateral leg remained innervated and was used ads control. Muscle tissue was harvested from both hind legs of DMSO and TSA-treated mice. RNA was isolated for quantitative RT-PCR analysis of myogenin, atrogin-1 and MuRF1 levels. Values represent the mean ± SEM. (*, $P < 0.05$; **, $P<0.01$;***, $P<0.001$).

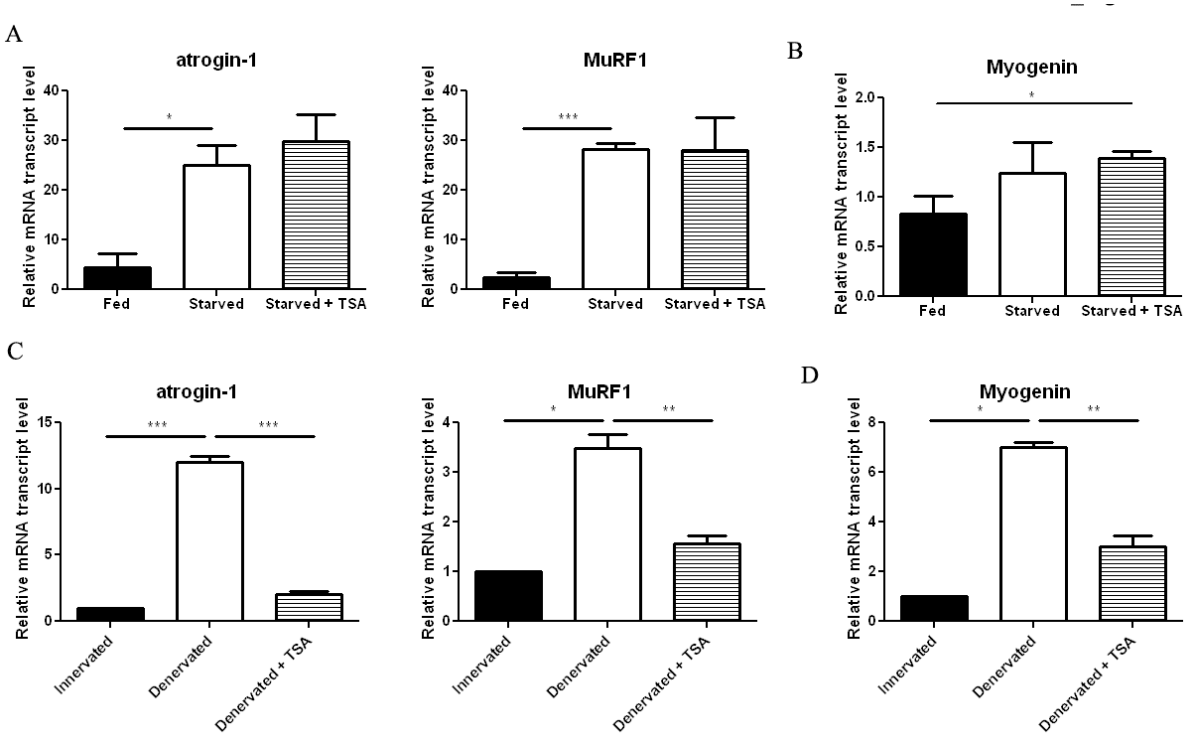
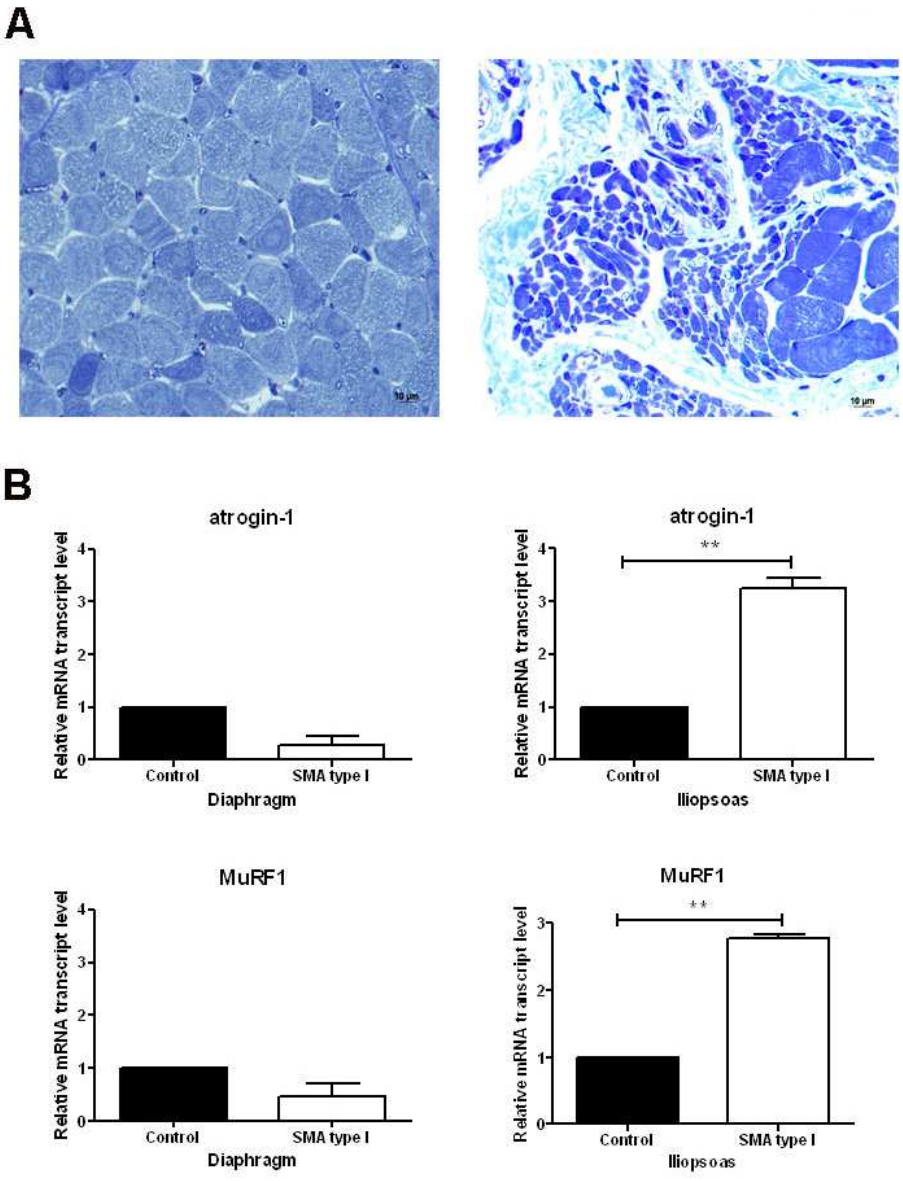


Figure 2.10. Atrophy related genes are activated in the iliopsoas muscle, but not in the diaphragm muscle isolated from type I SMA patients. (A) Toluidine-blue stained muscle cross sections of muscle show severe muscle atrophy in the iliopsoas (right), a clinically severely affected muscle, but not the diaphragm (left), a clinically relatively spared muscle. (B) RNA isolated from muscle tissues for quantitative RT-PCR analysis of atrogin-1, MuRF1, myogenin and HDAC4. Values represent the mean ± SEM. (*, $P < 0.05$; **, $P<0.01$).



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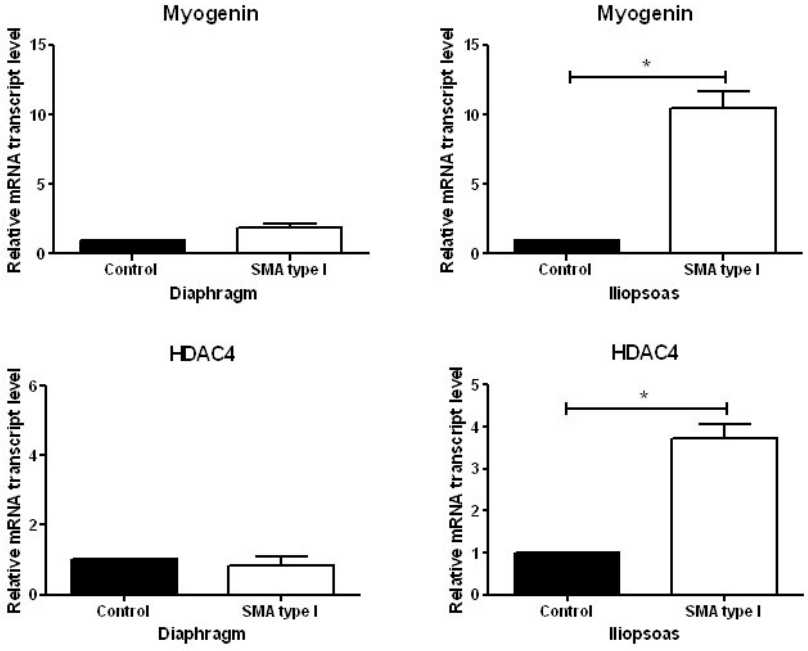


matched controls (Fig. 2.10A). Atrogin-1, MuRF1, myogenin, and HDAC4 were all significantly upregulated in SMA iliopsoas muscle, but unchanged in the diaphragm (Fig. 2.10B). These data are consistent with increased HDAC4 activity leading to increased expression of myogenin and upregulation of atrogenes in SMA human muscle (Fig. 2.11).

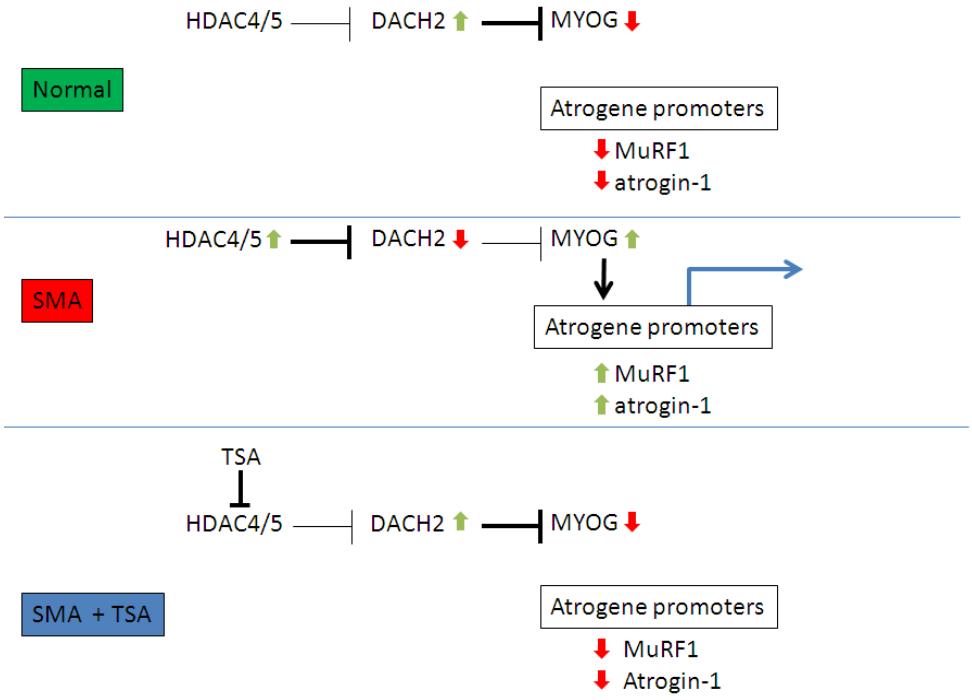
The atrogenes and myogenin are induced in human SMA muscle

To verify that myogenin-dependent atrogene activation is relevant to SMA in humans, we examined the expression of the atrogenes in the iliopsoas, a severely affected muscle, and the diaphragm, a relatively spared muscle, in type I SMA patients and age-matched controls (Fig. 2.10A). Atrogin-1, MuRF1, myogenin, and HDAC4 were all significantly upregulated in SMA iliopsoas muscle, but unchanged in the diaphragm (Fig. 2.10B). These data are consistent with increased HDAC4 activity leading to increased expression of myogenin and upregulation of atrogenes in SMA human muscle (Fig. 2.11).

