EVALUATING THE EFFECTS OF INHIBITORS OF THE SODIUM HYDROGEN EXCHANGERS ON SURVIVAL MOTOR NEURON EXPRESSION IN SPINAL MUSCULAR ATROPHY

by

Sambee Kanda

A thesis submitted to the Faculty of the University of Delaware in partial fulfillment of the requirements for the degree of Master of Science in Biological Sciences

Winter 2018

EVALUATING THE EFFECTS OF INHIBITORS OF THE SODIUM HYDROGEN EXCHANGER ON SURVIVAL MOTOR NEURON EXPRESSION IN SPINAL MUSCULAR ATROPHY

by

Sambee Kanda

Approved: _____
Donna S. Woulfe, Ph.D.
Co-Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
Matthew E.R. Butchbach, Ph.D.
Co-Professor in charge of thesis on behalf of the Advisory Committee

Approved: _____
E. Fidelma Boyd, Ph.D.
Interim Chair of the Department Biological Sciences

Approved: _____
George H. Watson, Ph.D.
Dean of the College of Arts and Sciences

Approved: _____
Ann L. Ardis, Ph.D.
Senior Vice Provost for Graduate and Professional Education

ACKNOWLEDGMENTS

I would to thank my thesis advisor Dr. Matthew Butchbach for allowing me to work on my thesis project under his mentorship and guidance. I truly appreciate all of the personal and professional knowledge I have gained during the time I have worked in the Motor Neuron Disease Research laboratory at Nemours. I would also like to acknowledge my committee members Dr. Donna Woulfe and Dr. Erica Selva for see potential in me and pushing me to do some much more than I could ever imagine. Thank you for your perspective and input on my thesis project as well as career as a researcher.

I would also would like to acknowledge the current and previous members of the Motor Neuron Research Laboratory; Ryan Kirk, Cinsley Gentillon, Andrew Connell, Kyle Hinkle, and Acadia Grimme. Thank you all so much for your encouragement and support during my time in the Butchbach lab. Special thanks to Cinsley and Ryan for training me in many of the techniques I used to complete my thesis and for troubleshooting advice.

Additionally, I would like to acknowledge Dr. Melinda Duncan and Betty Cowgill. Thank you, Dr. Melinda for encouraging me to complete my application to the graduate program and recommending me for the Graduate Scholars funding. Throughout my time at UD, Betty Cowgill has always made sure that I was on track with my degree requirements so thank you for all that you do behind the scenes. I would like to thank my mentor Conroy Fields for all of your help with my preliminary exam and for all the encouragement

To the students of Nemours Research and Education Program (SNERP), thanks for being my research family. Special thanks to Katie Robbins, Moni Phay, Effie Halakos, Cinsley Gentillion, and Francyne Kubasos for taking me under your wings and being my student mentors at Nemours. You are all such wonderful intelligent women and I am grateful for the knowledge I have gained from each of you and for your friendship. Thank you to my first graduate school friends Adebomi Adejuwon, Christian Escobar, and Alex Stuffer for giving me a support system to lean on as I worked to achieve my masters. To my wonderful family and friends thank you for all of the patience, love, support, and understanding you all have shown me during the ups and downs of my thesis project.

Finally, I would like to acknowledge the University of Delaware for awarding me the Graduate Scholars Fellowship which funded me for two years. I would also like to thank the Nemours Foundation and NIH COBRE (P30GM114736) which funded my thesis work.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABSTRACT

Spinal muscular atrophy (SMA) is an inherited neuromuscular disease caused by the degeneration of alpha motor neurons in the anterior horn of the spinal cord which leads to generalized muscle weakness, hypotonia, and atrophy. SMA is caused by mutations or the loss of the *survival motor neuron 1* (*SMN1*) which encodes the SMN protein. Unique to the human genome is a duplicate copy of the *SMN1* gene known as the *SMN2*. The major difference between *SMN1* and *SMN2* is a cytosine to thymine (C-to-T) nucleotide transition located in exon 7. This single C-to-T transition is positioned in the middle of an exonic splicing enhancer sequence (ESE) which is responsible for regulating the inclusion of exon 7 in the SMN transcripts. In *SMN1*, there is a cytosine located in this region allowing for the inclusion exon 7 producing mRNA that encodes full length SMN (FL-SMN) protein. Because *SMN2* has a thymine in this position instead of a cytosine, the majority of the *SMN2* mRNA transcripts (80-90%) lack exon 7 (SMNΔ7) forming a truncated product. Due to the majority of *SMN2* mRNA transcripts lacking exon 7, the *SMN2* gene is unable to produce enough FL-SMN protein to compensate for the loss of *SMN1* leading to the development of SMA. Numerous studies have concluded that a higher copy number of the *SMN2* gene correlates with a milder phenotype of the disease. A current target for modifying the severity of the SMA is developing therapeutics that enhance *SMN2* gene transcription and /or promote exon 7 inclusion. Inhibitors of the sodium hydrogen exchanger (NHE) like 5(N-ethyl-N-isopropyl)-amiloride (EIPA) have previously been studied for their ability to modulate exon 7 inclusion and *SMN2*

protein expression. In this study several NHE inhibitors were evaluated for their capability in modulating *SMN2* expression. As reported in the previous study, EIPA was identified as a modulator of exon 7 inclusion as well as another compound known as 5(N, N-hexamethylene)-amiloride (HMA). EIPA and HMA may modulate the inclusion of exon 7 due to their ability to specifically inhibit the NHE5 isoform which is the isoform found in neural and skeletal muscle tissues.

To improve current tools used to screen small molecular compounds that increase full length *SMN2* mRNAs through the activation of the *SMN2* promoter or by inducing the inclusion of exon 7 in *SMN2* pre-mRNAs two novel reporter cell lines were also developed. The two cell lines developed will more closely mimic *SMN2* gene regulation *in vivo* than the reporter assays currently used since it will be able to account for distal elements that may play a role in the regulation of *SMN2*. Development of these novel reporter cell lines will allow for a more accurate evaluation of previously discovered *SMN2* regulators like the NHE inhibitors as well as allowing for the development of new therapeutics for SMA patient

Chapter 1

INTRODUCTION

1.1 Spinal Muscular Atrophy

Spinal muscular atrophy (SMA) is an inherited neuromuscular disease primarily affecting children. SMA is caused by the degeneration of alpha motor neurons located in the anterior horn of the spinal cord. Loss of the alpha motor neurons is accompanied by severe generalized muscle weakness, hypotonia, and atrophy (Kolb and Kessel, 2015; Butchbach 2016). SMA is a leading genetic cause of infant death worldwide, as well as the second most common autosomal recessive disorder. Approximately 1 out of every 10,000 newborns is affected SMA with 1 in 25 to 1 in 50 people being carriers in most populations (Sugarman et al., 2012; Tisdale and Pellizzoni, 2015; Butchbach, 2016). Spinal muscular atrophy is categorized into five clinical grades (SMA 0-IV) based on the age of onset, as well as the severity of the disease (Table 1.1) (Russman 2007; Sugarman et al., 2012).

Type 0 SMA, also known as fetal SMA, is present in the neonate prior to birth causing serve hypotonia and required respiratory intervention from birth. Newborns with Type 0 SMA cannot survive pass the age of 6 months (Ahmad et al., 2016; Gabanella et al., 2007; Butchbach, 2016). Type I SMA (Werdnig-Hoffman disease) is the most prevalent form of SMA accounting for around 50% of patients diagnosed

with the disease. The onset of type I occurs before 6 months of age. Typical symptoms include weak intercostal muscles, inability to sit up without intervention, and hypotonia of the limbs. Due to their weak intercostal muscles, patients with type I SMA have a bell-shaped chest, but a fairly normal diaphragm. The shape of their chest causes type I SMA infants to have abnormal breathing patterns. These patients typically live to about 2 years of age (D'Amico et al., 2011; d'Ydewalle et al., 2015; Butchbach 2016). The onset of type II SMA usually occurs during 7 to 18 months of age. Children with type II SMA usually gain the ability to sit up without aid but are poor crawlers and unable to walk or stand without assistance. In most cases, their leg muscles are weaker than their arms. The life expectancy for patients with type II SMA is into early adulthood due in part to supported care. The mildest form of childhood SMA is known as type III SMA. The majority of patients with type III SMA are able to walk independently, but have difficulty doing so since the muscles in their legs tend to be weaker than their arms. Patients with type III SMA can live the normal life expectancy. The onset of type IV SMA occurs in early adulthood (18-21 years old) with patients displaying symptoms of slow progressive weakening of the limbs. Type IV is considered to be a very mild form of SMA with the disease being non-life threatening in most patients (Lunn et al., 2008; D'Amico et al., 2011; d'Ydewalle et al., 2015).

Type	*SMN2* Copy Number	Age of onset	Clinical symptoms	Life expectancy
0	1	Prenatal	Require respiratory assistance from birth, unable to sit up or walk	< 6 months
1	2	> 6 months	May require respiratory assistance cannot sit up, stand, or walk	< 2years
2	3	6-18 months	Can sit up with assistance cannot stand or walk	10-40 years
3	3-4	> 18 months	Can sit up but required assistance to walk	Adult
4	> 4	> 5 years	Symptoms are mild or benign	Adult

Table 1.1 Classification of SMA clinical grades. SMA patients are characterized into 5 different subgroups based on the age in which the disease presents its self and the severity of the symptoms [Adapted from (Butchbach, 2016)].

