Role of p38 and STAT5 Kinase pathways in the regulation of Survival of Motor Neuron gene expression for development of novel Spinal Muscular Atrophy therapeutics

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Abstract

Spinal muscle atrophy (SMA) is an autosomal recessive neurodegenerative disease which is characterized by the loss of α motor neurons from the anterior horn of the spinal cord, resulting in progressive muscle atrophy. The loss of functional Survival motor neuron (SMN) protein due to mutations or deletion in the *SMN1* gene is the cause of SMA. A potential treatment strategy for SMA is to upregulate levels of the SMN protein originating from the copy gene *SMN2* which can compensate in part for the absence of the functional *SMN1* gene. I have shown a novel therapeutic strategy for SMA treatment through the activation of the p38 pathway by the bacterial antibiotic anisomycin which stabilizes and increases SMN mRNA levels *in vitro*. Activation of the p38 pathway by anisomycin leads to cytoplasmic accumulation of HuR protein which binds to the 3'UTR of SMN transcript resulting in increased SMN levels. This opens up a novel potential therapeutic strategy for SMA. I have also identified and demonstrated a significant induction of SMN protein levels *in vitro* and *in vivo* upon treatment with FDA approved drug celecoxib, which also activates the p38 pathway. Celecoxib mitigates disease severity along with increasing the lifespan of SMA mice. Sodium valproate, trichostatin A and aclarubicin, all agents which effectively enhance SMN2 expression, have been recently shown to activate STAT5 in SMA-like mouse embryonic fibroblasts and human SMN2-transfected NSC34 cells. Given that prolactin is also known to activate the STAT5 signalling pathway, can cross blood brain barrier and is FDA approved, we elected to assess its impact on SMN levels. In this manner, I have demonstrated a significant induction in SMN mRNA and protein levels in neuronal NT2 and MN-1 cells upon treatment with prolactin. I have also demonstrated that activation of the STAT5 pathway by prolactin is necessary for this transcriptional upregulation of the

SMN gene. I have found that prolactin treatment induces SMN expression in brain and spinal cord samples and that it ameliorates the disease phenotype, improving motor neuron function and increasing survival in the SMA mouse model. Presently there is no cure for SMA. This study will help in the identification and characterization of potential therapeutic compounds for the treatment of SMA.

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List of Abbreviations

ARE	AU rich element
AREBP	ARE-binding protein
ATA	Aurintricarboxylic acid
ASO	Antisense oligonucleotide
BBB	Blood brain barrier
CAT	Chloramphenicol acetyltransferase
Cel	Celecoxib
CK	Creatine phosphokinase
CNS	Central nervous system
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DRB	1-β-D-ribobenzimidazole
EMG	Electromyography
ESE	Exon splicing enhancer
ESS	Exonic splice suppressor
FCS	Fetal calf serum
HDAC	Histone deacetylase
HET	Heterozygous
HuR	Human antigen R
hSMN	Human SMN

IP	Intraperitoneal
IPS	Induced pluripotent stem
LPS	Lipopolysaccharide
MAPK	Mitogen-activated protein kinase
MKK	MAPK kinase
MN-1	Motor neuron derived
mSMN	Mouse SMN
NAIP	Neuronal apoptosis inhibitor protein
NCS	Nerve conduction study
NEO	Neomycin
NMJ	Neuromuscular junction
NT2	Neuron-committed teratocarcinoma
PRL	Prolactin
PRLR	PRL receptor
RNS	Repetitive nerve stimulation
scAAV	Self complementary adeno-associated virus
SAHA	Suberoylanilide hydroxamic acid
SMA	Spinal muscular atrophy
SMN	Survival of motor neuron
snRNP	Spliceosomal small nuclear ribonucleoprotein
S.Seq	Scrambled sequence

STAT	Signal Transducers and Activator of Transcription
TNF	Tumour necrosis factor
TSA	Trichostatin A
Ub	Ubiquitin
UTR	Untranslated region
VPA	Valproic acid
WT	Wild type

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Chapter 1

Introduction

Preamble

This introductory chapter is presented in the format of an unpublished review article **"Spinal Muscular Atrophy: Classification, Diagnosis, Background, Molecular mechanism and Development of therapeutics"**. In this chapter, I summarize the clinical manifestations, molecular pathogenesis, diagnostic strategy and development of therapeutic regimes for the better understanding and treatment of SMA.

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Author Contribution: F.F. wrote the article. M.H and A.M. contributed ideas and editorial support.

Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease and the most common genetic cause of infant death. Loss or mutation of the *SMN1* gene, which results into reduced SMN protein level is the cause of the disease which leads to motor neuron death and progressive muscle atrophy. There is currently no cure for SMA. Recent progress has been made in our understanding of the molecular mechanisms underlying the pathogenesis of the disease. In this review, we summarize the clinical manifestations, molecular pathogenesis, diagnostic strategy and development of therapeutic regimes for the better understanding and treatment of SMA.

Epidemiology

Spinal muscular atrophy (SMA) is an autosomal recessive neuromuscular disorder characterized by the loss of motor neurons from the anterior horn of the spinal cord which leads to muscle weakness, hypotonia and ultimately muscle atrophy (1). With an incidence rate of 1:6000-10,000 live births and a carrier frequency of 1:40, SMA is the leading genetic cause of infant death globally (1-5).

Clinical classification

Due to heterogeneity of the disease and clinical variability in symptoms, SMA is broadly classified into four major categories characterized by the age of onset as well as severity of the disease (6-9). SMA type I, which was originally described by Werdnig and Hoffmann in the late 18^{th} century is the most severe and prevalent form of the disease and accounts for more than 50% of the known diagnosed cases of SMA. The onset of this form of the disease is within the first six months after birth and although historically patients succumbed within the first 2 years of life, with better health care and support the life expectancy of children with type I SMA can be increased to 5 years. Infants with type I SMA experience a rapid loss of skeletal muscle mass with profound hypotonia and general muscle weakness characterized by poor head control, difficulty with suckling, swallowing and an inability to sit without support. These children develop problems with breathing over time due to impaired bulbar function and respiratory muscle weakness leading to respiratory insufficiency. Respiratory failure due to aspiration pneumonia is an important cause of SMA mortality (6, 10, 11). The intermediate form of SMA, known as type II, has an onset between 6 and 18 months of age. Patients with type II SMA can sit unaided but still develop

progressive muscle weakness and can never stand or walk on their own. Other symptoms and physical signs include respiratory insufficiency due to reduced bulbar function, poor weight gain, fine hand tremors and joint contractures (6). SMA type III has an onset between 18 months to 30 years of age. Patients are able to stand and walk unaided, however they develop variable degree of muscle weakness which leads to a broad spectrum of physical signs and symptoms. While most walk independently, some lose ambulation during early adulthood and require wheelchair assistance. Others develop cramps and joint overuse problems; some develop scoliosis (6, 12, 13). Type IV SMA is the mildest form of the condition and is characterized by adult onset with normal mobility. They have mild muscle weakness in adulthood with normal longevity (6) (Table 1).

Table-1: Classification of SMA disease

SMA Type	Other Names	Age of Onset	Life Span	Highest Motor Activity
Type I (Severe)	Werdnig-Hoffmann disease	0-6 months	2-5 years	Never sit
Type II (Intermediate)	SMA, Dubowitz type	7-18 months	>2 years	Sit, Never stand
Type III (Mild)	Kugelberg-Welander disease	>18 months	Adult	Stand and walk (may require assistance)
Type IV (Adult)	----------	Adulthood	Normal	Walk during adulthood-unassisted (some muscle weakness)

Diagnosis and treatment

The diagnosis of SMA is made by a thorough patient history and physical examination followed by genetic testing. The survival of motor neuron (SMN) -1 genotyping has to a large degree replaced electromyography (EMG) and muscle biopsies (Fig 1.1) (2, 14). There is in 2012 no cure for SMA; current treatment is symptomatic and supportive. This includes clinical management through family education and counselling along with managing symptoms of the patients to prevent further complications.

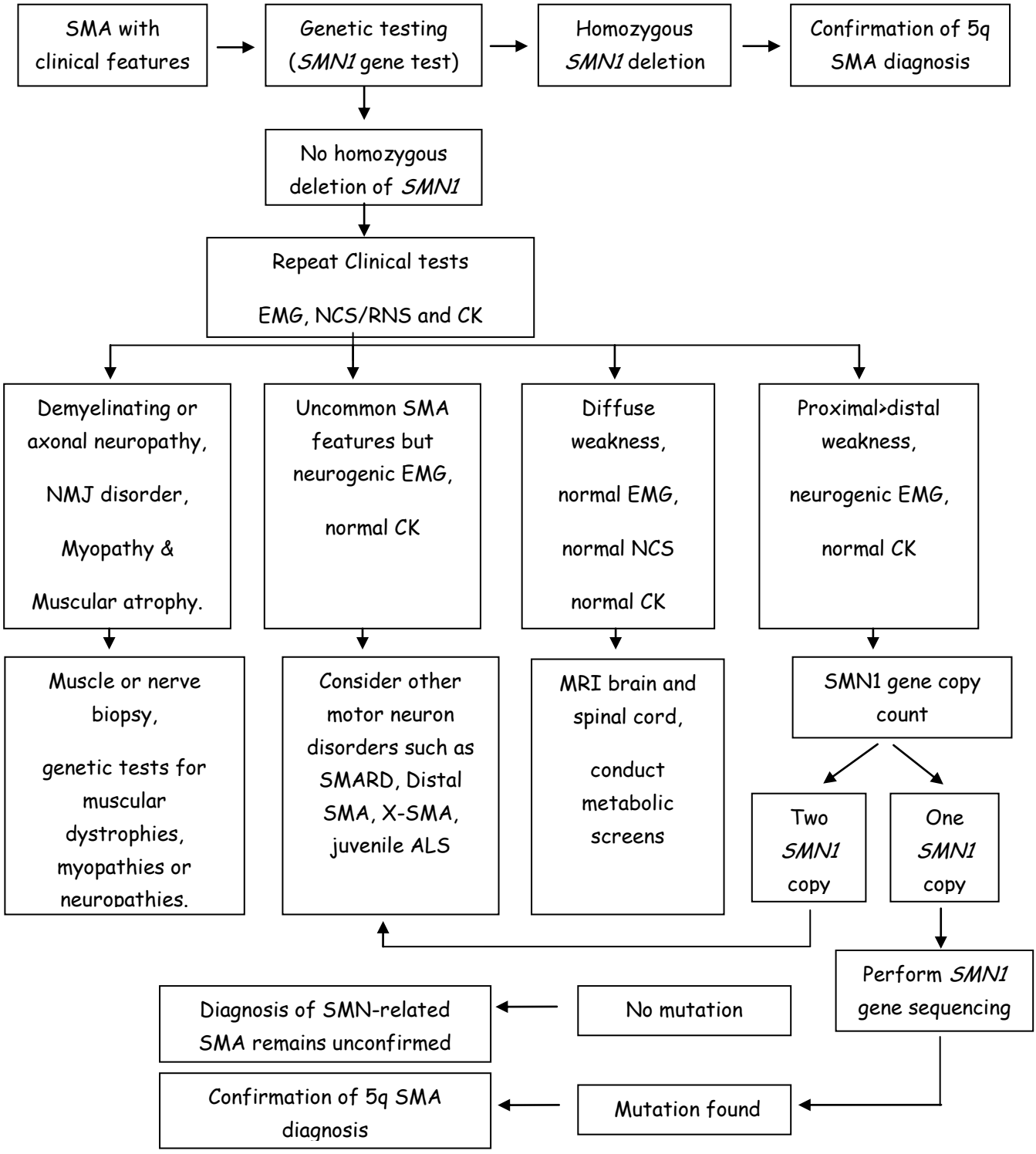

Figure 1.1: SMA diagnosis

Genetics of the disease

The SMA disease causing *SMN1* gene maps to a complex genomic region of chromosome 5q12-13. This region is characterized by an inverted duplication of the element with 4 genes (*SMN*, neuronal apoptosis inhibitor protein *{NAIP}, SERF and GTFH2*) present in telomeric and centromeric copies (Fig 1.2a) (15, 16). In 1995, it was reported that homozygous deletions of the *SMN1* gene were observed in and thus likely the cause of 95% of SMA patients (15). All SMA patients have a nearly identical twin centromeric copy of the *SMN1* gene, SMN2. These two genes are distinguished by five nucleotide changes in exon 7 and 8. The critical nucleotide difference which makes SMN2 only partially functional is a C to T transition at position 6 of exon 7. This change leads to the exclusion of exon 7 in the majority of transcripts. This mRNA is subsequently translated to form an unstable truncated isoform of SMN protein. However, the centromeric SMN2 copy gene still produces 5-10% functional full length SMN transcripts (Fig 1.2b) (15, 17, 18). All SMA patients have one or more copies of the SMN2 gene which, due to its partial functionality, acts as a positive disease modifier. The SMN2 gene is present in variable copy numbers in the population. There is an inverse correlation between the number of SMN2 gene (which can produce between 10-50% of SMN protein depending on copy number) and the severity of the disease (2). Low levels of SMN protein allows embryonic development but is not enough, in the long term, to allow motor neurons to survive in the spinal cord (19, 20). Type I patients usually have 1 or 2 copies whereas Type II have 2-3 copies of SMN2. Type III and IV have 3-4 copies of the *SMN2* gene. Individuals with 5 or more copies of the *SMN2* gene, despite having no functional *SMN1* gene are completely asymptomatic and are protected against the disease manifestation.

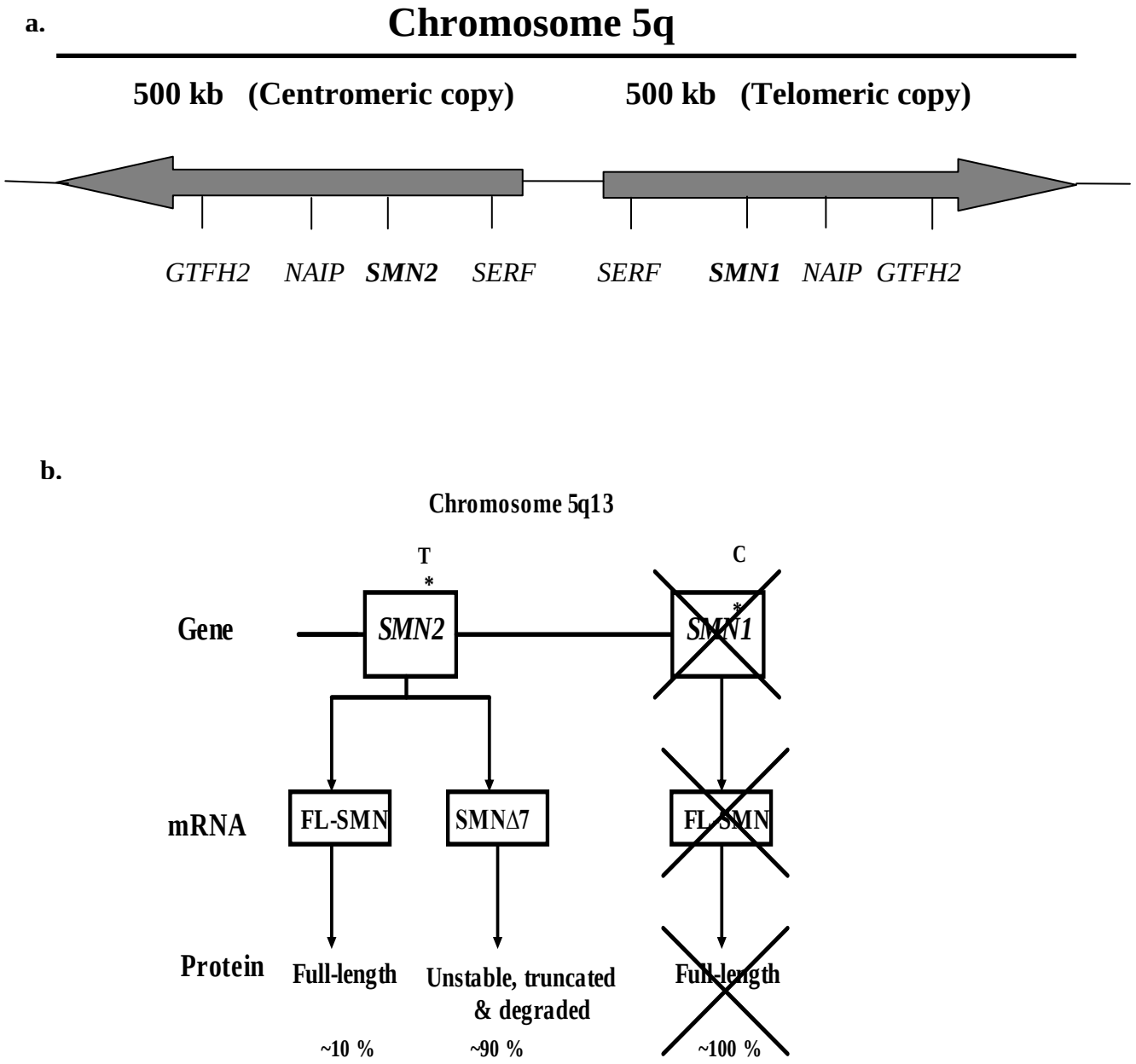

Figure 1.2: (a) Human SMN locus and (b) genetics of SMA patients

Pathology

The pathological hallmark of all forms of SMA is the loss of motor neurons from the lower brainstem and the anterior horn of the spinal cord (21). Anterograde axonal degeneration results in denervation of the myocytes within the motor unit. This sometimes leads to reinnervation of muscle, where adjacent uninjured motor neurons sprout leads to fiber type grouping of myocytes. The muscle in SMA patients consists of a large number of rounded atrophic fibers resulting from denervation of muscle in early childhood. The widely held notion had been that SMA is primarily a neuronopathy (involving the cell body) with secondary degeneration of the axons. However, more recent observations in the field have shifted the focus of SMA pathology from the motor neuron cell body to the distal axon (22, 23) and the possibility of a synaptopathic defect (20, 24). Regardless of the subcellular location of SMN mediated pathology, SMA is primarily considered as a motor neuron disease and consequently treatment strategies focus on drugs which can cross the blood brain barrier (BBB) to target the central nervous system (CNS). However, motor neuron autonomy of SMA pathogenesis has recently been called into question as many non-CNS pathologies (including cardiovascular, peripheral necrosis and liver defects) have been reported recently in both SMA patients and SMA mice models (25-33). In addition, one report has outlined the superiority of systemic SMN antisense oligonucleotide (ASO) therapy compared with intrathecal delivery in severe murine SMA calling into question the exclusive role of the motor neuron in disease causation (33).

Function of the SMN protein

SMN is a 294 amino acid long ubiquitously expressed protein with a molecular weight of 38 kilodaltons (kD). SMN is found in both the nucleus and cytoplasm. Within the nucleus, it is localized in nuclear structures called Gems and Cajal bodies (34). It is also found in abundance within the growth cones of the motor neurons (35). SMN has been implicated in ribonucleoprotein biogenesis (assembly, metabolism and transport of various ribonucleoproteins), as well as playing a major role in the splicing machinery. It is part of a multiprotein complex comprised of Gemins (2-8), spliceosomal U-snRNPs, Sm proteins and profilins called the SMN complex. This complex is essential for the biogenesis of snRNPs (36-45). Given the variety of roles that SMN has been implicated in, not surprisingly, the complete absence of SMN genes is embryonically lethal in virtually all metazoan life forms tested, indeed even cell cultures cannot survive without SMN (19, 20, 46).

Molecular mechanism: splicing defect in SMA.

Splicing is mediated by a complex called the spliceosome, the activity of which depends on a number of factors. In particular, various cis- and trans-acting elements regulate the splicing of both *SMN1* and *SMN2*. The C-T transition at position 6 of exon 7 in *SMN2* gene disrupts the function of an exonic splice enhancer (ESE; recognized by SF2/ASF to promote exon 7 inclusion) and/or creates an exonic splice suppressor (ESS; recognized by hnRNP A1/A2) which results in exon 7 skipping (Fig 1.3) (47-53).

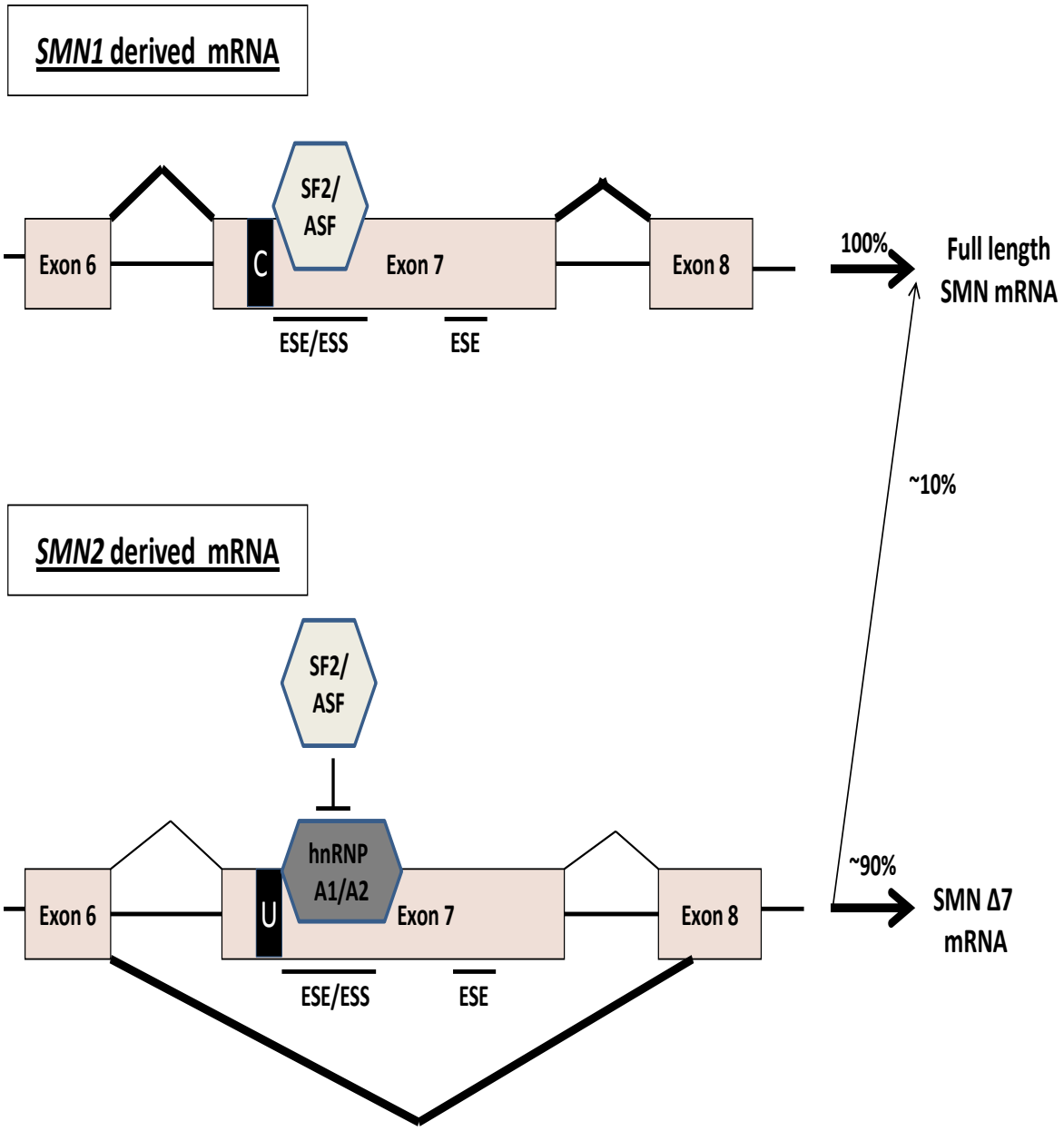

Figure 1.3: Splicing in SMA

Therapeutic strategies

Although there is no cure for SMA, the *SMN2* gene locus serves as a target for SMA treatment. The general treatment strategies for SMA are to compensate fully or in part for the absence of *SMN1* gene by increasing the levels of functional SMN2 protein levels though three distinct approaches: i) to induce the expression of *SMN2*, ii) to modulate splicing of SMN2 transcript, and iii) to stabilize the full length SMN2 mRNA and/or protein. In addition, gene and stem cell therapies are also under development for the treatment of SMA. These and other strategies are discussed below.

1. SMN dependent therapies: As outlined above, there is an inverse correlation between the *SMN2* gene copy number and disease severity (54, 55) which implies that directly targeting the *SMN2* gene in SMA patients through different pathways could be one key for the development of a SMA drug treatment. Alternatively, SMN protein can also be produced through gene replacement therapy.

a. Activation of *SMN2* promoter: Histone deacetylases (HDACs) repress transcription of genes including *SMN2* by chromatin condensation. Thus, HDAC inhibitors can increase transcription of the *SMN2* gene and can produce more full length SMN transcripts and protein which may have a beneficial effect in patients. Various HDAC inhibitors have been analyzed in cell culture, mouse models and in clinical trials as potential therapeutic for SMA. Sodium butyrate, Valproic acid (VPA) and phenylbutyrate showed promise in cell culture and mouse models and were also well tolerated by the patients (56-63). However, no clinical improvement was observed in SMA patients with HDAC inhibitors (61-63)**.**

Recent studies with other HDAC inhibitors, LBH589, Trichostatin A (TSA) and Suberoylanilide hydroxamic acid (SAHA) showed SMN2 gene induction in culture as well

as in a number of animal models of neurodegeneration (62, 64-66). In addition to these compounds, we have shown that the lactation hormone prolactin (PRL) which can both cross the blood brain barrier and, through binding to its receptor, activate the JAK2/STAT5 pathway upregulating *SMN2* gene transcription (68). Interestingly the degree of induction in SMN seen with the prolactin in the genetically engineered Δ7 SMA mouse model (where *SMN2* gene is the only source of SMN protein) is significantly greater than that seen in cell culture and wild type mice. We have determined that this is because of the difference between the promoter regions in *SMN1* and SMN2 genes, the latter uniquely having only STAT5a transcription binding motifs. This might prove beneficial as all SMA patients have SMN2 as the only source of SMN protein. Since PRL has been successfully tested and proven safe in humans for the treatment of lactation deficient mothers (67), it may bypass other compounds which are yet to be tested for clinical safety and join the short list of drugs which may have immediate potential SMA therapeutic potential (68).

b. Correction of splicing: The suppression of exon 7 skipping to produce more full length transcript from the *SMN2* gene is another treatment strategy being explored for SMA. HDAC inhibitors such as VPA, TSA and sodium butyrate appear to have a dual effect on SMN mRNA expression; they not only open chromatin structure and therefore increase the rate of transcription but also appear to affect the splicing process (56-58, 64). The antibiotic aclarubicin has been shown to increase full length SMN transcript by altering the splicing process *in vitro* (69). The most promising compounds which correct splicing by preventing SMN2 exon 7 skipping are Antisense oligos (ASOs). An ASO complementary to SMN2 exon 7 pre-mRNA sequences has been shown to inhibit binding of negative splicing factors and increase full length SMN2 transcript and protein production (30, 33, 70, 71). The major

hurdle in using ASOs for SMA therapeutics, however, is their inability to cross the blood brain barrier. However, Hua et al. 2011 documented a marked improvement in motor function along with an increase in survival in SMA mice with systemic delivery of ASO which results into increase in SMN levels mostly in peripheral tissues especially in liver. Interestingly, they documented only a slight increase in SMN levels in CNS tissues (33). However, there are a number of issues which need to be addressed before clinical introduction of ASOs for SMA treatment (clinical safety, quantity of ASO, cost, immune response etc) (72).

c. Full length SMN transcript stabilization: This is a relatively new approach which has been used in two studies to stabilize the full length SMN transcript. In the study by Singh et al., decapping enzyme DcpS, an integral part of the RNA degradation machinery, was targeted by C5-substituted quinazolines which interact and open the enzyme into a catalytically inactivated conformation. Full length SMN mRNA decay is in this fashion blocked, ultimately increasing SMN protein in cell culture (73).

In a different approach, SMN mRNA has been shown to have a specific AU rich element (ARE) region in its 3' UTR which marks the mRNA for degradation. Our laboratory has shown that activation of the p38 pathway results in the accumulation of RNA binding protein HuR in the cytoplasm which then binds to the ARE in 3'UTR region of SMN mRNA and stabilizes the transcript. Importantly, transcript stabilization is not associated with any discernible inhibition of SMN protein translation. This study provided a novel mechanism through which SMN mRNA could be stabilized using p38 activating compounds which can cross the blood brain barrier to develop new therapeutics for SMA treatment (74).

d. Full length SMN protein stabilization: Aminoglycosides are class of antibiotics which have been shown to mask premature stop codon mutations in some genes, allowing read through translation to occur. This moderates translation termination through an alteration in the conformation of the ribosomal reading site. Various aminoglycosides including tobramycin and amikacin have been used successfully in patient fibroblasts to increase SMN protein levels. However, their *in vivo* efficacy and safety has yet to be demonstrated (75-77).