DISCUSSION

In this study, we show that the atrogenes atrogin-1 and MuRF1 are activated in muscle isolated from both mice and human patients with the motor neuron disease SMA. This induction is associated with increased expression of the upstream regulators, HDAC4 and myogenin, which is consistent with the pattern of gene activation previously observed during acute denervation-induced muscle atrophy (Macpherson et al., 2011; Moresi et al., 2010). Importantly, this induction during both acute denervation and chronic SMA

Figure 2.11. Proposed model of muscle atrophy in SMA. In SMA muscle, upregulation of HDAC4/5 inhibits expression of the myogenin repressor Dach2 and results in increased myogenin expression. Myogenin then activates the atrogin-1 and MuRF1 promoters. TSA inhibits the activity of HDAC4/5, blocking the repression of Dach2. Increased Dach2 reduces myogenin expression and thus prevents the activation of atrogin-1 and MuRF1.



disease is markedly suppressed by the HDAC inhibitor TSA. In contrast, TSA does not suppress atrogene activation during fasting-induced muscle atrophy, which we show occurs independently of myogenin. These experiments highlight the distinct upstream pathways that regulate atrogene induction and show that myogenin-dependent atrogene activation may be suppressed pharmacologically with HDAC inhibitors. Thus these drugs may have therapeutic relevance in a number of denervating disorders.

SMA mice show a profound reduction in total muscle cross sectional area and myofiber diameter at disease endstage (Avila et al., 2007; Kong et al., 2009). Although specific muscle groups show structural denervation of NMJs (Ling et al., 2012), the NMJ endplates of many muscles retain presynaptic terminals (Kariya et al., 2008; Kong et al., 2009; Lee et al., 2011). Nonetheless, detailed structural analysis has revealed presynaptic neurofilament accumulations and reduced synaptic vesicle densities in most SMA NMJs and electrophysiological analysis has shown abnormalities of synaptic vesicle release. Furthermore, central synaptic inputs to motor neurons are also abnormal in SMA mice (Ling et al., 2012; Mentis et al., 2011) and this may have downstream consequences for NMJ function. Myofiber atrophy is likely activated in SMA muscle once these functional NMJ abnormalities reach a critical threshold, without the requirement for complete structural denervation of muscle. Here, we show that the atrogenes are induced at P11 in SMA mice, which corresponds well with the time period of decreasing muscle size (Kong et al., 2009) and weight loss. Importantly, this induction is nearly completely suppressed by treatment with the HDAC inhibitor TSA. We had previously shown that TSA improved the survival and motor function of SMA mice. Interestingly, in this study we observed a significant improvement in muscle and myofiber size without a change in

motor neuron numbers (Avila et al., 2007). Although TSA modestly increases SMN expression, we suspected that TSA may have independent beneficial effects on muscle. The current study indicates that suppression of myogenin-dependent atrogene induction with TSA is likely an important mechanism mitigating muscle atrophy in SMA mice.

Atrogin-1 and MuRF1 are induced in multiple models of skeletal muscle atrophy. While atrogin-1 targets transcription factors important for muscle growth and differentiation for degradation, MuRF1 mediates the degradation of structural proteins such as myosin heavy chain proteins (MHC), myosin light chains 1 and 2, myosin binding protein C, muscle creatine kinase, and other myofibrillar proteins (Clarke et al., 2007; Cohen et al., 2009; Koyama et al., 2008; Mearini et al., 2010; Witt et al., 2005). Together, these enzymes play essential roles in the loss of muscle mass.

The transcription factor myogenin regulates the induction of the atrogenes by binding their promoters and activating transcription. TSA prevents induction of atrogenes by modulating the upstream regulators of myogenin expression, HDAC4 and Dach2. HDAC4 inhibits expression of Dach2, which represses myogenin expression. We show here that HDCA4 is induced at P11 in SMA model mice, and this is associated with a marked reduction in the expression of Dach2. While TSA did not alter the expression of HDAC4 in TSA-treated mice, Dach2 expression was increased 2-fold, suggesting that TSA likely exerts its effect through HDAC4 inhibition rather than altering HDAC4 expression. Nevertheless, treatment with TSA almost completely blocked induction of myogenin in SMA mice, with only a modest increase in Dach2 levels. While it is possible that a 2-fold induction of Dach2 is sufficient to block induction of myogenin, it is likely

that TSA affects other myogenin modulators. Identifying these potential modulators could provide additional therapeutic targets.

In this study, we also demonstrate the relevance of this atrophy pathway to the human disease by examining atrogene and myogenin expression in human muscle samples isolated from type I SMA patients. In our study, we found upregulated expression of these genes in a clinically and pathologically severely affected muscle, the iliopsoas, but little induction in a relatively spared muscle, the diaphragm. Our data contrasts with a previous study in which type I and type III SMA patient muscle biopsy samples were examined by microarray and quantitative RT-PCR. A downregulation of atrogin-1expression was found in SMA patients (Millino et al., 2009). Expression changes in MuRF1 were not reported in this study. The difference in atrogin-1 expression levels in the two studies could be attributed to the different muscles examined and the stage of the disease when the muscle was collected. Iliopsoas and diaphragm muscles used in this study were collected at autopsy while the muscle samples used by Millino et al. came from biopsies of the quadriceps muscles of type I and type III patients. It is possible that the atrogenes were not induced at the time of biopsy, since atrogene induction occurs late in the SMA mice.

While motor neuron loss is central to SMA disease pathogenesis, therapeutic targeting of muscle may provide benefits to SMA patients. Previous studies in SMA mice investigated the effect of factors known to regulate muscle growth, such as myostatin, an inhibitor of muscle growth, and follistatin, an inhibitor of myostatin. Both genetic and therapeutic interventions to modulate these growth factors have not improved muscle mass or the overall SMA phenotype (Rindt et al., 2012; Rose et al., 2009; Sumner

et al., 2009). These approaches focused on muscle growth and protein synthesis to improve the disease phenotype. However, muscle maintenance is a balance between protein synthesis induced by growth factors and protein breakdown. Targeting protein synthesis without inhibiting atrophy-related protein breakdown may be insufficient to improve the disease phenotype. Thus, modulating expression of critical genes in the atrophic pathway may represent a strategy for mitigating atrophy in SMA and perhaps other denervating diseases. Recently, a MuRF1 inhibitor (P013222) was identified with an E3 ligase screening platform (Eddins et al., 2011). This small molecule selectively inhibits MuRF1 auto-ubiquitination and ubiquitination of MHC in a cellular atrophy model. If inhibiting MuRF1 in an animal model slows muscle wasting, then this would help to validate it as a therapeutic target for denervating diseases.

Considerable research has been done with animal models to investigate the role and regulation of atrogin-1 and MuRF1 in muscle wasting conditions, yet few investigations have shown that these pathways are conserved in human conditions of muscle atrophy. The gap in knowledge between atrogin-1 and MuRF1 regulation in animal and human models of skeletal muscle atrophy makes it difficult to fully assess the validity of atrogenes as potential therapeutic targets. In this study, we approached the molecular mechanisms underlying muscle atrophy in SMA mice and patients. Expression of the muscle-specific E3 ligases is increased in SMA muscle, and this is at least partly myogenin-dependent. We show that the HDAC4-Dach2-myogenin pathway can be pharmacologically targeted to suppress atrogene induction and muscle protein breakdown. TSA likely improves the SMA phenotype not only by increasing SMN gene expression, but also by attenuating myogenin expression and inhibiting muscle atrophy.

MATERIALS AND METHODS

Mouse treatment

Experiments were approved by the National Institute of Neurological Disorders and Stroke Animal Care and Use Committee. An SMA mouse breeder colony was maintained as described in (Avila et al., 2007). Treated SMA mice were given intraperitoneal injections of 10 mg/kg TSA (Enzo) in DMSO beginning on post-natal day 5 (P5). Daily injections were continued until mice were sacrificed at P9, P11, or P13. Control animals received equal volumes of vehicle alone. Mice were anesthetized with isoflurane and sacrificed by cervical dislocation. Distal hindlimb muscle was isolated and flash frozen in liquid nitrogen. Tissues were stored at -80°C and thawed before RNA or protein isolation. Adult wild-type littermates of SMA mice were used for starvation and denervation studies. For starvation studies, the mice were given TSA (as described above) 2 days, 1 day, and immediately before food removal. The mice were deprived of solid food for 18 hours, but given free access to water. For denervation studies, the mice were given TSA (as described above) 2 days and 1 day before denervation surgery and on the day of surgery. In anesthetized mice, fur covering the lower back and proximal thigh was removed. An incision was made near the sciatic notch. The facia was cut and the hamstring and gluteal muscles separated to reveal the sciatic nerve. In one leg, a 1 cm long piece of the sciatic nerve was removed. The other leg remained innervated and served as a control. The incision was closed with sutures. Post-operative mice were given either TSA or DMSO 1, 2, and 3 days after surgery and sacrificed on the third day.

Cell culture

C2C12 myoblasts were grown to confluency in DMEM with 10% fetal bovine serum, penicillin and streptomycin and differentiated for 4 days in DMEM with 10% horse serum, penicillin and streptomycin. Cells were treated with: 50 nM TSA in DMSO, 10 μM dexamethasone (Sigma) in ethanol or both for 24 hrs and then collected for RNA isolation. Control cells received vehicle only. HEK 293T cells were grown in DMEM with 10% fetal bovine serum, penicillin and streptomycin.

RNA isolation

Total RNA was isolated from muscle tissue and C2C12 cells using Trizol (Invitrogen), and the RNeasy kit (Qiagen) was used to purify the RNA following the manufacturer's instructions. 1 μg RNA was converted to cDNA using the High Capacity cDNA Reverse Transcription kit (Applied Biosystems), following manufacturer's instructions. Gene expression was determined by quantitative RT-PCR using TaqMan and SYBR Green reagents (Applied Biosystems). Primers are listed in Fig. 2.12. Transcript levels were quantified by threshold cycle values using phosphoglycerate kinase 1 as a control. For each gene, values were normalized to vehicle-treated, unaffected animals or cells. For patient samples, values were normalized to unaffected controls.

Western Blot

C2C12 cells were lysed in 1% NP-40, 50 mM Tris-HCl (pH 8), 150 mM NaCl and protease inhibitor cocktail (Roche) on ice for 10 minutes. The lysates were centrifuged at 4°C for 10 minutes and the supernatants collected. Protein concentration was determined

Figure 2.12.

Supplemental table 1: Primer information

Primer Name	**TaqMan Assay**
Atrogin-1	Mm00499523_m1
MuRF1	Mm01185221_m1
Myogenin	Mm00446194_m1
Pgk1	Mm00435617_m1
MITR	Fwd: GCGGTCCAGGTTAAAACAGA
	Rvs: GAGCTGAAGCCTCATTTTCG
	FAM Probe: CTTACTCAGGCGGAAGGATG
Primer Name	**Sequence**
Dach2	Fwd: GCAGCGGTGGCAGCAGCAACA Rvs: GCCCCCAGCCCACGGAGGAT

using the BCA Protein Assay kit (Pierce) according to the manufacturer's protocol. Protein extraction from hindlimb muscle was done as previously described (Kwon et al., 2011). Protein lysates (100 μg for C2C12 cells, 50 μg for mouse muscle) were resolved by SDS-PAGE (10%) and transferred to PVDF membranes. The membranes were blocked in 5% milk and probed with mouse anti-myogenin (1:500; clone F5D, Developmental Studies Hybridoma Bank), rabbit anti-atrogin 1 (1:1000, ECM Biosciences), rabbit anti-MuRF1 (1:500, Santa Cruz Biotechnology), and mouse anti-alpha tubulin (1:3000, Sigma).

Luciferase assay

C2C12 cell transfections were performed using GenJet Ver. III (SignaGen) following the manufacturer's instructions. HEK 293T cell transfections were performed using FuGENE (Roche) following the manufacturer's directions. The reporter plasmid, either atrogin-1 (Dr. Stewart Lecker, Beth Israel Deaconess Medical Center) or MuRF1 (Dr. Eric Olson, University of Texas Southwestern Medical Center) was used in a ratio of 5:1 with a pGL4-TK Renilla luciferase plasmid (Promega). 24 hrs after transfection, cells were treated for an additional 24 hrs with 10 nM, 50 nM, 100 nM, 200 nM or 500 nM TSA in DMSO or 0.5 mM, 2.5 mM or 12.5 mM valproic acid (Sigma) in water. Control cells received vehicle only. Following treatment, the cells were collected and luciferase assays were performed using the Dual Luciferase Reporter Assay system (Promega) following the manufacturer's instructions.

Protein breakdown assay

C2C12 cells were grown for 3 days in differentiation media, DMEM with 10% horse serum, penicillin and streptomycin. The media was supplemented with L[3,5-^{3}H]tyrosine (5 μCi/ml, PerkinElmer) for an additional 22 hrs. The cells were then washed with phosphate-buffered saline (PBS) and returned to differentiation media with 50 nM TSA in DMSO, 10 μM dexamethasone in ethanol or both. Control cells received vehicle only. 200ul aliquots of media were taken at 0, 1, 3, 9, 18 and 24 hrs for quantification of L[3,5-^{3}H]tyrosine release. Protein precipitation and radioactivity measurement were done as previously described (Sacheck et al., 2004).