1.2 Genetics of SMA

The spinal muscular atrophy locus has been identified to be positioned on the long arm of chromosome 5, specifically in the 5q13 region. Within this region are four coding genes which are the *survival motor neuron* (*SMN*), *neuronal apoptosis inhibitor protein* (*NIAP*), *general transcription factor IIH*, *p44*; *(GF2H2T),* and a small *EDRK- rich factor 1A*, *H4F5A*; (*SERF1A*) (Carter et al., 1997; Butchbach, 2016). SMA is an autosomal recessive disorder caused by a deletion or mutation in *SMN*. Unique to the human genome are two copies of the gene known as *SMN1* (telomeric copy) and *SMN2* (centromeric copy) due to a segmental duplication within chromosome 5 (Figure 1.1) (Rochette et al., 2001; Tisdale and Pellizzoni, 2015).

Multiple copies of the *SMN2* gene can exist in the human genome due to the instability of the 5q chromosomal duplication. The *SMN2* gene is virtually identical to *SMN1* other than a five nonpolymorphic nucleotide differences within the 3' region of gene (Lorson et al., 1999; Butchbach, 2016). Only the loss or mutation of *SMN1* has been shown to correlate with the development of spinal muscular atrophy supporting the fact that greater than 95% of patients diagnosed with SMA seem to retain their *SMN2* gene while the *SMN1* gene is lost (Butchbach, 2016). The remaining 5% of SMA cases are caused by small deletions, splicing mutations, and missense mutations (refer to Burghes and Beattie, 2009 for list of mutations within *SMN1* associated with SMA).

The major difference between *SMN1* and *SMN2* is a cytosine to thymine (C-to-T) nucleotide transition located on exon 7 that translationally silent (Monami et al., 1999; Lorson et al., 1999; Butchbach, 2016). This single transition is located in the middle of an exonic splicing enhancer sequence (ESE) within exon 7. This ESE is responsible for the regulation of the inclusion of exon 7 in the SMN transcripts. ESEs are binding sites for regulatory trans-acting proteins, such as the arginine/serine rich (SR) proteins. The SR proteins are recruited by CA- and purine-rich cis elements. SR proteins enable splicing through protein-to- protein and protein-to-RNA bridges essential in the recruitment of splicing factors and small nuclear ribonucleoproteins (Lorson and Androphy, 2000; Butchbach 2016). The SR proteins, which are required for proper RNA splicing and regulation, consist of an arginine/serine- rich domain and one or more RNA recognition motif (RRM) whose purposes are to assist in the mediation of protein-protein interactions and in RNA binding. ESEs are able to

compensate for suboptimal splice elements allowing constitutive inclusion of non-consensus exons. The ESEs contain two binding motifs, which are purine- rich enhancer consisting of guanine and adenosine rich regions (GAR regions with "R" being either a G or A) and ACEs which are cytosine and adenosine rich regions. The SR proteins can bind to these motifs located on the exonic splicing enhancers and recruit other splicing machinery and complexes initiating the formation of the splicing complex (Lorson and Androphy, 1999).

Three alternatively spliced *SMN2* transcripts have been observed: transcripts lacking exon 5, exon 7, or both exons 5 and 7 (Lorson et al., 1999). This C-to-T transition in exon 7 causes the binding of the SR protein and splicing activators ASF/SF2 not to be recruited while generating an inhibitory binding element for proteins such as hnRNPA1 and Sam68 that regulate *SMN2* pre-mRNA splicing (Cherry et al., 2014). In *SMN1*, the cytosine at this position allows for the inclusion of exon 7 leading to the production of mRNA that codes for full FL-SMN protein. Because *SMN2* has a thymine in this position instead of a cytosine, the majority of the *SMN2* mRNA transcripts (80-90%) lack exon 7 (SMNΔ7) forming a truncated product (Figure 1.1). Due to the majority of *SMN2* mRNA transcripts lacking exon 7, the *SMN2* gene is unable to produce enough full length SMN protein in order to compensate for the loss of *SMN1* leading to the development of SMA (Burghes and Beattie, 2009; Burnett et al., 2009; Butchbach,2016).

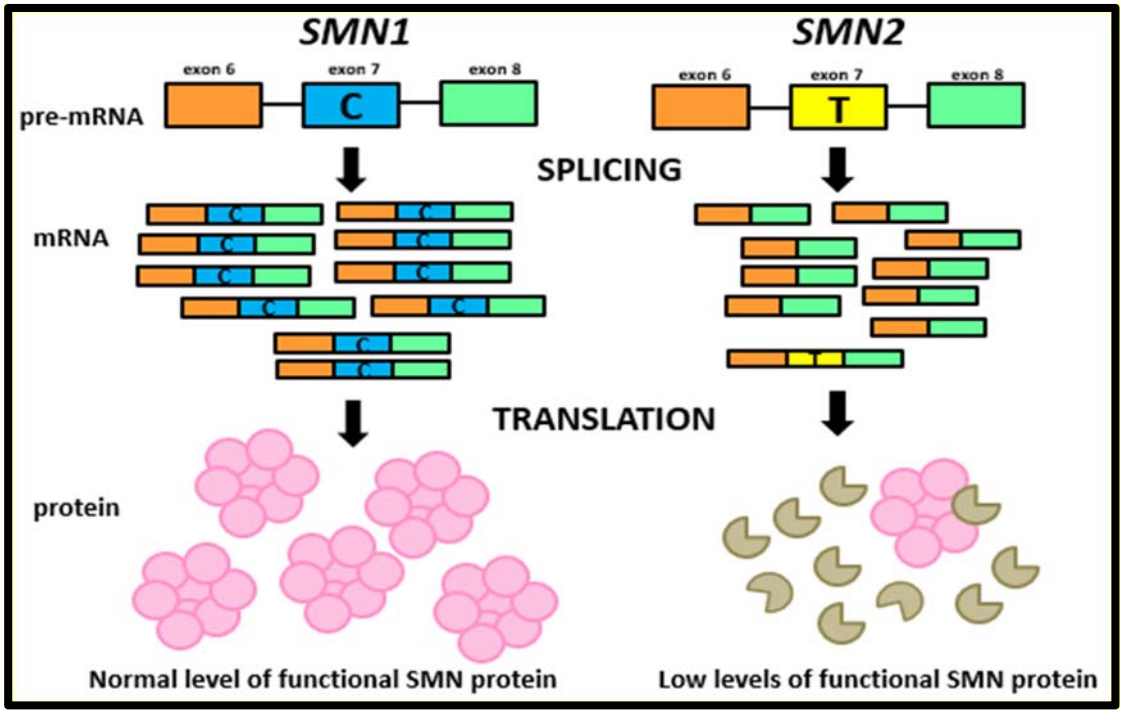


Figure 1.1 Survival Motor Neuron Gene. Majority of *SMN2* mRNA lack exon 7 due to a C-to-T nucleotide transition causing the exon to be spliced. mRNA lacking exon 7 produces truncated protein that is nonfunctional (Adapted from Butchbach 2016).

It has been shown that *FL-SMN* and *SMN*Δ*7* mRNA have comparable half-lives in primary fibroblasts culture, implying the difference in protein levels is not caused by mRNA instability (Heier et al., 2007; d'Ydewalle et al., 2015). The protein produced by the *SMN1* is able to form functional complexes while the bulk of protein produced from *SMN2* transcripts is truncated due to the loss of exon 7 causing the protein to be unstable and unable to associate with itself. Although loss of or mutations in the *SMN2* gene has not been shown to cause the development of SMA, it does play a role in modifying the severity of the disease. Various studies have shown an inverse relationship between the copy number of the *SMN2* gene and the severity of

the disease (Taylor et al., 1998). The number of *SMN2* copies in an individual's genome can vary from 0 to 8 copies (Butchbach, 2016). Patients with milder forms of SMA tend to have higher copy numbers of the *SMN2* gene in their genomes. The majority of type I SMA patients contains two copies of *SMN2*, SMA type II patients retain three copies, while type III and IV SMA patients generally have three or four copies of the *SMN2* (Table 1.1). Although in the majority of SMA this relationship holds true, there are reported cases in which patients with type II or type III SMA have only two copies of *SMN2* instead of the predict number. In these cases, a rare single nucleotide variant is present. This nucleotide variant allows for a greater ratio of the *SMN2* transcripts to contain exon 7 (Prior et al., 2009).

The inverse relationship between the SMA severity and *SMN2* copy number was further strengthened through studies performed on SMA mouse models. Unlike humans, mice have only one copy of the *SMN* gene, *mSmn* which shares 82% identity with the human *SMN1* gene (DiDonato et al. 1997; Viollet et al. 1997). Conditional *mSmn* knockout (*mSmn* $^{-/-}$) models lead to major cell death in the murine embryos resulting embryonic lethality (Schrank et al., 1997). Transgenic mice carrying human *SMN2* were then developed to test if *SMN2* has the ability to rescue the knockout mice from embryonic lethality creating a mouse SMA model that can be correlated to human SMA patients. *mSmn* knock-out mice expressing one copy of *SMN2* at birth looked phenotypically normal but postnatal 48 hours they began to show signs of decreased movement and decreased or lack of suckling. These mice died shortly after birth. Transgenic mice expressing two *SMN2* copies showed reduced number of motor

neurons by day 5 and showed poor activity with a variety of symptoms seen in SMA patients. These mice only survived for proximately 8 days postnatal (Hsieh-Li et al., 2000; Monani et al., 2000). Mice harboring three copies of *SMN2* were found to show a very mild SMA phenotype. These mice had a delayed onset of SMA symptoms as well as a slower disease progression and longer survival rates than SMN mice expressing two *SMN2* copies. Mice expressing four copies showed an even milder phenotype marked by a progressive degeneration of their extremities (Michaud et al., 2010). Transgenic mice expressing a high copy number of the *SMN2* gene (8-copies) seem to rescue the SMA phenotype (Monani et al., 2000). This finding further supported that SMN2 is a important disease modifier in SMA.