An alternative potential therapeutic approach involves targeting the ubiquitin-proteasome pathway which mediates intracellular protein turnover. Proteins are marked with poly ubiquitin (Ub) molecules by the action of the enzymes E1 (Ub activating enzyme), E2 (Ub conjugating enzyme) and E3 (Ub ligase). The polyubiquitin modification marks the protein for destruction by the proteasome complex. SMN is one of the many proteins degraded by the ubiquitin proteasome pathway. It has been shown that FDA approved proteasome inhibitor bortezomib increases SMN both *in vitro* and *in vivo* by blocking proteolysis of SMN protein. However, it should be noted that bortezomib cannot cross the BBB; thus, it must be used in combination with other drugs which can cross the BBB for the treatment of SMA (78).

e. Gene therapy: One of the most encouraging SMA therapeutic advances so far is the use of gene therapy which shows significant promise. In the last three years several groups have used self complementary adeno-associated virus (scAAV) 8 and 9 vectors carrying the *SMN1* cDNA to treat mice models of SMA, resulting in the most dramatic extension in the life span of mice yet observed combined with an overall amelioration of disease phenotype (79-82). However, early pre-symptomatic intervention is necessary for the success of this

therapy as is seen with other treatment strategies as well. Moreover, several challenges must be addressed for this mode of SMA treatment before bringing it to clinical application successfully. The most pressing issues are clinical safety, dealing with the cross-species barriers, the cost of virus production along with the possibility of an immune response to AAV which can neutralize its impact (83).

2. SMN-independent strategies: There have been some recent advances in SMN-independent strategies for the treatment of SMA. These include:

a. Stem cell therapy: Stem cell therapy has generated much attention as a treatment for motor neuron diseases, including SMA, through replacement of the lost motor neurons and, more realistically perhaps, supporting the existing neuron population. Primary murine neuronal stem cells as well as embryonic stem cell-derived neural stem cells injected into the spinal cord of animal models of SMA have been shown to ameliorate disease phenotype and increase survival (84, 85). It is unclear whether this is through motor neuron and other cell replacement and/or through neuroprotection of host motor neurons by the numerous factors released from the donor cells. Although induced pluripotent stem (iPS) cells from an SMA patient have been differentiated into motor neurons (86, 87), there are several obstacles which hinder their use as a therapeutic for SMA treatment. These challenges include the production of the large number of stem cells and their successful transplantation into the patients, which could populate and cover the entire nervous system. Also, lentivirus vectors are used to deliver the cocktail of factors, required to produce iPS cells *in vitro*; these would be unsuitable for use in patients as they have the potential for insertional mutagenesis which could result into oncogenesis. Finally, even if motor neurons could develop *in situ*, the

prospect that they would at a meaningful level connect with the host CNS must be viewed as highly unlikely at this time.

b. Modifying neuromuscular junctions through actin dynamics: The pharmacological Rho-kinase inhibitor (downstream effector of RhoA-GTP which plays role in actin dynamics) dramatically increases the life span of a mild SMA mouse model and improves disease phenotype. This improvement in the disease phenotype is independent of SMN increase, mainly through making neuromuscular junctions (NMJ) better, larger and more mature (88). This suggests that there are SMN independent novel avenues for the development of therapeutics for SMA.

The overall objective of this study is to investigate the role of p38 and STAT5 kinase pathway in the regulation of SMN gene expression as one possible avenue in the development new drugs for SMA therapeutics. My specific aims are to:

1. **To investigate the effect of modulation of p38 and Stat5 pathways on SMN mRNA and protein levels.**
2. **To determine whether any increase in the SMN mRNA levels following modulation of distinct signaling pathways is through mRNA stabilization and/or by transcriptional induction.**
3. **To characterize the 3ÚTR of SMN mRNA and identify & characterize the role of RNA binding proteins on SMN transcript stabilization.**
4. **To test the therapeutic effects of p38activator & prolactin treatment in a mouse model of SMA.**

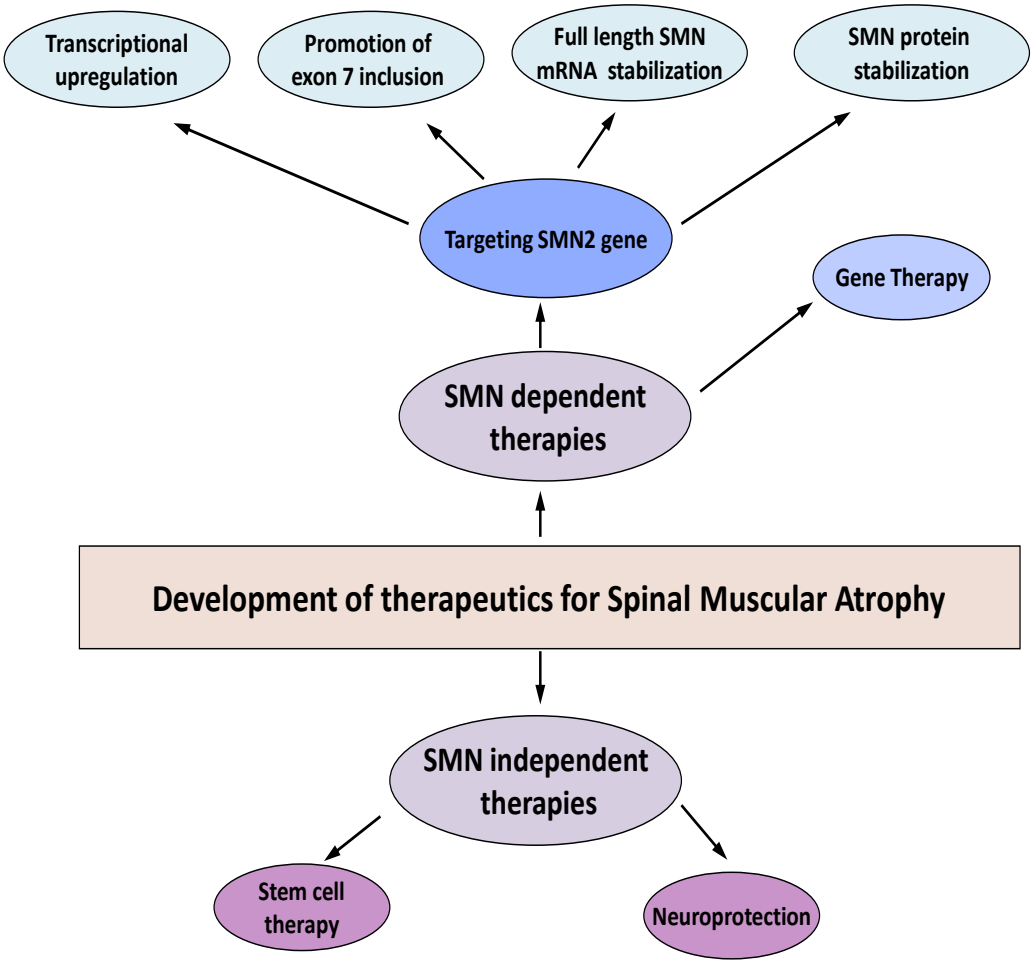

Figure 1.4: Development of therapeutics for SMA

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Chapter 2

p38 Mitogen-Activated Protein Kinase Stabilizes SMN mRNA Through RNA Binding Protein HuR.

Preamble

"p38 Mitogen-Activated Protein Kinase Stabilizes SMN mRNA Through RNA Binding Protein HuR" was first published as a research article in the journal Human Molecular Genetics (volume 18, number 21, July 2009). This articles identifies SMN gene regulation through p38 MAPK pathway. Activation of p38 pathway by bacterial antibiotic anisomycin stabilizes and increases SMN mRNA levels *in vitro* through RNA binding protein HuR.

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Author Contribution: F.F. wrote the article. S.B. initially started the project. X.L. did the initial screen. F.F, M.H and A.M. designed experiments. F.F performed experiments presented in Figures 2.1-2.8. M.H and A.M. contributed ideas and editorial support.

Abstract:

Spinal muscle atrophy (SMA) is an autosomal recessive neurodegenerative disease which is characterized by the loss of α motor neurons resulting in progressive muscle atrophy. Reduced amount of functional survival motor neuron (SMN) protein due to mutations or deletion in the *SMN1* gene is the cause of SMA. A potential treatment strategy for SMA is to upregulate levels of SMN protein originating from the *SMN2* gene compensating in part for the absence of functional *SMN1* gene. Although there exists a sizeable literature on *SMN2* inducing compounds, there is comparatively less known about the signalling pathways which modulate SMN levels. Here, we report a significant induction in SMN mRNA and protein following p38 activation by anisomycin. We demonstrate that anisomycin activation of p38 causes a rapid cytoplasmic accumulation of HuR, a RNA binding protein which binds to and stabilizes the AU-rich element within the SMN transcript. The stabilization of SMN mRNA, rather than transcriptional induction results in an increase in SMN protein. Our demonstration of SMN protein regulation through the p38 pathway and the role of HuR in this modulation may help in the identification and characterization of p38 pathway activators as potential therapeutic compounds for the treatment of SMA.

Introduction:

Spinal muscle atrophy (SMA) is a comparatively common autosomal recessive neurodegenerative disease, characterized by the loss of α motor neurons from the anterior horn of spinal cord resulting in progressive muscle atrophy (1). It has a prevalence of one in 10 000 live births and a carrier frequency of one in 50 (2). Reduced amount of functional survival motor neuron (SMN) protein due to mutations or, most commonly, deletion of the *SMN1* gene is the cause of SMA (3).

SMN, a 294 amino acid ubiquitously expressed protein is a key component of the SMN complex central to the biogenesis of spliceosomal small nuclear ribonucleoproteins including the removal of introns during pre-mRNA splicing (4). SMN also has diverse functions in the assembly, metabolism and transport of other ribonucleoproteins (5–14). Humans have two nearly identical *SMN* genes, *SMN1* and *SMN2* due to an evolutionary recent duplication event on chromosome 5 (15,16). *SMN1* gene produces full-length functional SMN protein, whereas *SMN2* due to a C to T transition at position 6 of exon 7 (one of five non-*SMN1–SMN2* nucleotide differences) produces mostly aberrantly spliced mRNA and produces only 10% of the full-length functional SMN protein (3,17,18).

A significant majority of SMA patients lack the functional *SMN1* gene due to homozygous deletion (3). Although the lack of functional full-length SMN protein ultimately leads to an apoptotic death to a subset of motor neurons (19), the precise mechanism of neuronal dysfunction remains unclear. One model suggests a reduced pre-synaptic transcriptome leading to anomalous neuromuscular junction (NMJ) architecture and functionality (19,20). All SMA patients have at least one copy of the *SMN2* gene which produces low levels of functional SMN protein. An inverse correlation is seen between the *SMN2* gene copy

number and SMA severity as higher *SMN2* copy numbers produce more functional SMN protein attenuating disease severity (21, 22). Presently, there is neither cure nor a particularly effective therapy for SMA. Potential treatment strategies for SMA include the induction of *SMN2* gene, the modulation of splicing of *SMN2* transcripts and the stabilization of either SMN mRNA and/or protein all of which might compensate in part for the absence of functional*SMN1* gene.

The p38 pathway comprises mitogen-activated protein kinases (MAPK) which are activated by a number of external stimuli such as UV light, osmotic shock, heat, growth factors and inflammatory cytokines (23–32). These stimuli activate two main upstream kinases, the MAPK kinases MKK3 and MKK6 which in turn phosphorylate and activate p38. The p38 pathway modulates a number of cellular responses including cell differentiation and apoptosis. One mechanism by which p38 works is the modulation of stability of various AU-rich element (ARE) containing mRNAs which comprise of as many as 4000 genes. The pentameric AUUUA instability motif in the 3′-UTR region of these transcripts marks them through specific ARE-binding proteins (AREBPs) (33–37) for a rapid degradation which, at least in part, is modulated by p38. Conversely, post-transcriptional stabilization of various ARE-rich transcripts through ARE-BPs is an important step in modulating the protein levels. For example, HuR [a member of embryonic lethal abnormal vision/Hu family of proteins] is a ubiquitously expressed ARE-BP that has a constitutive nuclear localization (38). Upon stimulation, HuR accumulates in the cytoplasm where it binds to the 3′-UTR of a variety of ARE containing cellular transcripts contributing to their stabilization (39). It has been shown that anisomycin, which is a known MAPK (p38, c-JNK) activator, can cause rapid nuclear to

cytoplasmic shuttling of HuR protein (40). Interestingly, SMN mRNA is classified as ARE containing mRNA (37) and contains a putative HuR binding motif (39).

Following preliminary data indicating p38 in SMN modulation in both cellular and *in vivo* models, we hypothesized that the activation of p38 pathway might stabilize SMN mRNA thus increasing functional SMN protein as a result of complex formation between 3′-UTR of SMN mRNA and HuR protein. P38 and HuR mediated regulation of SMN mRNA and protein level is demonstrated in this study representing novel avenues both for SMN regulation as well as SMA therapeutics.

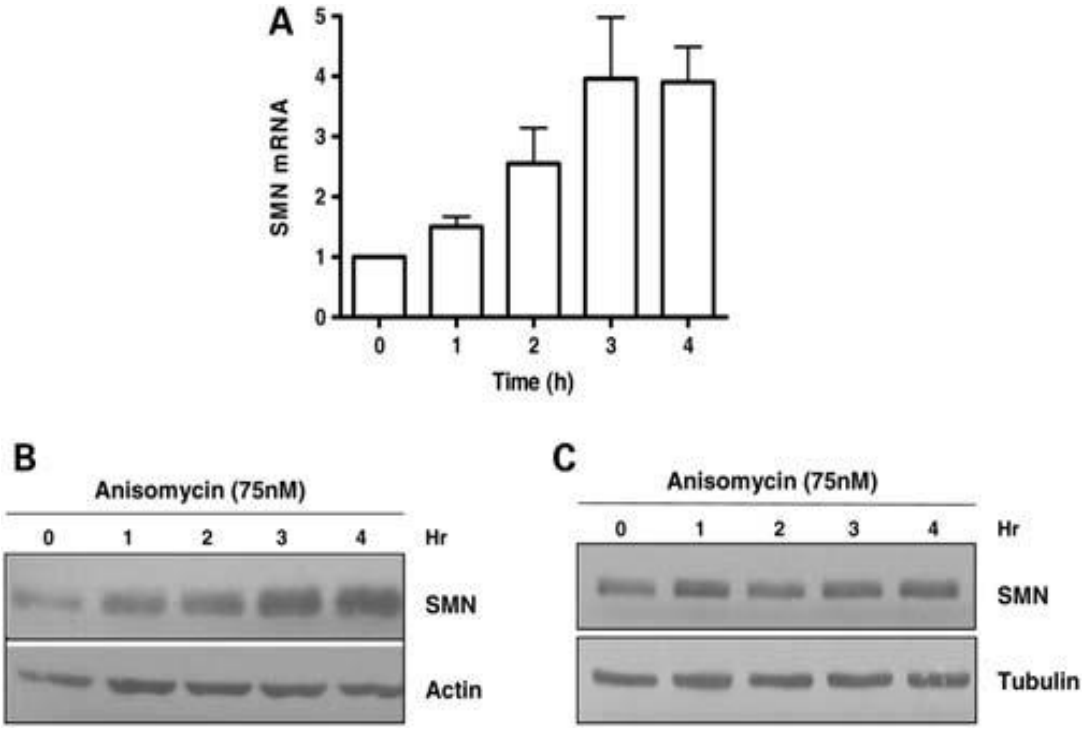
Results:

We have mined the Johnson & Johnson Pharmaceutical Research and Development archived gene expression profiles for SMN levels under different treatment conditions and with different compounds. SMN was found to be up-regulated in both cell lines and human white blood cells after systemic administration of lipopolysaccharide (LPS). The up-regulation of peripheral leukocyte SMN in response to LPS in human volunteers could be completely inhibited by a compound that inhibits p38 (unpublished data), indicating that the induction of SMN is downstream to the p38 MAPK pathway. Consonant with this interpretation, analysis of Connectivity Map data (41), revealed that of 1309 small molecules assayed, the p38 inducing antibiotic anisomycin was the most effective at upregulating SMN mRNA in three independent cell lines (Justin Lamb, pers commun).

Anisomycin treatment induces *SMN* gene expression

To investigate a potential p38 role in *SMN* gene regulation, Human neuron-committed teratocarcinoma (NT2) cells were treated with anisomycin. SMN transcript levels were found to be increased significantly (3–4-fold) in NT2 cells upon treatment with anisomycin (Fig. 2.1A). NT2 or motor neuron derived (MN-1) cells were then treated with anisomycin and then harvested for western blot analysis at 1 h intervals revealing a time-dependent increase of SMN protein in both NT2 and MN-1 cells (Fig. 2.1B and C). Taken together these results demonstrate that anisomycin treatment causes an increase in SMN steady-state mRNA and protein levels in neuronal-cell lines.

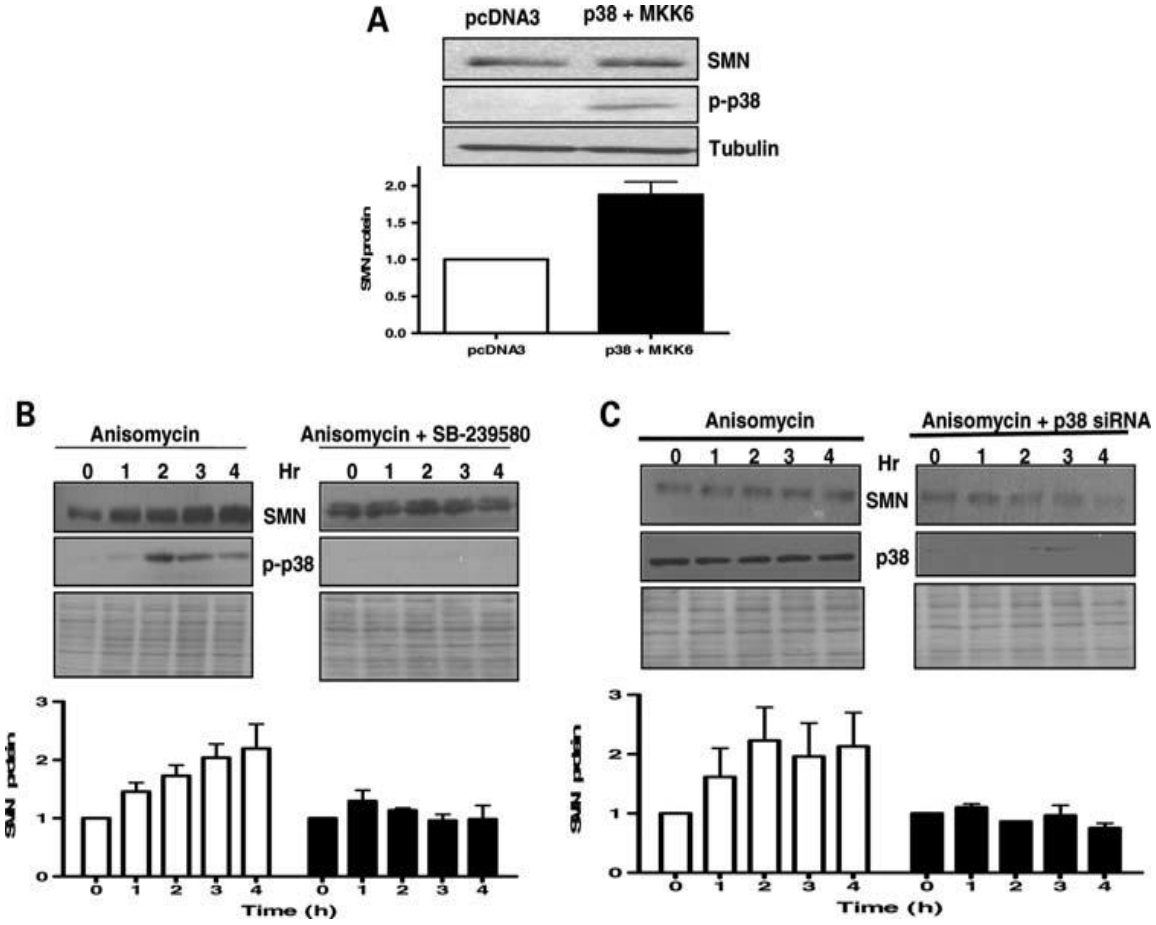
Figure 2.1. Anisomycin upregulates SMN mRNA and protein *in vitro*. Anisomycin treatment causes 2-3 fold induction in SMN mRNA and protein. NT2 or MN-1 cells were treated with anisomycin (75nM) and then harvested at indicated intervals for RT-PCR or Western blot analyses. (a) Quantification of SMN mRNA relative to β-actin in NT2 cells (the ratio at 0 hour was set as 1). Mean ± SD (bars) of three independent experiments. (b) Representative Western blot showing effect of anisomycin on SMN protein in NT2 cells. (c) Representative Western blot showing effect of anisomycin on SMN protein in MN-1 cells.


A
SMN mRNA
0
1
2
3
4
5
Time (h)
B
Anisomycin (75nM)
0 1 2 3 4
Hr
SMN
Actin
C
Anisomycin (75nM)
0 1 2 3 4
Hr
SMN
Tubulin

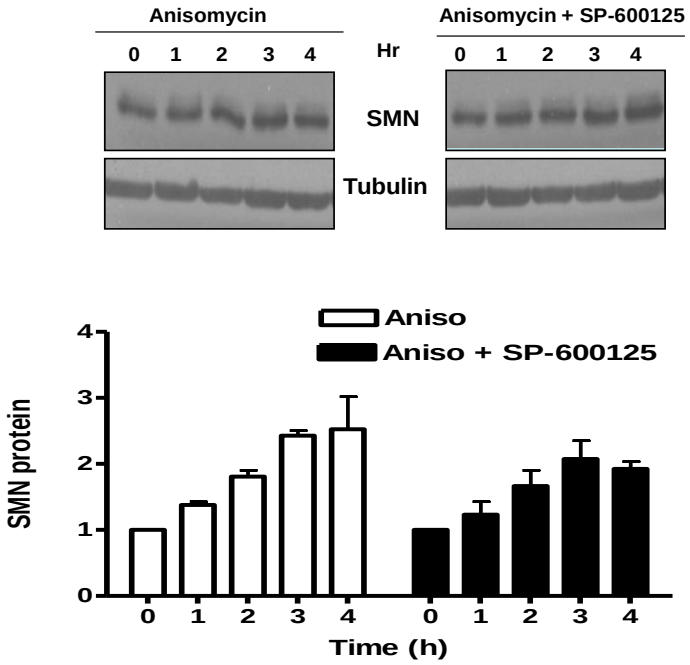
Activation of p38 pathway causes upregulation of SMN protein and is responsible for anisomycin conferred SMN protein increase

Anisomycin activates both the c-JNK and p38 pathways. To directly assay for the role of the p38 pathway in the regulation of SMN levels, MN-1 cells were transfected with either pcDNA3 or p38+MKK6 overexpression plasmid which constitutively phosphorylates and activates the p38 pathway. Cells were harvested for western blot analysis after 24 h of transfection. Overexpression of p38+MKK6 was found to increase the level of SMN protein by ~2-fold compared with pcDNA3 vector control (Fig. 2.2A). We next pre-treated NT2 cells with c-JNK inhibitor SP-600125 or p38 inhibitor SB-239580 for 1 h and then treated the cells with anisomycin for 4 h. Western blot analysis revealed that treatment with c-JNK inhibitor had no effect on anisomycin-induced increase in SMN protein levels (Supplementary Fig. 2.1), whereas p38 inhibition effectively blocked the increase in SMN protein (Fig. 2.2B). To further confirm the role of p38 in anisomycin-induced increase in SMN protein, NT2 cells were transfected with p38-specific siRNA or non-targeting control siRNA for 48 h, and then treated with anisomycin for 4 h. Cells were harvested and western blot analysis was conducted. siRNA-mediated abrogation of p38 expression blocked anisomycin-induced increase in SMN protein (Fig. 2.2C). These results strongly suggest that activation of p38 but not c-JNK pathway causes anisomycin-mediated upregulation of SMN protein.

Figure 2.2. p38 MAPK pathway regulates SMN protein level. (a) MN-1 cells were transfected with either pcDNA3 vector or constitutively activated p38+MKK6 overexpression plasmid (2 μg/ml). Cells were harvested for Western blot analysis 24 hours after transfection. Representative Western blot and densitometric quantification (Mean ± SD (bars) of three independent experiments) are shown. (b) Representative Western blots showing the effect of p38 inhibition on anisomycin induced increase in SMN protein. Quantification of SMN protein relative to β-actin (the ratio at 0 hour was set as 1). p38 inhibitor (SB-239580) blocked the anisomycin induced increase in SMN protein in NT2 cells. NT2 cells were treated with SB-239580 (3uM) for 1h and then treated with anisomycin (75nM) for up to 4 hours. Cells were harvested for Western blot analysis at hourly intervals. Mean ± SD (bars) of three independent experiments. (c) Representative Western blots showing both p38 knockdown and its effect on anisomycin induced increase in SMN protein. Quantification of SMN protein relative to β-actin (the ratio at 0 hour was set as 1). The siRNA knockdown of p38 protein attenuates anisomycin induced increase in SMN protein. NT2 cells were transfected with p38 siRNA (25nM) or scrambled sequence (25nM) for 48h and then treated with anisomycin (75nM) for up to 4 hours. Cells were harvested for Western blot analysis at hourly intervals. Mean ± SD (bars) of three independent experiments.


A
pcDNA3
p38 + MKK6
SMN
p-p38
Tubulin
SMN protein
pcDNA3
p38 + MKK6
B
Anisomycin
Anisomycin + SB-239580
Hr
SMN
p-p38
SMN protein
Time (h)
C
Anisomycin
Anisomycin + p38 siRNA
Hr
SMN
p38
SMN protein
Time (h)

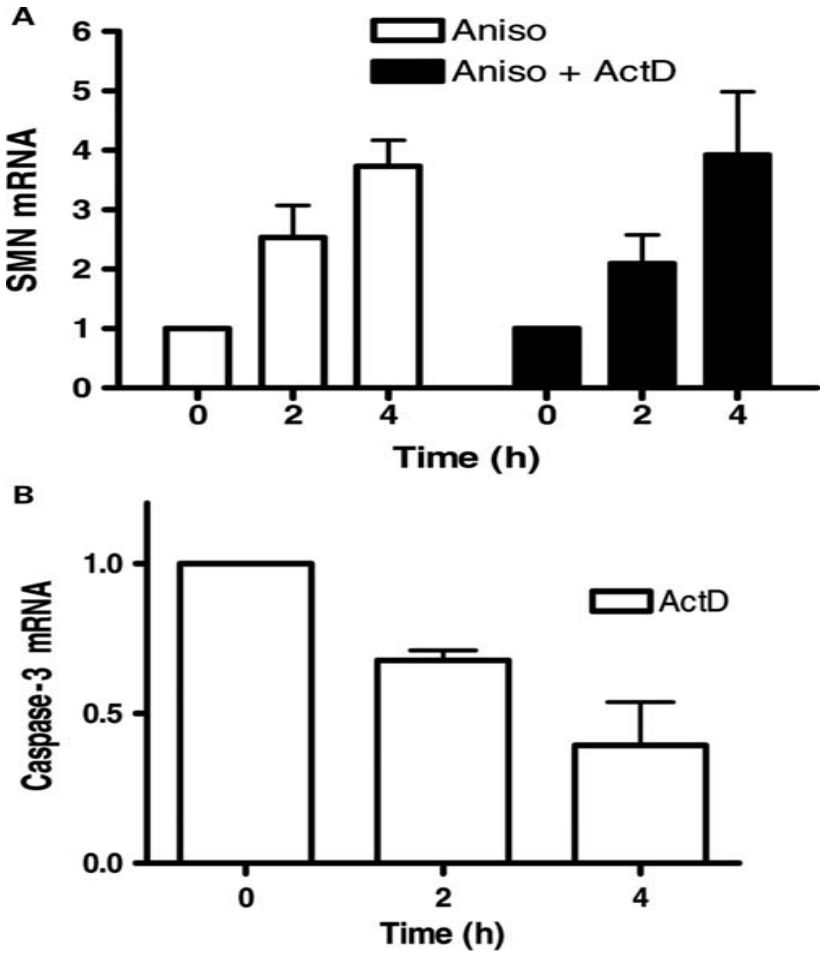
Supplementary Figure 2.1. Inhibition of c-JNK has no effect on anisomycin-induced increase in SMN. Representative Western blots showing effect of c-JNK inhibition on anisomycin-induced increase in SMN protein. Quantification of SMN protein relative to β-actin (the ratio at 0 hour was set as 1). c-JNK inhibitor (SP-600125) had no effect on anisomycin induced increase in SMN protein in NT2 cells. NT2 cells were treated with SP-600125 (20uM) for 1h and then treated with anisomycin (75nM) for up to 4 hours. Cells were harvested for Western blot analysis at hourly intervals. Mean ± SD (bars) of three independent experiments.