Chromatin immunoprecipitation

Flash frozen hindlimb muscle was minced on ice and fixed in 1% formaldehyde/PBS for 20 min and then quenched with glycine. Samples were centrifuged and washed twice in PBS with protease inhibitors. The cells were lysed and chromatin isolated using the EZ Magna ChIP A kit (Millipore) following the manufacturer's directions. Anti-myogenin antibody (M-225x, Santa Cruz) was used to immunoprecipitate the chromatin fragments. Primers for amplifying atrogin-1 and MuRF1 promoters were described previously (Moresi et al., 2010).

Patient tissue

Diaphragm and iliopsoas muscle tissues were obtained from autopsies of type I SMA patients (n=3) or age-matched controls (n=2) (National Institute of Child Health and Development Brain and Tissue Bank for Developmental Disorders at the University of Maryland, Baltimore, MD.). Cross sections of muscle were stained with toluidine-blue.

Statistical analysis

Data are presented as the mean +/- the standard error of the mean. T-tests were performed using GraphPad Prisim 5 software (GraphPad Software) with significance set at $p<0.05$.

Chapter 3: A role for survival motor neuron protein in myoblast fusion

Adapted from: Bricceno KV, Martinez T, Duguez S, Partridge TA, Fischbeck KH, Sumner CJ, and Burnett BG. A role for survival motor neuron protein in myoblast fusion. *In preparation*.

INTRODUCTION

Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by proximal muscle weakness and degeneration of anterior horn cells (Park et al., 2010a). It is caused by mutation of the *SMN1* gene and resulting deficiency of survival of motor neuron (SMN) protein (Lefebvre et al., 1995). There is a growing body of evidence to support a role for a muscle defect in SMA. SMN co-localizes with α-actinin in myofibers, indicating a possible muscle-specific function for SMN (Chang et al., 2008; Rajendra et al., 2007), and SMN-deficient C2C12 muscle cells have fusion and proliferation defects (Shafey et al., 2005). In SMA mouse models, different fiber groups are differently affected by SMN deficiency (Monani et al., 2000; Murray et al., 2008; Mutsaers et al., 2011). Targeted knock-out experiments in mice have shown that reducing SMN in muscle gives a dystrophic phenotype that is corrected with Smn expression in muscle progenitor cells (Cifuentes-Diaz et al., 2001; Nicole et al., 2003). Both SMA patients and model mice show have altered development of SMN-deficient muscle (Guettier-Sigrist et al., 2002; Lee et al., 2011). Myotubes grown from biopsies of patient muscle are smaller in size, consistent with data from the mouse model correlating muscle weakness with fewer and smaller fibers. This delay in post-natal muscle development occurs while neuromuscular junctions are occupied, though abnormalities in transmission may exist. These data suggest an intrinsic role for SMN in muscle

development and function. Recent work in primary muscle cells isolated from SMA model mice has shown SMN has a role in myogenesis and that normal muscle differentiation requires adequate levels of SMN (Hayhurst et al., 2012). Importantly, replacing SMN in SMA mouse muscle partially rescues muscle area and myofiber diameter (Martinez et al., 2012), further supporting of role for SMN in muscle growth and development.

In the formation and growth of myofibers, proliferating myoblasts normally differentiate, migrate, and fuse to form myotubes. Many factors affect the fusion process, including cell adhesion molecules, secreted molecules and their receptors, and molecules that regulate actin cytoskeleton remodeling (reviewed in (Abmayr and Pavlath, 2012)). Among the cytoskeletal structures involved in myoblast fusion are focal adhesions that directly bind to the β1-integrins of the extracellular matrix and to the actin cytoskeleton. The focal adhesion complex is made up of several proteins, including focal adhesion kinase, vinculin, α-actinin, and talin (Nayal et al., 2004). In the absence of cleavage, talin and other adhesion complex proteins remain longer in focal adhesions. In particular, the cleavage of talin by the protease calpain 2 is a mechanism by which adhesion dynamics are regulated (Franco et al., 2004). Therefore, focal adhesion disassembly may lead to reduced strength of integrin-mediated cell adhesions and thus enhance cell migration.

In this study, we show that SMN-deficient muscle cells have a fusion deficit and altered expression of differentiation markers, which are partially rescued by restoration of SMN. We provide evidence that talin-regulated focal adhesion dynamics are disrupted and are at least in part responsible for the fusion deficit. Therefore, it is likely that SMN deficiency impairs myoblast fusion through effects on focal adhesions.

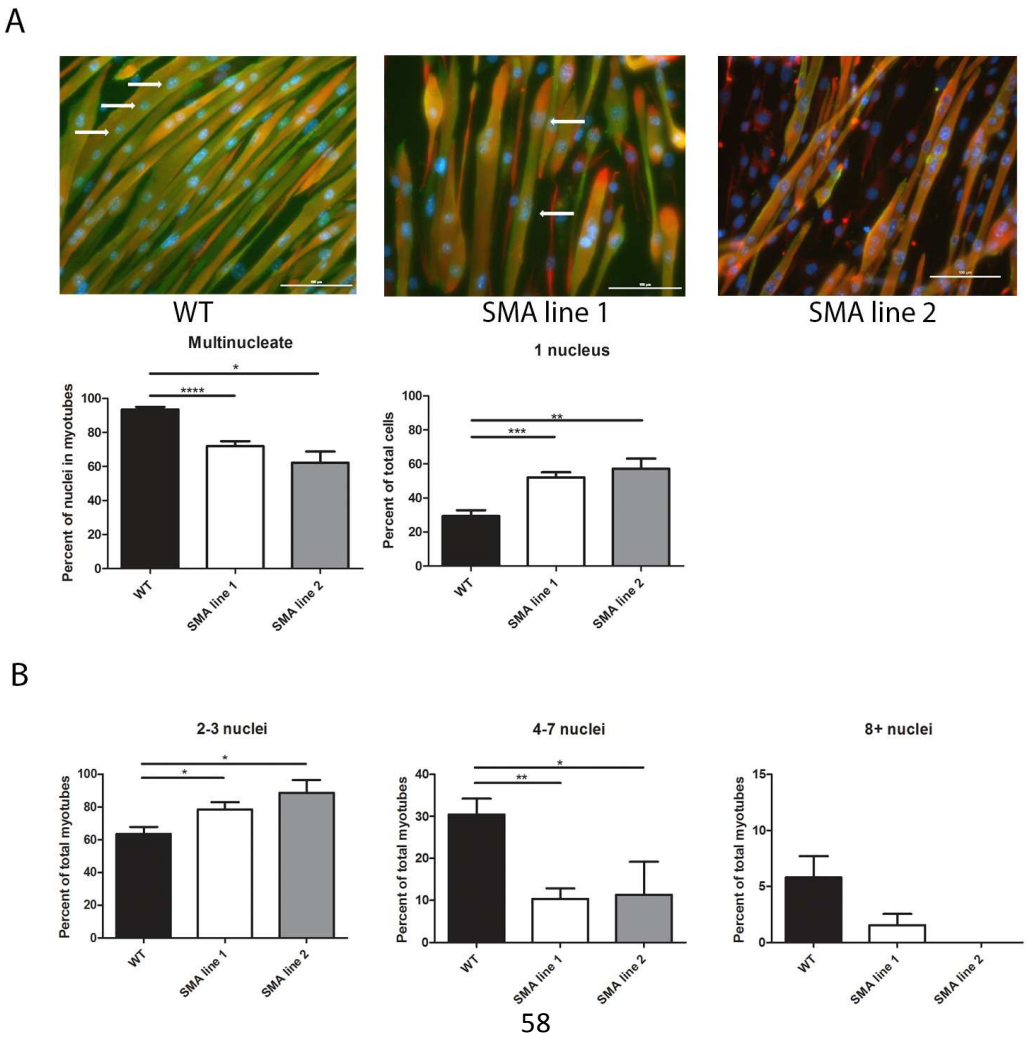
RESULTS

SMA muscle cell lines have a fusion deficit that is rescued by restoring SMN

It had previously been reported that SMN deficiency results in reduced myoblast fusion in cultured cells (Hayhurst et al., 2012; Shafey et al., 2005). In order to study the effects of SMN deficiency on myotube formation we established muscle cell lines from a SMA model mouse. The SMA delta 7 mice (Le et al., 2005) were crossed with mice overexpressing the H-2Kb-tsA58 (H2K) transgene (Jat et al., 1991), which encodes a thermolabile mutant of the large T antigen that allows the immortalization of cells when grown at 33°C in the presence of γ-interferon. When muscle cells are switched to non-permissive conditions, 37°C and absence of γ-interferon, they differentiate and form myotubes. This method of generating conditionally immortal cell lines has previously been used to generate cell lines from mouse models of other muscle diseases, including limb-girdle muscular dystrophy (Cohen et al., 2012). We established several monoclonal cell lines of immortalized SMN-deficient (Figure 3.2) and wild-type myoblasts from the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles of P4 pups. All lines were purified to remove fibroblasts and tested for myogenicity based on desmin staining.

Following derivation of the muscle cell lines we first sought to confirm the previous reports of a fusion deficit in an SMN-deficient cell line. On differentiation we found that fewer SMN-deficient myoblasts fused into multinucleate myotubes (Figure 3.1A), with more than 50% of cells failing to fuse. Overall, SMN-deficient myoblasts formed myotubes with fewer nuclei compared to wild-type muscle cells (Figure 3.1B).

Figure 3.1. SMA muscle cells have a fusion deficit. Muscle cells were differentiated for 6 days and stained for sarcomeric myosin (green) and desmin (red). DAPI (blue) was added to label nuclei. Representative images are shown in panel A. The percentage of total nuclei in myotubes and the percentage of total cells with one nucleus are quantified in A. Myotubes were defined as cells positive for desmin and MF-20 with two or more nuclei. The percentage of myotubes with 2-3, 4-7, and 8 or more nuclei are quantified in panel B. Nikon Elements software was used to count cells and nuclei. 6 experiments were done per cell line. The values represent the mean ± SEM. (*, P < 0.05; **, P<0.01; ***, P<0.001).



We confirmed the fusion deficits in an additional SMA line, indicating this effect was not limited to the clone selected (Figure 3.1A). To establish that the fusion deficit was due to reduced SMN levels we electroporated cDNA encoding human SMN into the SMN-deficient myoblasts to restore SMN protein levels (Figure 3.2). Restoring SMN levels rescued the fusion deficit in the SMN-deficient cells (Figure 3.3A) resulting in myotubes with nuclei numbers approaching those of wild-type myotubes (Figure 3.3B). These data suggest that SMN plays an important role in myoblast fusion and possibly muscle development.

SMA muscle cells have decreased expression of satellite cell markers and increased expression of differentiation markers.

Given the reduced capacity of SMN-deficient myoblasts to form multinucleate myotubes we sought to determine the effects of SMN deficiency on myoblast differentiation. Thus, we examined the differences in markers of muscle differentiation in SMN-deficient cells compared to wild-type controls. First, we investigated the expression levels of Pax7, a muscle progenitor cell marker; MyoD and myogenin, early differentiation markers; and myosin heavy chain (MHC), a terminal muscle differentiation marker, before differentiation in SMN-deficient cells and wild-type controls. Pax7 levels were lower in SMN-deficient cells, while MyoD and myogenin levels were elevated in comparison to wild-type cells (Figure 3.4 A). In addition, we found premature expression of the terminal differentiation marker MHC in SMN-deficient cells. These data are consistent with a previous report that SMN-deficient cells have premature expression of muscle differentiation markers (Hayhurst et al., 2012).

Figure 3.2. Electroporation of SMA cell line. A. Western blot of lysates from wild-type and SMA cell lines and the SMA cell line electroporated with cDNA encoding human SMN.

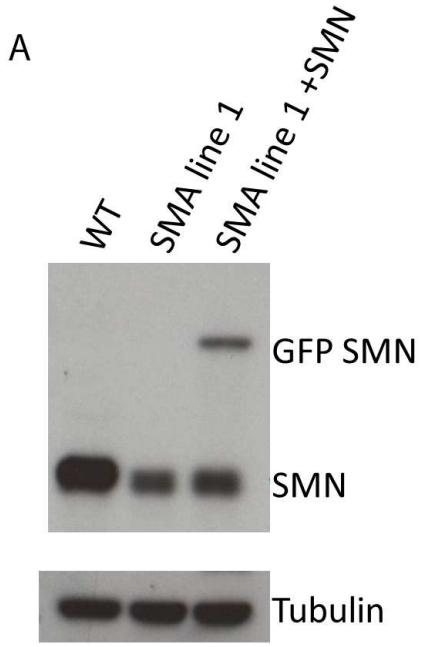


Figure 3.3. Restoration of SMN rescues the fusion deficit in SMA muscle cells. Electroporation was used to introduce either human SMN or empty vector. Muscle cells were allowed to recover in proliferation media for 6 hours and then switched to differentiation conditions. The cells were differentiated for 6 days and stained for sarcomeric myosin (green) and desmin (red). DAPI (blue) was added to label nuclei. Representative images are shown in panel A. The percentage of total nuclei in myotubes and the percentage of total cells with one nucleus are quantified in A. Myotubes were defined as cells positive for desmin and MF-20 with two or more nuclei. The percentage of myotubes with 2-3, 4-7 and 8 or more nuclei are quantified in panel B. Nikon Elements software was used to count cells and nuclei. 6 experiments each were done for wild-type (WT), SMA, and SMA + SMN. Values represent the mean ± SEM. (*, $P < 0.05$; **, $P<0.01$; ***, $P<0.001$).