1.3 Survival Motor Neuron (SMN) Protein

SMN protein is a 294-amino acid polypeptide projected to be around 38 kDa in size (Lorson et al., 1999). The *SMN* gene, does not seem to have any apparent sequence similarity to other known proteins (Fischer et al., 1997). SMN is a ubiquitously expressed protein found in both in the nucleus and cytoplasm. Contained in the SMN protein is a phylogenetically conserve sequence known as the tudor domain. The tudor domain is found in many proteins involved in RNA metabolism (Bühler et al., 1999; Meister et al., 2002). In the cell, the SMN protein is found in distinct spherical nuclear bodies (Lui et al., 1996; Carvalho et al., 1999; Lorson et al., 1999). Due to the fact that these domains are found in close proximity or overlap with

Cajal bodies, these structures are named gemini of Cajal bodies (gems). Cajal bodies are nuclear domains enriched with U- rich small nuclear RNPs (U snRNPs), U small nucleolar RNPs (snoRNPs) (Carvalho et al., 1999; Gubitz et al., 2004) and the three eukaryotic RNA polymerases (Gubitz et al., 2004). Because Cajal bodies lack DNA, nascent pre-mRNA, and non-snRNP essential splicing factors, they are not presumed to be the active sites for transcription or splicing. Instead Cajal bodies are believed to be the sites in which assembly and modifications of nuclear transcription and RNA processing machineries takes place (Gubitz et al., 2004) as well as trafficking and regeneration and histone mRNA 3' processing (Paushkin et al., 2002). In the nucleus of most adult tissue and cell lines, Cajal bodies and gems overlap, but are separate in fetal tissue. Methylation of coilin, a marker for Cajal bodies, was found to regulate the interaction between Cajal bodies and SMN with their associated gems. The number of gems per cell can vary from 2 to 8 depending upon the cell type (Pellizzoni, 2007).

Although SMN is ubiquitously expressed in all cell types, it is unclear why motor neurons seem to be more sensitive or vulnerable to loss of SMN. It is believed that the decreased level of full length SMN may affect the motor neurons' splicing machinery in a more serious manner than other cell types (Tizzano et al., 1998; Burghes and Beattie, 2009; Simone, 2016). There are currently two proposed hypothesis, which aim to account for the mechanism of SMA disease progression. The first hypothesis states that the loss of *SMN1* leads to a reduction in snRNP assembly. This disruption causes an alteration in the splicing in the motor neurons leads to a reduction in essential transcripts (Eggert et al., 2006; Gabanella et al., 2007; Pellizoni

et al.,2007). The second hypothesis proposes SMN function in the axon is to transport mRNA. Low levels of SMN protein disrupt this process drastically in neurons leading to the progression of SMA (Fan et al., 2002; Carrel et al., 2006; Eggert et al., 2006; Gabanella et al., 2007; Pellizoni et al.,2007). Although there have been multiple studies done providing evidence for these proposed hypothesis, the disease progression of SMA is still unclear. It may also be possible that snRNP assembly may preferentially affect the splicing of essential axonal mRNAs connecting the two leading hypothesis (Burghes and Beattie et al., 2009).

1.4 Functions of SMN Protein

The most characterized function of the SMN complex is in the assembly of small nuclear ribonucleoproteins (snRNPs) of the major (U2-dependent) and minor (U12-dependent) spliceosomes (Burghes and Beattie, 2009). The SMN complex includes the SMN protein as well as integral components called gemins. Gemins have a similar subcellular distribution as SMN and they also localize in gems (Burghes and Beattie, 2009; Pellizzoni, 2007). SMN directly interacts with the following gemin proteins: gemin 2(also known as SIP1), gemin3/dp103 (a DEAD-box RNA helicase), gemin5 (a WD-repeat protein), and gemin7 (Paushkin et al., 2002). Gemin4 and gemin6 indirectly associate with the SMN protein through gemin4 and gemin6 interacting with gemin3 and gemin7 (Pellizzoni, 2007). Gemin8 mediates the binding of the gemin6 and gemin7 to the SMN protein, as well as the binding of UNR

interacting protein (UNRIP also called STRAP) to the complex (Burghes and Beattie, 2009; Pellizzoni, 2007; Tisdale and Pellizzoni, 2015). Unlike the other protein components that bind to the SMN complex, UNRIP does not localize in gems or Cajal bodies. The assembly of gemin 2-8, unrip, and SMN requires ATP to occur (Burghes and Beattie, 2009; Carissimi et al., 2005; Carissimi et al., 2006).

During transcription, mRNA is first synthesized as pre-mRNA. After RNA polymerase II transcribes about 25-30 nucleotides, 7-methylguanosine cap is then added to the 5' end of the mRNA. RNA polymerase II continues to transcribe the mRNA until it reaches the termination sequence of the final exon. The transcript is then cleaved at a the 3' poly (A) site followed by the addition of around 250 adenine residues known as the poly-A tail (Moore and Proudfoot et al., 2001; Wahl et al., 2009). Noncoding sequences in pre-mRNA transcripts are removed by macromolecule complexes known as spliceosomes (Pellizzoni, 2007). Once RNA polymerase II finishes transcribing the mRNA, proteins including hnRNP and SR proteins will bind. Pre-mRNA will then be processed by uridine rich small nuclear RNPs (U snRNPs) (Wahl et al., 2009). The main components of spliceosomes are the U-type snRNPs which include U1, U2, U5, and U4/U6 (Burghes and Beattie, 2009; Tisdale and Pellizzoni, 2015) as well as a group of RNA-binding factors known as the SR proteins (Lorson and Elliot, 1999). While the splicing of pre-mRNA into mRNA takes place in the nucleus of a cell, the actual biogenesis of the spliceosome occurs in both the nucleus and cytoplasm of eukaryotes. Each spliceosomal snRNP consists of one (U1, U2, and U5) or two (U4/6) snRNAs.snRNPs (excluding U6) are composed of a small

nuclear RNA (snRNA), seven common Sm proteins, as well as proteins specific to that particular snRNAs (Wan et al., 2005; Gabanella et al., 2007).

Sm proteins belong to a family of proteins characterized by their retention of the Sm motif composed of two regions known Sm1 and Sm2. The seven common Sm proteins contained in snRNP are B/B′, D1, D2, D3, E, F, and G (Wan et al., 2005). These Sm proteins are organized into a very stable heptameric ring forming the Sm core. These Sm proteins bind to the snRNA in two steps. Sm proteins E, F, and G as well as D1 and D2 associate and then are immediately converted into the Sm core domain once B/B′ and D3 are added to the complex. The Sm core is positioned on a uridine-rich sequence motif flanked by two hairpin loops or the Sm site on snRNAs. The rest of the snRNA domains are binding sites for snRNA-specific snRNP proteins and for RNA to RNA interactions. Unlike the other small nuclear ribonucleoprotein particles, U6 contains a γ-monomethyl cap instead in place of the m3G cap which prevents the Sm proteins from binding to U6 directly since it does not contain a Sm site (Fischer et al., 1997; Burghes and Beattie, 2009; Wan et al., 2005).

Proper assembly of Sm cores is required for modifications of the snRNA cap, as well as for the stability and function of the snRNPs. Once assembly and modification of the snRNPs are completed, the snRNPs are imported into the nucleus where their specific proteins will associate to them creating the functional form of the snRNPs (Narayanan et al., 2002). The snRNP will continue to mature in the nucleus until it is needed for the splicing of pre-mRNA (Nesic et al., 2004; Stanek et al., 2004; Matera et al., 2006; Coady and Lorson et al., 2011). The role of the SMN in the

maturation of the snRNP in the nucleus is currently still poorly defined. It is currently believed that SMN helps to assist in the maturation of the small nuclear ribonucleoprotein through its association with the SMN-interacting protein 1 (SIP-1 also known as gemin2) and Sm proteins (Fisher et al., 1997; Liu et al., 1997). SMN and SIP1 can both be located in the cytoplasm and associate strongly as a heterogenic protein complex. It has already been established that the SMN/SIP1 complex are essential for spliceosomal snRNP biogenesis (Fisher et al., 1997; Pellizoni et al., 1998). There are two distinct domains located on the SMN protein allowing for its interaction with several Sm proteins and with SIP1; The SMN/SIP1 complex associate in the cytoplasm with snRNAs U1 and U5 snRNAs but have not been shown to associate with nuclear snRNPs other RNAs that have been looked at thus far showing that the complex is not involved in the mature nuclear snRNPs. Rather, the SMN/SIP1 complex only associates with the U1 and U5 snRNA during the cytoplasmic phase of their biogenesis (Fisher et al., 1997; Meister et al., 2002). SIP1 also permits the assembly of the Sm core domain of spliceosomal U snRNAs as well as their nuclear import (Fischer et al., 1997; Wang et al., 2001).