Anisomycin
Anisomycin + SP-600125
Hr
0 1 2 3 4
0 1 2 3 4
SMN
Tubulin
Aniso
Aniso + SP-600125
SMN protein
0
1
2
3
4
0 1 2 3 4 0 1 2 3 4
Time (h)

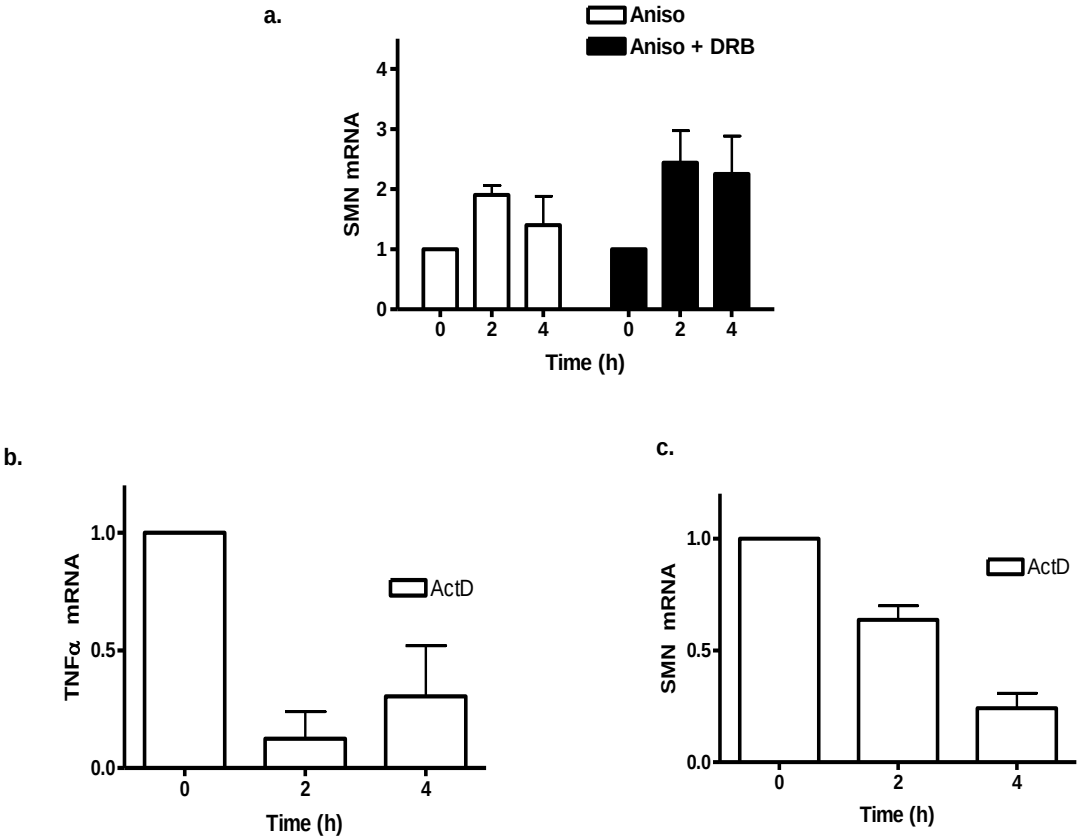
SMN mRNA stabilization by anisomycin in NT2 cells

To determine whether the increase in steady-state levels of SMN mRNA by p38 activation is through mRNA stabilization or by transcriptional upregulation, NT2 cells were treated with anisomycin in the presence or absence of transcriptional inhibitors Actinomycin D or 1-β-D-ribobenzimidazole (DRB) and total RNA was harvested at different time points (0, 2 and 4 h) for RT–PCR. Activation of p38 by anisomycin increased the SMN mRNA content in the presence of both transcriptional inhibitors, suggesting that anisomycin treatment does not increase SMN transcription, but most likely stabilizes SMN mRNA (Fig. 2.3A and Supplementary Fig. 2.2a). In contrast, levels of Caspase-3 and TNFα, two mRNA's which are known to be unstable genes and served as controls for transcriptional inhibition, decreased rapidly upon treatment with Actinomycin D (Fig. 2.3B and Supplementary Fig. 2.2b).

Figure 2.3. SMN mRNA stabilization by anisomycin in NT2 cells. Anisomycin-induced increase in SMN mRNA content is independent of transcriptional upregulation. (a) NT2 cells were treated with anisomycin (75nM) with/without transcriptional inhibitor Actinomycin D (2.5 μg/ml) at different time points (0, 2 & 4 h) and then harvested for RT-PCR. Quantification of SMN mRNA relative to β-actin. Mean ± SD (bars) of three independent experiments performed in triplicate. (b) NT2 cells were treated with transcriptional inhibitor Actinomycin D (2.5 μg/ml) at different time points (0, 2 & 4 h) and then harvested for RT-PCR. Quantification of Caspase-3 mRNA relative to β-actin. Mean ± SD (bars) of three independent experiments performed in triplicate.


A
Aniso
Aniso + ActD
SMN mRNA
6
5
4
3
2
1
0
0
2
4
0
2
4
Time (h)
B
ActD
Caspase-3 mRNA
1.0
0.5
0.0
0
2
4
Time (h)

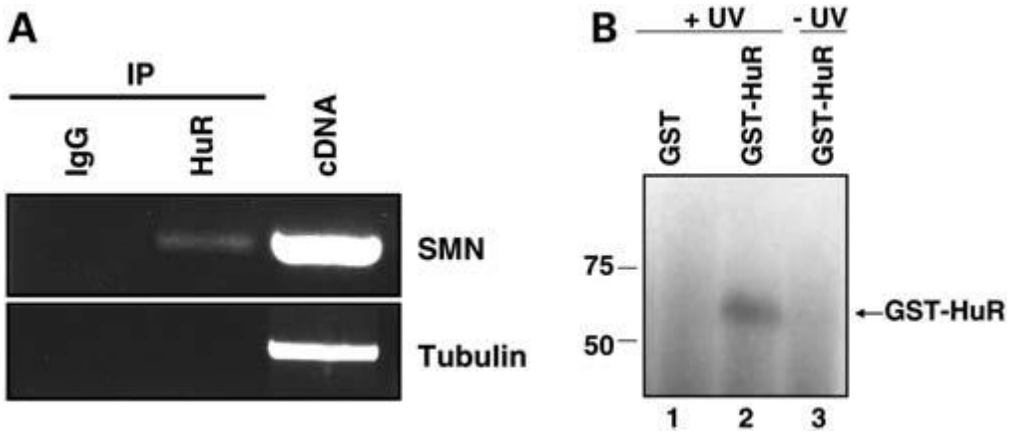
Supplementary Figure 2.2. (a) SMN mRNA stabilization by anisomycin in NT2 cells. Anisomycin induced increase in SMN mRNA content is independent of transcriptional upregulation. NT2 cells were treated with anisomycin (75nM) with/without transcriptional inhibitor DRB (100nM) at different time points (0, 2 & 4 h) and then harvested for RT-PCR. Quantification of SMN mRNA relative to β-actin (expressed as fold of internal control). Mean ± SD (bars) of three independent experiments performed in triplicate. **(b) Control for Actinomycin D treatment.** NT2 cells were treated with transcriptional inhibitor Actinomycin D (2.5 μg/ml) at different time points (0, 2 & 4 h) and then harvested for RT-PCR. Quantification of TNFα mRNA relative to β-actin (expressed as fold of internal control). Mean ± SD (bars) of three independent experiments performed in triplicate. (c) NT2 cells were treated with transcriptional inhibitor Actinomycin D (2.5 μg/ml) at different time points (0, 2 & 4 h) and then harvested for RT-PCR. Quantification of SMN mRNA relative to β-actin (expressed as fold of internal control). Mean ± SD (bars) of three independent experiments performed in triplicate.


a.
Aniso
Aniso + DRB
SMN mRNA
0
1
2
3
4
0
2
4
0
2
4
Time (h)
b.
TNFα mRNA
1.0
0.5
0.0
ActD
0
2
4
Time (h)
c.
SMN mRNA
1.0
0.5
0.0
ActD
0
2
4
Time (h)

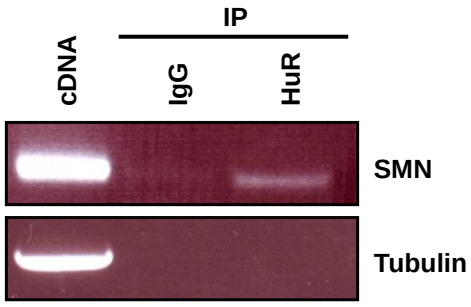
RNA binding protein HuR interacts with SMN mRNA

Trans-acting factors, such as HuR, AUF-1 and others have been shown to play an important role in the regulation and fate of an ARE-rich transcripts. It has been suggested previously that the 3′-UTR of SMN transcript contains an ARE-motif (37) and a sequence that resembles a HuR binding site (42). To assess whether endogenous HuR protein interacts with and potentially stabilizes endogenous SMN transcript, RNA–protein complexes were cross linked using formaldehyde in both NT2 and MN-1 cells. RNA–protein complexes were then immunoprecipitated with IgG or HuR antibody after cellular lysis. After cross-linking reversal, cDNA was reverse transcribed from RNA isolated from the immunoprecipitate followed by PCR amplification with SMN primers. In this fashion, we were able to RT–PCR amplify SMN mRNA from immunoprecipitated HuR but not with the IgG confirming that endogenous HuR associates with endogenous SMN mRNA in cells (Fig. 2.4A and Supplementary Fig. 2.3). To determine if HuR binds directly to the 3′-UTR of SMN mRNA, we performed a UV cross-linking experiment using a purified recombinant GST-HuR protein and radiolabeled probe of 3′-UTR of SMN mRNA. GST or GST-HuR were incubated with ^{32}P-labeled 3′-UTR of SMN mRNA probe followed by UV cross-linking and separation by SDS–PAGE. We found by autoradiography that 3′-UTR of SMN mRNA was cross-linked to purified recombinant GST-HuR protein *in vitro*, suggesting that HuR binds directly to the 3′-UTR of SMN mRNA (Fig. 2.4B).

Figure 2.4. HuR interacts with SMN mRNA. (a) Endogenous HuR interacts with SMN mRNA *in vivo*. In NT2 cells, RNA-protein complexes were cross-linked with formaldehyde and immunoprecipitated after cell lysis using antibodies against HuR and IgG. Following crosslink reversal the RNA was isolated from the immunoprecipitate and was used to produce cDNA by reverse transcription, followed by PCR amplification with SMN and Tubulin primers. A representative agarose gel of three independent experiments is shown. (b) HuR binds directly to the 3'UTR of SMN mRNA *in vitro.* GST (lane 1) or GST-HuR (lane 2) were incubated with ^{32}P-labelled 3'UTR of SMN mRNA probe, UV cross-linked and then separated by SDS-PAGE and visualized by autoradiography. As a negative control, GST-HuR (lane 3) was incubated with ^{32}P-labelled 3'UTR of SMN mRNA probe without UV cross-linking.


A
IP
IgG
HuR
cDNA
SMN
Tubulin
B
+ UV
- UV
GST
GST-HuR
GST-HuR
75
50
←GST-HuR
1
2
3

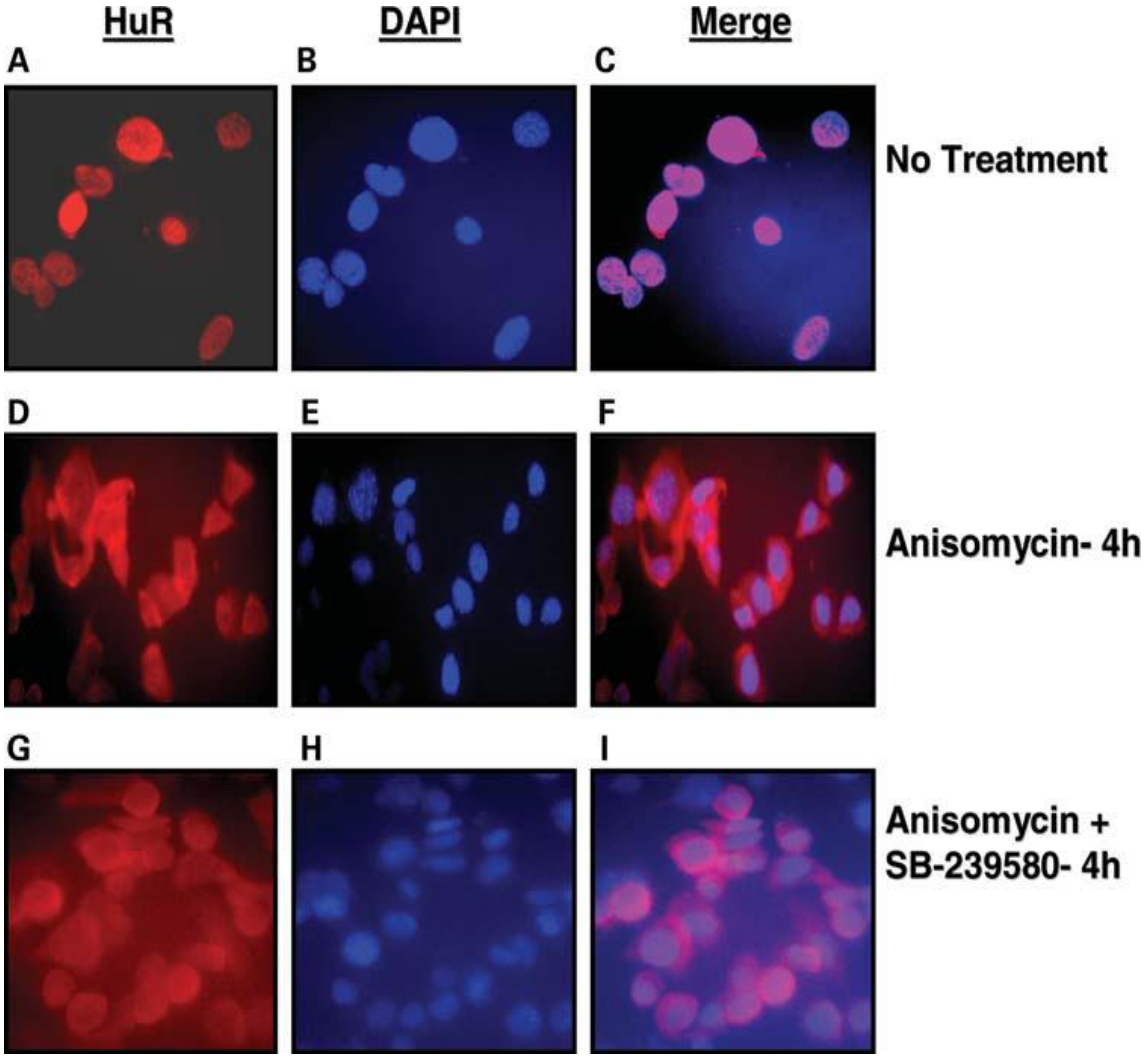
Supplementary Figure 2.3. HuR interacts with SMN mRNA. (a) Endogenous HuR interacts with SMN mRNA *in vivo*. In MN-1 cells, RNA-protein complexes were cross-linked with formaldehyde and immunoprecipitated after cell lysis using antibodies against HuR and IgG. Following crosslink reversal the RNA was isolated from the immunoprecipitate and was used to produce cDNA by reverse transcription, followed by PCR amplification with SMN and Tubulin primers. A representative agarose gel of three independent experiments is shown.


IP
cDNA
IgG
HuR
SMN
Tubulin

Anisomycin causes p38-dependent cytoplasmic accumulation of HuR

Although HuR is primarily localized in nucleus, it has been reported previously that in DDT1-MN2 cells, anisomycin can induce shuttling of HuR from nucleus to cytoplasm (38). To investigate whether there was a similar nuclear to cytoplasmic shuttling of HuR upon anisomycin treatment and if so, whether it was p38 dependent, MN-1 cells were treated with anisomycin for 4 h with or without 1 h pre-treatment with p38 inhibitor SB-239580. Immunohistochemistry results were consistent with previous reports showing that in untreated cells, HuR is primarily localized in the nucleus (Fig. 2.5C), whereas some relocalization of HuR from nucleus to cytoplasm is observed after 4 h anisomycin treatment (Fig. 2.5F). Importantly, HuR persists in the nucleus when cells are treated with the p38 inhibitor SB-203580 prior to anisomycin treatment. Thus anisomycin-induced relocalization of HuR from nucleus to cytoplasm is effectively inhibited by p38 inhibition (Fig. 2.5I).

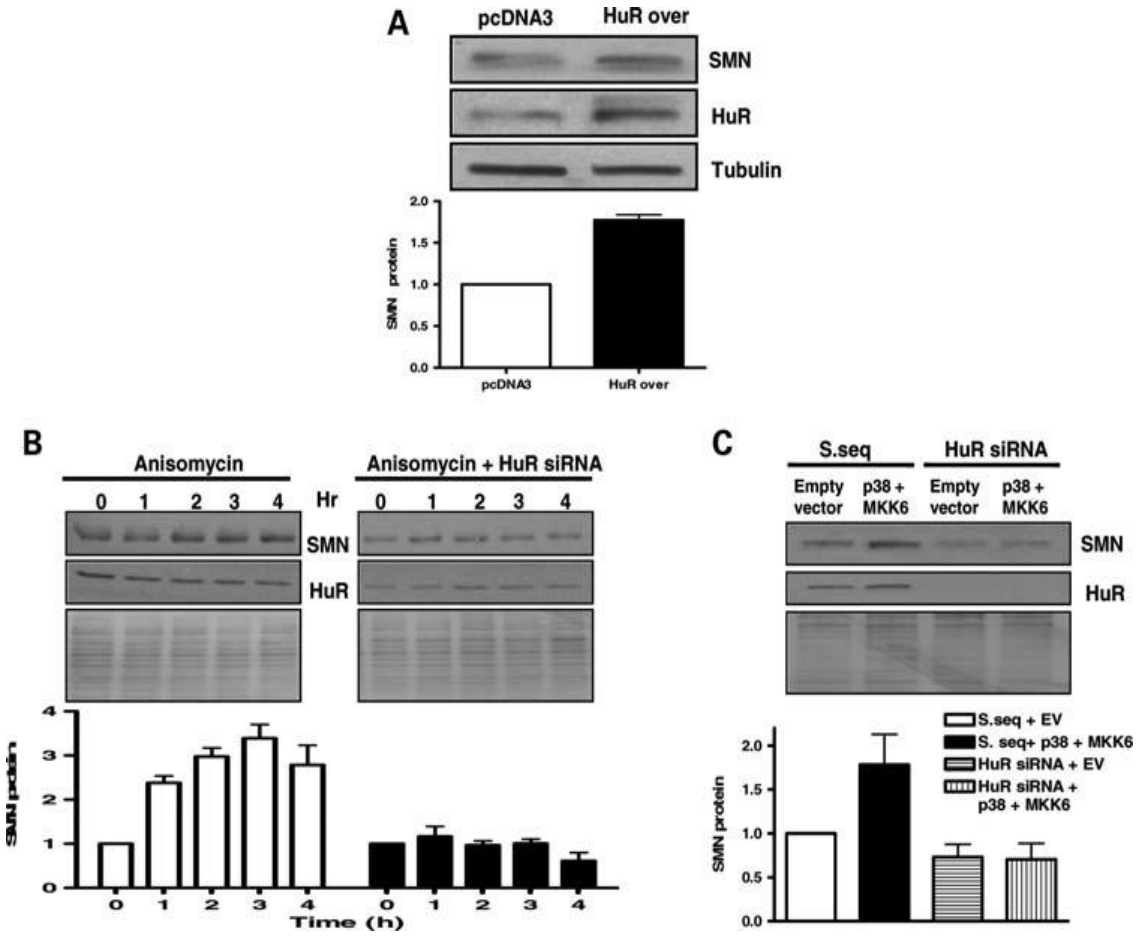
Figure 2.5. Anisomycin causes p38 pathway-mediated accumulation of HuR in the cytoplasm of MN-1 cells. Immunohistochemistry was performed in MN-1 cells which were treated with anisomycin for 4 h with or without one hour pre-treatment with p38 inhibitor SB-239580 (3uM). MN-1 cells were stained with monoclonal antibody for HuR (red, a, d, g), counter stained with DAPI (blue, b, e, h) with merged images of both shown in panel c, f and i. Representative images of three independent experiments are shown.


HuR
DAPI
Merge
A
B
C
No Treatment
D
E
F
Anisomycin- 4h
G
H
I
Anisomycin +
SB-239580- 4h

HuR is required for p38-mediated upregulation of SMN protein

To further elaborate the role of HuR in SMN transcript regulation, MN-1 cells were transfected with either empty pcDNA3 or HuR expressing plasmid. Cells were harvested for western blot analysis after 24 h of transfection. Overexpression of HuR protein (which increased both nuclear and cytoplasmic HuR content; data not shown) increased the level of SMN protein by ~2-fold compared with empty pcDNA3 vector control (Fig. 2.6A). To directly show that the increase in SMN protein levels mediated by anisomycin or p38+MKK6 overexpression plasmid is through the HuR-mediated stabilization of SMN mRNA, NT2 cells were transfected with HuR siRNA or non-targeting control siRNA for 48 h and then treated with anisomycin for 4 h. siRNA-mediated abrogation of HuR expression completely blocked the anisomycin-induced increase in SMN protein (Fig. 2.6B). Similarly, in MN-1 cells, HuR knockdown followed by overexpression of p38+MKK6 blocked the increase of SMN protein by p38 pathway activation. These results clearly indicate that HuR is required for p38-mediated upregulation of SMN protein (Fig. 2.6C).

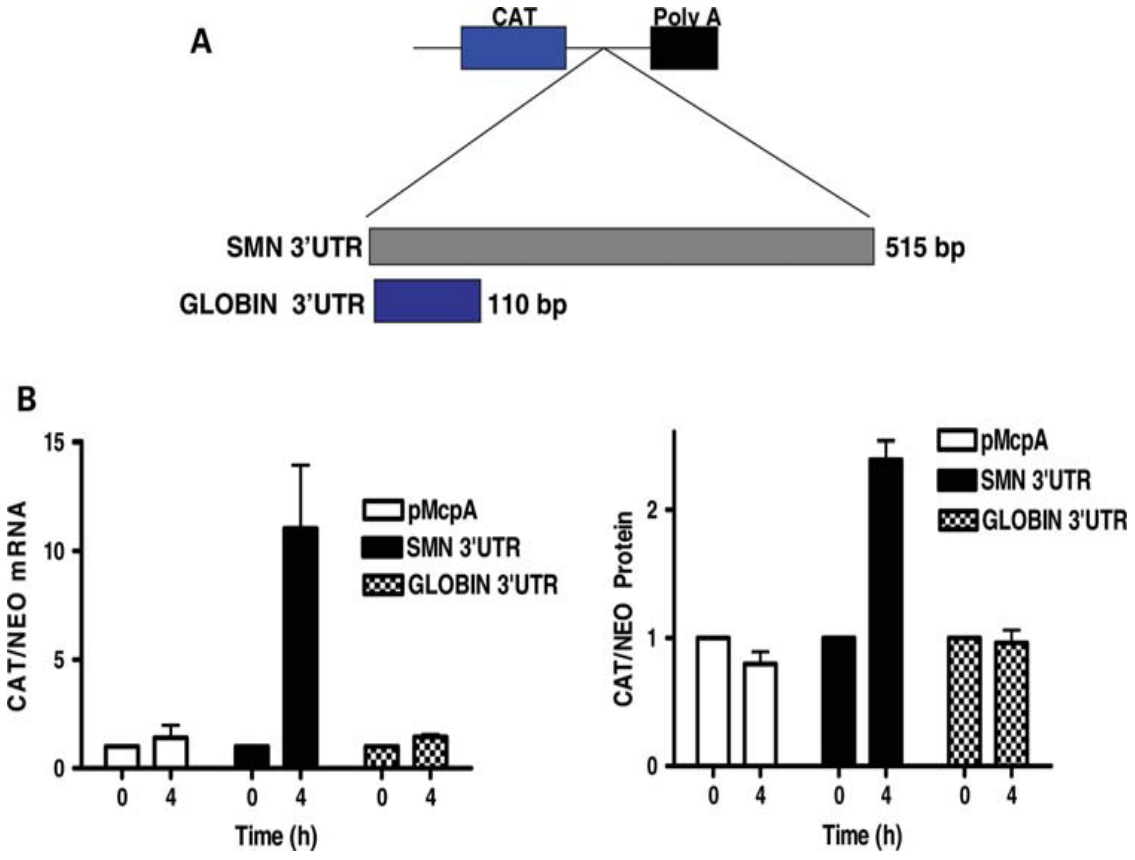
Figure 2.6. HuR is sufficient to induce SMN levels and is required for p38-mediated upregulation of SMN protein. (a) Overexpression of HuR protein increases SMN protein content. MN-1 cells were transfected with either pcDNA3 vector or HuR overexpression plasmid (2 μg/ml). Cells were harvested for Western blot analysis after 24 hours of transfection. Representative Western blot and densitometric quantification (Mean ± SD (bars) of three independent experiments) are shown. (b) The siRNA knockdown of HuR abrogates anisomycin-induced increase in SMN protein. NT2 cells were transfected with HuR siRNA (100nM) or non-targeting control sequence (100nM) for 48h and then treated with anisomycin (75nM) for up to 4 hours. Cells were harvested for Western blot analysis at indicated time points. Representative Western blot and densitometric quantification (Mean ± SD (bars) of three independent experiments; SMN protein relative to β-actin (the ratio at 0 hour was set as 1) is shown (c) The siRNA knockdown of HuR protein attenuates p38+MKK6 overexpression plasmid-induced increase in SMN protein. MN-1 cells were transfected with HuR siRNA (100nM) or non-targeting control sequence (100nM) for 48h followed by transfection with p38+MKK6 overexpression plasmid for 24 hours. Cells were harvested for Western blot analysis. Representative Western blot and densitometric quantification of SMN protein relative to Tubulin (the ratio at 0 hour was set as 1); Mean ± SD (bars) of three independent experiments) are shown.


A
pcDNA3
HuR over
SMN
HuR
Tubulin
SMN protein
2.0
1.5
1.0
0.5
0.0
pcDNA3
HuR over
B
Anisomycin
Anisomycin + HuR siRNA
Hr
0 1 2 3 4
0 1 2 3 4
SMN
HuR
SMN protein
4
3
2
1
0
0 1 2 3 4
0 1 2 3 4
Time (h)
C
S.seq
HuR siRNA
Empty vector
p38 + MKK6
Empty vector
p38 + MKK6
SMN
HuR
S.seq + EV
S. seq+ p38 + MKK6
HuR siRNA + EV
HuR siRNA + p38 + MKK6
SMN protein
2.0
1.5
1.0
0.5
0.0

Role of 3′-UTR of SMN mRNA in anisomycin-induced increase in *SMN* gene expression

The 3′-UTR of an mRNA can play an important role in modulating transcript stability. The p38 pathway stabilizes various ARE containing mRNA species through binding of ARE-BPs at the 3′-UTR of the mRNA. We have shown that HuR is involved in anisomycin-induced increase in SMN; HuR is a RNA binding protein (RBP) which is known to interact with 3′-UTR of its target mRNAs. The fact that SMN transcript also contains ARE element in its 3′-UTR region prompted us to explore the role of 3′-UTR of SMN mRNA in anisomycin-induced increase in SMN transcript. MN-1 cells were transfected with the different chloramphenicol acetyltransferase (CAT) reporter plasmids containing either the full-length 3′-UTR of SMN mRNA (SMN 3′-UTR), empty vector (pMcpA, control) or full-length 3′-UTR of α-globin mRNA (which has no ARE sequence and serves as negative control) with/without anisomycin treatment (Fig. 2.7A). CAT and Neomycin (that is expressed from an independent promoter on the same plasmid and was used as transfection efficiency control) expressions were assessed by RT–PCR and ELISA, respectively. An increase in the CAT expression in MN-1 cells transfected with SMN 3′-UTR CAT reporter plasmid upon treatment with anisomycin was observed at both mRNA and protein level. No increase in CAT expression was seen in MN-1 cells transfected with either pMcpA or α-globin upon treatment with anisomycin. These results demonstrate that the SMN 3′-UTR reporter construct mimics endogenous SMN mRNA behavior and that 3′-UTR of SMN mRNA is required for anisomycin-induced increase in SMN mRNA.

Figure 2.7. The 3' UTR of SMN mRNA is required for anisomycin induced increase in SMN mRNA and protein. MN-1 cells were transfected with CAT reporter plasmids containing either the full length 3'UTR of SMN mRNA (SMN UTR), α-globin mRNA (GLOBIN 3'UTR), or an empty vector (pMcpA) with/without anisomycin treatment (75 nM). CAT and Neomycin (used as transfection control) expression was assessed by RT-PCR and ELISA. Mean ± SD (bars) of three independent experiments performed in triplicate.