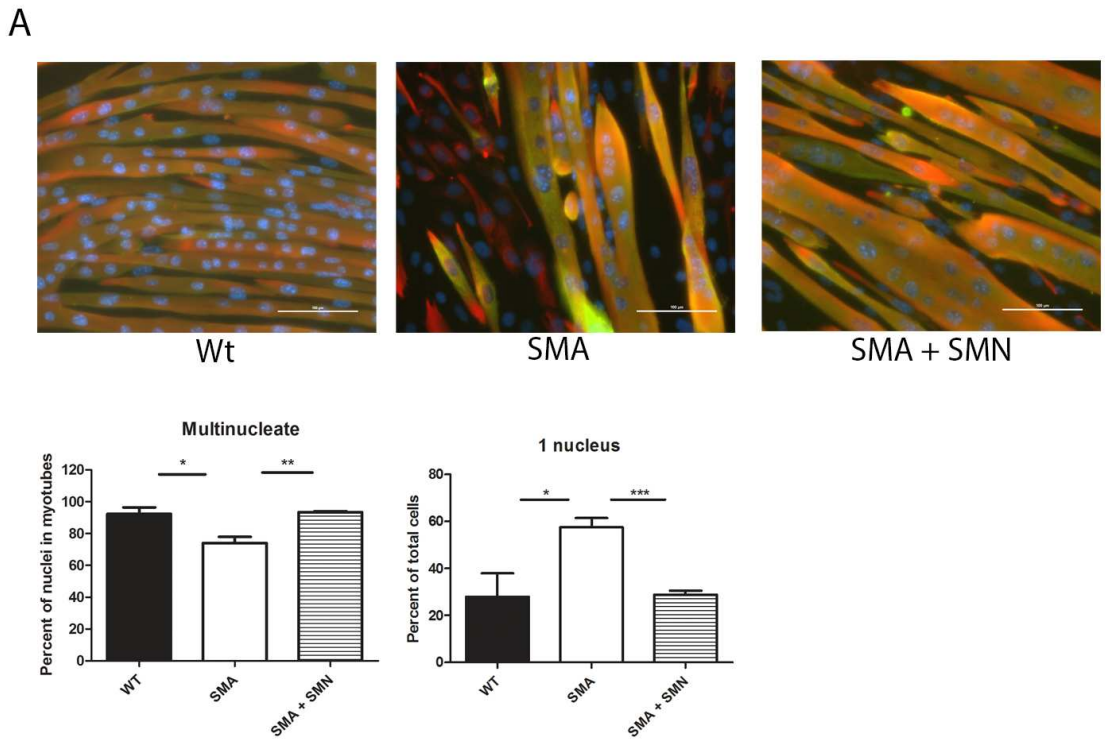


Figure 3.3 continued

B

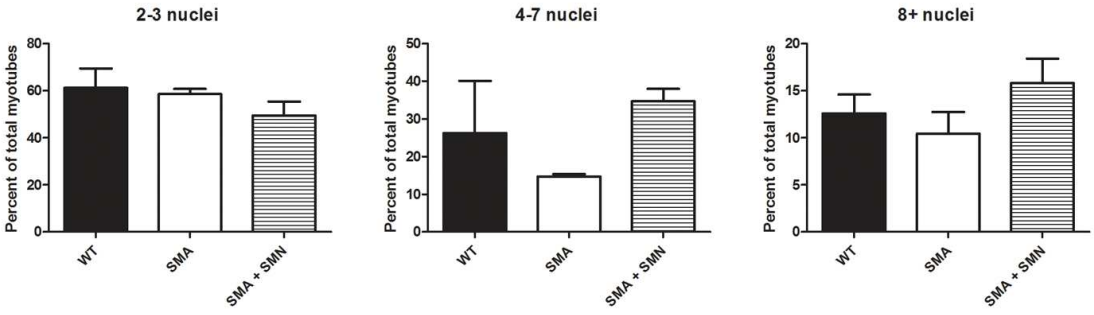
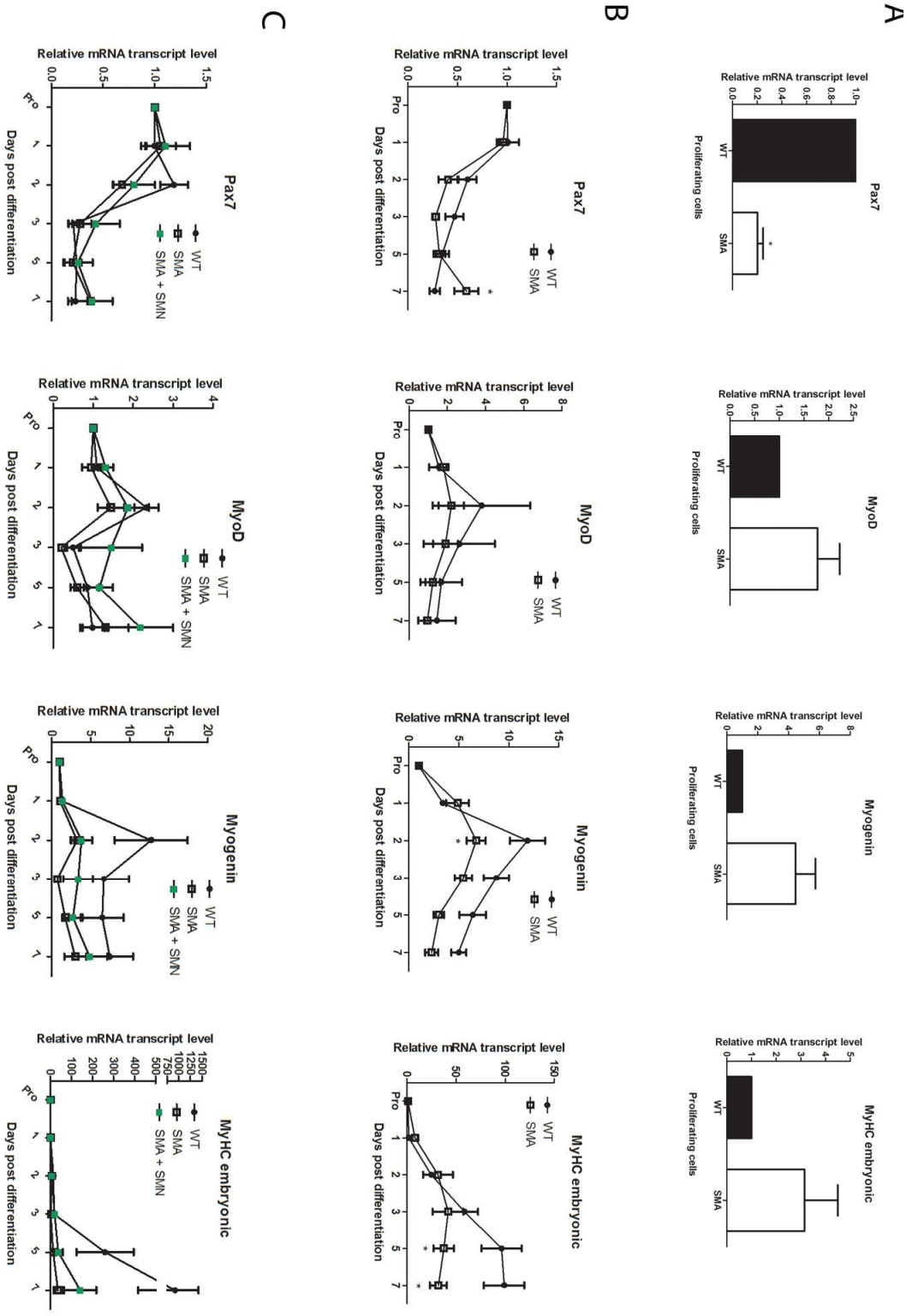


Figure 3.4. SMA muscle cells have lower expression of a satellite cell markers and higher expression of differentiation markers. A. Proliferating muscle cells were collected and RNA isolated for qRT-PCR. The data are represented as the fold change in compared to wild-type cells. B. Muscle cell lines were switched to differentiation conditions and samples were collected at multiple time points and RNA isolated for qRT-PCR. The data are represented as the fold change compared to proliferating cells of the same genotype. 5 time courses per genotype were run. C. Muscle cell lines were transfected with SMN or empty vector and switched to differentiation conditions. Samples were collected at multiple time points and RNA isolated for qRT-PCR. The data are represented as the fold change compared to proliferating cells of the same genotype. 4 time courses per genotype were run. Values represent the mean ± SEM. (*, $P < 0.05$).

Figure 3.4 continued



We next examined the change, relative to proliferating cells, in expression of muscle differentiation markers over 7 days following the initiation of differentiation in the SMN-deficient and wild type cells. The muscle progenitor cell marker Pax7 decreases once differentiation begins, and we found Pax7 levels were reduced similarly in both SMA-deficient and wild-type cell lines following initiation of differentiation (Figure 3.4B). The expression of the early differentiation markers MyoD and myogenin peaked in both SMA and wild-type cell lines after 2 days of differentiation, but induction was markedly lower in SMN-deficient cells compared to wild-type (Figure 3.4B). Given the reduced levels of these early markers of muscle differentiation, we sought to determine if the later markers of differentiation were also reduced. The level of induction of one terminal muscle differentiation marker, myosin heavy chain (MHC), was similar in the SMN-deficient and wild-type cell lines for the first 2 days of differentiation, but MHC expression increased in wild-type cells and remained unchanged in SMN-deficient cells. Based on these data, SMN-deficient cells have reduced induction of the early and late markers of muscle development compared to wild-type cells.

To determine whether the changes in gene expression we observed in SMN deficient myoblasts were due to reduced levels of SMN, we transfected SMA myoblasts with either GFP alone or GFP-tagged SMN. We found that restoring SMN levels brought MyoD, myogenin and MHC closer to wild-type levels (Figure 3.4C), indicating the altered induction may partially depend on SMN levels. Therefore, while levels of the muscle differentiation markers are prematurely elevated in SMN-deficient cells, the level of induction of these markers over the course of muscle differentiation is reduced compared to wild-type cells.

SMA muscle cells have decreased cleavage and increased retention of talin in focal adhesions.

In addition to the altered expression of muscle differentiation markers in the SMA myoblasts, we sought to investigate whether altered cytoskeletal dynamics contribute to the fusion deficit. To differentiate and fuse, proliferating satellite cells or myoblasts must migrate, align, and establish stable cell-cell contacts. Cell migration is thus a fundamental cellular function during skeletal muscle development. We sought to investigate whether altered cytoskeletal dynamics and cell migration contribute to the fusion deficit observed in SMN-deficient cells. Talin 1 and 2 connect integrins to the actin cytoskeleton and are required for myoblast fusion (Conti et al., 2009). Talin interacts with both focal adhesion proteins and actin filaments (Nayal et al., 2004), and cleavage of talin by calpain 2 regulates the turnover of focal adhesions (Franco et al., 2004), which is critical in cell migration. By western blot analysis we found reduced talin cleavage in SMN-deficient cells (Figure 3.5A). We performed time-lapsed analysis of talin localization to focal adhesions and found that talin is retained longer in adhesion complexes in SMN-deficient cells, indicating that adhesion turnover is disrupted (Figure 3.5B).

SMA muscle cells have altered motility and altered actin fiber organization.

Studies in Drosophila and C. elegans have shown that talin is needed for the interaction between the actin cytoskeleton and integrins, but not for the localization of integrins at the cell surface (Brown et al., 2002; Cram et al., 2003). Talin proteolysis affects the organization of other focal adhesion proteins, including vinculin (Franco et al., 2004). We

Figure 3.5. SMA muscle cells have decreased cleavage and increased retention of talin. A. Undifferentiated muscle cells were collected and cell lysates prepared for Western blot. The data are quantified as the ratio of cleaved to full length talin. B. Undifferentiated muscle cells were electroporated with a human GFP talin construct and analyzed by time-lapse microscopy. Duration measurements were made by counting the time elapsed between the first and last frames in which an adhesion was observed. At least 20 adhesions were counted for each genotype. Values represent the mean ± SEM. (*, P < 0.05; **, P<0.01; ***, P<0.001).