The biogenesis of snRNPs is an intricate process requiring bidirectional transport of the snRNAs across the nuclear envelope snRNAs are first transcribed in the nucleus by RNA polymerase II where they will acquire a 5′-terminal monomethyl (m7G) cap (Fischer et al., 1997; Sleeman et al 1999; Kiss, 2004; Ohno et al., 2000; Pellizoni et al., 2007). Even though the Sm proteins are synthesized in the cytoplasm, they are unable to migrate on their own into the nucleus. Alternatively, snRNAs are

exported from the nucleus to the cytoplasm (Lührman et al., 1990; Fischer et al., 1993; Hamm et al 1999; Ohno et al., 2000) where the Sm proteins bind to the Sm site on an snRNA to form the Sm core. The assembly of Sm core occurs around a conserved sequence of snRNAs (Sm site) in the nucleus (Meister et al., 2002; Pellizoni et al.,2007). Before binding to the SMN complex, the Sm proteins will associate with both protein arginine methyltransferase 5 (PRMT5) complex (also referred to as methylsome) and the chloride-conductance regulatory protein (pICln also referred to as ICLN) in the cytoplasm (Meister et al., 2000; Pellizzoni et al., 2002; Gubitz et al., 2004; Pellizoni et al., 2007). PRMT5 proportionally dimethylates the SMN protein increasing its affinity for binding to the SMN complex. Hypermethylation of the m7G cap leads to the formation of the m3G cap allowing for the assembled U snRNP to be imported into the nucleus (Narayanan et al., 2002). The m3G capped spliceosomal snRNPs is believed to be the crucial step allowing the snRNP to be imported by generating a nuclear import signal only when both the m3G cap as well as the Sm core domain are formed (Fischer et al., 1993; Massenet et al.,2002).

The SMN complex will then bind to the seven Sm proteins and interact with spliceosomal snRNA mediating the assembly of the Sm core. The binding of SMN to Sm core is mediated directly by the tudor domain. This binding is enhanced by a SMN oligomerization located in exon 6. During snRNP biogenesis, the SMN protein also interacts with trimethyl guanosine synthase 1 (TGS1) which is believed to aid to the hyper methylation of snRNA after the assembly of the Sm core occurs (Mouaikel et al., 2003). Together with a protein known as snurportin, the SMN protein binds

importin-β which then allows the nucleus to import the snRNP (Narayanan et al., 2004). Although Sm protein have the ability to form Sm core on snRNA, that are thermodynamically stable without the assistance of additional proteins (Raker et al., 1999), the Sm site does not contain the adequate information needed for the Sm proteins to distinguish the site from other RNAs located in the same vicinity (Pellizoni et al., 2002). SMN protein works as an RNP chaperone by binding directly to both the Sm proteins and snRNAs ensuring that the Sm proteins only assemble on their target RNAs increasing the specificity as well as the efficiency of snRNP assembly (Pellizzoni et al., 2002, Young et al., 2004; Golembe, 2005; Pellizzoni, 2007).

SMN is sometimes referred to as the master ribonucleoprotein assembler even though it still remains poorly defined what other RNP SMN is responsible for assembling (Terns MP and Terns RM, 2001). A majority of the SMN RNA--binding protein targets have RG and RGG domains including the Sm-like proteins (LSm proteins) which are very similar to the Sm proteins used in the assembly of the snRNP. Like the Sm proteins, the LSm proteins are also form a heptameric ring that will bind to the snRNA (Friesen et al., 2000; Kiss,2004; Khusial et al., 2005; Burghes and Beattie, 2009). LSm10 and LSm11 are able to form the heptameric ring structure with Sm proteins that will then go on to bind with the U7 snRNA with the assistance of the SMN protein (Pillai et al., 2003; Tisdale and Pellizzoni, 2015). During this time, LSm protein 2-8 located in the nucleus will also form a ring structure on U6 (Kiss,2004; Khusial et al.,2005). LSm proteins 1-7 ring formation takes place in the cytoplasm where its primary function consists of mRNA decay and stability (Kiss, 2004; Khusial

et al., 2005; Tharun et al., 2005; Bergman et al., 2007). The LSm1 and LSm4 have more recently been shown to be present in dendrites and particular axons including the axons of the spinal cord (di Penta A et al., 2009). The RNP complexes located in these axons and dendrites contain SMN protein as well as being connected with RNAs transport (di Penta A et al., 2009). The function of SMN in the assembly of the LSm ring has not clearly been established, but both pICln and SMN have been shown to be able to bind LSm4 which could potentially mean that SMN plays a role in the assembly of the LSm core (Friesen et al., 2000; Brahms et al., 2001; Gandini et al., 2008; Burghes and Beattie, 2009).

The RG rich regions of the SMN complex substrates play and important role in their association with the SMN complex which was seen when deletions of the RG rich domains in SMN complex substrates were shown to inhibit their ability to bind the SMN complex. SMN complex is thought to interact with its substrates by methylation of the arginine residues in these RG domains like in the case of the symmetrical dimethylarginine-modified RG domains that occurs in the Sm proteins (Pellizoni et al., 1999; Friesen et al., 2001; Pellizoni and Baccon et al., 2001). Although the majority of SMN-complexes have RG and RGG rich domains, the SMN complex is able to associate with substrates that do not contain RG-rich motifs and have no known interaction with ribonucleoproteins including ZRP1, profilin, p53 and FUSE-binding protein (Giesemann et al., 1999; Williams et al., 2000; Gangwani et al., 2001; Young et al., 2002; Paushkin et al., 2002).

Subsequently after each assembly of the Sm core, the Sm protein used must be replenished with new Sm proteins in order for the SMN complex to regain function. Recent findings have shown that this essential step may be regulated by the PRMT5 complex (WT Pu et al., 1999; Friesen et al., 2001; Meister et al., 2001). pICln, and components of the PRMT5 complex were once believed to be nucleotide-sensitive factors of chloride channel (Krapivinsky et al., 1994) but were recently found to be part of a complex containing Sm proteins, PRMT5, and WD45. In *Xenopus* oocytes, pICln is an inhibitory factor of U snRNP assembly. Once bound to the Sm motif, the pICln prevents formation of the Sm core domain (WT Pu et al., 1999; Friesen et al., 2001; Meister et al., 2001). *Xenopus* oocytes were also seen to compile Sm proteins for biogenesis of snRNPs during different stages of development, suggesting the PRMT5 complex as the storage site for Sm proteins (WT Pu et al., 1999). The PRMT5 sequence has homology with arginine methyltransferases (PRMTs) (Frankel and Clark,2000). The most well-known PRMTs are classifies as type I based on their common ability catalyzes the formation of asymmetric dimethylarginines in proteins with the Arg-Gly-Gly tripeptide motifs. PRMT5 has been shown to act similarly as a methyltransferase, but is considered to be a type II enzyme that because it converts arginine to symmetrical dimethylarginines (sDMAs) (Branscombe et al., 2001; Friesen et al., 2001; Meister et al., 2001). These findings on PRMT5 are further supported by the fact that the Arg-Gly-Gly rich sequences in the carboxyl terminal of Sm B/B′, D1, and D3 contain sDMAs and the PRMT5 complex is able to catalyze this modification. Interestingly, sDMA modification increases the affinity of these Sm proteins. Overall,

it is believed that the PRMT5 complex is able to activate Sm proteins by modifying the sDMA allowing for their transfer to the SMN complex. Expanding the function of the PRMT5 complex to activating the Sm proteins for transfer to SMN complex and stimulating the activity of the SMN complex for the assembly reaction (Meister et al., 2002).

While it has been shown that snRNP biogenesis in SMA patients capacity is decreased, it is still occurring since it is required in all cell types. The need for snRNP biogenesis to sustain life has created debate on why SMA is a neuromuscular disease and if snRNP biogenesis is truly its main function of SMN (Monani et al., 2005; Gabanella et al., 2005; Pellizonni et al., 2007). In regard to the transport of mRNA being disrupted in neurons due to the decreased function of the SMN protein, previous *in vivo* studies have already documented the presence and localization of the SMN in axons and dendrites of spinal cord neurons (Rossoll et al., 2003; Todd et al., 2010; Zhang et al., 2006; Sharma et al., 2005; Fan et al., 2002). The hypothesis that SMN played a role in the transport of mRNA was reinforce in an *Smn* knockdown in zebrafish showing motor neuron axonal defects such as a truncated axon, a branched axon, or a combination of the two (McWhorter et al., 2003). Due to the fact that during visualization of cultured neuron actions it is hard to distinguish between whether granules in axons are in fact a part of the axon or located in a nearby cell, it is hard to pinpoint if these observed defects are related to the lack of snRNP assembly or due to another function of SMN (McWhorter et al., 2003; Breise et al., 2006; Burghes and Beattie, 2009). Smn was also shown to localized within granules in neurons and

is constantly being transported into developing neurites and growth cones (Rossoll et al., 2003). A study using primary hippocampal, primary motor neurons and ES cell-derived motor neuron further proved this by showing the colocalization of SMN, gemin2, and gemin3 in granules supplying neuronal processes and growth cones with SMN associating with gemin2 about 40% and gemin3 about 48% in the growth cones (Zhang et al., 2006). SMN and gemin particles that do not colocalize were also found to be present suggesting that there is a diversity of SMN-containing multiprotein complexes in neuronal processes. Due to the lack of spliceosomal Sm proteins in these processes, it is assumed that the SMN complexes that are located in these neuronal processes have an additional function than the biogenesis of snRNPs that has been characterized in other cell types. One speculation is that these SMN/gemin complexes may play a role in some aspect of messenger ribonucleoprotein (mRNP) assembly in particularly, the assembly of a localized β-actin mRNP complex (Briese et al., 2005; Monani et al., 2005). Motor neurons taken from SMA transgenic mouse models with relatively low levels of SMN were shown to have a decreased localization of β-actin mRNA as well as a low level of protein product in axonal growth cones. Axons also were determined to be shorter in length with growth cones smaller in size. No indication of dendritic impairments were seen (Rossoll et al., 2003).