A
CAT
Poly A
SMN 3'UTR
515 bp
GLOBIN 3'UTR
110 bp
B
CAT/NEO mRNA
15
10
5
0
pMcpA
SMN 3'UTR
GLOBIN 3'UTR
0 4 0 4 0 4
Time (h)
CAT/NEO Protein
2
1
0
pMcpA
SMN 3'UTR
GLOBIN 3'UTR
0 4 0 4 0 4
Time (h)

Discussion:

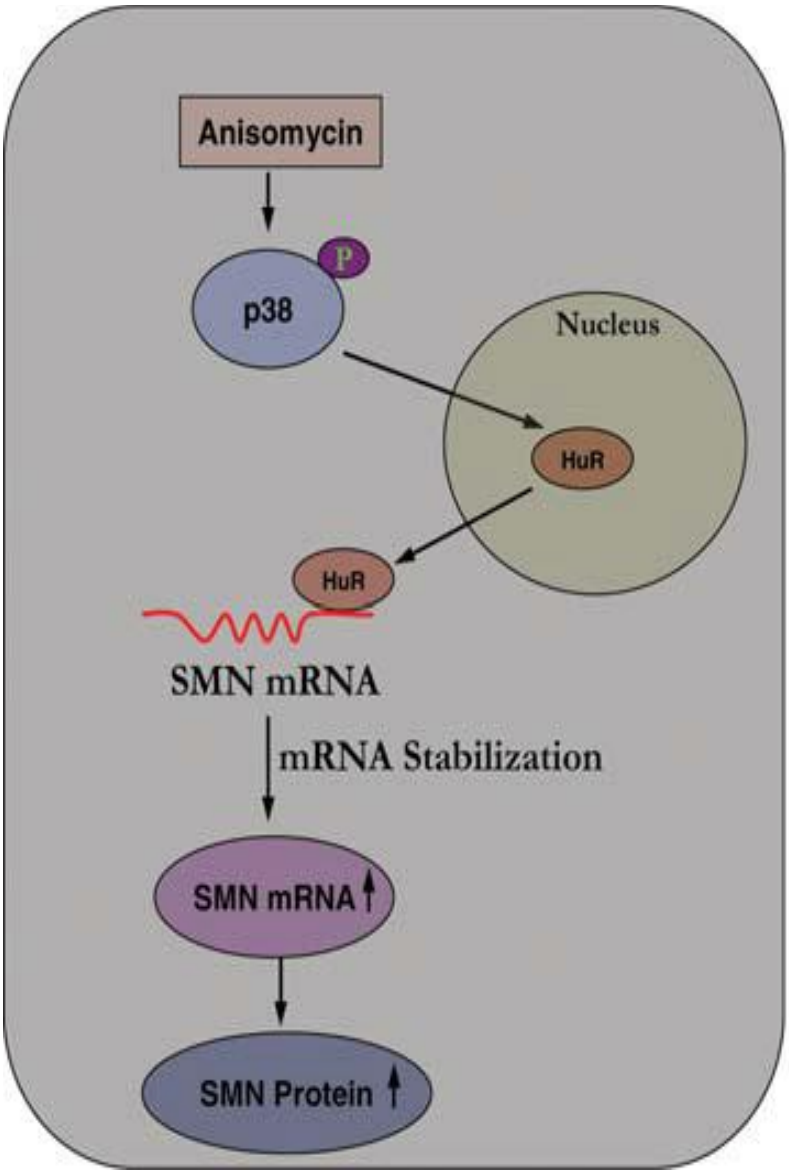
The p38 MAPK pathway which in addition to its role in the regulation of cell responses such as cell cycle arrest and apoptosis also plays an additional important role in the post-transcriptional regulation of gene expression. P38 has been found to regulate the stability of various ARE-containing mRNAs such as COX-2 and TNFα. The antibiotic anisomycin has been shown to activate MAPK (p38, c-JNK) stabilizing some ARE-containing mRNA such as β_2-adrenergenic receptor mRNA (43). The p38 pathway is activated by anisomycin in an RAS-independent manner and has been shown to mediate the activation of several early response genes known to be induced anisomycin. It should be noted that the low concentration of anisomycin used does not appear to inhibit protein synthesis, affect cell growth or cause other toxic effects on cells (44). In this study, we found that when NT2 and MN-1 cells were treated with anisomycin, there was a significant time-dependent increase in the levels of SMN mRNA and protein levels. SMN mRNA has been suggested as an ARE-rich transcript that contains an AUUUA motif in its 3′-UTR. We have demonstrated that an activation of p38 regulates SMN mRNA and protein by relocalizing HuR protein from nucleus to cytoplasm whereafter it binds SMN mRNA 3′-UTR thereby stabilizing the SMN transcript. Previously, Singh *et al*. have shown that C5-substituted quinazolines inhibit DcpS decapping activity by interacting and opening the enzyme in a catalytically incompetent conformation thereby inhibiting SMN2 mRNA decay notionally increasing SMN protein levels. These results along with those presented here suggest that post-transcriptional modulation of SMN mRNA may be a productive means of modulating SMN levels (45) and may play a significant impact on SMN levels.

The ARE motifs in 3′-UTR are well-established targets of ARE-BPs regulating the post-transcriptional expression levels of diverse mRNAs by either promoting or suppressing their stabilization. HuR is well-characterized ARE-BP which is known to bind and stabilize various p38-regulated ARE containing mRNAs (33,39,46,47). Here we report that HuR directly binds to SMN transcript in the 3′-UTR region of the mRNA (Fig. 2.4). Our findings are consistent with the previous reports that anisomycin can cause rapid accumulation of HuR protein in the cytoplasm (40). However, blocking of p38 pathway using specific p38 inhibitor prevents the shuttling of HuR protein from nucleus to cytoplasm after anisomycin treatment, which indicates that activation of p38 pathway is necessary for anisomycin-induced nuclear to cytoplasmic movement of HuR protein in NT2 cells (Fig. 2.5). Overexpression of HuR protein, even in the absence of additional triggers, is sufficient to increase SMN protein level which is consistent with the previous reports that HuR, through transcript stabilization, can increase the levels of various p38 regulated proteins (33,39,46,47). The p38-mediated upregulation of SMN protein levels by anisomycin or p38 overexpression plasmid is abrogated when HuR protein is knocked down using siRNA, indicating that HuR protein is required for p38-mediated induction in SMN protein (Fig. 2.6).

We have shown that the 3′-UTR of SMN mRNA is critical in anisomycin-induced increase in SMN expression. The exact binding motif for HuR in ARE transcripts has not been characterized yet. It has been suggested by Silanes *et al*. that Hu binding sequences comprise short stretches of U-rich nucleotides which also contains A and G nucleotides (48). These sequences are present in 3′-UTR regions of both human *SMN1* and mouse *Smn* mRNA which we have shown to be required for anisomycin-induced increase in SMN induction, an

observation which is consistent with HuR binding to this region of SMN mRNA. The most parsimonious model developing from our data is that anisomycin activates p38 pathway which in turn relocalizes HuR from nucleus to cytoplasm. In the cytoplasm, HuR binds to 3′-UTR of SMN mRNA thus stabilizing SMN transcript resulting in increased protein levels (Fig. 2.8).

Figure 2.8. Proposed Model for anisomycin-mediated induction of SMN. Anisomycin treatment causes phosphorylation and activation of p38 which results in accumulation of HuR in the cytoplasm where it is able to interact with the 3'UTR of SMN transcript. This binding stabilizes SMN mRNA which results in an increase in SMN steady-state mRNA levels and ultimately increases SMN protein expression.



Motor neurons

Interestingly, HuR has been shown to be present in murine motor neurons and to undergo translocation to cytoplasm in the presence of ALS causing SOD1 mutation (49). In SMA, anomalous NMJ structure and function is observed, possibly compounded by a loss of axonal ability to regenerate and ultimately a loss of motor neurons. In this regard, it has been shown that the p38 pathway is required for efficient growth cone regeneration after axon injury (50,51), suggesting that activation of p38 pathway is required for the maintenance of motor neuron function and its integrity. A recent study showed an under-expression of p38 pathway in SMA I muscles, suggesting that p38 may be regulator of protein synthesis in SMA I (52).

If one is to realistically consider p38 activation as an SMN enhancing SMA therapeutic there are a number of issues which need to be addressed. First, the *in vivo* recapitulation of our *in vitro* observations shall be critical; this is underway in our laboratory. There is no clearly defined time that SMN absence causes SMA or conversely when SMN repletion may ameliorate SMA. Extensive analysis of the robust SMA mouse models and electrophysiological analysis of clinical SMA cases appears to point to a dysfunction which antecedes actual denervation and ultimately cell loss that occurs early in the lifespan even in the milder forms of SMA. A picture of a window of motor neuron vulnerability which may span the antenatal period to the early weeks and months in mice and years in human emerges. This coincides with the greatest level of SMN expression in both humans and mice. There is the risk that the SMN induction achieved both in this work and others is to some extent mimicking what already happens physiologically early in development; induction achieved so readily in these systems may not be so readily attained in the disease setting. However, if the high levels of SMN observed developmentally are a result of transcriptional

induction, then the HuR mediated stabilizing a pool of transcripts appears to be a more realizable goal.

The other issue is that of p38 activation itself. Although p38 is involved in a large number of biological phenomena, in the majority of cases its stimulation appears to be driven by cellular stress. Assuming that the motor neuron of a child with SMA has a pool of SMN mRNA that are less than 100% occupied by HuR, achieving an innocuous means of upregulating p38 to activate/translocate more HuR may represent a challenge.

In conclusion, we present here direct mechanism for SMN induction; p38 pathway activation triggering HuR protein shuttling resulting in SMN mRNA stabilization and SMN protein level increase. This may lead to novel treatments for SMA.

Materials and methods:

Reagents

anisomycin was purchased from Alomone laboratories and c-JNK inhibitor SP-600125, p38 inhibitor SB-239580, Actinomycin D and DRB were from Sigma. Non-silencing siRNA control, p38 siRNA and HuR siRNA were supplied by Qiagen, Cell Signaling and Dharmacon, respectively. CAT ELISA kit was from Roche and NEO ELISA kit was supplied by Agdia. The antibodies used in this study are SMN (BD Transduction Laboratories), Actin (Abcam), Tubulin (Abcam), Phospho-p38 (Cell Signaling), p38 (Cell Signaling), Phospho-c-JNK (Cell Signaling) and HuR (Santa Cruz).

Primer sequences

- SMN—Human
  - Forward: 5′-GCTATCATACTGATACTGGCTATTATATGGGTTTTT
  - Reverse: 5′-CTATAACGCTTCACATTCCAGATCTG
- SMN (3′-UTR) Human
  - Forward: 5′-ATGGATCCGGAGAAATGCTGGCATAGAG
  - Reverse: 5′- ATTCTAGAACAGTACAATGAACAGCCATG
- SMN—Mouse
  - Forward: 5′- ATGGATCCGAAGTTCAGCTCTGTCTCA
  - Reverse: 5′-ATTCTAGACTAAGAAAATGACAATTGCAC
- Actin
  - Forward: 5′-CTGGAACGGTGAAGGTGACA
  - Reverse: 5′-AAGGGACTTCCTGTAACAATGCA
- Tubulin—Human
  - Forward: 5′-ATGGCCAGATGCCAAGTGA
  - Reverse: 5′- CACCAGGTTGGTCTGGAAT
- Tubulin—Mouse

- Forward: 5′- ATGGCCAGATGCCAAGTGA
- Reverse: 5′- TACCAGGTTGGTCTGGAATT

- CAT
  - Forward: 5′-GCGTGTTACGGTGAAAACCT
  - Reverse: 5′-GGGCGGAAGAACTTGTCCATA
- NEO
  - Forward: 5′-TGAATGAACTGCAGGACGAG
  - Reverse: 5′-CAATAGCAGCCAGTCCCTTC
  - Caspases-3 (Hs_CASP3_1_SG QuantiTect Primer Assay, Qiagen).

Cell culture and drug treatment conditions

NT2 or MN-1 cells were maintained in standard conditions (37°C in a 5% CO_2humidified atmosphere) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% antibiotics (100 U/ml penicillin–streptomycin) and 2 mM glutamate.

NT2 or MN-1 cells were seeded in 12-well plates (2.5×10^5 cells/well) and treated 24 h later with anisomycin (75 nM) for up to 4 h. For c-JNK and p38 inhibitor treatment, NT2 cells were seeded in 12-well plates (2.5×10^5cells/well) and treated 24 h later with c-JNK inhibitor SP-600125 (10 µM) or p38 inhibitor SB-239580 (3 µM) for 1 h followed by anisomycin (75 nM) treatment for up to 4 h. For transcriptional inhibitor treatment, NT2 cells were seeded in 12-well plates (2.5×10^5 cells/well) and treated 24 h later with Actinomycin D (2.5 µg/ml) or DRB (100 nM) for up to 4 h.

Transfection

MN-1 cells were seeded in 12-well plates (2.5×10^5 cells/well) and transfected on the following day in serum-free DMEM with 2 µg of DNA per well using LipofectAMINE 2000

transfection reagent (Invitrogen, Carlsbad, CA, USA). The transfection mixture was supplemented 3 h later with 1 ml DMEM containing 10% FCS, antibiotics and glutamate. Cells were harvested 24 h after transfection for analysis.

For siRNA transfections, NT2 or MN-1 cells were seeded in 12-well plates (2.0×10^5 cells/well) and transfected on the following day in serum-free DMEM with p38 siRNA(25 nM) or HuR siRNA (100 nM) or non-silencing control siRNA (25 or 100 nM), using LipofectAMINE 2000 transfection reagent for 48 h.

Western blot analysis

Cells were washed two times with 1 ml PBS (1×) and lysed in 75 µl RIPA buffer containing 10 mg/ml each of aprotinin, PMSF and leupeptin (all from Sigma), 5 mM β-Glycerophosphate, 50 mM NaF and 0.2 µM sodium orthovanadate for 30 min at 4°C, followed by centrifugation at 13 000*g* for 15 min and supernatants were collected and kept frozen at −20°C. Protein concentrations were determined by Bradford protein assay using a Bio-Rad protein assay kit (Richmond, CA, USA). For western blot analysis, protein samples were boiled for 5 min and equal amounts of protein extract were separated by 10% SDS–PAGE. Proteins were subsequently transferred onto nitrocellulose membrane and the membrane was incubated in blocking solution (PBS, 5% non-fat milk, 0.2% Tween-20) for 1 h at room temperature followed by overnight incubation with primary antibody at 4°C at the dilution prescribed by the manufacturer. Membranes were washed with PBS-T (PBS and 0.2% Tween-20) two times followed by incubation with secondary antibody (anti-mouse or rabbit, Cell Signaling) for 1 h at room temperature. Antibody complexes were visualized by autoradiography using the ECL Plus and ECL western blotting detection systems (GE

Healthcare). Quantification was performed by scanning the autoradiographs and signal intensities were determined by densitometry analysis using Odyssey v1.1 program.

Quantitative RT–PCR

Total RNA was isolated according to the protocol provided by the manufacturer using the RNAeasy kit (Qiagen). For quantitative RT–PCR, cDNA was reverse transcribed from isolated RNA with oligo dT_{18} primer using First-Strand cDNA Synthesis kit from GE Healthcare following manufacturer's instructions. The synthesized c-DNA template was used for quantitative PCR employing the QuantiTect SYBR Green PCR kit (Qiagen) and analysed on an ABI Prism 7000 sequence detection system using the ABI Prism 7000 SDS Software. Quantitative PCR was carried out to detect SMN, Caspase-3, Actin, CAT and NEO genes using primers listed above.

***In vivo* RNA–protein complex immunoprecipitation**

In vivo RNA–protein complex cross linking and co precipitation was performed as described previously (53). RNA–protein complexes were immunoprecipitated with IgG or HuR antibody at 1:50 dilution after cellular lysis. After cross-linking reversal, RNA was isolated from the immunoprecipitate using a Stratagene kit. cDNA was reverse transcribed from isolated RNA with oligo dT_{18} primer using a First-Strand cDNA Synthesis kit from GE Healthcare according to the protocol provided by the manufacturer. The partial sequence of SMN and Tubulin was PCR amplified with the cDNA using SMN and Tubulin primers. PCR products were run on 1% agarose gel and visualized by ethidium bromide staining.

UV cross-linking of RNA–protein complexes

UV cross-linking of 3′-UTR of SMN mRNA with HuR was performed as previously described (54).

Immunohistochemistry

Cells were washed two times with 1 ml PBS (1×) and fixed with 4% PFA for 10 min. The cells were incubated in blocking solution (PBS, 10% normal goat serum, 0.3% Triton X-100) for 1 h at room temperature followed by overnight incubation with primary antibody at 4°C. Cells were washed three times with PBS (1×) followed by incubation with fluorescence dye conjugated secondary antibody (Alexa Fluor 546, anti-mouse, Invitrogen) for 1 h at room temperature. 4′,6-diamidino-2-phenylindole was used for nuclear staining.

Construct preparation and CAT analysis

The CAT reporter plasmids pMcpA was described previously (55). The 3′-UTR of α-globin and SMN was generated by PCR amplification from cDNA from HeLa and NT2 cells, respectively, and insertion of the 3′-UTR downstream of CAT in a pMcpA construct. Transiently transfected cells were washed with 1 ml PBS (1×) followed by lysis with CAT ELISA kit lysis buffer. CAT ELISA kit was used according to the manufacturer's protocol to determine CAT levels in the cell lysate. NEO levels were determined also using NEO ELISA kit according to the protocol provided by the manufacturer.

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Conflict of Interest:

No conflict of interest are reported

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Chapter 3

Celecoxib increases *SMN* and survival in a severe spinal muscular atrophy mouse model via p38 pathway activation

Preamble

" **Celecoxib increases *SMN* and survival in a severe spinal muscular atrophy mouse model via p38 pathway activation"** is an unpublished manuscript. This articles identifies p38 pathway activating, FDA approved drug celecoxib as potential therapeutic compound for the treatment of SMA.

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Author Contribution: Author contributions: FF designed and executed the experiments, analyzed the data, and wrote the manuscript; DM, JH, FS and SO assisted with the experiments and analysis; MH and AM assisted with analysis and writing.

Abstract:

The loss of functional Survival Motor Neuron (SMN) protein due to mutations or deletion in the *SMN1* gene causes autosomal recessive neurodegenerative spinal muscle atrophy (SMA). A potential treatment strategy for SMA is to upregulate the amount of SMN protein originating from the highly homologous *SMN2* gene, compensating in part for the absence of the functional *SMN1* gene. We have previously shown *in vitro* activation of the p38 pathway stabilizes and increases SMN mRNA levels leading to increased SMN protein levels. In this report we identify FDA approved, blood brain barrier permeating compound celecoxib as an activator of the p38 pathway and assess its impact on SMN levels *in vitro* and in mouse model of SMA. We demonstrate a significant induction of SMN mRNA and protein levels in human and mouse neuronal cells upon treatment with celecoxib. We show that activation of the p38 pathway by celecoxib increases SMN mRNA in an HuR protein dependent manner. Furthermore, celecoxib treatment induces SMN expression in brain and spinal cord samples of wild type mice *in vivo*. Critically, celecoxib treatment increased SMN levels, improved motor function and enhanced survival in a severe SMA mouse model. Our results identify celecoxib as a potential new member of the SMA therapeutic armamentarium.

Introduction:

Childhood spinal muscular atrophy (SMA) is one of the most common genetic causes of infant death globally. The autosomal recessive neurodegenerative disease is caused by the loss of motor neurons from the anterior horn of the spinal cord leading to muscle weakness, muscle atrophy and respiratory insufficiency (1). The estimated heterozygote frequency is 1/50 with an incidence of 1:11,000 in newborns (2,3). SMA is broadly classified into four major categories based on age of onset as well as clinical severity. SMA type I is the most severe and frequent form of the disease accounting for more than half of the known diagnosed cases of SMA; children with Type 1 SMA usually succumb by the age of five.

The loss of the *SMN1* gene due to homozygous deletion or mutations is documented in 95% of the SMA patients (4). All patients harbor a nearly identical twin centromeric copy of the *SMN1* gene, *SMN2* (5,5,6,6). C to T transition at position 6 of SMN2 exon 7 leads to exclusion of this exon in ~90% of the transcripts. However, the centromeric copy gene still produces 5-10% of functional full length SMN transcript (4,7,8). All SMA patients have one or more copies of the *SMN2* gene; in general the higher the *SMN2* copy number, the milder the SMA.

Post-transcriptional regulation of the *SMN* genes are mediated by the presence of AU-rich elements (ARE) in the 3'untranslated region (UTR) which act as a signal for mRNA degradation (9). The p38 MAPK pathway is known to play an important role in post-transcriptional regulation observed in ARE containing mRNAs, regulating the abundance and/or activity of RNA binding proteins that control mRNA stability (10,11,12,13,14). The RNA binding protein HuR (a ubiquitously expressed member of the

ELAV family of proteins) is a known ARE binding protein (AREBP) which binds to ARE sequences and, in some cases, antagonizes the degradation of these mRNA by stabilizing them (15,16,17,18). Activation of the p38 pathway has been shown to lead to a cytoplasmic accumulation of HuR protein with its increased binding to target mRNAs, resulting in there stabilization (9,19). Our group has previously shown that the p38 MAPK activation induces SMN expression in this fashion triggering HuR mediated stabilization of SMN mRNA and increases the pool of transcripts available for translation thus increasing functional SMN protein levels (9).

In our previous work, the known p38 MAPK activator anisomycin was used; however this drug cannot cross the blood brain barrier (BBB) thus limiting its ability to act *in vivo* on the central nervous system in particular the motor neurons, where the lack of SMN contributes to SMA pathophysiology. A literature search for an alternative to anisomycin identified the FDA approved COX-2 (cycloxygenase 2) selective inhibitor non steroidal anti-inflammatory drug celecoxib as a known p38 activator. Its capacity to cross the BBB made celecoxib a good candidate for further exploration as an SMA therapeutic (20,21,22).

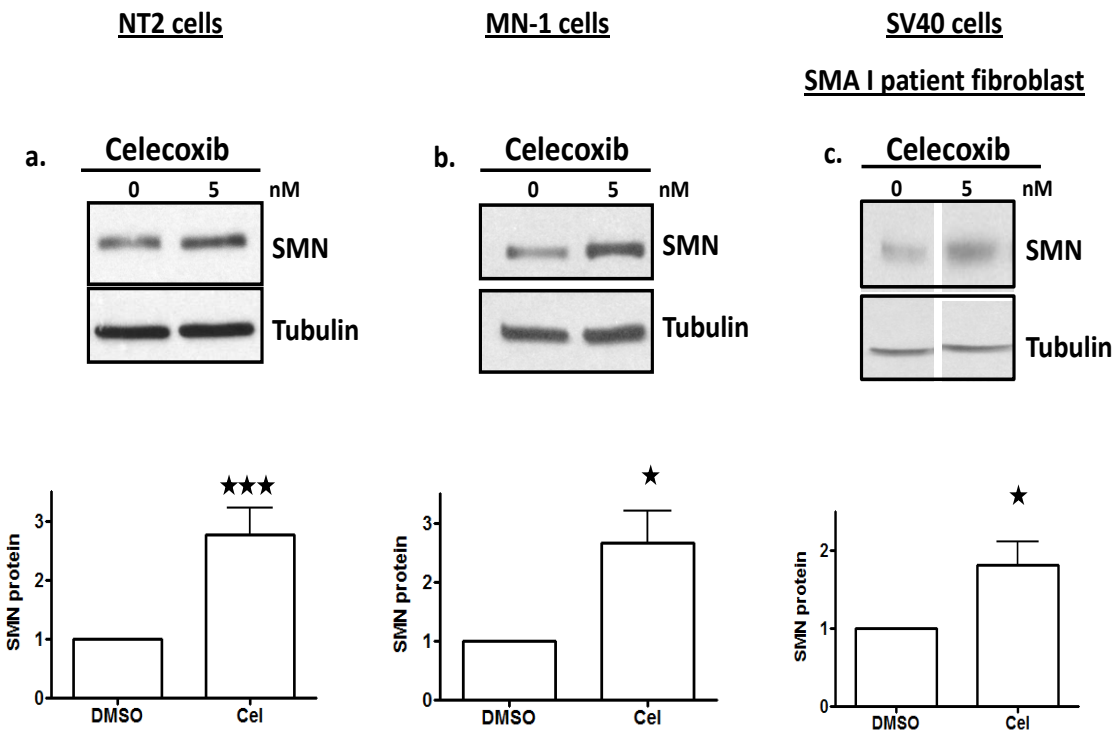
We show here a celecoxib conferred increase in SMN protein in both neuronal cells and wild type (WT) mice. We present evidence that celecoxib activates the p38 pathway leading to increase SMN mRNA and a subsequent increase in SMN protein. Importantly, we show that treatment with celecoxib increases SMN levels, improves motor function and survival in a severe SMA mouse model. Our results confirm earlier work proposing p38 MAPK pathway activators as potential therapeutic compounds for the treatment of SMA and identifies celecoxib as one such promising agent.

Results:

Celecoxib treatment upregulates SMN protein *in vitro*

To investigate a potential role for p38-activating, FDA approved drug celecoxib in the regulation of the *SMN* gene expression *in vitro*, the human neuron-committed teratocarcinoma (NT2), mouse motor neuron derived (MN-1) cells and SMA I patient fibroblasts were treated with celecoxib (5nM) for 24 h and subsequently harvested for Western blot analysis. SMN protein levels were found to be increased in all cell lines upon treatment with celecoxib (Fig. 3.1 a-c). Taken together these results demonstrate that low dose of celecoxib leads to an increase in SMN protein levels in both human and mouse neuronal cell lines as well as in SMA patient fibroblasts.

Figure 3.1. Celecoxib treatment upregulates SMN protein *in vitro*. NT2, MN-1 and SMA I patient fibroblasts were treated with celecoxib (5 nM) and then harvested at 24 hours for Western blot analyses. (a) Representative Western blots showing the effect of celecoxib on SMN protein in NT2 cells. Densitometric quantification of SMN relative to Tubulin [mean + SEM (bars) of three independent experiments] are shown for NT2 cells. (b) Representative Western blot showing effect of celecoxib on SMN protein in MN-1 cells. Densitometric quantification of Smn relative to Tubulin [mean + SEM (bars) of three independent experiments] are shown for MN-1 cells. (c) Representative Western blots showing the effect of celecoxib on SMN protein in SMA I patient fibroblasts (all lanes were run on the same gel but were non-contiguous). Densitometric quantification of SMN relative to Tubulin [mean + SEM (bars) of three independent experiments] are shown for SMA I patient fibroblasts. *P< 0.05; ***P< 0.001, log-rank test.


NT2 cells
MN-1 cells
SV40 cells
SMA I patient fibroblast
a.
Celecoxib
0
5
nM
SMN
Tubulin
b.
Celecoxib
0
5
nM
SMN
Tubulin
c.
Celecoxib
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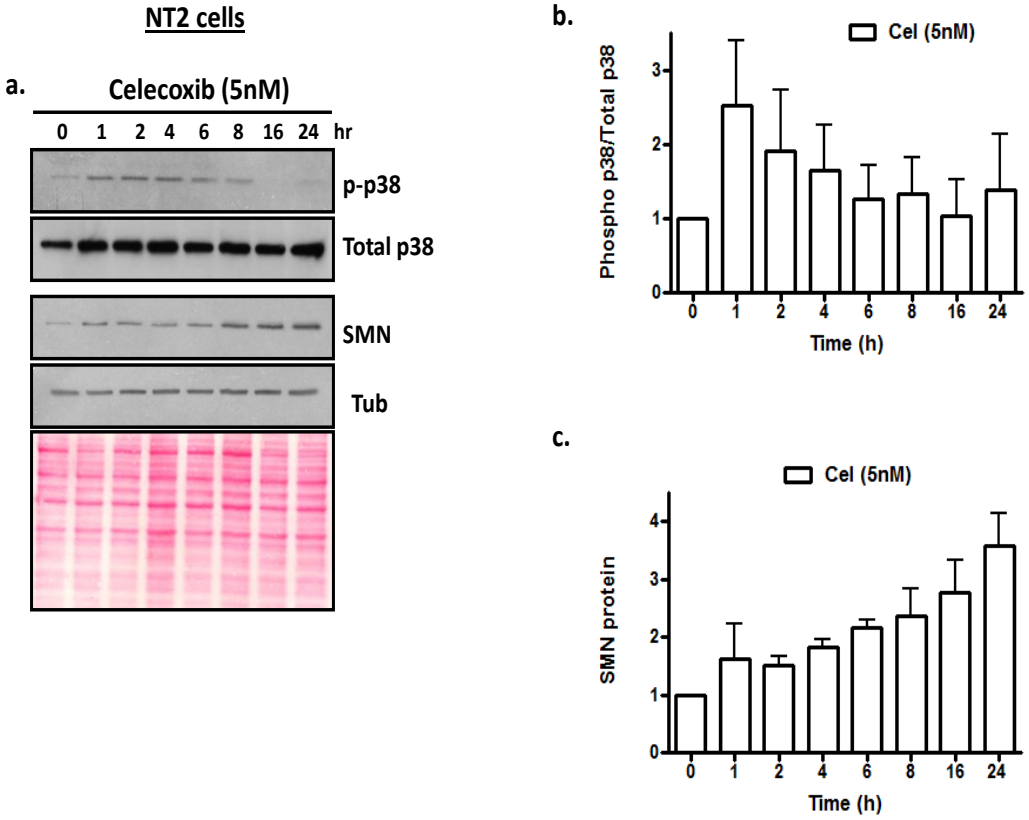
Celecoxib activates the p38 MAPK and increases SMN protein levels in a time dependent manner *in vitro*

Since the p38 pathway has been implicated in the regulation of the *SMN* gene, and given that celecoxib is a known activator of this pathway, we wished to confirm that at concentrations we have shown induce SMN, celecoxib activates the p38 MAPK pathway. NT2 cells were thus treated with celecoxib at 5 nM and then harvested for Western blot analysis at the indicated time intervals revealing a time-dependent increase in the ratio of phosphorylated-p38/ total p38 protein (up to 8 hr after celecoxib treatment). This confirms that celecoxib activates the p38 MAPK pathway in NT2 cells (Fig 3.2 a-b). We also found that activation of the p38 MAPK pathway by celecoxib treatment was followed by a time dependent increase in SMN protein level in NT2 cells (Fig 3.2 a & c).