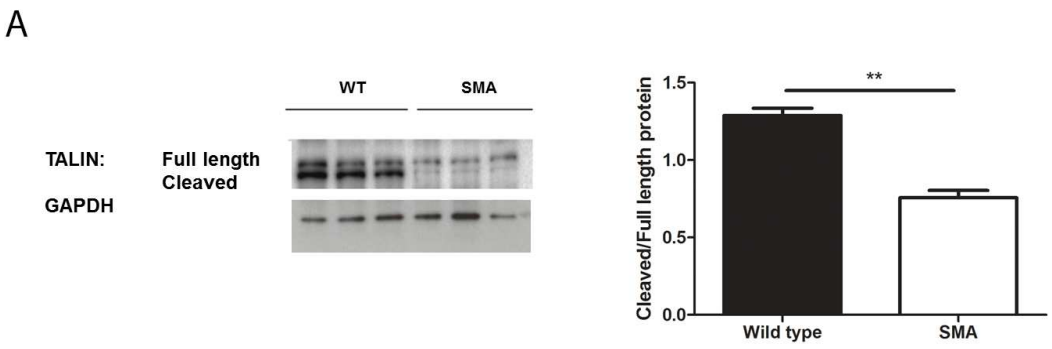
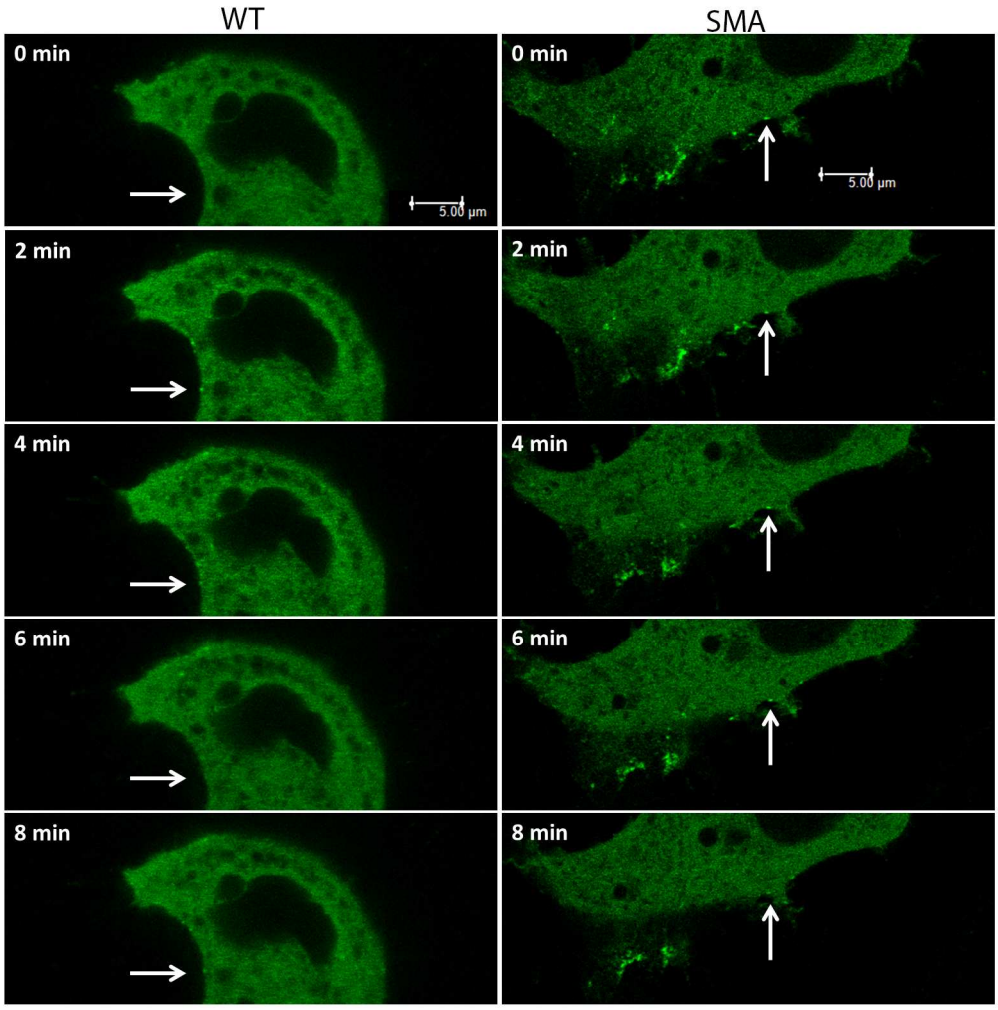


Figure 3.5 continued.

B



thus examined the distribution of vinculin in SMN-deficient and wild-type cells by immunostaining. In SMN-deficient cells vinculin is recruited to focal adhesions, but they are larger and more elongated than those in wild-type cells (Figure 3.6A) indicating that reduced talin proteolysis also reduces the turnover of other adhesion proteins in the focal adhesions.

We found that the organization of the actin cytoskeleton, critical for cell migration and membrane fusion, is altered in SMN-deficient muscle cells (Figure 3.6B). We did not find a difference in staining for the actin cleaving protein gelsolin, suggesting actin filament turnover is not a problem. To determine whether the decrease in adhesion turnover and altered actin cytoskeleton affects muscle cell migration, we examined cell motility in SMN-deficient and wild-type cells. An area of culture was denuded, and the number of cells migrating into the denuded region was counted after 24 hours. SMN-deficient cells had a dramatically reduced migration rate compared to wild-type cells (Figure 3.6C). We did not find a difference in BrdU incorporation between wild-type and SMN-deficient cells (data not shown). These data suggest that SMN levels affect cytoskeletal dynamics, at least in part, through effects on talin cleavage and cell migration.

DISCUSSION

In this chapter we addressed three questions: 1) does SMN deficiency cause a myoblast fusion deficit, 2) can we reverse this deficit by replacing SMN, and 3) what are the

molecular mechanisms underlying the changes in fusion. We showed that SMA myoblasts are incorporated into multinucleate myotubes at decreased frequency,

Figure 3.6 SMA muscle cells have altered motility and actin fiber organization. A. Representative images of undifferentiated muscle cells stained for vinculin (green). Size bar = 25 μm. The smaller panels are 1.6× magnified images of the adhesions. B. Representative images of undifferentiated muscle cells stained for F-actin (red). DAPI (blue) was added to label the nuclei. Size bar = 50 μm. The smaller panels are 1.6× magnified images of the adhesions. C. After 1 day in differentiation conditions, confluent muscle cells were removed with a cell scraper, and the media was replaced. Cells that moved into the denuded area after 24 hours were counted. The data represent the average of at least 3 fields per experiment from 3 experiments, shown as percentages of the wild-type average. The values represent the mean ± SEM. (**, P<0.01).

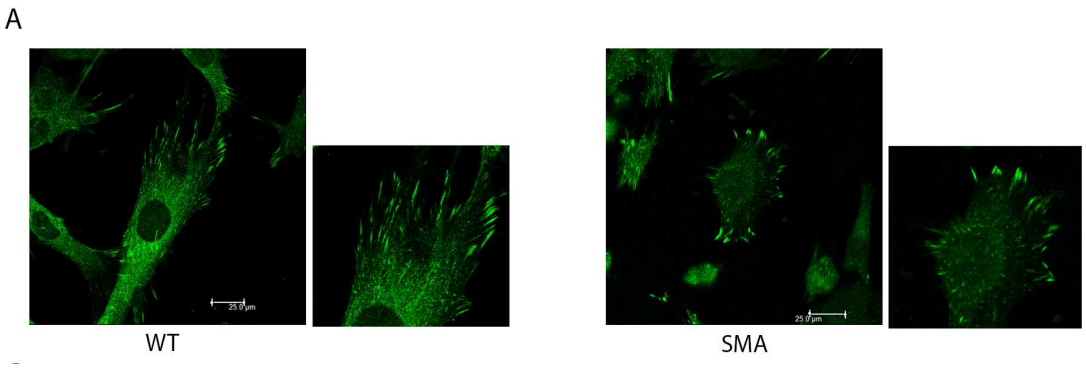
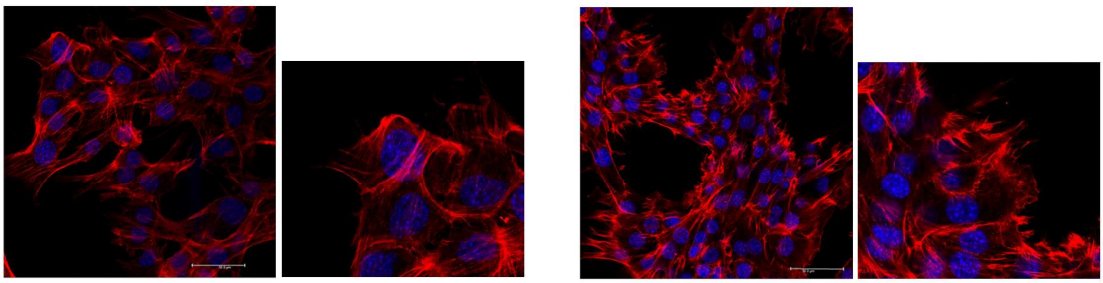


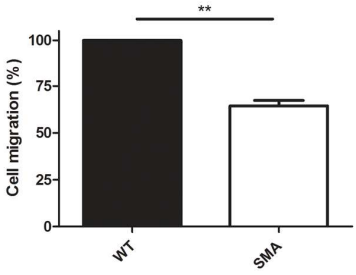
Figure 3.6 continued.

B



WT SMA

C



consistent with the fusion deficit reported by other groups using primary cells from SMA patients and SMA model mice, and C2C12 mouse muscle cell lines with SMN knockdown (Arnold et al., 2004; Hayhurst et al., 2012; Shafey et al., 2005). We extended these previous studies by showing that the fusion deficit can be rescued by restoring SMN, which indicates that SMN plays an important role in this cellular activity. We also show SMN-dependent alterations in the actin cytoskeleton and in focal adhesion dynamics, which are potential contributing factors. For these experiments we used muscle cell lines from a SMA model mouse. SMA delta 7 mice (Le et al., 2005) were crossed with mice overexpressing the H-2Kb-tsA58 (H2K) transgene (Jat et al., 1991), which allows conditional immortalization of the cells. Cell lines from mouse models of other muscle diseases, including limb-girdle muscular dystrophy (Cohen et al., 2012) have been generated using this method.

To better understand the mechanism behind the fusion deficit in SMN-deficient cells, we looked for differences in the expression of muscle differentiation markers. MyoD and myogenin are members of a family of basic helix-loop-helix transcription factors that regulate muscle differentiation. These factors bind to regulatory regions, E-boxes, in many genes expressed in skeletal muscle (Blackwell and Weintraub, 1990) and also to their own regulatory sequences, creating a positive feedback loop that drives cells toward a myogenic fate (Brennan et al., 1990; Thayer et al., 1989). Changes in the expression or activity of these transcription factors are critical to muscle differentiation (Brennan et al., 1990; Molkentin and Olson, 1996). We found that the level of myogenin is higher in SMN-deficient proliferating cells and that the SMN-deficient cells have reduced induction of this marker during muscle differentiation.

Restoring SMN levels partially rescued MyoD, myogenin, and MHC levels, suggesting that SMN has an effect on the expression of these genes and thus has a role in early muscle differentiation. It is unclear whether the high level of premature expression of myogenin affects myotube formation, or whether the low level of induction is important, or both.

A possible cause of the reduced fusion of SMN-deficient myoblasts is impairment in the cytoskeletal dynamics needed for the cells to migrate and fuse. The findings presented here suggest that SMN deficiency results in disruption of cell migration, an important factor in myoblast fusion. We found that SMN-deficient myoblasts have decreased talin cleavage and retention of talin-positive focal adhesions, with increased concentration of vinculin at the cell perimeter. Talin mediates the interaction between integrins and the actin cytoskeleton, and we found altered actin organization in SMA muscle cells. Focal adhesion turnover and actin dynamics are involved in cell motility and migration, and SMN-deficient cells have alterations in both compared to wild-type cells. Overall, our findings show SMA muscle cells have SMN-dependent changes in the level of of factors critical for muscle development and cytoskeletal defects that reduce cell motility.