A reduction in the level of β-actin mRNA transport furthermore correlated in a changed distribution of the N-type voltage-gated dependent calcium ($Ca_v2.2$ Ca^{2+}) channel supported by an altered electrophysiological environment. This alteration of the availability and distribution of Ca^{2+} channels at the neuromuscular junction could

potentially impacting the release of neurotransmitters, as well as active zone development. This is similar to results seen in mouse models lacking laminin-β2 and in SMA mouse models along with aborization of the neurons (Noakes et al., 1995; Nishimune et al., 2004; Le et al., 2005; Jablonka et al., 2007; Kariya et al., 2008; Kong et al., 2009). Conflicting issues with these finds are that a reduction of folds in the neuromuscular junction do not always have an effect on the function of the neuromuscular junction, as well as the fact that the abnormalities seen in the neuromuscular junction does not account for the severe phenotypes that occur in SMA (Burghes and Beattie, 2009).

Another piece of support for the importance of β-actin in modifying the severity of SMA is a recently study showing that plastin 3, which is an important protein crucial for the stabilization of actin filaments was found to be a modifier of SMA It is reported that the SMN protein and plastin 3 are able to form a complex, but it is unclear if SMN is in fact associated with filamentous actin (Oprea et al., 2008; Burghes et al., 2009). Increased expression of plastin 3 was shown to rescue the axonal defects that have been observed in both zebrafish and mice cultured motor neurons with an *Smn* knockdown, but this may be due to the fact that it promotes axonal growth or correlated to SMA (Oprea et al., 2008). Plastin 3 studies performed on SMA human subjects showed major inconsistencies. A discordant SMA family with affected and unaffected siblings showed comparable levels of Plastin 3 with the male family members being more severely affected compared to their female counterparts. Furthermore, in the male patients with two copies of *SMN2*, high plasma

levels of plastin 3 was not shown to modify SMA. The researchers accounted for these inconsistency by stating that plastin may be a sex-linked modifier as well as mediating incomplete penetrance, making it hard to definitely say if plastin 3 is correlated to the cause of modifying SMA (Cobben et al., 1995; Jedrejowska et al., 2008).

In severe SMA mouse models motor neurons were shown to grow at normal rates without any axonal branching and or misdirection, but where shown to have neuromuscular junctions that were unoccupied by a motor neuron (Le et al., 2005; McGovern et al., 2008). In comparison, mice with an intermediate SMA phenotype showed either the same results as the severe SMA mice as well as synapses that were partially occupied with. Low amounts of SMA seem to affect the synapses of motor neurons that mature faster during development being affect more significantly than their slower counterparts and cause the neuromuscular junction to have poor terminal branching, tend to be immature, and are abnormally development (Murray et al., 2008; Kariya et al., 2008; Narver et al., 2009; Kong et al., 2009). Through electrophysiology studies it was revealed that SMA mice with a mild phenotype had functional deficit in their neuromuscular junction, as well as irregular neurotransmission while mice with an intermediate SMA phenotype had synaptic vesicle release with reduce quantal content at the NMJ preceding degeneration of the nerve terminal (Kong et al., 2009). The major similarity seen in neuromuscular junction studies utilizing mouse models is that there is an unusual buildup of neurofilaments that can be seen in the motor neurons of these mice as well as the neuromuscular junction in some cases causing

varicosities in the motor neurons similar to those seen in human SMA patients (McGovern et al., 2008; Kariya et al., 2008; Murray et al., 2008)

Although the SMN has been identified as the disease-causing gene of SMA, the mechanism in which the reduced amount of full length function SMN protein causes SMA is yet to be determined. What has been determined is that the duplicate copy of the SMN gene known as *SMN2* plays an important role in modifying the severity of this disease. *SMN2* plays a compensatory role when there is a mutation or deletion of the *SMN1* gene. While the *SMN2* gene cannot fully replace the function of the SMN1 gene due to a nucleotide difference that causes the *SMN2* gene not to produce large quantities of functional SMN protein, it still has the ability to produce full length survival motor neuron protein. The SMN protein is then able to form complexes needed for proper pre-mRNA processing by the spliceosome machinery. Behaving as a chaperone, the SMN protein regulates the assembly of Sm proteins on to their target RNAs ensuring the specificity and efficiency of snRNP assembly. Beyond its well characterized role in snRNP assembly, the survival motor neuron protein has also been implicated in the transport of mRNA in these motor neurons affected in the SMA disease. Further considerations into how the alterations caused by decreased SMN levels lead to SMA will need to be examined to connect the molecular targets of the SMN protein to the development and progression of SMA.

1.5 Use of Reporter Assays in SMA Therapeutics Discovery

With the majority of SMA treatment efforts being focused on finding ways to modify expression of *SMN2*, the need of developing methods in which to screen for changes in *SMN2* expression has become more crucial. One of the challenges of motoring changes in SMN protein due endogenous *SMN2* expression is that even a single copy of the *SMN1* gene can mask its expression, making it hard to correlate a small molecules effect on *SMN2*. SMA derived cell lines where first considered to be used for screening of small molecules since SMA patients have a loss of *SMN1*, but retain at least one copy of *SMN2*. SMA patient derived cell lines have been found to be an effective tool in the discovery of and validation of therapeutic approaches in the treatment of SMA. Primary culture cell lines however are not as effective in high throughput screening applications which allow for the screening of hundreds of thousands of compounds. Primary culture cells show variability in their expression of SMN. This variability can be due to a number of factors such as the age, density, composition of culture medium, and passage number of the cells. The use of immortalized reporter cell lines as a surrogate of endogenous *SMN2* expression, therefore, is the preferred method of monitoring changes in SMN protein to identify small molecules (Figure 1.2) (Cherry et al., 2014).

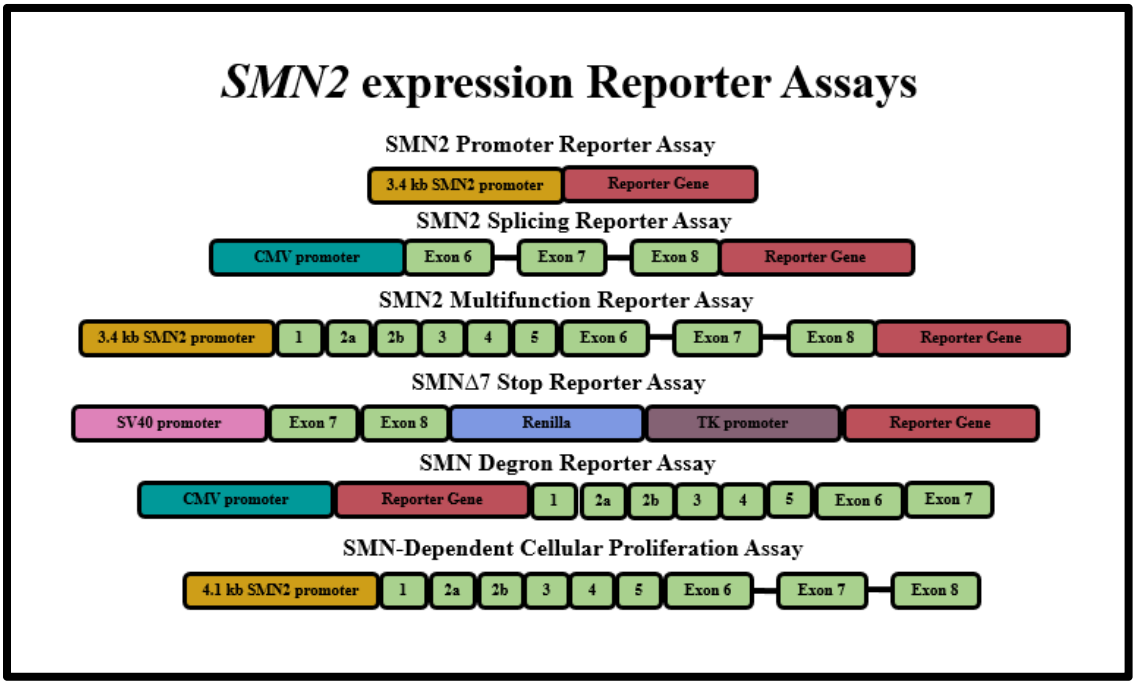


Figure 1.2 *SMN2* expression Reporter Assays. Illustration of the currently developed reporter assays for measuring changes in *SMN2* expression. Reporter assays color code: *SMN2* sequences are colored green, reporter gene are magenta, the *SMN2* promoter is orange, and non-SMN promoters are teal, pink, or purple (Adapted from Cherry et al., 2014).

One of the first reporter assays created focuses on identifying compounds that affect the regulation of the SMN transcription. This reporter assay system is known as the *SMN2* promoter-based assay. This reporter utilizes a 3.4 kb fragment of *SMN2*. This 3.4 kb fragment contains cis positive and negative regulatory elements that control *SMN2* expression. This 3.4 kb fragment was cloned upstream of the reporter gene, β-lactamase, this construct was then stably transfected into the NSC-34 cell line (Jarecki et al., 2005). NSC-34 cells are a hybrid of mouse neuroblastoma cells and mouse spinal cord cells. The NSC-34 cell line is a good model cell line to use for

studying *SMN2* modifiers since they display many of the same characteristics of motor neurons (Cashman et al., 1992). The *SMN2* promoter assay was able to reproduce previous work showing a two-fold increase observed in *SMN2* fibroblasts expression in response to compounds that act as non-selective histone deacetylase (HDAC) inhibitors, trichostatin A, and sodium butyrate, showing the validity of this new tool. With this new assay, novel modifiers of *SMN2* expression such as the C5-substituted 2,4-diaminoquinazolines were identified (Jarecki et al., 2005).