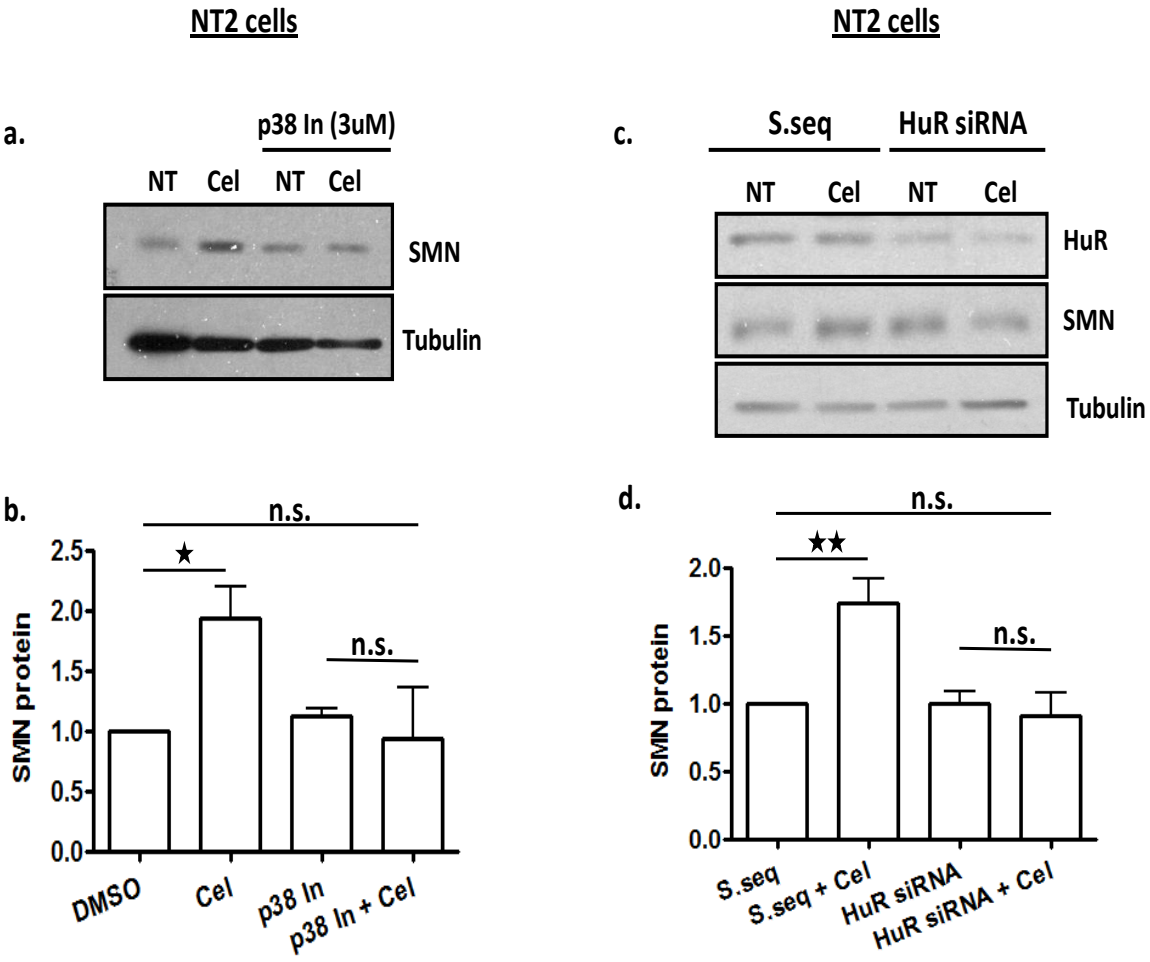
Figure 3.2. Celecoxib treatment activates p38 MAPK pathway. (a) Representative Western blot showing activation of p38 MAPK pathway upon celecoxib (5nM) treatment in NT2 cells. NT2 cells were treated with celecoxib at indicated times and then harvested for Western blot analysis. Activation of p38 pathway by celecoxib is followed by increase in SMN protein. (b) Densitometric quantification of phospho-p38 relative to total-p38 [mean + SEM (bars) of three independent experiments] are shown for NT2 cells. (c) Densitometric quantification of SMN relative to Tubulin [mean + SEM (bars) of three independent experiments] are shown for NT2 cells.


NT2 cells
a.
Celecoxib (5nM)
0 1 2 4 6 8 16 24 hr
p-p38
Total p38
SMN
Tub
b.
Cel (5nM)
Phospho p38/Total p38
0 1 2 3
0 1 2 4 6 8 16 24
Time (h)
c.
Cel (5nM)
SMN protein
0 1 2 3 4
0 1 2 4 6 8 16 24
Time (h)

Activation of p38 pathway and HuR protein are required for celecoxib-conferred SMN protein increase

To investigate a potential role for p38 in *SMN* gene regulation by celecoxib, NT2 cells were pre-treated with the p38 inhibiting agent SB-239063 for 2 h followed by treatment with celecoxib for 24 h. Western blot analysis revealed that p38 inhibition effectively blocked the celecoxib-mediated increase in SMN protein (Fig 3.3 a & b). We have shown previously that the p38 pathway mediates increase in SMN levels through HuR protein. To further elaborate the role of HuR protein in celecoxib-induced increase in SMN protein, NT2 cells were transfected with HuR-specific siRNA or control siRNA for 48 h, and then treated with celecoxib for 24 h. siRNA-mediated attenuation of HuR expression blocked the celecoxib-mediated increase in SMN protein expression (Fig 3.3 c & d). These observations strongly implicate the p38 pathway and HuR protein in the celecoxib-induced increase of SMN levels.

Figure 3.3. Celecoxib increases SMN expression via p38 MAPK pathway. (a) Representative western blots showing the effect of p38 inhibition on celecoxib-induced increase in SMN protein. p38 inhibitor (SB-239580) blocked the celecoxib-induced increase in SMN protein in NT2 cells. NT2 cells were treated with SB-239580 (p38 In; 3 μM) for 2 h followed by treatment with celecoxib (Cel; 5 nM) for 24 h and than harvested for Western blot analysis. (b) Quantification of SMN protein relative to tubulin (the ratio at control treatment was set as 1). Mean ± SEM (bars) of three independent experiments. (c) Representative Western blots showing both HuR knockdown and its effect on celecoxib-induced increase in SMN protein. The siRNA knockdown of HuR protein attenuates celecoxib-induced increase in SMN protein. NT2 cells were transfected with HuR siRNA (100 nM) or scrambled sequence (S.seq; 100 nM) for 48 h and then treated with celecoxib (5 nM) for 24 h. Cells were harvested for Western blot analysis. (d) Quantification of SMN protein relative to Tubulin (the ratio at control treatment was set as 1). Mean ± SEM (bars) of three independent experiments. *P< 0.05; **P< 0.01, log-rank test.

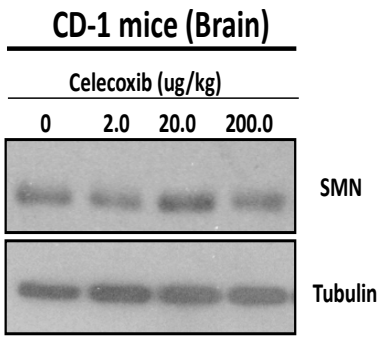

NT2 cells
NT2 cells
a.
p38 In (3uM)
NT
Cel
NT
Cel
SMN
Tubulin
c.
S.seq
HuR siRNA
NT
Cel
NT
Cel
HuR
SMN
Tubulin
b.
n.s.
★
n.s.
SMN protein
2.5
2.0
1.5
1.0
0.5
0.0
DMSO
Cel
p38 In
p38 In + Cel
d.
n.s.
★★
n.s.
SMN protein
2.0
1.5
1.0
0.5
0.0
S.seq
S.seq + Cel
HuR siRNA
HuR siRNA + Cel

Celecoxib treatment upregulates SMN protein levels in wild type mice

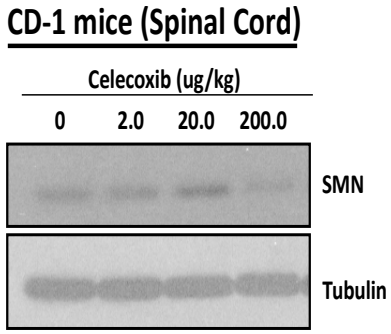
In order to both confirm that celecoxib-mediated SMN induction extends to the *in vivo* setting, and to begin to explore celecoxib treatment in animals, CD-1 mice were given daily intraperitoneal (IP) celecoxib injections for 5 days over a range of doses. Brain and spinal cord samples were subsequently isolated for Western blot analysis. Celecoxib treatment (20 μg/kg) increased SMN protein levels in brain (Fig 3.4 a & b) and spinal cord samples (Fig 3.4 c & d) in CD-1 mice.

Figure 3.4. Celecoxib upregulates Smn protein in wildtype mice. 4 weeks old CD-1 wild type mice were treated daily with celecoxib (2.0, 20.0, 200.0 µg/kg) for 5 days, then sacrificed. Brain and spinal cord tissues were harvested for Western blot analysis. (a) Representative Western blot showing the effect of celecoxib on Smn protein in brain samples of CD-1 mice treated with saline (control, lane-1) or celecoxib (lane 2, 3 & 4 respectively) (n=3). (b) Densitometric quantification of SMN relative to Tubulin [mean + SEM (bars)] is shown for brain samples. (c) Representative Western blot showing the effect of celecoxib on SMN protein in spinal cord samples of CD-1 mice treated with Saline (control, lane-1) or celecoxib (lane 2, 3 & 4) (n=3). (d) Densitometric quantification of SMN relative to Tubulin [mean + SEM (bars)] is shown for spinal cord samples. *P< 0.05, log-rank test.

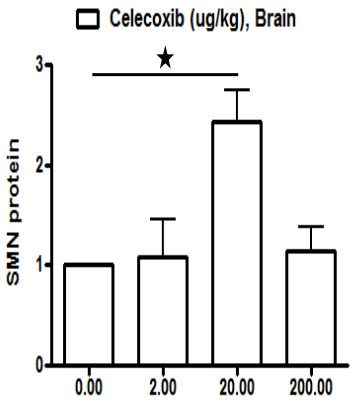
a.


CD-1 mice (Brain)
Celecoxib (ug/kg)
0
2.0
20.0
200.0
SMN
Tubulin

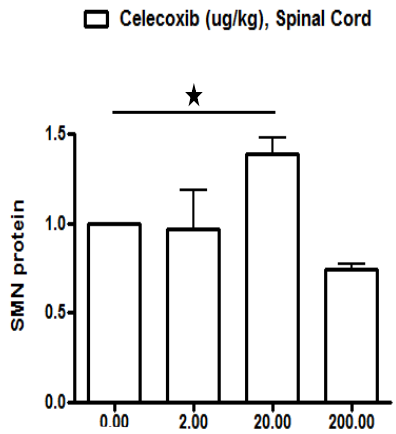
c.


CD-1 mice (Spinal Cord)
Celecoxib (ug/kg)
0
2.0
20.0
200.0
SMN
Tubulin

b.


Celecoxib (ug/kg), Brain
SMN protein
0
1
2
3
0.00
2.00
20.00
200.00

d.

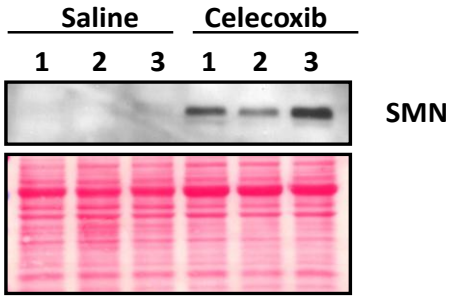

Celecoxib (ug/kg), Spinal Cord
SMN protein
0.0
0.5
1.0
1.5
0.00
2.00
20.00
200.00

Celecoxib treatment upregulates SMN protein levels in a SMA mice model

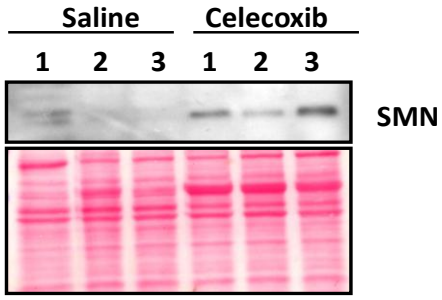
In order to explore the *in vivo* impact of celecoxib-induced SMN upregulation, commonly used SMNΔ7 SMA mice (*mSmn*-/-;*hSMN2*+/+, *hSMNΔ7*+/+(23)) were given 20 μg/kg celecoxib IP injections every day from P1 until P6. Mice were euthanized 24 hours after their last treatment. Brain, spinal cord, muscle and heart samples were then harvested for Western blot analysis. Importantly, celecoxib treatment was observed to increase *SMN2*-derived full length SMN protein levels significantly in both brain and spinal cord samples when compared with vehicle treated animals (Fig 3.5 a & b). No induction was observed in skeletal muscles and a lesser degree of induction in SMN protein was observed in the hearts of SMA mice following celecoxib treatment when compared with vehicle treated mice (Fig 3.5 c & d).

Figure 3.5. Celecoxib upregulates SMN protein in SMA mouse model. SMAΔ7 mice were treated daily with saline or celecoxib (20 μg/kg) from P1 for 6 days, then sacrificed at P7. Brain, spinal cord, heart & muscle tissues were harvested for Western blot analysis. Representative Western blots showing effect of celecoxib on SMN protein in brain, spinal cord, heart & muscle samples (a, b, c & d respectively) of SMAΔ7 mice treated with Saline (control, lane 1,2 & 3) or celecoxib (lane 4, 5 & 6 respectively) (each lane represents individual animal).

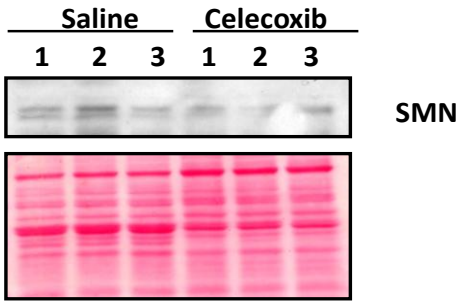
a. SMA mice (P7) - Brain



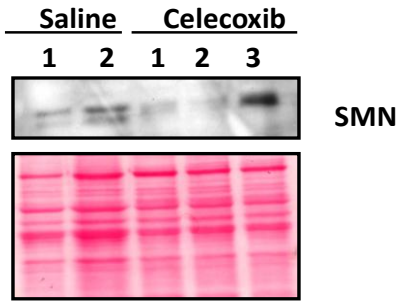
b. SMA mice (P7) – Spinal Cord



c. SMA mice (P7) - Muscle



d. SMA mice (P7) - Heart



Celecoxib treatment improves disease phenotype in SMA mice model

We next examined the impact of celecoxib-induced increase in SMN levels on disease phenotype in SMAΔ7 mice. The SMAΔ7 mice have severely impaired righting time and muscle weakness detectable by P5. They are also significantly underweight compared to heterozygous and WT littermates with a median survival of 13 days. SMAΔ7 mice were given daily celecoxib or vehicle IP injections starting at P1and their weight, motor function and longevity were assessed daily. SMAΔ7 mice treated with celecoxib showed no improvement in weight loss as compared with vehicle-treated SMAΔ7 mice (Fig 3.6). However, SMAΔ7 mice treated with celecoxib showed a dramatic improvement of motor function (as assessed by righting time), and significant extension of survival (median survival of 18 days) as compared with vehicle-treated SMAΔ7 mice (median survival of 13 days, Fig 3.7 a & b).

Figure 3.6: Celecoxib treatment does not attenuate weight loss in SMA mouse model. SMAΔ7 mice were treated daily with intraperitoneal injections of celecoxib (20.0 μg/kg) from P1 onward. (a) Weights of SMAΔ7 mice treated with celecoxib (black filled square) or saline (black filled circle) (n =5); weights for heterozygous mice treated with saline (black filled triangle) are also shown for comparison [mean ± SEM (bars)].

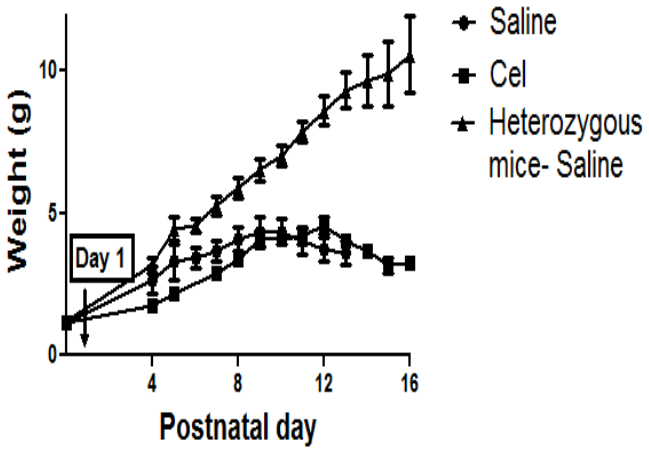
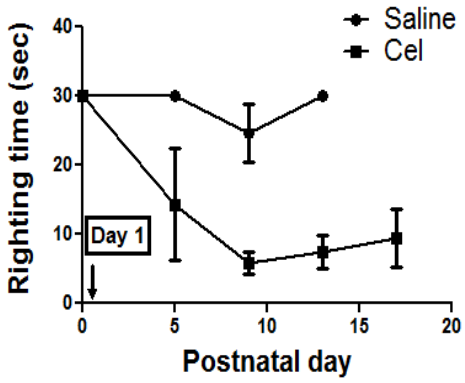
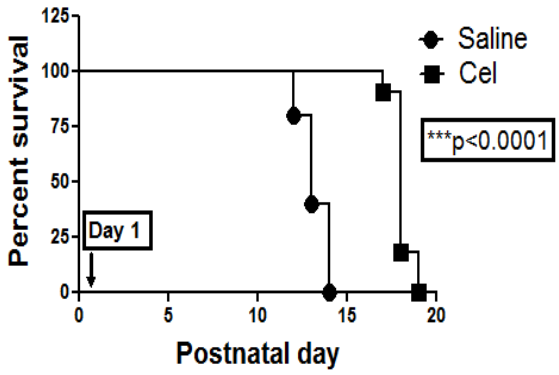

Saline
Cel
Heterozygous mice- Saline
Weight (g)
10
5
0
Day 1
4
8
12
16
Postnatal day

Figure 3.7: Celecoxib ameliorates disease phenotype and increases survival of SMA mouse model. SMAΔ7 mice were treated daily with intraperitoneal injections of celecoxib (20.0 μg/kg) from P1 onward. (a) Righting times of SMAΔ7 mice treated with celecoxib (black filled square) or saline (black filled circle) (n =5) [mean ± SEM (bars)]. (b) Kaplan-Meier survival curves of SMAΔ7 mice treated with celecoxib (black filled square) or vehicle (black filled circle) (n =10); $P < 0.0001$, log-rank test.

a.


Saline
Cel
Righting time (sec)
40
30
20
10
0
Day 1
0
5
10
15
20
Postnatal day

b.


Saline
Cel
***p<0.0001
Percent survival
125
100
75
50
25
0
Day 1
0
5
10
15
20
Postnatal day

Discussion:

SMA is an incurable neurodegenerative disease which mostly affects children, many of whom die before the age of two. Increasing SMN levels through the *SMN2* gene can partially rescue the disease phenotype (24). One strategy for SMA therapy is to increase levels of SMN protein from *SMN2* gene through stabilization of the full length SMN transcript which ultimately leads to an increase in SMN mRNA and protein levels (9). The p38 pathway regulates a number of cell processes including post transcriptional regulation of a certain class of transcripts with an ARE sequence in their 3'UTR (10,11,12,13,14). We have previously documented that anisomycin, a known p38 activator, increases SMN levels through mRNA stabilization *in vitro* (9) . However a number of issues need to be addressed in order to recapitulate our *in vitro* results with anisomycin in a SMA mouse model. The transient increase in SMN levels observed with anisomycin treatment, along with its inability to cross BBB limits its use as a therapeutic for SMA, a motor neuron disease (25). An alternative to anisomycin is p38 activating, FDA approved COX-2 inhibitor drug celecoxib which can cross the BBB thus making it an ideal candidate to test as a therapeutic for the treatment of SMA. In this study we have demonstrated that celecoxib induces SMN expression *in vitro* in a p38 and HuR protein dependent fashion.

We document here a celecoxib mediated increase in SMN protein levels in human neuronal NT2 cell line and murine motor neuron MN-1 cells as well as in SMA I patient fibroblasts. It was shown in previous studies that low dose celecoxib activates the p38 pathway (20,21,22) although not higher doses (22,26,27,28). Here we treated neuronal cells with a low concentration (nanomolar range) of celecoxib observing a rapid increase in phospho p38 levels followed by an increase in SMN protein levels. The p38 pathway

regulates the stability of COX-2, TNFα, p21 and SMN transcripts which are known to contain ARE in their 3'UTR region (9,10,12,29). To confirm the role of p38 activation in celecoxib-mediated increase in SMN protein, cells were pre-treated with a p38 inhibitor before celecoxib treatment. This resulted in an attenuation of SMN induction consistent with a role for celecoxib based activation of p38.

We have shown in an earlier report that *in vitro* p38 activation-conferred SMN induction is through transcript stabilization *via* binding of AREBP HuR to the 3'UTR region of SMN transcripts (9). A second report showed a similar mechanism for the regulation of p21 mRNA by the p38 pathway and HuR protein (29). To explore whether a similar role exists for HuR protein in the celecoxib mediated increase of SMN, NT2 cells were pre-treated with HuR siRNA followed by celecoxib treatment. Celecoxib mediated SMN induction was blocked when cells were pretreated HuR siRNA suggesting that HuR is required in celecoxib mediated increase in SMN protein.

Treatment of WT mice with different doses of celecoxib revealed a induction of SMN protein in the brain and spinal cord samples at 20 μg/kg celecoxib dose compared to saline treated control mice. We have previously documented a difference in the responsiveness of mouse Smn (low) and human SMN2 (high) to STAT5 kinase activation (30) and thus wondered if the same distinction might exist in the case of p38 activation. To further explore this possible effect, SMA Δ7 mice were treated with celecoxib for 5 days postnatally. Celecoxib treatment resulted in a significant and sustained increase in SMN protein levels in CNS tissues (brain & spinal cord) and, to a lesser degree or no increase, in muscle tissues (heart & skeletal muscle) compared to saline treated SMA mice. It has been reported that the p38 pathway is not activated in SMA I muscles (31).

Some studies have shown a direct correlation between SMN levels in CNS and ultimate motor neuron survival. Gavrilina *et al.* have shown that a small increase of SMN in neurons correlates with an increased survival of SMA mice whereas a high SMN level in mature skeletal muscle alone has no impact (25). Also, recent CNS-targeted gene therapy studies showed that early induction of neuronal SMN is required to improve survival and motor function in a mouse model of SMA (32,33,34,35). We have documented here that celecoxib treatment increases SMN levels in SMA mice which results into an attenuation in disease phenotype.

There are a number of factors which we need to take into account when using SMA mice as a disease model, particularly when comparing results between laboratories (e.g. health of mice at birth, competition within cage between littermates, housing conditions). Therefore, to account for this variability, we used the ratio of median survival of treated to non treated animals to assess drug response on survival. With celecoxib we have achieved a ratio of 18d/13d or 1.38, a number that compares favourably with the 1.2 (19d/16d) observed with TSA (albeit P1 celecoxib initiation versus P5 TSA initiation) and 1.3 (12.9d/9.9d) seen with SAHA (36,36,37,37).

In addition to HDAC inhibitors, antisense oligonucleotides (ASOs), have shown promise for the treatment of SMA by preventing alternative splicing of the SMN2 transcript and ultimately resulting in more full length SMN transcript (38,39). A major hurdle with these compounds is their failure to cross the BBB. However, a recent study shows a marked improvement in motor function along with increase in survival in SMA mice with systemic delivery of ASO which results into increase in SMN levels largely in peripheral tissues (39); whether this observation extends beyond mice is at present unclear. The other encouraging

treatment of SMA is gene therapy using sc-AAV9-SMN resulting in a marked increase in longevity of SMA mice along with a significant improvement in motor function (32). Similar results were seen by other groups who used similar a gene therapy (33,34,35,39). However, there are a number of issues which need to be addressed before clinical introduction of these modes of treatment for SMA (clinical safety, species barrier, quantity of virus and ASO, cost, immune response etc) (40,40,41,41).

Off-target drug effects, either harmful or useful, are commonly observed occurrences. Even non-synthetic compounds known to have certain functions in some tissues often have unanticipated roles elsewhere in the body. In the current study, celecoxib is used as an alternative to the p38 activating compound anisomycin. Celecoxib was the first of the class of selective COX-2 inhibitors to be FDA approved for the treatment of rheumatoid arthritis and osteoarthritis. Although celecoxib causes a lower incidence of gastrointestinal ulceration and other complications than other non-steroidal anti-inflammatory drugs (42,43), the effects are nonetheless real. A recent study demonstrated possible damage of hepatic and renal tissue when rats were treated with 10 and 50 mg/kg celecoxib doses (44). However, the doses of celecoxib (20 μg/kg) which we have used for treatment in this study are three orders of magnitude lower than doses currently used for osteoarthritis (1-2 mgs/kg) in humans, and much lower than levels shown by toxicology reports to be harmful (42) making it likely that this would be a safe intervention for the vulnerable demographic comprised by infants and children diagnosed with SMA.

Our results demonstrate clear amelioration of the SMA disease phenotype in mouse model using BBB penetrant, FDA approved celecoxib raising the prospect of its use in clinical trial studies. As many recent studies have shown that early timing of *SMN* gene

therapy is critical for maximum benefit in SMA mouse model, diagnosing newborn pre-symptomatic SMA infants and their early treatment will be critical. It may even be beneficial for SMA type II and III patients as a recent study showed that increasing SMN levels even post- symptomatically ameliorates SMA disease phenotype (45). It may be of value to combine the effect of celecoxib with SMN2 transcriptional activator prolactin (30) and/or neuroprotective compounds such as Y-27632 and fasudil (Rho kinase inhibitors) (46,47).

Presently there is no cure of SMA. This study provides a novel mechanistic insight of how SMN protein is regulated through celecoxib via p38 MAPK pathway and its effect on the phenotype of the disease as well as its potential for future therapeutic use for the treatment of SMA.

Materials and methods:

Animals:

All protocols were approved by Animal Care and Veterinary Services (ACVS) and Ethics board of University of Ottawa. All experiments were carried out in accordance with the Canadian Institute of Health Research (CIHR) Guidebook and ACVS legislation. CD-1 mice were obtained from Charles River Laboratory. The original breeding pair of heterozygous SMAΔ7 mice (*mSmn*+/-;*hSMN2*+/+, *hSMNΔ7*+/+) on the FVB background were provided by the Jackson Laboratory. The animals were maintained in an air-conditioned ventilated animal facility. Survival, righting time and weight were monitored daily as described by Aviva *et al* (37).

Celecoxib administration

Celecoxib was diluted in DMSO and administered through IP injection using a 30-gauge needle. Control animals received equal volumes of vehicle alone. SMAΔ7 mice were genotyped at P0 and celecoxib treatment was started from P1. Animals were sacrificed within twenty four hours of final celecoxib dose.

Reagents

Celecoxib was purchased from Toronto Research Chemicals. p38 inhibitor SB239063 was purchased from Sigma. Non-silencing siRNA control and HuR siRNA were purchased from Qiagen and Dharmacon respectively. The antibodies used in this study were SMN/Smn (BD Transduction Laboratories), Actin (Abcam), Tubulin (Abcam), Phospho-p38 (Cell signalling) and Total p38 (Cell signalling).

Primer sequences

For genotyping

Genotyping was performed as previously described by Aviva *et al* (37,37) using the following primers

mSmn WT

Forward: 5'-TCTGTGTTCGTGCGTGGTGACTTT-3'

Reverse 1877: 5'-CCCACCACCTAAGAAAGCCTCAAT-3'

Lac Z

Forward: 5'-CCAACTTAATCGCCTTGCAGCACA-3'

Reverse: 5'-AAGCGAGTGGCAACATGGAAATCG 3'

Human *SMN2 trans*gene

Forward: 5'-CAAACACCTGGTATGGTCAGTC-3'

Reverse: 5'-GCACCACTGCACAACAGCCTG-3'

Product sizes:

mSMN: 372 bp

Lac Z: 626 bp

SMN2 transgene: 250 bp

Cell Culture and Drug Treatment conditions

Human neuron-committed teratocarcinoma (NT2) or mouse motor neuron derived (MN-1) cells were maintained in standard conditions (37^{o}C in a 5% CO_2 humidified atmosphere) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% antibiotics (100 units/ml penicillin-streptomycin) and 2mM glutamate.

NT2 or MN-1 cells were seeded in 12 well plates (2.5 x 10^5cells/well) and treated 24h later with celecoxib (5, 50, and 500 nM) for 24h. For time course experiment, NT2 cells were seeded in 12 well plates (2.5 x 10^5cells/well) and treated 24h later with celecoxib (5 nM) for up to 24h. For p38 inhibitor treatment, NT2 were seeded in 12 well plates (2.5 x 10^5cells/well) and pre-treated with p38 inhibitor SB239063 for 2h followed by celecoxib treatment (5 nM) for 24h.

Transfection

For siRNA transfections, NT2 cells were seeded in 12 well plates (2.0 x 10^5cells/well) and transfected on the following day in serum-free DMEM with HuR siRNA(1000 nM) or non-silencing control siRNA (1000 nM), using LipofectAMINE 2000 transfection reagent for 48h.

Western Blot Analysis

Cells were washed 2 times with 1 ml PBS (1X) and lysed in 75µl RIPA buffer containing 10 mg/ml each of aprotinin, PMSF and leupeptin (all from Sigma), 5 mM β-Glycerolphosphate, 50 mM NaF and 0.2 µM sodium orthovanadate for 30 min at 4°C, followed by centrifugation at 13 000 x g for 15 min; supernatants were then collected and kept frozen at -20°C. Tissue samples were homogenized in 1 ml RIPA (10 mg/ml each of aprotinin, PMSF and leupeptin) and then sonicated for 15 seconds. Total protein concentrations were determined by

Bradford protein assay using a Bio-Rad protein assay kit. For Western blot analysis, protein samples were separated by 10% SDS-PAGE. Proteins were subsequently transferred onto nitrocellulose membrane and incubated in blocking solution (PBS, 5% non-fat milk, 0.2% Tween-20) for 1 h at room temperature followed by overnight incubation with primary antibody at 4°C at the dilution prescribed by the manufacturer. Membranes were washed with PBS-T (PBS, and 0.2% Tween-20) 3 times followed by incubation with secondary antibody (anti-mouse or rabbit, Cell signalling) for 1 h at room temperature. Antibody complexes were visualized by autoradiography using the ECL Plus and ECL Western blotting detection systems (GE Healthcare). Quantification was performed by scanning the autoradiographs and signal intensities were determined by densitometric analysis using the Odyssey v1.1 program.

Statistical methods

GraphPad Prism software package (version 5.04 for Windows, GraphPad Software, San Diego, California, USA, www.graphpad.com) was used for the Kaplan–Meier survival analysis. The log-rank test was used and survival curves were considered significantly different at $P < 0.0001$.