An increasing body of evidence has suggested that SMA muscle pathology could at least be due in part to deficiency of SMN in muscle. *In vitro* experiments showed that SMA patient muscle can be successfully innervated with embryonic rat spinal cord explants, but the innervated myofibers underwent marked degeneration after 1-3 weeks. Such degeneration was not observed in cultures using muscle from aged-matched, healthy controls (Askanas et al., 1987; Guettier-Sigrist et al., 2001). Furthermore, SMA

patient muscle cells do not fuse into multinucleate myotubes whereas controls do (Arnold et al., 2004). Muscle cell lines deficient in SMN show decreased proliferation and fusion (Shafey et al., 2005). Studies in primary SMA myoblasts (Hayhurst et al., 2012) and SMN-deficient muscle cell lines (our data) also show decreased fusion. However, Hayhurst and colleagues found no difference in cell proliferation, and we did not find a difference in BrdU incorporation in our cell line. The increase in absolute levels of MyoD and myogenin transcripts in SMN-deficient cells (Figure 3.4A) that we report are consistent with a previous report which found increased nuclear staining for MyoD and myogenin (Hayhurst et al., 2012). Studies in mouse models of SMA have found altered morphology and transmission in the neuromuscular junctions (Kariya et al., 2008; Kong et al., 2009; Murray et al., 2008). Together, these findings suggest that while motor neuron degeneration is characteristic of SMA, a contribution of muscle to the disease pathology cannot be excluded.

Burghes and colleagues addressed the question of cell-autonomy by over-expressing SMN in striated muscle with a skeletal actin promoter and did not find a significant improvement in lifespan (Gavrilina et al., 2008). However, the muscle-specific skeletal actin promoter drives expression only in post-mitotic myotubes, not proliferating myoblasts or satellite cells. If SMN indeed has a role in early muscle development, then SMN expression in proliferating cells is likely needed to see improvement in the phenotype. Consistent with this theory, recent work shows that early expression of SMN in muscle tissue, driven by MyoD promoter (active at E9.75), improves survival and motor function (Martinez et al., 2012) suggesting the possibility of an intrinsic role of SMN in muscle.

The phenotype of mice with muscle-specific deletion of exon 7 in murine Smn supports this idea. Deletion of Smn exon 7 in actively dividing muscle precursors resulted in muscle necrosis, motor paralysis, and earlier death than in mice with exon 7 deleted only in fused myotubes (Nicole et al., 2003). In response to muscle damage, regeneration was impaired in mice with exon 7 deleted in muscle precursor cells and fused myotubes. In contrast, regeneration in mice with exon 7 deleted in myotubes is not significantly different from controls. The muscle necrosis could indicate that SMN has an intrinsic role in muscle development or that SMN is a critical protein in the development of all tissues and its loss results in increased cell death. In either case, amelioration of muscle pathology and lifespan in SMA mice may depend on correcting levels of SMN in muscle early in development.

The data presented here support a mechanism for SMN in muscle development, specifically in regulating actin and focal adhesion dynamics involved in cell migration and fusion. Improved understanding of the normal function of SMN in muscle and other tissue allows us to identify critical functions of SMN that may contribute to the SMA phenotype and therefore be potential targets for therapeutic intervention.

MATERIALS AND METHODS

H2K-SMA mice

Experiments were approved by the NINDS Animal Care and Use Committee. H-2Kb-tsA58 (H2K)-SMA mice were purchased from Jackson Laboratories, stock number 006553. An H2K-SMA mouse breeder colony was maintained as described in (Avila et al., 2007).

H2K-SMA cell lines

To generate H2K-SMA cell lines, P4 mice were sacrificed and myofibers were isolated from the extensor digitorum longus (EDL) and tibialis anterior (TA) muscles as described in (Rosenblatt et al., 1995). To purify the cell population, the cells were pre-plated on a Petri dish and incubated at room temperature for 20 minutes. Fibroblasts should quickly attach to the plastic, while myoblasts do not attach as quickly to plastic and remain in solution. After incubation, the supernatant was transferred to a Matrigel-coated culture dish. The cells were allowed to grow (see proliferation conditions described below) until they were at least 50% confluent, and then the pre-plating process was repeated. The cells were pre-plated at least three times and then tested for myogenicity based on staining for desmin as described in (Rosenblatt et al., 1995). Once established, muscle cell lines were maintained in proliferation media [Dulbecco's Modified Eagle Medium with pyruvate and glutamine (Life Technologies, Grand Island, NY), 20% fetal bovine serum (Atlanta Biologicals, Lawrenceville, GA), 2% chick embryo extract (Accurate Chemical, Westbury, NY), 20U/ml γ-interferon (Millipore, Billerica, MA), 1X penicillin and streptomycin mixture (Life Technologies, Grand Island, NY)] on culture dishes coated with Matrigel (BD Biosciences, San Jose, CA) at 33°C and 10% CO_2. For differentiation into myotubes, cells were switched to differentiation media [Dulbecco's Modified Eagle Medium with pyruvate and glutamine (Life Technologies, Grand Island, NY), 5% horse serum (Life Technologies, Grand Island, NY), 1X penicillin and streptomycin mixture (Life Technologies, Grand Island, NY)] and cultured at 37°C and 5% CO_2.

Myoblast fusion assays

Muscle cells were grown on Matrigel-coated plastic in differentiation conditions for 6 days. The cells were fixed in 4% formalin at 37°C for 15 minutes, washed in phosphate-buffered saline (PBS) and blocked in 0.5% Triton X, 20% goat serum and 2% bovine serum albumin in tris-buffered saline with 0.1% Triton X (TBST). Cells were immunostained with mouse anti-sarcomeric myosin (1:200; MF-20, Developmental Studies Hybridoma Bank, Iowa City, IA), rabbit anti-desmin (1:200; Abcam, Cambridge, MA) in 0.2% Triton X, 2% goat serum and 2% bovine serum albumin in TBST. Cells were washed in TBST and incubated in goat-anti mouse Alexa Fluor 488 (1:200; Life Technologies, Grand Island, NY), goat anti-rabbit Alexa Fluor 555 (1:200; Life Technologies, Grand Island, NY), and DAPI in the same solution as used for the primary antibodies. Following the incubations, the cells were washed in TBST. Pictures were taken with a Nikon Eclipse Ti microscope using Nikon Elements software. For the fusion index, the investigator taking the pictures did not know the genotype of the cells. Nikon Elements software was used to count cells and nuclei. Myotubes were defined as cells positive for desmin and MF-20 with two or more nuclei. The fusion index was calculated as the number of nuclei in myotubes divided by the total number of nuclei.

RNA isolation

Total RNA was isolated from muscle cells using Trizol (Life Technologies, Grand Island, NY), and the RNeasy kit (Qiagen, Germantown, MD) was used to purify the RNA according to the manufacturer's instructions. 1 μg RNA was converted to cDNA using

the High Capacity cDNA Reverse Transcription kit (Life Technologies, Grand Island, NY), following the manufacturer's instructions. Gene expression was determined by qRT-PCR using TaqMan reagents (Life Technologies, Grand Island, NY). Primers are listed in Figure 3.7. Transcript levels were quantified by threshold cycle values using phosphoglycerate kinase 1 as a control. For each gene, values were normalized to unaffected cells.

Western blots

Muscle cells were lysed in 1% NP-40, 50 mM Tris-HCl (pH 8), 150 mM NaCl and protease inhibitor cocktail (Roche, Indianapolis, IN) on ice for 10 minutes. The lysates were centrifuged at 4°C for 10 minutes and the supernatants collected. Protein concentration was determined using the BCA Protein Assay kit (Pierce, Rockford, IL) according to the manufacturer's protocol. Protein lysates (50 μg) were resolved by SDS-PAGE (10%) and transferred to PVDF membranes. The membranes were blocked in 5% milk and probed with mouse anti-talin (1:500; clone 8d4, Sigma, St. Louis, MO), rabbit anti-calpain 2 (1:1000, Cell Signaling, Danvers, MA), mouse anti-GAPDH (1:4000, EnCor Biotechnology, Gainesville, FL) and mouse anti-alpha tubulin (1:3000, Sigma, St. Louis, MO).

Electroporation of H2K-SMA cells

DNA was transfected into H2K-SMA cells by electroporation using the Amaxa Cell Line Nucleofection Kit V (Lonza, Valais, Switzerland) following the manufacturer's

Figure 3.7.

Supplementary Table 1: Primer information

Primer Name	TaqMan Reagent
MyoD	Mm00440387_m1
Myh3*	Mm01332452_m1
Myogenin	Mm00446194_m1
Pax7	Mm00834079_m1
Pgk1	Mm00435617_m1

*Myh3: myosin heavy chain embryonic isoform

instructions with the following modifications for the H2K cells. For each transfection, 500,000 cells were used. Following transfection, the cells were returned to proliferation conditions (see above). If myotubes were required for the experiment, the cells were switched to differentiation conditions once they adhered to the dish 6 hours post-electroporation. 2 µg of GFP SMN or GFP empty vector or GFP-talin (Addgene, Cambridge MA) were used for each transfection. For fusion assays, myc constructs described in (Burnett et al., 2009) were used.

Cell migration assays

Muscle cells were grown on Matrigel-coated plastic in differentiation conditions for 1 day. An area of the well of the same size for all wells was marked on the bottom of dish and cells were removed from within this area. The cells were washed in PBS and fresh media added. The cells were allowed to grow for an additional 24 hours in differentiation conditions. The cells were fixed in 4% formalin at 37°C for 15 minutes, washed in TBST and the number of cells that moved into the denuded area were counted. The average of 3 fields was determined for each genotype. This process was repeated for a total of 3 experiments. The average of the 3 experiments for wild-type was set as 100% migration. The average of the 3 experiments for SMA was expressed as a percentage of the wild-type average and graphed with error bars representing the SEM of the 3 experiments.

Immunostaining

Muscle cells were grown in proliferation conditions for 2 days on glass chamber slides coated with poly-D-lysine (PDL) (Sigma, St. Louis, MO). One day before plating, the slides were coated overnight at 37°C with 50 µg/ml of PDL in PBS. The next day, the slides were washed with PBS and dried for 1 hour at 37°C. The slides were washed once with DMEM before plating the cells. The cells were fixed in 4% cold paraformaldehyde in PBS for 30 minutes at 4°C, washed in PBS, and blocked in 0.3% Triton X and 10% goat serum in PBS. The cells were immunostained with Alexa Fluor Phalloidin 555 (1:50, Life Technologies, Grand Island, NY) or mouse anti vinculin (1:400, Sigma, St. Louis, MO) in 0.1% Triton X and 5% goat serum in PBS. The cells were washed in TBST and incubated in goat anti-mouse Alexa Fluor 488 (1:200; Life Technologies, Grand Island, NY) (vinculin only) and DAPI in the same solution used for the primary antibodies. Following the incubations, the cells were washed in PBST. Pictures were taken on a Leica TCS SP5 II confocal microscope using Leica LAS AF software.

Time lapse microscopy

Time lapse fluorescence imaging of live cells was done with a 63x objective on a Leica TCS SP5 II confocal microscope using Leica LAS AF software. The cells transfected (see above) with GFP-talin and grown on glass bottom dishes coated with poly-D-lysine (PDL) (Sigma, St. Louis, MO). One day before plating, the slides were coated overnight at 37°C with 50 µg/ml of PDL in PBS. The next day, the slides were washed with PBS and dried for 1 hour at 37°C. The slides were washed once with DMEM before plating the cells. Fluorescent images were captured every 2 minutes for 2 hours. Time-lapse sequences were used to determine the duration of focal adhesions by counting the amount

of time elapsed between the first and last frame when an adhesion was observed. For each cell type, at least 20 individual adhesions were counted in five separate cells by a blinded evaluator.

Statistical Analysis

Data are presented as the mean +/- the standard error of the mean. T-tests were done using GraphPad Prisim 5 software (GraphPad Software) with significance set at $p<0.05$.

Chapter 4: Discussion

STUDY DESIGN AND FINDINGS

SMA is caused by deletions and other mutations in *SMN1*, resulting in reduced expression of the ubiquitously expressed SMN protein. SMN has several reported functions, the best characterized being its role in snRNP biogenesis and spliceosome assembly. SMN has also been localized to neurites where it may have a role in mRNA trafficking (Peter et al., 2011; Rossoll et al., 2003; Rossoll et al., 2002; Zhang et al., 2003). In muscle SMN also interacts with alpha actinin (Rajendra et al., 2007), a component of Z-disk. Although several functions of SMN have been characterized, it is still unclear how SMN deficiency results in the specific pathology of SMA.

Given the marked weakness of SMA patients, several studies have examined SMA muscle *in vitro*. In a nerve-muscle system, SMA patient muscle cells were innervated with rat embryonic spinal cord explants, and the innervated myofibers degenerated after 1-3 weeks. This degeneration was not observed with muscle from age-matched controls (Askanas et al., 1987; Guettier-Sigrist et al., 2001). In another study, SMA patient muscle cells did not fuse into multinucleate myotubes as efficiently as controls do (Arnold et al., 2004). Muscle cell lines deficient in SMN showed decreased proliferation and fusion (Shafey et al., 2005). The neuromuscular junctions of SMA mice have altered morphology and transmission (Kariya et al., 2008; Kong et al., 2009; Murray et al., 2008). Although motor neuron degeneration is the hallmark of the disease, the contribution of muscle and other peripheral tissues to the disease cannot be excluded. Based on previous work, we hypothesized that deficiency of SMN in muscle alters its development and maintenance in SMA.