Another approach to increasing SMN protein produced by the *SMN2* gene is by increasing the splicing efficiency of *SMN2* so that a greater ratio of the mRNA transcripts produced contain exon 7. An *in vivo* reporter system was created to recapitulate the splicing patterns of endogenous *SMN2* gene while measuring changes in exon 7 incorporation. The *SMN2* splicing utilizes a mini-gene cassette of the *SMN2* gene that contains exon 6-8 as well as their introns. An enzymatic reporter gene was introduced to the 3' end of exon 8 reading frame in order to prevent any potential alternations of exon 7 splicing that could occur if the reporter gene was directly associated with exon 7. To prevent any internal translation or background expression from occurring, the 5' end of the initiation codon of the reporter gene was modified. The translation termination code for exon 7 was then inactivated by the addition of a single nucleotide in a region of exon 7 previously characterized for SMN exon 7 splicing that is less sensitive to mutations. The addition of the single nucleotide in exon 7 causes exon 7 to now be in frame with the reporter gene instead of exon 8 of the *SMN2* mini cassette. When exon 7 in the *SMN2* splicing reporter constructs is

skipped, exon 6 is spliced on to exon 8 causing the reporter gene to be out of frame and not expressed. On the other hand, when exon 7 is included, the reporter gene will be in frame and transcribed allowing for its expression to be monitored and correlated to a particular compounds ability to modulate the exclusion of exon 7 in *SMN2* transcripts (Zhang et al., 2001). Indoprofen and aclarubicin were identify as exon 7 inclusion modifiers through the *SMN2* splicing reporter (Andreassi et al., 2001; Lunn et al., 2004).

Unlike the other previously described reporter systems that were designed to quantify a specific step in *SMN2* gene expression separately, the *SMN2* multifunctional reporter assay was a next generation reporter designed to simultaneously detect changes in *SMN2* expression through multiple mechanism including activation of the promoter, as well as exon 7 inclusion. The *SM2* promoter sequence including exon 1-5 cDNA was used with the addition of the reporter gene splicing cassette which includes exon 6-8 (Cherry et al., 2012). Drug compounds dihydroquinolones, heterocyclic carboxamides, and a series of 4-arylthiazolyl piperidines were identified as compounds with the ability to modify *SMN2* expression (Cherry et al., 2013; Xiao et al., 2011).

A third strategy for increasing SMN protein expression is salvaging the truncated protein already expressed by the *SMN2* gene. *SMN2* mRNA transcripts lacking exon 7 have a loss of the C-terminal 16 amino acids and an addition of four amino acids (EMLA) encoded for by exon 8 (Lefebvre et al., 1995) resulting in a truncated and unstable SMNΔ7 protein with a half-life shorter than full-length SMN

protein. Although the SMNΔ7 has a reduced ability oligomerize with other proteins (Lorson et al.,1998), it is still believed to retain some of its function (Le et al.,2005; Burghes and Beattie 2009). It has been shown that addition of amino acids to the N-terminus of the SMNΔ7 protein causes the stabilization of the protein and corrects its cellular distribution leading researchers to hypothesize that compounds that promote readthrough of the stop codon will allow for the *SMN*Δ*7* transcripts to produce more stable SMNΔ7 protein (Cherry et al., 2014). The SMNΔ7 stop reporter assay utilizes the *SMN2* sequence and contains the desired stop codon inserted on the 5' end of the reporter gene. The reporter gene therefore is only expressed if termination of the stop codon is suppressed. This reporter assay contains a second reporter gene placed just upstream of the reporter gene measuring stop codon termination as an internal control for the assay. The antibiotic geneticin (G418) was identified through the SMN 7 stop reporter assay due to its ability to suppress termination at the target SMNΔ7 stop codon in a dose-dependent manner (Heier and DiDonato 2009).

Another explanation for the lack of stability of SMNΔ7 protein is that the juxtaposition of the final 10 amino acids in exon 6 (YG-box) in combination with the four amino acids encoded for by exon 8 (EMLA) form a protein degradation signal also known as a degron. A degron reporter assay, based on this hypothesis was developed in order to detect changes in the stability of the SMN protein in comparison to the SMNΔ7 protein. To create the degron reporter assay, a reporter gene was fused to the N-terminus of either full length or delta 7 SMN protein encoding cDNA and transfected into a cell line. The validity of the degron reporter was shown by an

increase half life of SMN and SMNΔ7 reporters when treated with two previously discovered compounds, MG132 and lactacystin (Cho et al., 2010). Currently no new compounds have been identified with the degron reporter assay.

Expression of the SMN protein has been shown to be essential for cell proliferation (Grice et al., 2011). Although the SMN protein is needed for cell proliferation, decreasing the levels of the fully functional SMN has not been shown to affect the viability of most cells types in culture causing a need to develop a phenotypic screen that could recapitulate altered cell proliferation due to the loss of SMN protein. The SMN-dependent cellular proliferation assay utilizes the mouse embryonic fibroblast cell line NIH3T3. The NIH3T3 has an inducible SMN RNAi-mediated knockdown cell line. In the presence of doxycycline, the endogenous mouse SMN in the NIH3T3 fibroblast is knockdown leading the growth arrest and senescence of these cells (Lotti et al., 2012; Li et al., 2013). The SMN-dependent cellular proliferation reporter assay was generated by using the NIH3T3 cell line and introducing the human *SMN2* gene. The 35.5 kb *SMN2* gene *BamHI* fragment was cloned into a vector with neomycin selection under the control of the SV40 promoter and then transfected into the NIH3T3 cells. Inserting *SMN2* to this cell line allowed for the generation of a system where cell proliferation is sensitive to changes in functional SMN levels produced by *SMN2* giving a direct phenotypic readout of SMN function. Two clonal cell lines were obtained from this process with one containing a high copy number of *SMN2* and the other containing a low copy number. The low copy number cell line was then used to develop a high-throughput screening system

scaled for a 96-well plate. The validity of the cellular proliferation assay was shown by a three-fold increase of cell survival when a lentiviral overexpression of SMN was introduced. The SMN-dependent cellular proliferation as was also treated with a histone deacetylase inhibitor valproic (VPA) known to increase the expression of SMN. VPA treatment was able to increase the number of viable cells by 2.5-fold when compared to the untreated low copy cell line (Li et al.,2013).

1.6 Sodium hydrogen Exchangers

The sodium hydrogen exchangers (NHE, NHA, or NHX), also known as the sodium hydrogen antiporters are a group of proteins responsible for maintaining intracellular pH and regulate cellular volume in mammalian cells through uptake of sodium ions in exchange for protons (Masereel et al., 2003). The sodium hydrogen exchangers are encoded by the SLC9 gene family of transporters. To date 11 isoforms of the sodium hydrogen exchanger have been identified (NHE 1-11) (Alexander et al., 2017). The SLC9 family is divided into three subgroups known as SLC9A, SLC9B, and SLC9C. The first subgroup, SLC9A, contains nine paralogs of the Na^+/H^+ antiporter known as *SLC9A1-9* (*NHE 1-9*). This group also consist of one possible NHE1 splice variant as well as five pseudogenes. SLC9B encodes *NHA1* and *NHA2*(*SLC9B1* and *SLC9B2*). NHA1 was discovered to have a splice variant. *NHE10* and *NHE11*(*SLC9C1* and *SLC9C2*) belong to the SLC9C subgroup and encode the sperm NHEs. Although the full structure of the sodium hydrogen exchanger is

unknown, each NHE consist of a 450-amino acid 10-12 membrane spanning (M segments) domain responsible for mediating the ion exchange, as well as a hydrophilic C-terminal domain (125-444 amino acids depending on isoform) (Donowitz et al., 2013). The C-terminal domain serves a regulatory role in mediating the ion exchange (Nakamura et al., 2005).

The NHE isoforms share approximately 20-60% amino acid similarity and have a molecular mass of 74 to 99 kDa. Segments M3-M12 have high sequence homology in each of the NHE isoforms with M6-M7 sharing about 97% identity which is why there are believed to be the domains in which sodium ions and protons are transported across the membrane (Masereel et al., 2003). NHE 1-5, 10, 11 are distributed and expressed on the cell membrane while NHE 6-9 are localized to the recycling endosomes and *trans*-Golgi (Masereel et al., 2003; Nakamura et al., 2004). The NHEs are believed to exist as dimers to provide stability to molecule although it appears that the exchangers function as monomers during transport (Donowitz et al., 2013).

The NHE1 protein is 815 amino acids long the N-terminal membrane consist domain of 500 amino acids while the hydrophilic intracellular C-terminus is 350 amino acids long. The majority of the NHE1 protein resides on the surface of cells, but there are cell types in which the NHE1 accumulates in microdomains of the plasma membrane. NHE1 is ubiquitously expressed in mammalians cells except for in the macula densa and α-and β-intercalated cells of the kidney (Donowitz et al., 2013).