Data in figures (histograms, points on graphs) are mean values with the standard error mean (SEM) shown as error bars. The Student's two-tail *t* test for paired variables was used to test for statistical differences between samples. The log-rank test was used and were considered significantly different at $P<0.05$

Acknowledgments: We thank all those at the Animal Care and Veterinary Service at the University of Ottawa who have been so helpful. M. Holcik is a CHEO Volunteer Association Endowed Scholar. This work was supported by operating grants from the National Science and Engineering Research Council (NSERC) (to M. Holcik) and from Tori's Buddies, CML Healthcare, FightSMA, the SMA Foundation, the Canadian Gene Cure Foundation, Physicians Services Incorporated, Ilsa Mae SMA Research Fund and the Canadian Institutes of Health Research (to A. MacKenzie).

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Chapter 4

Prolactin increases *SMN* expression and survival in a severe SMA mouse model via STAT5 pathway

Preamble:

" **Prolactin increases *SMN* expression and survival in a severe SMA mouse model via STAT5 pathway"** was first published as a research article in the Journal of Clinical Investigation (volume 121, number 8, August 2011). This articles provides a good mechanistic insight of how SMN protein is regulated through PRL via STAT5 pathway and its effect on the phenotype of the disease as well its potential for future therapeutic use for the treatment of SMA.

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Author Contribution: F.F. wrote the article. F.F, M.H and A.M. designed experiments. F.F. and F.A.M. performed experiments presented in Figures 4.5 and Supplementary Figures 4.5. F.F. and J.H. performed experiments presented in Figures 4.3. F.F and D.M. performed experiments presented in Figures 4.5. F.F. performed experiments presented in Figures 4.1, 4.2, 4.4, 4.7, 4.8 and Supplementary Figures 4.1, 4.2 and 4.4. M.H and A.M. contributed ideas and editorial support.

Abstract:

Spinal muscle atrophy (SMA) is an autosomal recessive neurodegenerative disease which is characterized by the loss of motor neurons resulting in progressive muscle atrophy. Loss of functional Survival motor neuron (SMN) protein due to mutations or deletion in the *SMN1* gene is the cause of SMA. A potential treatment strategy for SMA is to upregulate levels of SMN protein originating from the *SMN2* gene compensating in part for the absence of the *SMN1* gene. Sodium valproate, trichostatin A (TSA) and aclarubicin, all agents which effectively enhance *SMN2* expression, have been recently shown to activate STAT5 in SMA-like mouse embryonic fibroblasts and human SMN2-transfected NSC34 cells. Given that prolactin (PRL) is also known to activate the STAT5 signalling pathway, we elected to assess its impact on SMN levels. In this manner we have demonstrated a significant induction in SMN mRNA and protein levels in human and mice neuronal cells upon treatment with PRL. We have shown that activation of the STAT5 pathway by PRL mediated this transcriptional upregulation of the *SMN* gene. Furthermore, PRL treatment induces SMN expression in brain and spinal cord samples of wildtype mice *in vivo*. Critically, PRL treatment increased SMN levels, improved motor function and enhanced survival in a severe SMA mouse model. The SMN2 gene shows enhanced sensitivity to PRL induction when compared with *SMN1*. This study thus shows SMN protein regulation through PRL via the STAT5 pathway and its effect on the phenotype of the disease. Our results confirm earlier work suggesting STAT5 pathway activators as potential therapeutic compounds for the treatment of SMA and identify PRL as one such promising agent.

Introduction:

Autosomal recessive Spinal muscular atrophy (SMA) is a leading inherited cause of infant death worldwide. It is characterized by the loss of motor neurons from the anterior horn of spinal cord, attendant paresis leading to respiratory insufficiency (1). The incidence of SMA is one in 10,000 live births with a carrier frequency of approximately 1:35 to 1:50 (2). Based on disease onset and severity, SMA is broadly classified into four categories. Type I, the most common and severe form in which there is a profound weakness by six months often resulting in death by the first 2 years of life; types II and III which are milder forms and type IV characterized by mild proximal weakness at adulthood and normal longevity.

Low levels of functional Survival motor neuron (SMN) protein due to mutations or deletion of the *SMN1* gene causes SMA (3, 4). SMN is a ubiquitously expressed 294 amino acid protein evolutionarily conserved which through a myriad of interactions is required for subcellular RNA localization and processing (4-12). Chief among these is a complex with Gemins, central to biogenesis of spliceosomal small nuclear ribonucleoproteins (snRNPs) a major component of pre-mRNA splicing machinery (13). SMN has other functions as well in the assembly, metabolism and transport of other ribonucleoproteins (8, 10, 12). Due to an evolutionarily recent duplication event on chromosome 5q13, humans uniquely have two nearly identical *SMN* genes, *SMN1* and *SMN2* (14, 15). The *SMN1* gene is the primary source of full length functional SMN protein, its loss or mutation is both necessary and sufficient for SMA (3). There are 5 nucleotides which distinguish *SMN1* and *SMN2* coding regions (3); none are translationally significant although the C to T transition at position 6 of exon 7 results in SMN2 mRNA being alternatively spliced producing only 5-10% of the full length functional SMN protein (3, 16, 17).

All SMA patients have at least one copy of the *SMN2* gene which produces low levels of functional SMN protein. Complete absence of *SMN* genes is embryonically lethal in both human and mice (18-20). Individuals with SMA have variable number of *SMN2* genes; the greater the *SMN2* gene copy number, the milder disease severity (21, 22). Presently there is no effective therapy for SMA. A potential treatment strategy SMA is to upregulate levels of SMN protein originating from the *SMN2* gene compensating in part for the absence of functional *SMN1* gene.

A number of small molecules have been proposed as *SMN2* inducers; among them are sodium valproate, trichostatin A (TSA) and aclarubicin which have all been suggested to activate STAT5 in SMA-like mouse embryonic fibroblasts and human SMN2-transfected NSC34 cells (23). STAT5 is member of Signal Transducers and Activator of Transcription proteins family which control diverse cellular responses such as, cell growth, differentiation and apoptosis, through modification of gene expression of cytokine inducible genes (24, 25). STATs are direct targets of Janus kinase (JAK) which is activated by the binding of ligand to specific cytokine receptors. Prolactin (PRL) is a 199 amino acid long 23 kD polypeptide hormone which binds to PRL receptor (PRLR) and activates the JAK2/STAT5 pathway (25, 26). PRLRs are present in metazoan central nervous system including motor neurons (25, 27) and the protein is blood brain permeant, enticing us to explore its impact on *SMN2*.

We show here a PRL conferred SMN increase in both neuronal cells and wild type (WT) mice and present evidence for a STAT5 pathway activation of SMN transcription underlying this increase in functional SMN protein. Importantly we show that PRL also increases SMN levels, improved motor function and survival in a severe SMA mouse model. Our results

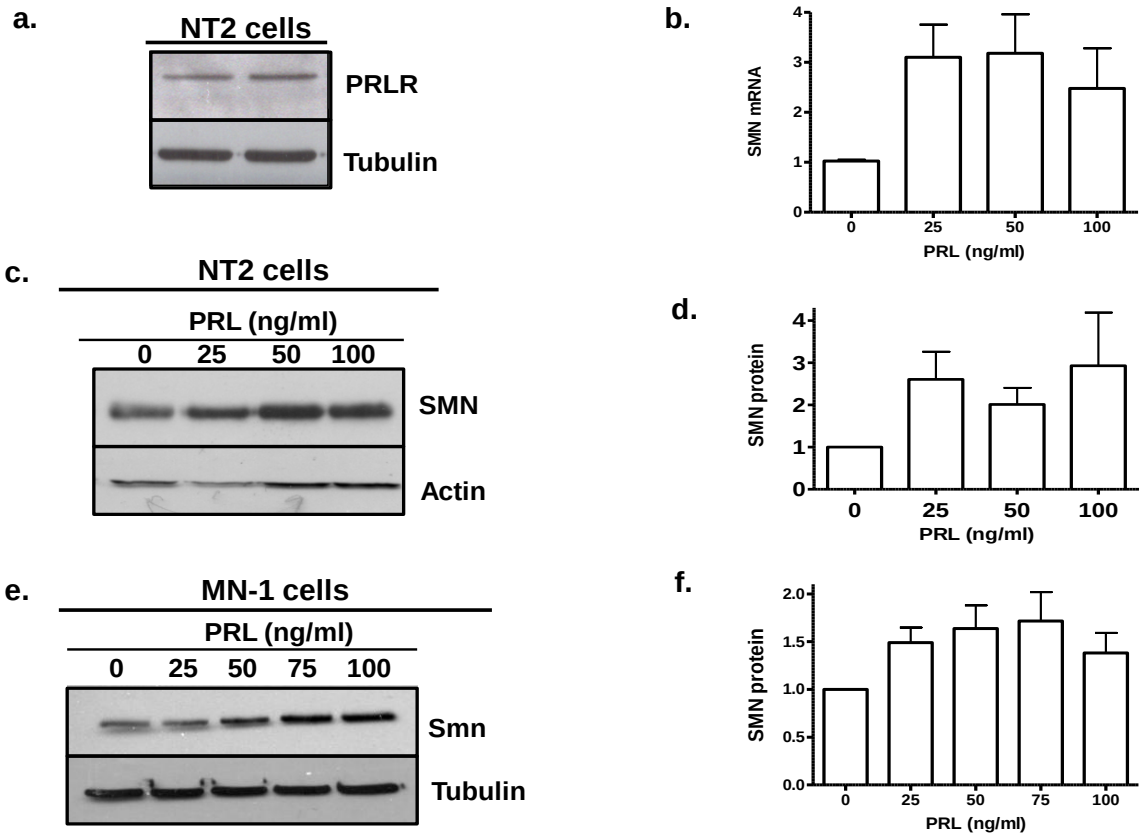
confirm earlier work suggesting STAT5 pathway activators as potential therapeutic compounds for the treatment of SMA and identify PRL as one such promising agent.

Results:

PRL treatment upregulates *SMN* gene expression *in vitro*.

In order to explore a role for PRL in the regulation of the *SMN* gene *in vitro*, the expression of PRL receptor in human neuron-committed teratocarcinoma (NT2) cells was first confirmed (Fig 4.1a). The cells were next treated with a range of PRL doses (25-100ng/ml) for 48 h. SMN transcript levels were found to be increased significantly (3–4 fold) in NT2 cells upon treatment with all PRL doses (Fig. 4.1b). NT2 or motor neuron derived (MN-1) cells were then treated with similar PRL doses for 48h and harvested for Western blot analysis. All PRL doses tested led to a significant increase in SMN protein levels in both NT2 and MN-1 cells (Fig. 4.1 c-f). Taken together these results demonstrate that PRL treatment causes an increase in SMN steady-state mRNA and protein levels in both human and mice neuronal-cell lines.

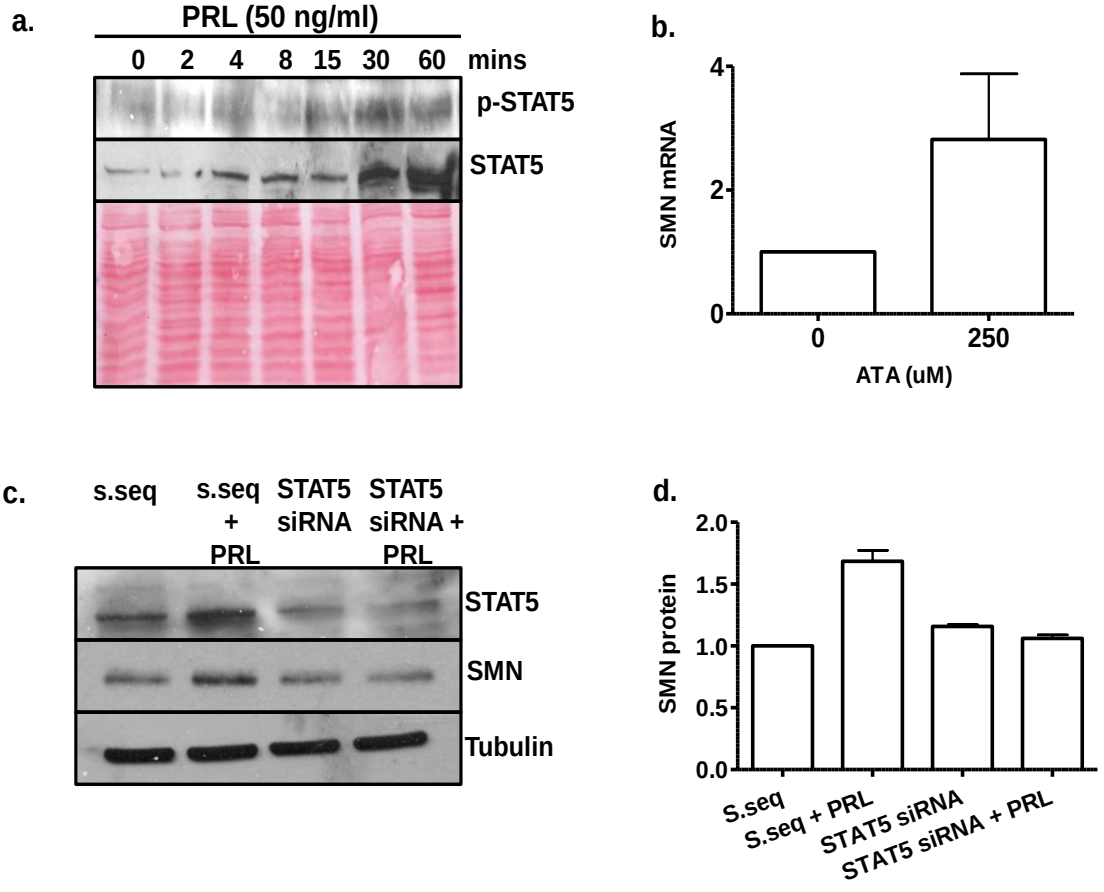
Figure 4.1. PRL treatment upregulates SMN mRNA and protein *in vitro*. NT2 or MN-1 cells were treated with PRL (0, 25, 50, & 100 ng/ml) and then harvested at indicated intervals for RT-PCR or Western blot analyses. (a) Presence of PRL receptor in NT2 cells. (b) Quantification of SMN mRNA relative to β-actin in NT2 cells after PRL treatment (fold induction; the ratio at 0 hour was set as 1). Mean + SD (bars) of three independent experiments. (c) Representative Western blot showing effect of PRL on SMN protein in NT2 cells. (d) Densitometric quantification of SMN relative to β-actin [mean + SD (bars) of three independent experiments] are shown for NT2 cells. (e) Representative Western blot showing effect of PRL on SMN protein in MN-1 cells. (f) Densitometric quantification of Smn relative to Tubulin [mean + SD (bars) of three independent experiments] are shown for MN-1 cells.


a.
NT2 cells
PRLR
Tubulin
b.
SMN mRNA
0
1
2
3
4
0
25
50
100
PRL (ng/ml)
c.
NT2 cells
PRL (ng/ml)
0
25
50
100
SMN
Actin
d.
SMN protein
0
1
2
3
4
0
25
50
100
PRL (ng/ml)
e.
MN-1 cells
PRL (ng/ml)
0
25
50
75
100
Smn
Tubulin
f.
SMN protein
0.0
0.5
1.0
1.5
2.0
0
25
50
75
100
PRL (ng/ml)

Activation of STAT5 pathway causes upregulation of *SMN* gene and is responsible for PRL conferred SMN protein increase.

Since the STAT5 pathway has been implicated in the regulation of the *SMN* gene (23) and given that PRL is a known activator of the pathway, we confirmed the previously published results that PRL increases total STAT5 levels and also activates STAT5 pathway by phosphorylating STAT5 protein (Fig 4.2a). To further investigate a potential STAT5 role in *SMN* gene regulation, NT2 cells were treated with Aurintricarboxylic acid (ATA; known STAT5 activator which mimics PRL activity (28)) for 48 h. Treatment with ATA led to a significant induction in SMN transcript levels similar to that seen with PRL treatment (Fig 4.2b). To further confirm the role of STAT5 pathway in PRL-induced increase in SMN protein, NT2 cells were transfected with STAT5-specific siRNA or control siRNA for 24 h, and then treated with PRL for 48 h. siRNA-mediated abrogation of STAT5 expression completely blocked the PRL-induced increase in SMN protein (Fig 4.2c & d). The results when viewed in aggregate strongly implicate the STAT5 pathway in the PRL-induced increase of SMN levels.

Figure 4.2. PRL increases SMN expression via STAT5 pathway. (a) Representative Western blot showing activation of STAT5 pathway upon PRL treatment in NT2 cells. (b) ATA (STAT5 pathway activator) treatment upregulates SMN mRNA *in vitro*. NT2 cells were treated with ATA (250 uM) and then harvested after 48 hrs for RT-PCR; Quantification of SMN mRNA relative to β-actin in NT2 cells after ATA treatment (fold induction; the ratio at 0 hour was set as 1). Mean + SD (bars) of three independent experiments. (c) The siRNA knockdown of STAT5 protein attenuates PRL induced increase in SMN protein. Representative Western blots showing both STAT5 knockdown and its effect on PRL induced increase in SMN protein. (d) Densitometric quantification of SMN relative to Tubulin [mean + SD (bars) of three independent experiments] is shown.

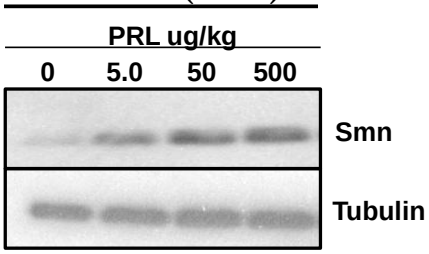

a.
PRL (50 ng/ml)
0 2 4 8 15 30 60 mins
p-STAT5
STAT5
b.
SMN mRNA
4
2
0
0
250
ATA (uM)
c.
s.seq
s.seq + PRL
STAT5 siRNA
STAT5 siRNA + PRL
STAT5
SMN
Tubulin
d.
SMN protein
2.0
1.5
1.0
0.5
0.0
S.seq
S.seq + PRL
STAT5 siRNA
STAT5 siRNA + PRL

PRL treatment upregulates SMN protein level in WT mice.

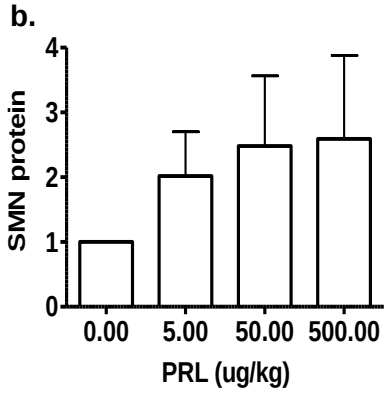
In order to both confirm that PRL-mediated SMN induction extends to the *in vivo* setting and to begin to optimize the dose for PRL treatment, CD-1 mice were given daily intraperitoneal (IP) PRL injections for 5 days over a range of doses. Brain and spinal cord samples were isolated for Western blot analysis. PRL treatment increased SMN protein levels in a dose dependent manner in brain (Fig 4.3a & b) and at higher doses in spinal cord samples (Fig 4.3c & d).

Figure 4.3. PRL upregulates Smn protein in wildtype mice. Male CD-1 wild-type mice were treated daily with PRL (5.0, 50.0, 500.0 µg/kg) for 5 days, then sacrificed. Brain and spinal cord tissues were harvested for Western blot analysis. (a) Representative Western blot showing the effect of PRL on Smn protein in brain samples of CD-1 mice treated with Saline (control, lane-1) or PRL (lane 2, 3 & 4 respectively) (n=3). (b) Densitometric quantification of Smn relative to Tubulin [mean + SD (bars)] is shown for brain samples. (c) Representative Western blot showing the effect of PRL on SMN protein in spinal cord samples of CD-1 mice treated with Saline (control, lane-1) or PRL (lane 2, 3 & 4) (n=3). (d) Densitometric quantification of Smn relative to Tubulin [mean + SD (bars)] is shown for spinal cord samples.

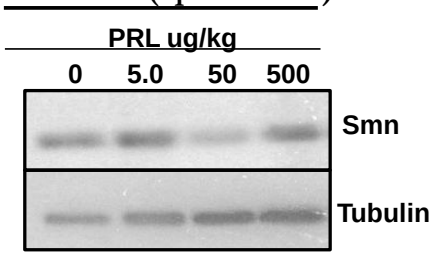
a.


CD-1 mice (Brain)
PRL ug/kg
0
5.0
50
500
Smn
Tubulin

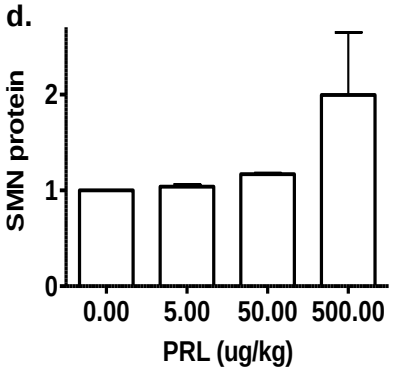
b.


SMN protein
4
3
2
1
0
0.00
5.00
50.00
500.00
PRL (ug/kg)

c.


CD-1 mice (Spinal Cord)
PRL ug/kg
0
5.0
50
500
Smn
Tubulin

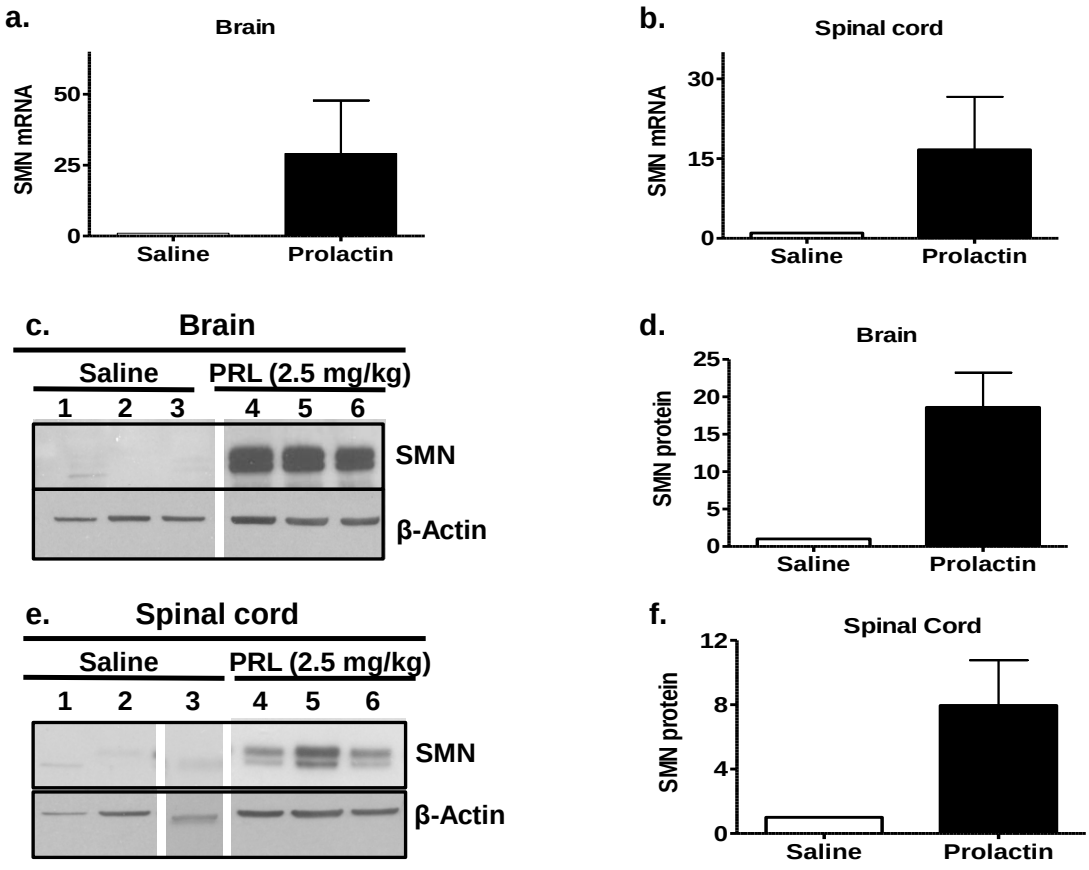
d.


SMN protein
2
1
0
0.00
5.00
50.00
500.00
PRL (ug/kg)

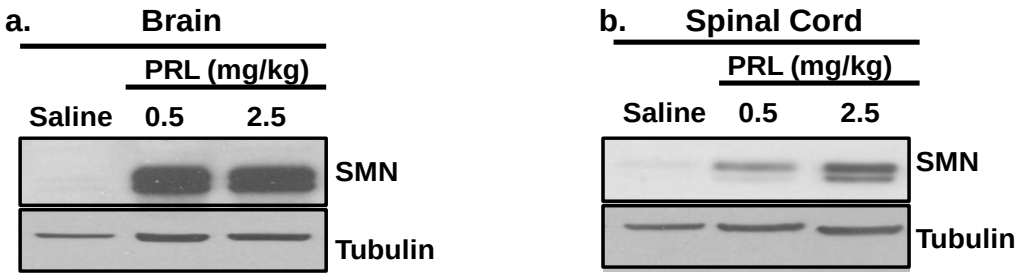
PRL treatment upregulates SMN mRNA and protein levels in a SMA mice model.

In order to explore the impact of PRL-induced SMN upregulation on SMA, genetically faithful transgenic (*mSmn*-/-;*hSMN2*+/+, *hSMNΔ7*+/+) SMA mice (SMAΔ7) (29) were given 2.5mg/kg PRL IP injections every day from P1 till P6. Mice were euthanized 24 hours after last treatment. Brain and spinal cord samples were then harvested for RT-PCR and Western blot analysis. Importantly, PRL treatment was observed to increase *SMN2* derived full length SMN transcript (Fig 4.4 a & b) as well as protein levels significantly in both brain and spinal cord samples when compared with vehicle treated mice (Fig 4.4 c-f & Supplemental Fig 4.1).

Figure 4.4. PRL upregulates SMN protein in SMA mice model. SMAΔ7 mice were treated daily with saline or PRL (2.5 mg/kg) from P1 for 6 days, then sacrificed at P7. Brain and spinal cord tissues were harvested for RT-PCR and Western blot analysis. (a) Quantification of SMN mRNA relative to β-actin in brain tissue after PRL treatment (fold induction; the ratio at saline treatment was set as 1). Mean + SD (bars) is shown (n=5). (b) Quantification of SMN mRNA relative to β-actin in spinal cord tissue after PRL treatment (fold induction; the ratio at saline treatment was set as 1). Mean + SD (bars) is shown (n=5). (c) Representative Western blot showing effect of PRL on SMN protein in brain samples of SMAΔ7 mice treated with Saline (control, lane 1,2 & 3) or PRL (lane 4, 5 & 6 respectively) (each lane represents individual animal; all lanes were run on the same gel but were non-contiguous). (d) Densitometric quantification of SMN relative to Tubulin [mean + SD (bars)] is shown for brain samples. (e) Representative Western blot showing effect of PRL on SMN protein in spinal cord samples of SMAΔ7 mice treated with Saline (control, lane 1,2 & 3) or PRL (lane 4, 5 & 6 respectively) (each lane represents individual animal; all lanes were run on the same gel but were non-contiguous). (f) Densitometric quantification of SMN relative to Tubulin [mean + SD (bars)] is shown for spinal cord samples.


a.
Brain
SMN mRNA
50
25
0
Saline
Prolactin
b.
Spinal cord
SMN mRNA
30
15
0
Saline
Prolactin
c.
Brain
Saline
PRL (2.5 mg/kg)
1 2 3 4 5 6
SMN
β-Actin
d.
Brain
SMN protein
25
20
15
10
5
0
Saline
Prolactin
e.
Spinal cord
Saline
PRL (2.5 mg/kg)
1 2 3 4 5 6
SMN
β-Actin
f.
Spinal Cord
SMN protein
12
8
4
0
Saline
Prolactin

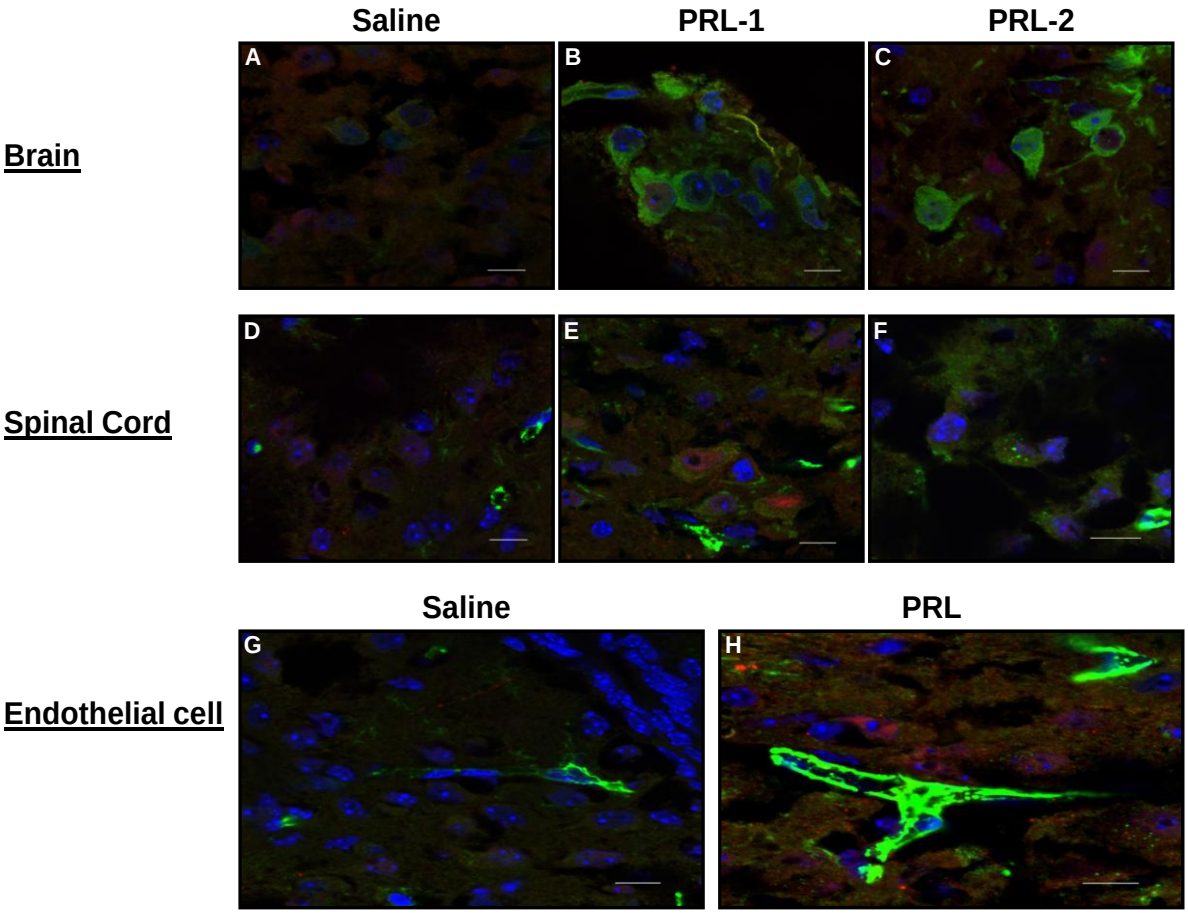
Supplemental figure 4.1. PRL dose optimization for SMN induction in SMA mice model. SMAΔ7 mice were treated daily with PRL (0.5 and 2.5 mg/kg) from P1 for 6 days, then sacrificed at P7. Brain and spinal cord tissues were harvested Western blot analysis. (a) Representative Western blot showing effect of PRL on SMN protein in brain samples of SMAΔ7 mice treated with Saline (control, lane 1) or PRL (0.5 & 2.5 mg/kg; lane 2 & 3 respectively) (each lane represents individual animal). (b) Representative Western blot showing effect of PRL on SMN protein in spinal cord samples of SMAΔ7 mice treated with Saline (control, lane 1) or PRL (0.5 & 2.5 mg/kg; lane 2 & 3 respectively) (each lane represents individual animal).


a.
Brain
PRL (mg/kg)
Saline
0.5
2.5
SMN
Tubulin
b.
Spinal Cord
PRL (mg/kg)
Saline
0.5
2.5
SMN
Tubulin

PRL treatment upregulates SMN expression within motor neurons and endothelial cells in a SMA mice model.