To address this hypothesis, we used *in vivo* and *in vitro models* of SMA and muscle atrophy. Our first aim (Chapter 2) focused on the underlying molecular mechanism of atrophy in SMA muscle. We discovered that the atrophy pathway involving HDAC4/5, Dach2, and myogenin is induced in SMA muscle, and we examined whether an HDAC inhibitor, TSA, improved muscle pathology in SMA mice by inhibiting the induction of muscle atrophy. We examined muscle tissue for changes in the expression of critical genes involved in this pathway in SMA mice in comparison to wild-type littermates. We found that myogenin expression is increased in SMA mice, resulting in induction of atrogenes and muscle atrophy. Similar changes in gene expression were also observed in post-mortem human SMA muscle tissue.

Overall, the changes in atrogin-1, MuRF1, myogenin, and HDACs we found are consistent with neurogenic atrophy (Figure 2.11). However, SMA mice are weak and have decreased motor function as early as P5 (Avila et al., 2007), while atrogin-1 and MuRF1 expression does not increase until P11. This suggests effects of SMN deficiency in muscle development before the effects of denervation set in. It has been reported that early over-expression of SMN in SMA mouse muscle, driven by the MyoD promoter (active at E9.75), improves survival and motor function (Martinez et al., 2012) indicating an intrinsic role of SMN in muscle. Chapter 3 addresses this potential role by examining the effects of SMN deficiency on muscle development. In particular, we asked three key questions: i) does SMN deficiency cause a fusion deficit, ii) can we reverse this deficit by replacing SMN, and ii) what are the molecular mechanisms underlying the changes in fusion?

We showed that a decreased percentage of nuclei in SMN-deficient cells are incorporated into multinucleate myotubes, consistent with a fusion deficit. When SMN was restored, the fusion index increased to wild-type levels, suggesting that SMN has a role in myoblast fusion and muscle development. To better understand the mechanism behind the fusion deficit in SMN-deficient cells, we looked for differences in the expression of markers of muscle differentiation and found that SMN-deficient muscle cells have reduced induction of early and late differentiation markers, consistent with a delay in differentiation.

In addition to expression changes, we found that cytoskeletal dynamics, important in membrane fusion and cell motility, are disrupted in SMN-deficient cells. The fusion impairment could thus result from the disruption of focal adhesions; this suggests a role for SMN in muscle development through regulation of the actin cytoskeleton and focal adhesions.

Overall, we have shown a myogenin-dependent mechanism for altered development and atrophy in SMN-deficient muscle. Understand this mechanism could lead to interventions that mitigate the manifestations of SMA. Also, understanding the intrinsic role of SMN in muscle could prompt further investigations into the pathophysiology of other SMN-deficient tissues, such as whether migration defects play role in motor neurons.

ADDITIONAL FINDINGS

Pharmacological effects on muscle atrophy

In chapter 2, we delineate an atrophy pathway, measured by atrogin-1 and MuRF1 induction, which is induced by both acute (sciatic nerve cut) and chronic (SMA) denervation. We also found atrogin-1 and MuRF1 were induced in a starvation model of atrophy, but this induction occurred independently of myogenin activation. The myogenin-dependent atrophy pathway is amenable to pharmacological intervention with HDAC inhibitors.

HDACs and histone acetyltransferases affect gene expression by deacetylating and acetylating, respectively, the histone tails that extend from the nucleosome. Changes in the acetylation state of histones affect the conformation of chromatin and regulate the expression of genes (reviewed in (Minucci and Pelicci, 2006). HDACs can be divided into four classes; class III or sirtuin-like HDACs are structurally unrelated to the other classes. A variety of compounds that inhibit HDACs have been identified and most work on class I, II and IV HDACs (Johnstone, 2002; Marks et al., 2001), but a few can distinguish between class I and class II or even HDACs of the same class (Haggarty et al., 2003; Heltweg et al., 2004; Mai et al., 2003; Park et al., 2004). Inhibition of HDACs does not necessarily increase transcription of genes. HDACs work in concert with other enzymes, such as histone acetyltransferases, and regulatory factors to modify gene expression. Approximately 20 percent of known genes are affected by HDAC inhibitors, and the number of up and down regulated genes is similar (Glaser et al., 2003; Mitsiades et al., 2004; Peart et al., 2005; Van Lint et al., 1996).

Both TSA and valproic acid are pan-HDAC inhibitors that do not specifically inhibit HDAC4 or HDAC5. We show that the effect of TSA on the atrogin-1 and MuRF1 promoters can be reproduced with valproic acid, a structurally unrelated HDAC inhibitor,

indicating that this not an off-target effect. As HDAC4 and HDAC5 are class II HDACs, a class-specific inhibitor such as MC1568 (Nebbioso et al., 2009) could have a higher therapeutic index.

Muscle development

We show that focal adhesions and actin stress fibers are altered in SMN-deficient muscle cells. The organization of these structures is regulated by several signaling proteins, one of which, Rho kinase, modulates actin filament re-organization in response to Rho A (reviewed in (Pellegrin and Mellor, 2007; Salhia et al., 2005)). Dysregulation of the actin cytoskeleton, with increased Rho A activition, has been correlated with the SMA phenotype in model mice (Bowerman et al., 2010). Inhibition of Rho kinase with the inhibitor Y-27632 reduces focal adhesion formation (Adachi et al., 2011) and improves SMA mouse neuromuscular junctions and muscle fiber size. Y-27632 treatment may improve Rho kinase-mediated regulation of actin filaments and focal adhesions, resulting in improved myoblast fusion and more mature myofibers.

In addition to the candidate gene expression studies presented in chapter 3, we also used a muscle-specific qRT-PCR profile array to identify other genes that are alternatively expressed in SMN-deficient versus wild type myoblasts. Based on the time course of muscle cell differentiation (Figure 3.4A), we chose to examine cells 2 days after differentiation because we saw differences in cell morphology and changes in the muscle differentiation markers between SMN-deficient and wild type cells at that timepoint. Of the 84 genes examined, 17 were upregulated and 10 were downregulated greater than 2 fold in SMN-deficient muscle cells compared to wild type (Figure 4.1A).

Figure 4.1. Differentially expressed transcripts in SMA muscle cells. Muscle cells were differentiated for 2 days, collected and RNA isolated. 4 experiments per genotype were run a muscle-specific qRT-PCR profile array. Genes that were up or down regulated 2 fold in SMA versus wild type muscle cells were included and are shown with p values.

A

Gene name	Accession	Description	Fold difference[a]	p value
Mstn	NM_010834	Myostatin	16.44	0.023
Cast	NM_009817	Calpastatin	13.27	0.188
Igfbp5	NM_010518	Insulin-like growth factor binding protein 5	5.33	0.043
Tnni2	NM_009405	Troponin I, skeletal, fast 2	4.72	0.015
Tnf	NM_013693	Tumor necrosis factor	3.86	0.552
Ppargc1b	NM_133249	Peroxisome proliferative activated receptor, gamma, coactivator 1 beta	3.58	0.028
Il1b	NM_008361	Interleukin 1 beta	2.8	0.516
Casp3	NM_009810	Caspase 3	2.72	0.170
Neb	NM_010889	Nebulin	2.71	0.030
Dag1	NM_010017	Dystroglycan 1	2.51	0.607
Prkag3	NM_153744	Protein kinase, AMP-activated, gamma 3 non-catatlytic subunit	2.43	0.268
Tnnt3	NM_011620	Troponin T3, skeletal, fast	2.34	0.079
Camk2g	NM_178597	Calcium/calmodulin-dependent protein kinase II gamma	2.21	0.073
Myod1	NM_010866	Myogenic differentiation 1	2.2	0.000
Acta1	NM_009606	Actin, alpha 1, skeletal muscle	2.16	0.114
Myog	NM_031189	Myogenin	2.16	0.066
Mapk8	NM_016700	Mitogen-activated protein kinase 8	2.09	0.001
Igfbp3	NM_008343	Insulin-like growth factor binding protein 3	0.49	0.047
Capn2	NM_009794	Calpain 2	0.4	0.342
Bcl2	NM_009741	B-cell leukemia/lymphoma 2	0.37	0.040
Tnnt1	NM_011618	Troponin T1, skeletal, slow	0.36	0.000
Dmd	NM_007868	Dystrophin, muscular dystrophy	0.35	0.038
Capn3	NM_007601	Calpain 3	0.32	0.359
Mmp9	NM_013599	Matrix metallopeptidase 9	0.3	0.919
Fgf2	NM_008006	Fibroblast growth factor 2	0.26	0.013
Pax7	NM_011039	Paired box gene 7	0.12	0.013
Lep	NM_008493	Leptin	0.06	0.499

[a]Relative to wild type

B

Fold change in SMN-deficient vs. wild-type cells		
	by qRT-PCR	by profile array
Pax7	0.13	0.12
MyoD	1.96	2.2
Myogenin	2.4	2.16

We also confirmed the expression changes in Pax7, MyoD, and myogenin that were detected previously in our samples (Figure 4.1B).

The increases in MyoD and myogenin levels in SMN-deficient cells compared to wild-type muscle cells are consistent with a previous report comparing their absolute levels (Hayhurst et al., 2012). We found that, compared to wild-type muscle cells, Pax7 expression is decreased and MyoD and myogenin expression are increased in SMN-deficient cells before differentiation (Figure 3.4A). Following differentiation, the levels of myogenin and MyoD remain higher in the SMN-deficient cells (Figure 4.1B), but further induction is ablated (Figure 3.4B). Thus, while reduced SMN results in increased levels of myogenin and MyoD in muscle precursor cells, it appears that adequate SMN is required for normal myogenin and MyoD induction during muscle differentiation. The increased levels of myogenin and MyoD in muscle precursor cells may result in reduced induction of these markers in SMN-deficient cells during differentiation as a compensatory mechanism. Nevertheless, although myogenin and MyoD levels are higher in SMN-deficient cells the induction of these genes during differentiation may be more important to myoblast fusion than their absolute levels. This is not surprising, given that myoblast fusion is a complex process requiring the temporal regulation of multiple genes and the coordinated rearrangement of the actin cytoskeleton. A possible explanation is that SMN-dependent altered expression of myogenin and MyoD results in premature expression of muscle differentiation markers, offsetting the differentiation process. It is also possible that other genes which contribute to muscle differentiation are affected by reduced SMN protein levels. Follow-up studies involving targeted disruption or temporal

control of the expression of the muscle specific genes that we found altered in SMN-deficient myoblasts will help to further reconcile these findings.

We also found differential expression of calcium signaling genes including the calcium-dependent proteases calpains 1, 2 and 3 and their natural inhibitor, calpastatin. Calpains co-localize with focal adhesions (Franco et al., 2004), and in the presence of calpain inhibitors retraction of the cell membrane at the rear of migrating cells is impaired (Huttenlocher et al., 1997; Palecek et al., 1998). Calpain 2-deficient myoblasts have defects in migration and fusion (Dedieu et al., 2004; Honda et al., 2008). Calpain 3 functions in the dysferlin complex and may affect membrane remodeling and regeneration (Huang et al., 2008). In addition, mutations in calpain 3 cause limb girdle muscular dystrophy type 2A (Richard et al., 1995). Given the role of these proteins in muscle function, altered expression of these genes may be relevant to the impaired fusion and increased atrophy we observed in SMA muscle.

CHOICE OF MODEL

The H2K SMA mice have an additional transgene encoding a thermolabile mutant of the large T antigen that allows the immortalization of the cells when grown at 33°C with interferon gamma. Therefore, primary cells may be isolated and maintained as a cell line. When switched to non-permissive conditions, the cells differentiate (Jat et al., 1991). We can reproducibly generate myotubes from these lines after a minimum of 4 days in non-permissive differentiating conditions. These cell lines are potentially useful for

screens. For example, small molecule screens could identify inhibitors of atrogin-1, and RNAi screens could identify genes where knockdown increases levels of SMN.

The large T antigen in these cell lines may have an effect on transcription independent of differentiation or SMN levels. Large T antigen may affect gene expression by altering mRNA levels of transcription factors, interacting with and regulating the activity of transcription factors, substituting endogenous transcription factors, binding to DNA or regulating signaling pathways (reviewed in (Moens et al., 1997). We are mindful of this possible confounding factor and sought to confirm our findings in primary myoblasts.