High levels of NHE1 found mainly in cardiomyocytes, platelets, and the basolateral membrane of renal tubules (Masereel et al., 2003). Numerous studies have shown that the loss of NHE1 seems to alter the expression and activity of other membrane transport proteins the brain, resulting in increased neuronal excitability while increased NHE1 activity has been shown to induce cardiac hypertrophy and heart failure (Gumina et al., 1999; Masereel et al., 2003; Nakamura et al., 2008).

NHE2 is 812 amino acids long an is known as the epithelial Na+/H+ exchanger. NHE is found in the brush border of the small intestine, colon, and gallbladder. It is also present in the kidney, basolateral membrane of stomach and endothelial cells that form the blood brain barrier. The third sodium hydrogen exchanger, NHE3, consist of 834 amino acids. The NHE3 isoform uniquely traffics between the cells recycling system and the plasma membrane, but they function mainly at the plasma membrane. NHE3 predominately is found in the renal tubules, but can also be found in the intestine, colon, gallbladder, thymus, and ovary. NHE4 has 798 amino acids. Its expression is highest in stomach, but found in kidney medulla, hippocampus, zymogen granule of pancreas, and salivary gland (Donowitz et al., 2013). NHE5 is 896 amino acids long and shares the highest homology with NHE3 (50% in amino acid identity) as well as having similar pharmacological, regulatory, and cellular localization, Like NHE3, NHE5 is localizes in both plasma membrane and recycling endosomes. NHE5 is expressed in high concentrations in non-epithelial tissue like the brain as well as the spleen, testis and skeletal muscle

(Masereel et al., 2003; Donowitz et al., 2013). In fact, NHE5 is believed to be highly restricted to brain due to the multiple regions of the brain (gyrus, cerebral cortex, hippocampus, amygdala, caudate nucleus, hypothalamus, subthalamic nucleus, and thalamus) in which NHE5 is highly enriched suggesting that it is a neuron specific sodium hydrogen exchanger (Donowitz et al., 2013).

The intracellular NHEs consist of NHE 6-9. NHE6 is a recycling endosomal protein, NHE7 trans-Golgi, NHE8 is located in mid to late trans-Golgi, but can also be found in the brush border of some epithelial cells, while NHE9 is present in recycling endosomes as well as late endosomes/lysosome (Nakamura et al., 2005). NHE6 is highly expressed in brain, heart, and skeletal muscle (Numata et al.,2001). Similar to NHE1, NHE7 is ubiquitously expressed in mammalian cells, but is mainly centralized in the brain, skeletal muscle, stomach, and glands (Masereel et al., 2003). NHE8 consist of 581 amino acids and shares around 25% homology with the other NHE isoforms. NHE8 is also ubiquitously expressed in most human tissues, but its highest expression is seen in skeletal muscle and kidney (Brett et al., 2005).

NHE function based on the Na^+ and H^+ gradients of the cell through exchanging at a 1:1 stoichiometry one extracellular Na+ for one intracellular H^+. NHE1, NHE2, NHE3 and NHE5 display a hyperbolic dependence on extracellular sodium concentration while NHE4 has a sigmoidal dependence based on Michaelis-Menten kinetics (Mahnensmith et al., 1985; Orlowski et al., 1997; Wakabayashi et al., 1997). Sodium influx is competitively inhibited by influxes of extracellular Li^+ and or

H^+ binding. NHE1, can also be inhibited by nonphysiological levels of extracellular K^+ (Yu et al., 1993; Szabo et al., 2000) while NHE7 and NHE4 can mediate influxes of K^+ and/or Na^+ in exchange for H^+ (Chambrey et al., 1997; Numata and Orlowski, 2001). In the presence of low H^+, the intracellular pH will decrease causing an enhancement of the gradient resulting in the activation of NHE isoforms while in the presence of decreased extracellular Na^+ the NHE isoforms will shift to their reverse mode and expel sodium (Aronson, 1985).

1.7 Regulation of the sodium hydrogen antiporter

The NHE isoforms are modulated primarily by tyrosine kinases and agonists of Ser/Thr kinases including protein kinases A (PKA) and protein kinases C (PKC) (Fliegel et al., 1993; Orlowski et al., 1997). More recently, the NHEs were found to be sensitive to increases in cytosolic Ca^{2+} and to changes in cell volume. The NHE primary sequence has binding sites for phosphorylation by PKA and/or PKC. There are also multiple binding sites for the substrates CaM kinase and proline directed Ser/Thr kinase which includes the mitogen-activated kinases. NHE1 and NHE3 are found to be constitutively phosphorylated in resting cells and are further phosphorylated in response to phorbol esters, growth factors, and phosphatase inhibitors (Fliegel et al., 1993; Levine et al., 1993; Aharonovitz et al 1996; Blanchini et al., 1997; Wakabayashi et al., 1997).

NHE activity can also be regulated by both heterotrimeric and small GTP-binding proteins. The active form of Gαq, Gα12, and Gα13 have been shown to stimulate the Na^+/H^+ exchanger (Dhanasekarn et al., 1994; Lin et al., 1996; Hooley et al.,1996). NHE1 was found to contain domains capable of binding calmodulin. These CaM-A domains are believed to be important in transport regulation. It was found that removing the CaM-A domain causes the exchanger to be constitutively stimulated, suggesting this domain autoinhibits the exchanger until a ligand binds. Although only NHE1 has been convincingly shown to be regulated by CaM, the transmembrane regions of other isoforms can respond to conformational changes of the tail induced by CaM (Bertrand et al., 1994; Wakabayashi et al., 1997; Orlowski et al 1997; Massereel et al., 2003).

The NHEs are secondary active transporters since the cation flux through NHEs are driven by the combined transmembrane chemical gradients of the substrates and not ATP. Although ATP is not required for transport, plasma membrane NHEs require normal physiological levels. Cellular depletion of ATP has been shown to reduce the activities of NHE1 and NHE2 while NHE3 and NHE5 were found to have almost no activity. The NHE isoforms were unable to recover even in the presence of a large transmembrane H^+ gradient (Wakabayashi et al., 1997; Kapus et al., 1994; Szabo et al., 2000).

1.8 Inhibitors of the sodium hydrogen antiporter

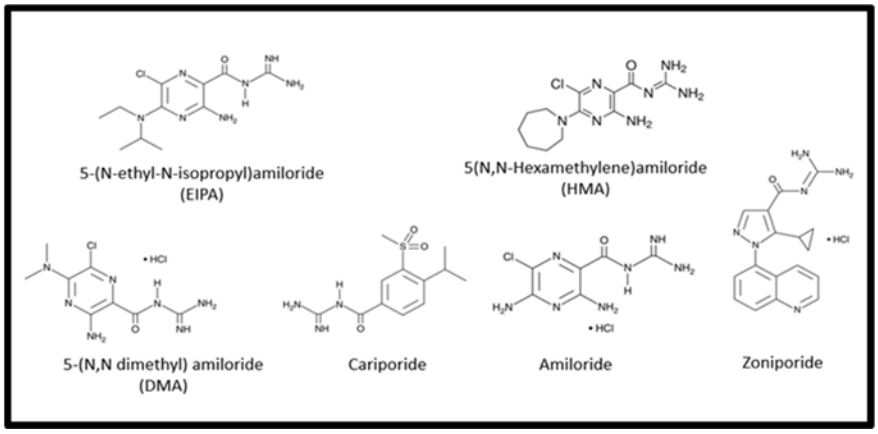
Inhibitors of the sodium hydrogen antiporter were originally developed to counteract the negative effects of excessive activation of the NHEs with NHE1 primarily being focused on to provide cardioprotective effects. The first described NHE1 inhibitor is a K^+ sparing diuretic known as amiloride (Benos, 1982). Amiloride is able to inhibit a sodium conductive channel and the Na^+/Ca^{2+} exchanger NHE1 and NHE2 are the most sensitive to amiloride with NHE5 having an intermediate response and NHE3 and NHE4 being insensitive to the compound (Chambrey et al., 1997; Szabo et al., 2000). To make NHE inhibitors more potent and selective for the different NHE isoforms, derivatives of the amiloride compound were developed (Figure 1.2). Among the first compounds developed were 5-(N, N-hexamethylene) amiloride (HMA), 5(N,N-dimethyl)-amiloride (DMA), 5-(N-methyl-N-isobutyl) amiloride (MIBA) and 5-(N-ethyl-N-isopropyl) amiloride (EIPA) which all have double substitutions on the nitrogen of the 5-amino group. Unlike amiloride, these derivatives no longer have the potency to inhibit the sodium conductive channel and Na^+/Ca^{2+} exchanger, but have more of an effect on the different NHE isoforms than amiloride (Table 1.1) (Masereel et al., 2003).

	Inhibitory Potency (IC_{50} or K_i in μM)				
Drug Compounds	**NHE1**	**NHE2**	**NHE3**	**NHE4**	**NHE5**
Amiloride	1-1.6	1	>100	836	21
Cariporide	0.03-3.4	4.3-62	1->100		>30
DMA	0.023	0.25	14		
EIPA	0.01-0.02	0.08-0.5	2.4	>10	0.42
HMA	0.013		2.4		0.37
Zoniporide	0.059	12	>500		Inactive

Table 1.2 Potency of NHE inhibitors for various NHE isoforms. Table shows the potency of each inhibitor for NHE1-5. IC_{50}(K_i) tell how much of the inhibitor is needed in μM to inhibit the NHE by 50%. HMA and EIPA have the lowest IC_{50} for NHE1 and NHE5 (Adapted from Masereel et al., 2003).