To check the expression of SMN protein within motor neurons after treatment with PRL, SMAΔ7 mice were given daily 2.5mg/kg PRL IP dose from P1 till P6. Mice were euthanized 24 hours after the last treatment. Brain and spinal cord samples were harvested for immunohistochemistry analysis. Motor neurons were labelled with HB9 antibody (motor neuron marker). PRL treatment increased SMN protein levels significantly in motor neurons of both brain and spinal cord samples when compared with vehicle treated mice (Fig 4.5 A-F). Also SMN levels were significantly higher in endothelial cells among treatment group (Fig 4.5 G & H).

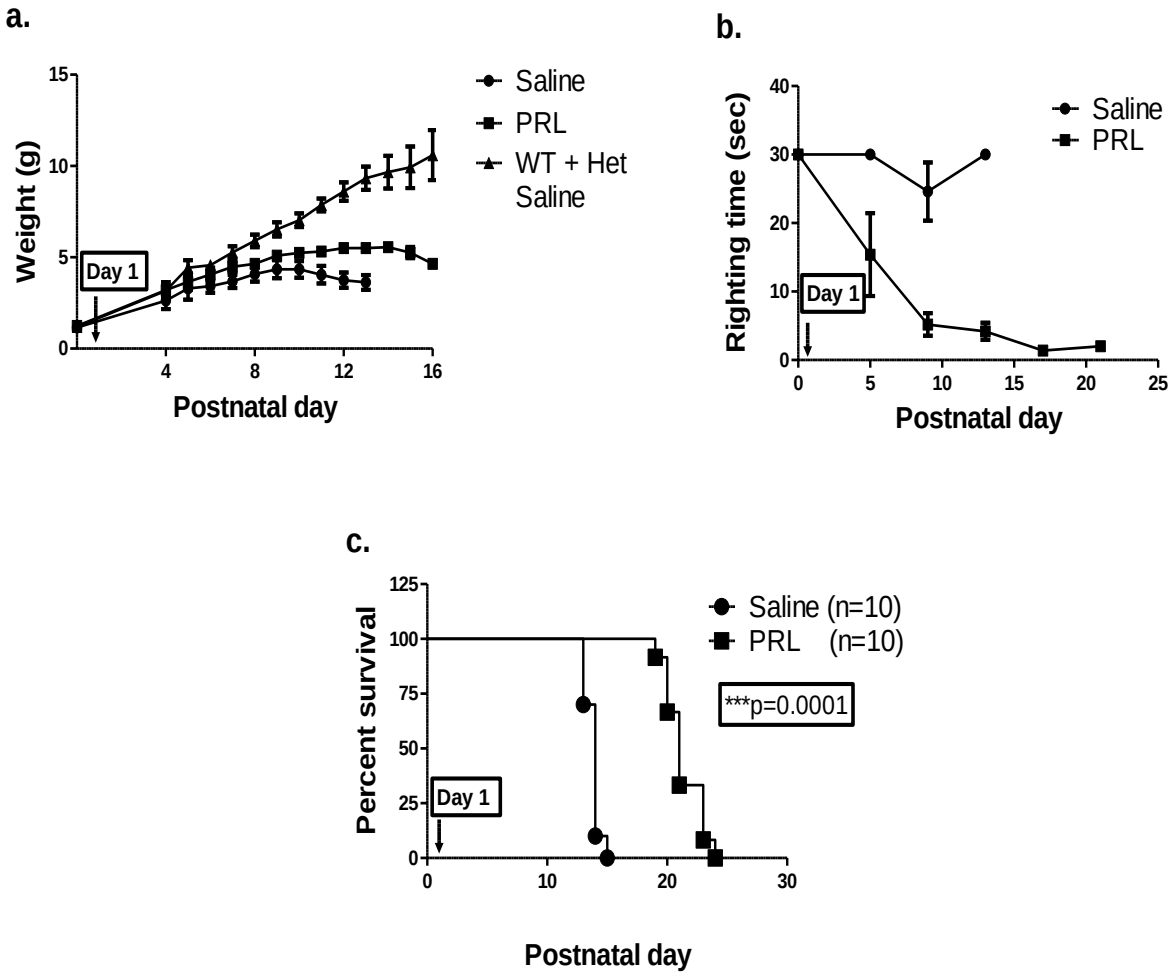
Figure 4.5. PRL upregulates SMN protein expression in motor neurons and endothelial cells in SMA mice model. SMAΔ7 mice were treated daily with saline or PRL (2.5 mg/kg) from P1 for 6 days, then sacrificed at P7. Brain stem and spinal cord tissues were harvested for Immunohistochemistry analysis. Representative merged Confocal images [SMN/alexa488 (green) + HB9/alexa 568 (red; motor neuron marker) + Hoechst (blue)] for different tissues are shown. Representative Confocal images showing effect of PRL on SMN protein expression in brain stem and spinal cord motor neurons samples of SMAΔ7 mice treated with Saline (control, A & D) or PRL (B,C & E,F) respectively (n=3). Scale bars: 10μM. Representative Confocal images showing effect of PRL on SMN protein expression in endothelial cells of SMAΔ7 mice treated with Saline (control, G) or PRL (H) (n=3). Scale bars: 10μM.


Saline
PRL-1
PRL-2
A
B
C
Brain
D
E
F
Spinal Cord
Saline
PRL
G
H
Endothelial cell

PRL treatment improves disease phenotype of SMA mice model.

The impact of PRL-induced increase in SMN levels on disease phenotype in SMAΔ7 mice was next examined. The SMAΔ7 mice have severely impaired righting time and muscle weakness detectable by P5. They are also significantly underweight compared to heterozygous and WT littermates with a median survival of 13 days. In order to assess the impact of PRL on the phenotype of the disease, SMAΔ7 mice (P1) were given daily PRL or vehicle IP injections and their weight, motor function and longevity assessed. SMAΔ7 mice treated with PRL showed a considerably slower weight loss, dramatic improvement of motor function (as assessed by righting time), and significant extension of survival (median survival of 21 days) as compared with vehicle-treated SMAΔ7 mice (median survival of 14 days, Fig 4.6 a, b & c respectively and supplemental video).

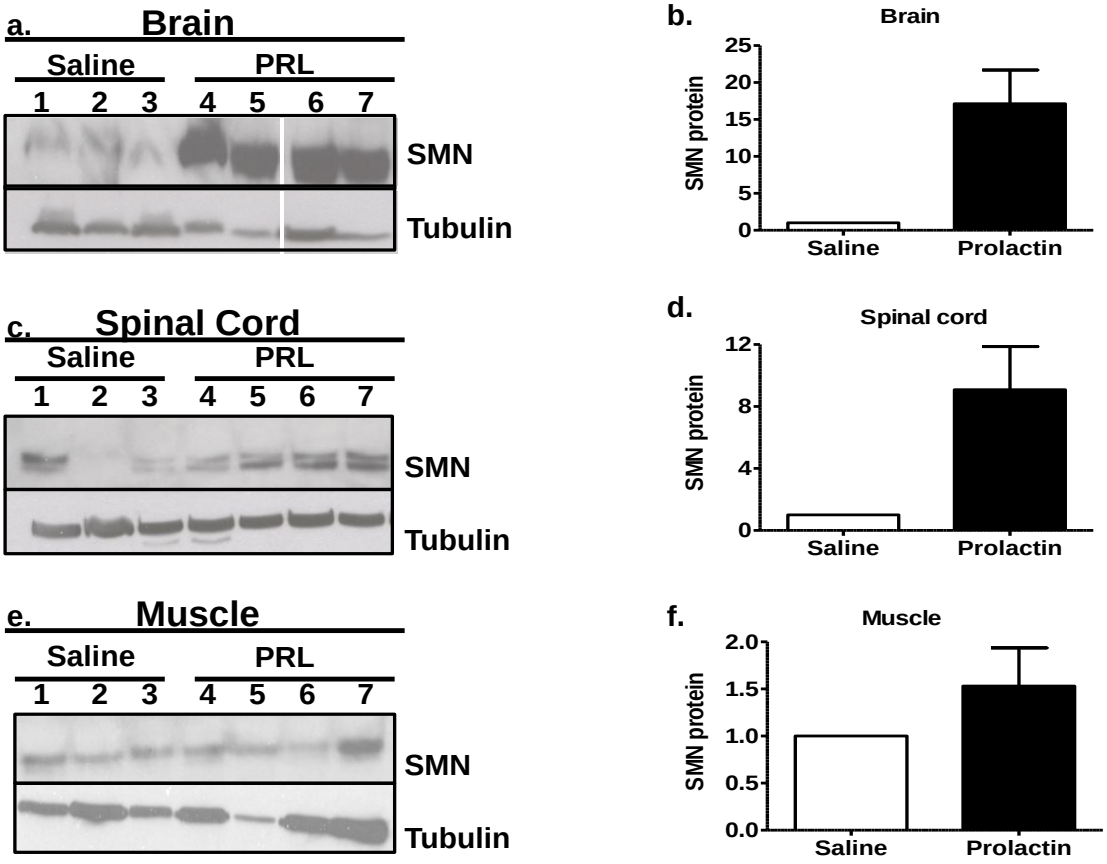
Figure 4.6: PRL ameliorates disease phenotype in SMA mice model. SMAΔ7 mice were treated daily with intraperitoneal injections of PRL (2.5 mg/kg) from P1 onward. (a) Weights of SMAΔ7 mice treated with PRL (black filled square) or saline (black filled circle) (n =5); weights for heterozygous mice treated with saline (black filled triangle) are also shown for comparison [mean ± SD (bars)]. (b) Righting times of SMAΔ7 mice treated with PRL (black filled square) or saline (black filled circle) (n =5) [mean ± SD (bars)]. (c) Kaplan-Meier survival curves of SMAΔ7 mice treated with PRL (black filled square) or vehicle (black filled circle) (n =10); $P < 0.0001$, log-rank test.


a.
Weight (g)
15
10
5
0
Day 1
4
8
12
16
Postnatal day
Saline
PRL
WT + Het
Saline
b.
Righting time (sec)
40
30
20
10
0
Day 1
0
5
10
15
20
25
Postnatal day
Saline
PRL
c.
Percent survival
125
100
75
50
25
0
Day 1
0
10
20
30
Postnatal day
Saline (n=10)
PRL (n=10)
***p=0.0001

PRL treatment upregulates SMN protein level in a SMA mice model.

In order to investigate whether SMN is upregulated throughout the life span, SMAΔ7 mice were given daily 2.5mg/kg PRL IP doses from P1 till death (P23). Brain, spinal cord and muscle samples were harvested for Western blot analysis. SMN protein levels in brain and spinal cord tissues were found to be significantly increased at the time of death (Fig 4.7a-d), with smaller increases observed in muscle (Fig 4.7e & f).

Figure 4.7. SMN protein level is upregulated in SMAΔ7 mice until time of death. SMAΔ7 mice were treated daily with PRL (2.5 mg/kg) from P1 onward. Brain, spinal cord and muscle tissues were harvested upon death for Western blot analysis. (a) Representative Western blot showing the effect of PRL on SMN protein in brain samples of SMAΔ7 mice treated with saline (control, lane 1,2 & 3) or PRL (lane 4, 5, 6 & 7) (each lane represents individual animal; all lanes were run on the same gel but were non-contiguous). (b) Densitometric quantification of SMN relative to Tubulin [mean + SD (bars)] is shown for brain samples. (c) Representative Western blot showing effect of PRL on SMN protein in spinal cord samples of SMAΔ7 mice treated with Saline (control, lane 1,2 & 3) or PRL (lane 4, 5, 6 & 7 respectively) (each lane represents individual animal). (d) Densitometric quantification of SMN relative to Tubulin [mean + SD (bars)] is shown for spinal cord samples. (e) Representative Western blot showing the effect of PRL on SMN protein in muscle samples of SMAΔ7 mice treated with Saline (control, lane 1,2 & 3) or PRL (lane 4, 5, 6 & 7) (each lane represents individual animal). (f) Densitometric quantification of SMN relative to Tubulin [mean + SD (bars)] is shown for muscle samples.


a.
Brain
Saline
PRL
1 2 3 4 5 6 7
SMN
Tubulin
b.
Brain
SMN protein
25
20
15
10
5
0
Saline
Prolactin
c.
Spinal Cord
Saline
PRL
1 2 3 4 5 6 7
SMN
Tubulin
d.
Spinal cord
SMN protein
12
8
4
0
Saline
Prolactin
e.
Muscle
Saline
PRL
1 2 3 4 5 6 7
SMN
Tubulin
f.
Muscle
SMN protein
2.0
1.5
1.0
0.5
0.0
Saline
Prolactin

Discussion:

A potential treatment strategy for SMA is the upregulation of SMN protein originating from *SMN2*, a gene which is present in all affected SMA individuals, compensating in part for the absence of a functional *SMN1* (3). One of a number of pathways which increase SMN is STAT5 (23), and one of the more potent known inducer of STAT5 is PRL. The facts that PRL passes the blood brain barrier (BBB) and its receptor is expressed throughout the central nervous system (26, 27) increase its potential as an SMA therapeutic.

We document here a PRL mediated increase in both SMN mRNA and protein levels in human neuronal cell line and murine motor neuron MN-1 cells. We also show an associated rapid increase in both phospho and total STAT5 levels upon PRL treatment. SMN mRNA induction was also seen when cells are treated with the small molecule STAT5 activator, ATA (28). Conversely an attenuation of PRL mediated SMN induction was observed when cells were pretreated with STAT 5 RNAi. An earlier report showing *in vitro* STAT5 conferred SMN induction revealed the induction to be transcriptional in nature (23) (rather than transcript stabilization or altered splicing); in keeping with this we have found that Actinomycin D (a transcription inhibitor) treatment effectively abrogated the SMN induction in NT2 cells (Data not shown).

Treatment with different doses of PRL in WT mice next revealed, as with cell culture, a sustained induction of SMN protein in brain and, to a lesser degree, in spinal cord. An increased and optimized PRL dose in SMA mice resulted in a significant and sustained increase in SMN mRNA and protein levels, surpassing that observed in previously identified

SMN inducing agents (23, 30-34). Immunohistochemical analysis revealed a significant SMN induction in motor neurons. The second site of profound SMN upregulation appeared to be capillaries and endothelial cells. Although recent work suggests a central nervous system mediated role in this phenomenon (35), whether the modulation of SMN in the endothelium also has implications for the peripheral necrosis recently observed in both mouse model of the disease (30) and in patients (36, 37) is an open question.

Although the *mSmn*-/-;*hSMN2*+/+, *hSMNΔ7*+/+ mice (null for mouse *Smn* gene rescued with 2 transgenes; human *SMN2* and an *SMN* cDNA deleted for exon 7) with a phenotype that closely resembles type I SMA in humans, is one of the most widely used in preclinical assessment of SMA therapeutics (29), inter-laboratory comparisons of the impact of drug or other intervention poses a challenge. The sole sure commonality, in addition to it being the same species, are the absence of endogenous mouse *Smn* and presence of presumably identical human *SMN2* and *SMNΔ7* transgenes. Otherwise the genetic background, housing conditions (e.g. temperature, dark light cycle) and not least, feeding conditions can vary widely as can be seen by survivals ranging from 12 to 17 days. Thus we find that taking the ratio of median survival of treated to non treated is a useful metric by which to assess effectiveness of a given intervention, irrespective which lab is conducting the analysis. We have achieved a ratio of 21d/14d or 1.6, a number that compares favourably with the 1.2 (19d/16d) observed with TSA (albeit P1 PRL initiation versus P5 TSA initiation) and 1.3 (12.9d/9.9d) seen with SAHA (32, 33).

In addition to apparently being more effective than the comparatively toxic TSA treatment, the distinct groups of responders and non-responders observed to TSA treatment (33) was not observed with PRL treatment. Another compound that has shown promise for

the treatment of SMA is an antisense oligonucleotide which prevents alternative splicing of SMN2 transcript and ultimately results into more full length transcript (30). The most encouraging report so far in the field of SMA therapeutics is the use of self complementary AAV9 gene therapy with SMN as a payload which resulted in an extension in longevity of SMA mice from 2 weeks to 250 days plus (38). Similar results have been seen by other groups who used similar gene therapy approach to treat SMA mice (39-41). However, the clinical introduction of this treatment for SMA must await resolution of clinical safety, potential species barrier including immune response, preparing adequate quantity of GMP grade virus and overall cost (42).

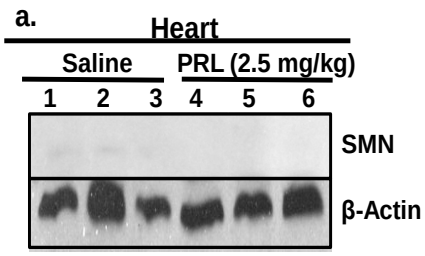
We were struck by the dramatically greater SMN induction observed in SMA mice when compared with WT mice; we also noted that SMN induction in human cell line surpassed that observed in murine cell line. The sole source of SMN protein in the SMA mice are human *SMN* genes. This prompted an analysis of the putative STAT5 binding sites in murine *Smn* and human *SMN* genes. In the original STAT5 SMN paper, promoter sequence analysis of both murine and human *SMN* gene showed two conserved Stat5 binding sites (TTCNNNGAA/ TTCNNNTAA) in murine SMN promoter (NCBI accession no. AF027668) but none in human *SMN2* promoter (NCBI accession no. AF027688). Three similar CTCNNNTAA elements were detected uniquely in *SMN2* promoter (−413 to −409 bp, −2338 to −2330 bp and −3881 to −3873 bp downstream of the *SMN2* start codon (+1) (23)). However our further analysis using the more less stringent online database known as DECODE **(**the Champion ChiP Transcription Factor Search Portal based on SABiosciences' proprietary database; http://www.sabiosciences.com/chipqpcrsearch.php?app=TFBS) revealed a total of twelve

STAT5 binding for the human *SMN2* and none for mouse *Smn* promoter. We believe that this difference may account for the profound SMN induction we see when SMN2 is the source of SMN protein.

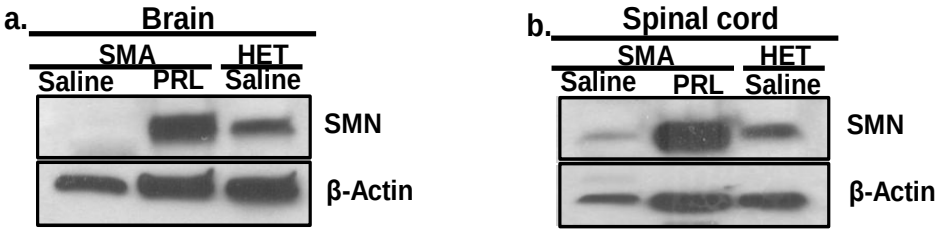
PRL treatment in SMA mice has revealed a significant *in vivo* induction of SMN protein which correlates with an overall improvement in the phenotype of the disease. PRL treatment attenuated the weight loss and improved motor neuron function considerably as well resulted in a ~70% increase in life span in SMA mice. However the degree of SMN induction, greater than that observed in heterozygote mice which have a normal life span (Supplemental Fig 4.4 & 4.5), is at odds with the significant but in contrast comparatively modest improvement in longevity. Four obvious sources of this disconnect are 1.) Notwithstanding the P1 inception of treatment, a delay in SMN induction in the target motor neuron, 2.) A role for SMN in other neuronal cells beside motor neurons refractory to PRL mediated induction, 3). A role for SMN in other tissues refractory to PRL mediated induction and 4.) Inconsistent SMN induction. Given the small size of P1 and P2 pups, there can be technical challenges in ensuring complete administration of the total PRL dose in the first few days of life. However, the recent AAV9 *SMN* gene therapy rescue of the same mouse model work has shown that motor neuron transduction as late as five days still results in survival extension to 40 days suggesting that a failure to induce SMN in other neuronal cells or in tissues other than the motor neuron, may be the more likely source of the mortality (38). In this regard, the recent observation of significant cardiac pathology in this mouse model (43-45) combined with the lack of SMN induction we observed in the myocardium tissue (Supplemental Fig 4.2) suggests that cardiac failure may underlie the early mortality. However it has been shown that induction of SMN in heart tissue itself does

not rescue SMA mice whereas neuronal specific transgenic expression of SMN ameliorates disease phenotype with increase in survival of SMA mice (46). This suggests that may be higher levels of SMN is required in the neurons which innervates heart tissue.

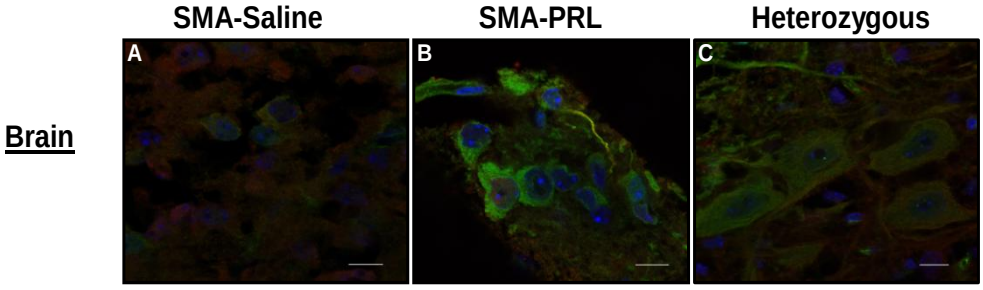
Supplemental figure 4.2. PRL treatment does not affect SMN protein level in heart tissues of SMAΔ7 mice until time of death. SMAΔ7 mice were treated daily with PRL (2.5 mg/kg) from P1 onward. Heart tissues were harvested upon death for Western blot analysis. (a) Representative Western blot showing the effect of PRL on SMN protein in heart samples of SMAΔ7 mice treated with saline (control, lane 1,2 & 3) or PRL (lane 4, 5 & 6).


a.
Heart
Saline
PRL (2.5 mg/kg)
1 2 3 4 5 6
SMN
β-Actin

Supplemental figure 4.4. Comparison of SMN induction in SMA mice model (*mSmn*-/-;*hSMN2*+/+, *hSMNΔ7*+/+) after PRL treatment with carrier treated heterozygous transgenic mice (*mSmn*+/-;*hSMN2*+/+, *hSMNΔ7*+/+). SMAΔ7 and heterozygous mice were treated daily with saline or PRL (2.5 mg/kg; SMAΔ7 mice only) from P1 for 6 days, then sacrificed at P7. Brain and spinal cord tissues were harvested Western blot analysis. (a) Representative Western blot showing effect of PRL on SMN protein in brain samples of SMAΔ7 and heterozygous mice treated with Saline (control, lane 1 & 3 respectively) or PRL (2.5 mg/kg; lane 2) (each lane represents individual animal). (a) Representative Western blot showing effect of PRL on SMN protein in spinal cord samples of SMAΔ7 and heterozygous mice treated with Saline (control, lane 1 & 3 respectively) or PRL (2.5 mg/kg; lane 2) (each lane represents individual animal).


a.
Brain
SMA
HET
Saline
PRL
Saline
SMN
β-Actin
b.
Spinal cord
SMA
HET
Saline
PRL
Saline
SMN
β-Actin

Supplementary figure 4.5. Comparison of SMN induction in motor neurons in SMA mice model (*mSmn*-/-;*hSMN2*+/+, *hSMNΔ7*+/+) after PRL treatment with carrier treated heterozygous transgenic mice (*mSmn*+/-;*hSMN2*+/+, *hSMNΔ7*+/+). SMAΔ7 and heterozygous mice were treated daily with saline or PRL (2.5 mg/kg; SMAΔ7 mice only) from P1 for 6 days, then sacrificed at P7. Brain tissues were harvested for Immunohistochemistry analysis. Representative merged Confocal images [SMN/alexa488 (green) + HB9/alexa 568 (red; motor neuron marker) + Hoechst (blue)] for different tissues are shown. Representative Confocal images showing effect of PRL on SMN protein expression in brain stem motor neurons samples of SMAΔ7 and heterozygous mice treated with Saline (A & C respectively) or PRL (B). Scale bars: 10μM.

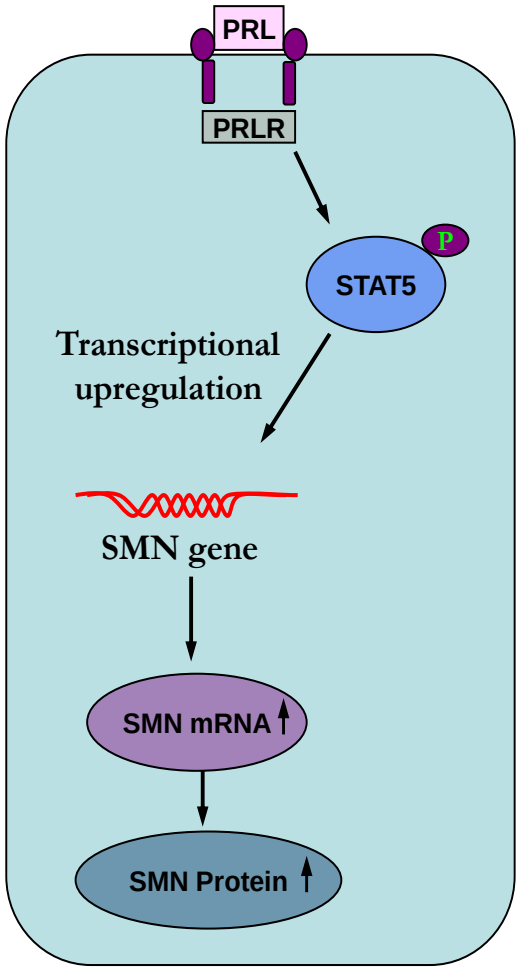

SMA-Saline
SMA-PRL
Heterozygous
Brain
A
B
C

Clinical experience with PRL is limited although a recent study has demonstrated the safe and effective use of recombinant PRL for lactation deficient mothers (47). Moreover PRL has been shown to regulate oligodendrocyte precursor proliferation and mimics the regenerative effects of pregnancy. PRL's striking ability to repair demyelination identifies it as a potential therapeutic agent in multiple sclerosis (48). There could be some potential side effects of higher levels of PRL. A condition called hyperprolactinaemia (resulting from higher level of blood PRL) can lead to hypoestrogenism which may result into infertility and osteoporosis (49). Higher levels of PRL also decreases dopamine release therefore some antipsychotic and antidepressant drugs can elevate PRL levels as well.

Our results demonstrate a clear promise for PRL use in clinical trial studies; amelioration of disease phenotype in mouse model, BBB penetration and safe and FDA approved status. Given recent work showing that early timing of *SMN* gene therapy is critical for maximum benefit in murine SMA, diagnosing newborn pre-symptomatic SMA infants and their early treatment might be a key for trial with PRL. We hope that SMA type II and III patients will also be benefitted with PRL treatment as hopefully increase in SMN levels upon treatment will ameliorate disease progression and will make the remaining motor neurons better. It will also be interesting to combine the effect of PRL with SMN2 transcript stabilizers (50) and/or neuroprotective compounds such as Y-27632 (Rho kinase inhibitor) (51).