In collaboration with Dr. Charlotte Sumner, we confirmed the fusion deficit findings in myoblasts isolated from conditional SMN-deficient mice that express a Cre-inducible Smn allele knocked in at the mouse Smn locus and a Cre transgene driven by the MyoD promoter (Martinez et al., 2012). This allows expression of full-length SMN in muscle progenitor cells and myofibers. The MyoDCre mice have full length SMN mRNA and protein rescue specifically in muscle. In cultured primary myoblasts isolated from leg muscles at P10, there was a reduced number of multinucleated myotubes in myoblasts from SMA MyoDCre negative mice compared to wild-type mice, but myoblast fusion was substantially improved in SMA mice expressing MyoDCre (Martinez, T and Sumner, S, unpublished data). We are currently working to confirm the expression changes described in chapter 3 in these primary cells. The primary cells have limited replication in culture, and myoblast isolations and often yield small amounts of RNA, which increases variability in the data and limits the number of experiments we can run. We have therefore made complementary use of the two systems, with larger scale

experiments in the H2K SMA cell lines and confirmation of positive findings in the primary SMA myoblasts.

While the life span of the SMA mice allows us sufficient time for pharmacological studies, such as TSA treatment, it is too short to study reformation of neuromuscular junctions in response to treatment. A milder model of SMA mouse with a longer life span or one with conditional expression of SMN would be useful to study regeneration of muscle and neuromuscular junctions.

We used C2C12 mouse muscle cell lines to to study TSA's effect on normal muscle biochemistry. The C2C12 cell lines are easily manipulated for promoter activity assays and radioactive pulse-chase assays to assess protein breakdown. The addition of dexamethasone to the culture media allowed us to reproducibly induce atrophy in a well characterized model, glucocorticoid-induced atrophy.

CHOICE OF TECHNIQUE

We used qRT-PCR and Western blots to assess changes in gene expression and protein levels during the phenotypic progression of SMA mice and during the differentiation of myoblasts to myotubes. Muscle regulatory transcription factors (MyoD and myogenin) as well as their upstream transcriptional regulators (Pax7 and Dach2) were assayed by qRT-PCR. Protein levels were assayed for targets with enzymatic (atrogin-1 and MuRF1) or structural (talin) functions. The current study focused on changes in mRNA levels during muscle differentiation. In subsequent experiments, we could also determine protein levels of the muscle regulatory factors in our muscle cell lines.

In addition to specific muscle regulatory factors, the retinoblastoma family of proteins, including pRb, p130, and p107, are needed for myogenic differentiation and to promote withdrawal from the cell cycle. The retinoblastoma proteins act primarily through the E2F family of transcription factors which regulate genes involved in proliferation (Nevins, 1992). The role of pRb and E2F in regulating transcription in muscle cells has been reviewed in detail (De Falco et al., 2006). In actively dividing cells, hypophosphorylated pRb binds to and inhibits the E2F transactivation domain, while phosphorylation of pRb by cyclin-dependent kinases releases E2F allowing entry into S phase. In differentiating cells, MyoD binds to target promoter sequences, recruits chromatin remodeling complexes, and promotes polymerase II activity. Among its many targets, MyoD activates the expression of pRb and p21, a cyclin-dependent kinase inhibitor. The increased level of hypophosphorylated pRb promotes MyoD expression, creating a positive feedback loop, and p21 prevents cyclin, cyclin-dependent kinase, and E2F-containing complexes from initiating DNA synthesis. Ultimately, the increased expression of pRb and p21 promotes the exit of the cell from the cell cycle. Therefore, in addition to levels of muscle regulatory factors, we could determine the levels of p21 and pRb protein, and its phosphorylation state, in lysates from our SMN-deficient and wild-type cells. Increased levels of p21 and hypophosphorylated pRb in SMN-deficient cells compared to wild-type would indicate the cells have prematurely left the cell cycle. In contrast, increased levels of hyperphosphorylated pRb in SMN-deficient cells compared to wild-type would indicate the cells are not differentiating and leaving the cell cycle.

To show the effects of transcription factors on promoter activity, we used promoter sequences coupled to luciferase reporters. Luciferase activity allowed us to

determine changes in promoter activity in response to different small molecules. Demonstrating the direct interaction of transcription factors with target genes required chromatin immunoprecipitation (ChIP). This technique allowed us to delineate the myogenin-dependent atrophy pathway induced in SMA muscle and inhibited by TSA treatment.

We used histological sections to show changes in SMA patient muscle morphology. While it is standard practice to stain muscle sections with hematoxylin and eosin, we have found that toluidine-blue better preserves the muscle architecture of the autopsied tissue we collected.

Immunocytochemistry allowed us to assess myotube formation and changes morphology in wild type and SMN-deficient muscle cells. Imaging software allowed us to count cells and nuclei automatically, thereby reducing potential bias in counting. The images were also collected by an investigator who did not know the genotypes of the cells. Imaging was also used to detect changes in the organization of actin filaments and focal adhesions, as visualized by the marker vinculin. After observing reduced cleavage of talin on Western blot, we suspected the turnover of focal adhesions would be disrupted as has been reported by others (Franco et al., 2004). To determine the turnover of focal adhesions, an inherently dynamic process, we used live-cell time-lapse imaging at high magnification.

FUTURE DIRECTIONS

In chapter 2, we described a myogenin-dependent pathway involved in the muscle atrophy observed in SMA mice. We showed that the induction of this pathway could be blocked by treatment with the pan-HDAC inhibitor TSA, indicating that this pathway is amendable to pharmacological intervention. Now that we know this pathway is induced in SMA muscle, small molecule screens can be done to identify inhibitors acting on more specific targets, for example the MuRF1-inhibitor P013222 (Eddins et al., 2011). The SMN-deficient cell lines described in chapter 3 could be useful for such screens. We showed that induction of the myogenin-dependent pathway in response to acute denervation (sciatic nerve cut) can also be blocked by TSA. Thus, inhibitors of this pathway may be useful in other denervating diseases.

Since all SMA patients have at least one copy of *SMN2*, increasing the expression of *SMN2* is a potential treatment. HDAC inhibitors can increase gene expression by modifying chromatin (Minucci and Pelicci, 2006). Previous work has shown that several HDAC inhibitors increase the expression of SMN2 through alteration in the acetylation of histones at its promoter (Kernochan et al., 2005). Valproic acid, sodium butyrate, and phenylbutyrate increase the levels of full-length SMN transcripts in lymphoid cells of SMA patients (Andreassi et al., 2004; Brichta et al., 2003; Chang et al., 2001; Sumner et al., 2003) in part by increasing expression of the splicing factor Htraβ1 (Brichta et al., 2003).

SMA model mice treated with sodium butyrate before birth showed improvements in lifespan and reduced muscle atrophy based on histology (Chang et al., 2001). However, a randomized, double-blind, placebo-controlled trial of 100

symptomatic, pediatric SMA patients did not find a significant improvement in the primary outcome, Hammersmith functional motor scale, in patients receiving phenylbutyrate (Mercuri et al., 2007). Results with secondary outcome measures, myometry, and forced vital capacity, were also similar between the two groups. The drug was well tolerated in the children, and there is currently a clinical trial (ClinicalTrial.gov ID: NCT00528268) underway in pre-symptomatic SMA infants.

Valproic acid treated SMA model mice have shown improved motor function, increased neuromuscular junction innervation, less muscle atrophy, and less motor neuron loss (Tsai et al., 2008). The treatment increased levels of SMN protein and the anti-apoptotic factors, Bcl-2 and Bcl-x(L), in the spinal cord. These data suggest valproic acid improved the SMA phenotype by increasing SMN levels and reducing apoptosis in the spinal cord. Despite improvements in the mice, a phase II, double-blind clinical trial investigating treatment with valproic acid in combination with L-carnitine in non-ambulatory SMA patients did not find a benefit in the primary outcome, measure, motor function, in the treatment group compared to the placebo group (Swoboda et al., 2010). After 6 months, the placebo group began receiving treatment as well, and patients in both groups received treatment for as additional 6 months. The youngest children in the group, ages 2 to 3, who received 12 months of treatment were found to have significantly improved motor function scores and age was found to significantly influence the scores making age a confounding variable in the assessment of motor function.

In addition to the double-blind study, an open label trial of valproic acid in combination with L-carnitine was conducted in ambulatory SMA patients. Similar to the study in non-ambulatory patients, no improvement in the primary outcome measure was

found after 6 and 12 months of treatment (Kissel et al., 2011). In both studies, more than 80 percent of patients were reported to have adverse events, most commonly weight gain, and in the double-blind trial, adverse events were more common in patients receiving treatment and weight gain was a confounding variable in the assessment of motor function.

A third study of valproic acid and L-carnitine in type II and type III SMA patients found an improvement in one primary outcome measure, motor function, but not the other, muscle strength, after 12 months of treatment (Darbar et al., 2011). The improvement in motor function occurred only in type II SMA patients. The study was open label with all patients receiving treatment. The authors reported that side effects were unremarkable, but did note 5 of 22 patients completing the study reported weight gain and additional patients had increased tremor in their fingers. Overall, 2 out of 3 clinical trials did not find a benefit to valproic acid and L-carnitine treatment based on motor function as assessed by the Hammersmith Functional Motor Scale. Weight gain was reported in all three trials and found to be a confounding variable with age in at least one trial, suggesting the need for carefully controlled studies in subsequent trials of drugs to treat SMA patients. Also, none of these studies showed a pharmacodynamic effect of valproic acid treatment on SMN levels.

As described in chapter 2, pervious work has shown TSA treatment of SMA mice improves the disease phenotype (Avila et al., 2007). TSA treatment increased SMN protein levels and improved the life span and motor function of SMA mice. Treated mice had increased myofiber size and number and increased motor neuron size. In combination with nutritional support, TSA further increased the life span of the mice and improved

their motor function. A structurally similar HDAC inhibitor, vorinostat (suberoylanilide hydroxamic acid), has also been shown to increase life span and improve motor function in SMA mice (Riessland et al., 2010). Treated mice also had reduced motor neuron degeneration, and increased neuromuscular junction and myofiber size.

TSA and vorinostat are approximately 1000 times more potent than valproic acid and phenylbutyrate as HDAC inhibitors. Neither has yet been administered to patients, and both may represent potential therapies for SMA patients in the future. Increased understanding of the pathways affected by HDAC inhibitor treatment in SMA would be helpful. Such understanding could allow for more targeted approaches to therapeutics development as described in the preceding sections of this chapter.

In chapter 3, we characterized the differentiation of wild-type and SMA muscle *in vitro* and presented mechanisms by which fusion is impaired in SMN-deficient muscle. Restoration of SMN improved the fusion of SMN-deficient myoblasts and brought the levels of MyoD, myogenin, and embryonic myosin heavy chain closer to wild-type levels. Follow up studies characterizing the role of SMN in regulating expression of critical muscle differentiation markers would be worthwhile. The luciferase reporter constructs described in chapter 2, could be used to assay promoter activity in the presence and absence of SMN. Given that SMN interacts with alpha-actinin (Rajendra et al., 2007) and we show abnormalities in the organization of cytoskeletal proteins actin and vinculin, SMN may interact directly with proteins involved in focal adhesion formation and disassembly. Co-immunoprecipitation assays could be used to test this theory.

In addition to protein-protein interactions, we could look at the mRNA levels of actin, vinculin, and focal adhesion proteins such as talin. SMN deficiency may affect the

mRNA levels or splicing of these proteins or their upstream regulators. Exon splicing arrays in the SMN-deficient cells could identify candidates for further study. Another way to assess changes in gene expression that are needed for muscle differentiation and fusion would be to treat our SMN-deficient cells with TSA and determine whether the fusion index and cell migration are improved. If we do find an improvement, we could then perform RNASeq on samples from SMN-deficient cells with and without TSA treatment to identify changes at the RNA level that promote muscle differentiation and fusion. This analysis could allow us to identify differentially expressed genes and non-coding RNA such as miRNAs, and subsets of genes with recognition sites for transcription factors critical for differentiation such as MyoD. ChIPSeq would be another option to assess enrichment of MyoD (or other transcription factors) at the promoters of genes needed for differentiation. Ultimately, a deeper understanding of the normal function of SMN in muscle allows us to identify critical functions of SMN that are lost in SMA and are therefore targets for therapeutic intervention.

Overall, we have delineated an atrophy pathway induced in SMN-deficient muscle and shown that it can be repressed with pharmacological intervention. We have presented an *in vitro* model of SMN-deficient muscle development and shown SMN-dependent changes in fusion and muscle differentiation markers. In addition, we found abnormalities in myoblast migration in these cells that may contribute to the fusion deficit. Better understanding of the normal role of SMN in muscle and the pathway contributing to muscle atrophy in SMA informs research on therapeutic interventions that may be beneficial to patients with SMA and other neuromuscular diseases.

Chapter 5: References

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