Presently there is no cure of SMA. This study provides a good mechanistic insight of how SMN protein is regulated through PRL via STAT5 pathway (Fig 4.8) and its effect on the phenotype of the disease as well its potential for future therapeutic use for the treatment of SMA.

Figure 4.8. Proposed Model for PRL-mediated induction of SMN in motor neurons. PRL treatment causes phosphorylation and activation of STAT5 pathway which results in transcriptional upregulation of *SMN* gene, resulting in an increase in SMN mRNA levels and ultimately increases SMN protein expression.



Motor Neuron

Materials and methods:

Animals:

All experiments were performed in accordance with ACVS legislation. All protocols were approved by Animal Care and Veterinary Services and Ethics of University of Ottawa and all experiments were carried out in accordance with the CIHR (Canadian Institute of Health Research) Guidebook. CD-1 mice were obtained from Charles River Laboratory. The original breeding pair of heterozygous SMAΔ7 mice (*mSmn*+/-;*hSMN2*+/+, *hSMNΔ7*+/+) on the FVB background were provided by the Jackson Laboratory. The animals were maintained in an air-conditioned ventilated animal facility. Survival, righting time and weight were monitored daily as described by Aviva *et al* (33).

PRL administration

The mouse or human recombinant PRL was diluted in phosphate buffered solution (PBS) and administered through IP injection using a 30-gauge needle. Control animals received equal volumes of vehicle alone. SMAΔ7 mice mice were genotyped at P0 and PRL treatment was started from P1. Animals were sacrificed within twenty four hours of final PRL dose.

Reagents

PRL was purchased from Cedarlane labs and ATA was from Sigma. Non-silencing siRNA control and STAT5 siRNA (#6275) were supplied by Qiagen and Cell signaling respectively. The antibodies used for in this study are SMN/Smn (BD Transduction Laboratories), PRLR

(Abcam), Actin (Abcam), Tubulin (Abcam), Phospho-STAT5 (Cell signaling), Total STAT5 (Cell signaling) and HB9 (Abcam).

Primer sequences

For q-PCR

SMN - Human (amplifies full length transcript only)

Forward: 5'-GCTATCATACTGATACTGGCTATTATATGGGTTTTT-3'

Reverse: 5'-CTATAACGCTTCACATTCCAGATCTG-3'

Actin

Forward: 5'-CTGGAACGGTGAAGGTGACA-3'

Reverse: 5'-AAGGGACTTCCTGTAACAATGCA-3'

For genotyping

Genotyping was performed as previously described by Aviva *et al* (33) using the following primers

mSmn WT

Forward: 5'-TCTGTGTTCGTGCGTGGTGACTTT-3'

Reverse 1877: 5'-CCCACCACCTAAGAAAGCCTCAAT-3'

Lac Z

Forward: 5'-CCAACTTAATCGCCTTGCAGCACA-3'

Reverse: 5'-AAGCGAGTGGCAACATGGAAATCG 3'

Human *SMN2 trans*gene

Forward: 5'-CAAACACCTGGTATGGTCAGTC-3'

Reverse: 5'-GCACCACTGCACAACAGCCTG-3'

Product sizes:

mSMN: 372 bp

Lac Z: 626 bp

SMN2 transgene: 250 bp

(Supplemental Fig 3)

Cell Culture and Drug Treatment conditions

Human neuron-committed teratocarcinoma (NT2) or motor neuron derived (MN-1) cells were maintained in standard conditions (37^{o} C in a 5% CO_2 humidified atmosphere) in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS), 1% antibiotics (100 units/ml penicillin- streptomycin) and 2mM glutamate.

NT2 or MN-1 cells were seeded in 12 well plates (2.5×10^5cells/well) and treated 24h later with PRL (25, 50, 75 and 100 ng/ml) for 48h. For time course experiment, NT2 cells were seeded in 12 well plates (2.5×10^5cells/well) and treated 24h later with PRL (50 ng/ml) for up to 1h. For ATA Treatment; NT2 were seeded in 12 well plates (2.5×10^5cells/well) and treated 24h later with ATA (125 μM) for 24h.

Transfection

For siRNA transfections, NT2 cells were seeded in 12 well plates (2.0 x 10^5cells/well) and transfected on the following day in serum-free DMEM with STAT5 siRNA(50 nM) or non-silencing control siRNA (50 nM), using LipofectAMINE 2000 transfection reagent for 48h.

Quantitative RT-PCR

Total RNA was isolated according to the protocol provided by the manufacturer using the RNeasy kit (Qiagen). For quantitative RT-PCR, cDNA was reverse transcribed from isolated RNA with oligo dT_{18} primer using the First-Strand cDNA Synthesis kit from GE healthcare following manufacturer's instructions. The synthesized c-DNA template was used for quantitative PCR employing the QuantiTect SYBR green PCR kit (Qiagen) and analyzed on an ABI Prism 7000 sequence detection system using the ABI Prism 7000 SDS Software. Quantitative PCR was carried out to detect SMN and Actin genes using primers listed above.

Western Blot Analysis

Cells were washed 2 times with 1 ml PBS (1X) and lysed in 75µl RIPA buffer containing 10 mg/ml each of aprotinin, PMSF and leupeptin (all from Sigma), 5 mM β-Glycerolphosphate, 50 mM NaF and 0.2 µM sodium orthovanadate for 30 min at 4°C, followed by centrifugation at 13 000 x g for 15 min; supernatants were then collected and kept frozen at -20°C. Tissue samples were homogenized in 1 ml RIPA (10 mg/ml each of aprotinin, PMSF and leupeptin) and then sonicated for 15 seconds. Total protein concentrations were determined by Bradford protein assay using a Bio-Rad protein assay kit. For Western blot analysis, protein samples were separated by 10% SDS-PAGE. Proteins were subsequently transferred onto nitrocellulose membrane and incubated in blocking solution (PBS, 5% non-fat milk, 0.2% Tween-20) for 1 h at room temperature followed by overnight incubation with primary

antibody at 4°C at the dilution prescribed by the manufacturer. Membranes were washed with PBS-T (PBS, and 0.2% Tween-20) 3 times followed by incubation with secondary antibody (anti-mouse or rabbit, Cell signaling) for 1 h at room temperature. Antibody complexes were visualized by autoradiography using the ECL Plus and ECL Western blotting detection systems (GE Healthcare). Quantification was performed by scanning the autoradiographs and signal intensities were determined by densitometry analysis using Odyssey v1.1 program.

Immunofluorescence staining and confocal microscopy:

Brain stems and spinal cords where briefly rinsed in PBS, fixed for 6 hours in 2% paraformaldehyde in PBS and then transferred for cryopreservation into 30% sucrose/PBS prior to the making of the cryostat blocks. 20 µm sections were obtained with a cryostat, collected onto positively charged slides and air-dried for 1 hour at room temperature. The slides were then incubated for 30 minutes with 0.2% Triton X-100/PBS, briefly rinsed with PBS and then incubated with 10% normal goat serum in PBS. Blocking solution was discarded and the slides were then incubated overnight at 4 Cº with the mouse anti-SMN antibody diluted in PBS at 1:1000 (BD Transduction Laboratories™) and the rabbit anti-HB9 neuronal marker diluted in PBS at 1:1000 (Abcam). After incubation with the primary antibodies, the slides were rinsed 3 times for 10 minutes with PBS and then incubated for 1 hour at room temperature with goat anti-mouse Alexa Fluor® 488 and goat anti-rabbit Alexa Fluor® 568 (Invitrogen™) diluted at 1:1000 in PBS. The slides were then rinsed 3 times for 10 minutes with PBS, counterstained for 5 minutes with Hoechst 33342 (Invitrogen™) diluted at 10µg/mL in PBS and mounted with Dako Fluorescent Mounting Medium. Confocal microscopy was performed with an Olympus FluoView™ FV1000 confocal

microscope. Confocal microscope settings remained constant for each of the channels imaged. Channels were acquired in a sequential mode, the lasers output was set at 5% and the confocal aperture was set at 176 µm.

Statistical methods

GraphPad Prism software package (version 5; GraphPad Software) was used for the Kaplan–Meier survival analysis. The log-rank test was used and survival curves were considered significantly different at $P < 0.0001$. Data in figures (histograms, points on graphs) are mean values with the standard deviation (SD) shown as error bars.

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Chapter 5

General Discussion

SMA is one of the leading genetic causes of infant death worldwide. This neuromuscular disorder is caused by deletion or mutations in the *SMN1* gene resulting in reduced expression of full length SMN mRNA and full length SMN protein (1). Low levels of SMN protein leads to motor neuron dysfunction and death, resulting in muscle atrophy (1, 2), thus SMA is primarily considered as a motor neuron disease (3). However, the motor neuron exclusive autonomy of SMA pathogenesis is being challenged as a number of groups have recently documented the importance and requirement of SMN in peripheral tissues for SMA treatment in mouse models of the disease (4-7). Three 2010 reports documented significant cardiac defects in severe SMA mice; 1.) Beven *et al.*, reported compromised cardiac function resulting from early bradycardia, which is likely attributable to aberrant autonomic signalling. These defects were partially corrected by sc-AAV9-*SMN1* delivery in severe SMA mouse model (4). 2.) Heier *et al.*, showed that SMA mice suffer from severe bradyarrhythmia and impaired ventricular depolarization (5) and 3.) Shababi *et al.*, in severe SMA mouse model identified early-stage developmental defects along with pathological responses (fibrosis and oxidative stress markers) (6). Although mild congenital heart defects including septal defects have been documented in a subset of SMA patients (especially type I patients), the severity of the cardiac defects appears to be significantly greater in the mouse model of the disease. Further research is required to understand the underlying pathways which result in cardiac defects in human SMA patients and to clarify the relevance of these findings from SMA mouse model as we move new SMA therapeutics towards clinical trials. Hua et al, 2011 suggested the importance of the liver in SMA pathogenesis in a severe SMA mouse model (7). Developing pancreatic and glucose metabolism defects are also reported in a mild mouse model of SMA (unpublished data from Dr Kothary's lab). Hepatic and

pancreatic defects have not yet been documented in SMA patients suggesting the need for more research to unmask any unappreciated non CNS defects in human SMA patients, to determine the extent to which SMA mice models mimics the human SMA pathogenesis.

There is presently no cure for SMA. The most common experimental treatment strategy for SMA is the upregulation of SMN protein levels originating from the SMN2 gene compensating in part for the absence of functional *SMN1* gene.

New therapeutic approach for SMA via SMN RNA stabilization.

A considerable number of genes including SMN mRNA (8, 15) contain single or multiple copies of mRNA stability modulating AU rich elements (ARE) in the 3' UTR. Trans-acting factors such as ARE-binding proteins (AREBPs) can positively or negatively affect the stability of these ARE-rich mRNAs by either blocking or facilitating the AU-rich-mediated RNA decay pathway. I have shown here a role for an ARE in the stabilization of SMN2 with a consequent increase in SMN protein levels. I also document as the centrality of the AREBP, HuR, a ubiquitously expressed member of the ELAV protein family (9,10) in the post transcriptional stabilization of ARE containing SMN mRNAs through its binding to the 3'UTR region of the transcript (8). The p38 MAPK pathway is often involved in the post transcriptional regulation of ARE-rich mRNAs either through stabilizing/destabilizing them and determining their fate (11-15). We have documented that *in vitro* activation of the p38 pathway by the bacterial antibiotic anisomycin stabilizes and increases SMN mRNA. The activation of the p38 pathway causes cytoplasmic accumulation of the HuR protein which binds to the 3'UTR of SMN transcript resulting in increased SMN levels (8). This opens up a

novel potential therapeutic strategy for SMA. However, the inability of anisomycin to cross the blood brain barrier (BBB) and target CNS tissues may limit its potential as a therapeutic for SMA *in vivo* (although the motor neuron autonomy of SMA pathogenesis has recently been called into question). Another p38 activating drug, celecoxib shows promise for the treatment of SMA. Celecoxib activates p38 as does anisomycin, inducing SMN expression both *in vitro* in neuronal cells and *in vivo* in SMA mice model. It also moderates disease progression along with increasing the lifespan of SMA mice. Given that celecoxib can cross the BBB and is FDA approved, it may surpass drugs which are currently in the pipeline to treat SMA. However, the drug only increases the lifespan of SMA mice by 30-40% and the SMN induction appears to be limited to brain and spinal cord. The fact that there was very little increase in SMN levels in peripheral tissues in mice treated with celecoxib compared to the untreated group of mice suggests that celecoxib might be a good adjunct drug rather than to be in isolation for the treatment of SMA.

Prolactin as a therapeutic for SMA.

I have also documented that prolactin (PRL) through activation of the STAT5 pathway increases SMN levels transcriptionally both *in vitro* and *in vivo*. Treatment of SMA mice with PRL attenuates disease phenotype by reducing weight loss and improving motor function. The greatest upregulation in SMN mRNA and protein upon treatment with PRL was specific to the cells and mice transgenic for *SMN2* and was greater than that seen with any other drug to date (and was comparable to SMN levels in HET mice) (16). Despite such significant induction in SMN mRNA and protein levels in the neuronal tissues, PRL

treatment increased the life span of the mice only up to 70% longer than untreated mice. This raises a number of questions about its use as a therapeutic: whether it should be used earlier or at more frequent dosing or with a drug that targets other peripheral tissues or some pairing or combination of all three approaches. We have shown that PRL failed to increase SMN levels in heart; importantly SMA mice show severe cardiomyopathy which could be contributory or even causal of their death (16). There are some distinctions between murine SMA and humans; as outlined above in SMA patients the cardiomyopathy is not as severe as that observed in the SMA mouse. The treatment window is also likely much wider in humans than that of SMA mice which makes it easier to intervene early to treat pre-symptomatic infants (as long as they are diagnosed early in postnatal period). PRL is already used in human subjects and has a good safety profile (17), making it an ideal candidate to move forward to clinical trials for SMA. Small compounds which can activate the STAT5 kinase pathways should also be screened in SMA mouse model as an alternative for PRL, as production of GMP grade recombinant human PRL for clinical trials will be very expensive.

Future directions:

Combination therapy: The impressive results seen so far with gene therapy in the field of SMA will be difficult to equal with a monotherapy approach. However, unless and until gene therapy is cleared for clinical safety as a therapeutic option for SMA treatment, combinatorial approaches for SMA shall likely be necessary to target not only CNS but also other tissues which are affected because of a lack of SMN. As outlined above, SMA can be targeted through different approaches, we can in a safe combination therapy use compounds

which are already FDA approved and can increase SMN levels through SMN2 gene activation (such as PRL) along with SMN2 transcript stabilizers (p38 pathway activators such as celecoxib) and/or SMN protein stabilizer (proteasome inhibitor bortezomib) (18) and/or neuroprotective compounds (Rho kinase inhibitor) (19), or a cocktail of the best suitable combination of these compounds (Fig 5.1). I believe that this approach will speed up the process of finding the best possible and safest treatment of SMA. This approach is currently being assayed in our laboratory and others, showing some positive and promising results in the severe mouse model of the disease. More work is required to assess the potential drug interactions and their side effects in the animal models of the disease before pushing this approach for human clinical trials.

Designing clinical trials for SMA: In the last 5 years, a tremendous amount of promising translational work has been done using animal models of the SMA which is progressing rapidly towards the pre-clinical stage. However there are major challenges for designing a perfect clinical trial for SMA which includes 1) Variability of the disease phenotype, 2) lack of molecular biomarkers, 3) Accessibility of treatment centers and 4) lack of agreement for standard of care and disease management. However these issues are likely to be resolved as recently there has been a remarkable coorperation and collaboration between researchers, clinicians, industry, government and volunteer organizations which is bringing everyone on the same page to address these issues and reach a consensus for designing standard human clinical trials for SMA internationally.

Early intervention: New born screening: We and others have seen, irrespective of the modality, that early timing of the treatment is critical for maximum benefit in the mouse model of the disease. Presymptomatic identification of infants with SMA through new-born

screening represents an important step in the effective treatment of SMA. In essence we shall need to intervene before the damage is done; to do so we need to rapidly identify infants with SMA, cases who will also serve as the best candidates to show the efficacy of promising therapeutic treatments in the near future. Children in which the disease has already progressed may also benefit with the use of best combinational approach, however the aim will be more towards ameliorating the disease progress and preserving the function of remaining motor neurons and other tissues rather than a complete reversal of the disease phenotype.

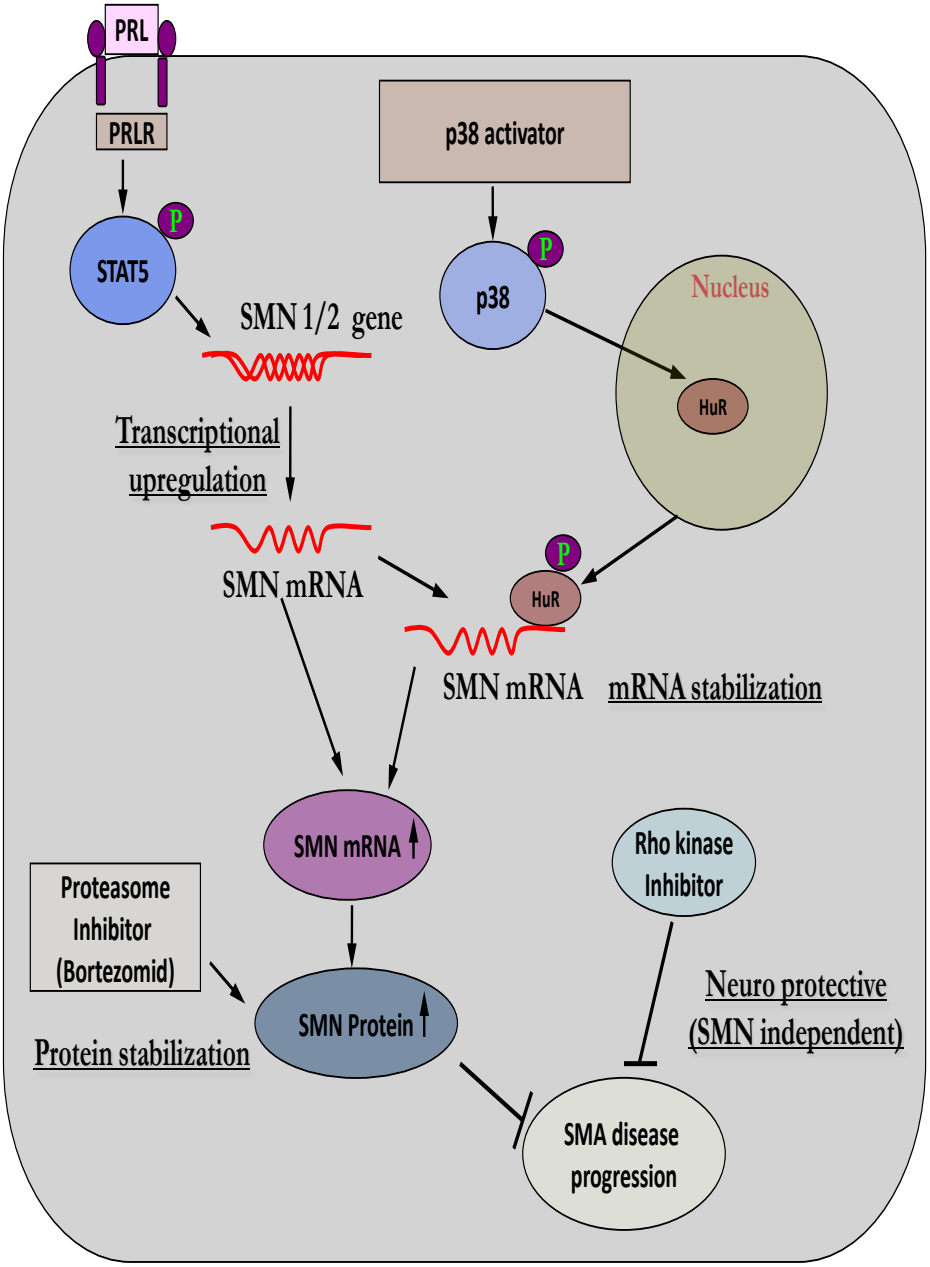

Figure 5.1: Model for combination therapy for SMA treatment.

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Appendix A:

Statistical methods for figures from Chapter-2 (Figures 2.1, 2.2, 2.3, 2.6 & Supplementary figure 2.1)

The Student's two-tail *t* test for paired variables was used to test for statistical differences between samples. The log-rank test was used and were considered significantly different at . *P< 0.05; **P< 0.01 and ***P< 0.0001 log-rank test.

Figure 2.1

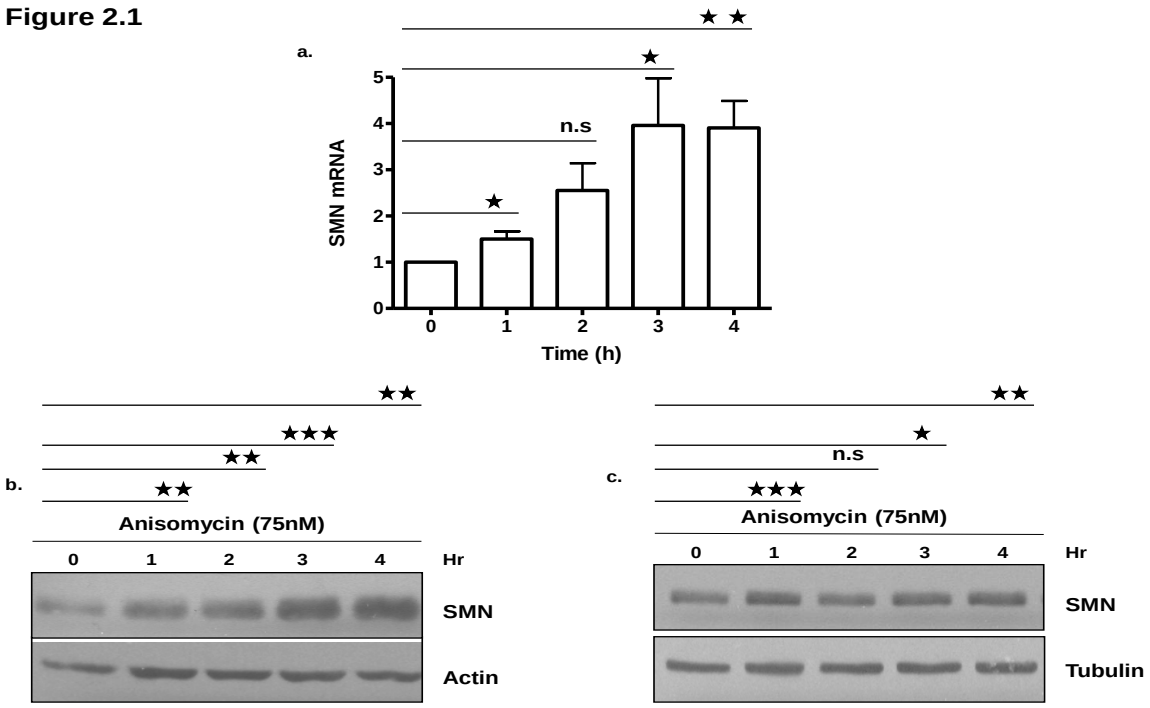


Figure 2.2

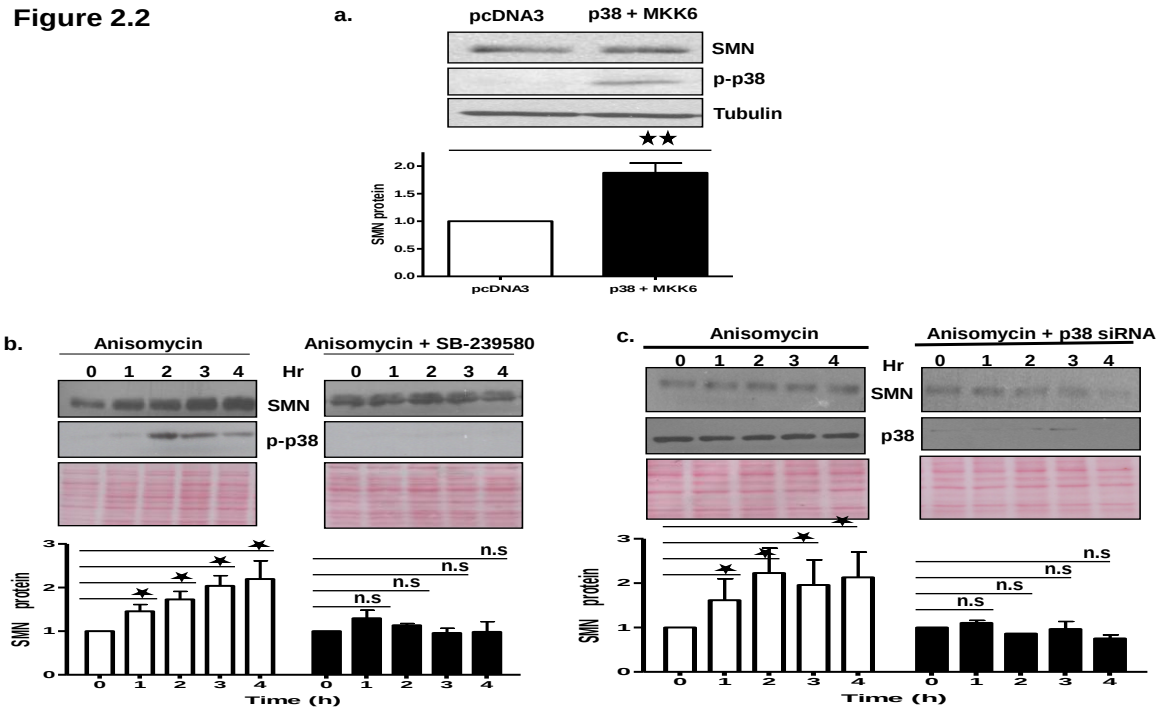


Figure 2.3

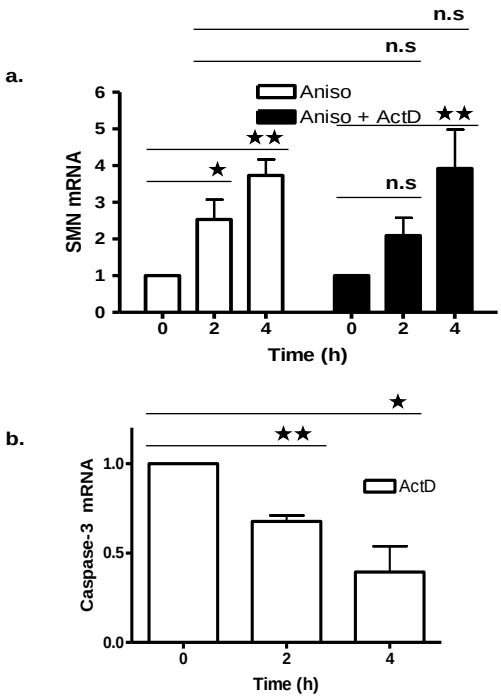
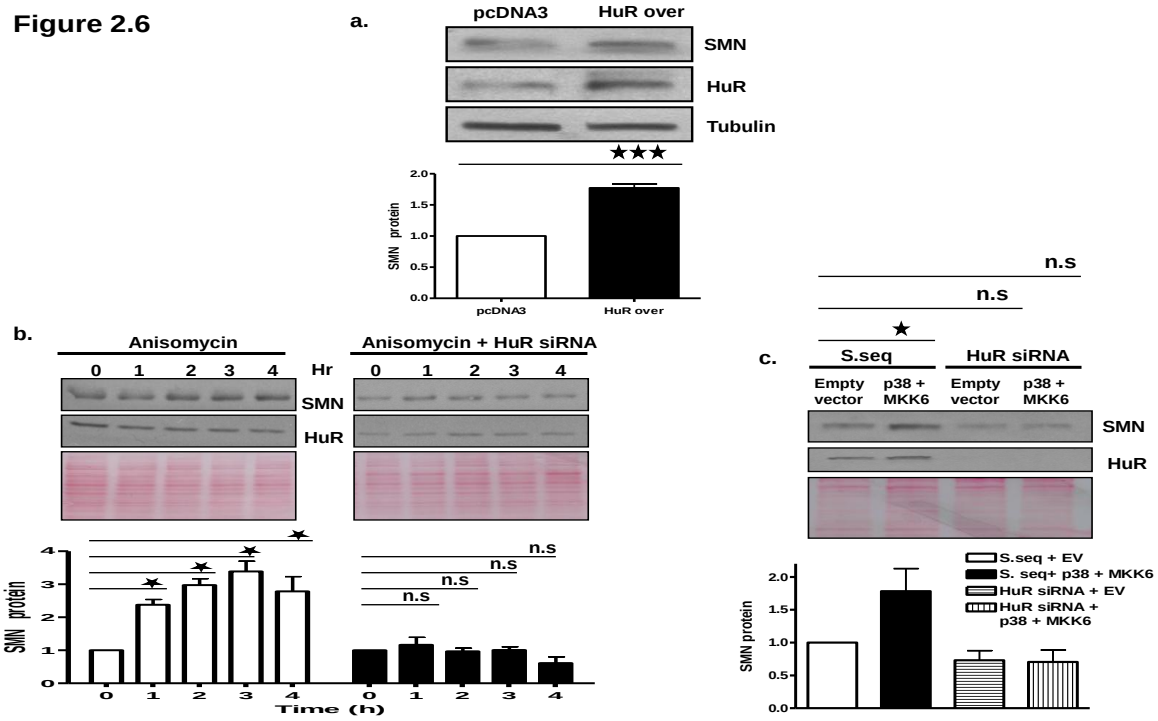


Figure 2.6



Supplementary Figure 2.1