The next set of amiloride derivatives were created by replacing the pyrazine ring on amiloride with a pyridine ring or phenyl. The replacement with the phenyl and pyridine in the heterocyclic nitrogen in the meta position of acylguanidine were greater than 30 times more active than amiloride on inhibiting NHE1 found on human platelets. The pyridine derivative was further modified in the heterocyclic nitrogen in the ortho position of the acylguanidine was active. Modification made to the phenyl derivative included a sulfomethyl substitution of the 6-chloro and a 2-amino deletion or replacement by methyl group (Laeckmann et al., 2002). All of these modifications led to the development of the benzoylguanidines. The benzoylguanidines includes the compounds cariporide, eniporide, HOE-694, and BIIB-513. Like the first step derivatives of the amiloride compounds, the benzoylguanides are also unable to block sodium channels and Na^+/Ca^{2+} exchanger (Scholz et al., 1995; Baumgarth et al., 1997; Gumina et al., 1999). The remainder of the NHE inhibitors were based on a bicyclic template with the bicyclic ring being a quinoleine. This group includes zoniporide,

MS31038, SMP-300, SM 20220, SM 20550, KB-R9032, BMS-284640, T-162559, and TY-12533 (Banno et al 1999; Harada et al., 1997; Kuribayashi et al., 1999; Aihara et al., 2000; Ahmad et al., 2001; Guzman et al., 2001; Fukumoto et al., 2002). With the exception of T-162559 all the bicyclic compounds have unsubstituted acylguanidine group (Fukumoto et al., 2002). Potency of the inhibitors is directly affected by the chemical structure and ionization of the guanidine function. It has been shown that amiloride, as well as a subgroup of amiloride derivatives inhibition of the NHEs is reduced in conditions of increase sodium causing researchers to infer that their cationic form is binding to the external Na^+ binding site (Aihara et al., 2000; Fukumoto et al., 2002). Dependent on the pH of the cell under physiological conditions compared to ischemia or reperfusion phase different amiloride compounds are protonated allowing for them to more efficiently inhibit NHEs under their cationic form (Aihara et al., 2000; Guzman et al., 2001; Fukumoto et al., 2002).



1.3 NHE Inhibitors. Chemical structure of amiloride and derivatives of amiloride (EIPA, HMA, DMA, cariporide and zoniporide).

1.9 EIPA promotes exon 7 inclusion in *SMN2*

The NHE inhibitors were previous study to evaluate if changes in the cellular pH microenvironment regulate *SMN2* pre-mRNA alternative splicing *in vivo* (Yuo et al., 2008). Previous studies performed have shown that extracellular pH is a modulator of *SMN2* exon 7 alternative splicing. A decrease in pH was shown to increase exon 7 skipping and reduce the expression of SMN protein while an increase in the extracellular pH was shown to promote exon 7 inclusion. A decrease in cellular pH was then suggested to induce the skipping of SMN2 exon 7 through the upregulation of hnRNP A1 (Chen et al., 2008) IT was eluded to that several NHE inhibitors were studied but only the data on EIPA was reported. EIPA was discovered to enhance exon 7 inclusion in *SMN2* mRNA and expression of SMN protein in SMA lymphoid cells in a dose dependent and time dependent manner (Yuo et al., 2008). In effort to explain the mechanism of EIPA on modifying exon 7 inclusion, changes in SR proteins, intracellular pH, and cell viability was assessed in EIPA treated SMA lymphoid cells.

SR and hnRNP were previous shown to regulate alternate splicing of *SMN* (Cartegni et al., 2002; Hoffmann,2002; Young et al., 2002), in this study changes in SRp20, hnRNP A1, and SF2/ASF protein were measured. EIPA was shown to significantly increase the expression of the SRp20 (Yu et al., 2008). Although previous studies have not shown SRp20 to interact with exon 7 of *SMN2*, SRp20 has been shown to modify the splicing of many pre-mRNA transcripts (Galina et al.,2003; Jumaa et al., 2000; Yu et al., 2004). SRp20 expression levels were also significantly

increased in drug studies with a deacetylase inhibitor, M334 which has been shown to promote exon 7 inclusion and increase SMN protein (Riessland et al., 2006).

EIPA was found to reduce the intracellular pH of SMA patient derived lymphoid cells from 7.40 to 7.15 and 7.03 after four hours of treatment with 2 μM and 10 μM, EIPA respectively (You et al., 2008). SMA lymphoid cells were 90% viable after 24 hours of EIPA treatments at a concentration of 20μM or lower, while at 50μM of EIPA, only 60% of the cells were viable. A five-day dose response curve of EIPA treated SMA lymphoid cells showed at the concentration of 8 μM, EIPA caused 50% growth inhibition of the lymphoid cells. Cytotoxic and pH effects exhibited on EIPA treated SMA lymphoid cells were comparable to a study done using the EIPA for hypertensive patients (Rosskopf et al., 1995) The mechanism behind specific effect of EIPA was not determined but it may be due to the different potencies of the NHE inhibitors, features unique to EIPA, or through increasing SRp20. EIPA ability to promote the *SMN2* exon 7 inclusion EIPA has sparked the potential of a new class of potential compounds for the treatment of SMA (Yuo et al., 2008).

1.10 Hypothesis and Aims

SMN2 copy number variations have been shown to modulate the disease severity of SMA patients. The inverse relationship seen between *SMN2* copy number and disease severity has made *SMN2* the primary target of SMA treatment and drug discovery. In an aim to cure or alleviate the symptoms seen in SMA, numerous drug

compounds are being assed for their ability to induce the *SMN2* promoter or increase exon 7 inclusion. In the first portion of this study, compounds that modify cellular pH (NHE inhibitors) will be assessed for their ability to modulate *SMN2* expression. Two new reporter assays will also be developed to improve on currently used reporter assays.

The NHE inhibitor EIPA was previously found to increase the inclusion of exon 7 in SMN mRNA transcripts in a dose dependent and time dependent manner (Yuo et al., 2008). In this study six NHE inhibitors will be assessed at various concentrations for their ability to modulate the inclusion of exon 7 through drug treatments utilizing three different type II SMA patient derived cell lines to evaluate changes in mRNA and protein levels. The motor neuron like cell line, NSC-34, containing the *SMN2* β-lactamase report cell line was also used to examine the direct effect of these compounds in regard to their ability to promote exon 7 inclusion. The mechanism in which the NHE inhibitors exude their effects in modifying the splicing of *SMN2* transcripts by assessing the different isoforms of the sodium hydrogen exchangers. The toxicity of each drug was also monitored using various concentrations during the treatment of patient derived fibroblast to asses if compounds identified as modifiers of exon 7 inclusion can be further developed for SMA patient treatment.

The second aim of this project is to establish novel tools that can be used to screen small molecular compounds that increase levels of full length *SMN2* mRNAs through the activation of the *SMN2 p*romoter or by inducing the inclusion of exon 7 in

SMN2 pre-mRNAs. The *SMN2* reporter assay that are currently available were designed using short regions of the *SMN2* gene in order to examine the effects of small molecules on the *SMN2* promoter or splicing. Rather than using a small fragment of the *SMN2* promoter or a mini-gene cassette containing exon 7, the reporter cell lines generated will contain the entire *SMN2* locus allowing for the two cell lines to show changes in *SMN2* gene regulation in context to the entire locus of the gene in a motor neuron intracellular environment. We hypothesize that the two cell lines developed will more closely mimic *SMN2* gene regulation *in vivo* than the reporter assays currently used since it will be able to account for distal elements that may play a role in the regulation of *SMN2*. Here we will describe the development of our reporter cell line that can simultaneously monitor the activation of the *SMN2* promoter and inclusion of exon 7 using a sensitive enzyme assay. Additionally, a second cell line will be created by transfecting the complete *SMN2* genomic sequence into NSC-34 motor neuron like cells. Once these novel tools are developed, of these reporter cell lines will allow for a more accurate evaluation of previously discovered *SMN2* regulators like the NHE inhibitors, as well as allowing for the development of new therapeutics for SMA patients.

Chapter 2

METHODS

2.1 Cell Culture

Fibroblast cells derived from type II SMA patient (GM03813, GM22592, and AIDHC-SP22) and non SMA patient (GM03814) as well as mouse derived motor neuron cell line NSC-34 (Cashman et al., 1992) and NSC-34 reporter based cell line-clone 5.3 (Andreassi et al., 2001) were grown in DMEM containing 10% EquaFETAL (Atlas Biologicals), 2mM L-glutamine (Life Technologies) and 1% penicillin-streptomycin (Life technologies). Fibroblast cell lines were acquired from Coriell Cell Repositories (Camden, NJ). All cell lines were maintained to a confluency of 80-90% in a humidified chamber at 37°C with 5% carbon dioxide. The three-patient derived SMA fibroblasts were derived from patients with type II SMA. These fibroblasts have a homozygous for deletion of *SMN1* and three copies of *SMN2*. GM03814 is the maternal parent of patient GM03813 making this a carrier/non-SMA cell line. GM03814 contains one copy of *SMN1* and five copies of *SMN2* (Stabley et al., 2015). GM03814 cell were used as references to compare changes in SMN mRNA and protein expression in amiloride treated samples.

2.2 Drug Compounds

Amiloride, cariporide, 5-(N-ethyl-N-isopropyl) amiloride(EIPA),5-(N,N-dimethyl)-Amiloride (hydrochloride) (DMA), and zoniporide were acquired from Cayman chemicals while 5-(N, N-Hexamethylene) amiloride (HMA) was acquired from Sigma-Aldrich. All compounds were dissolved with DMSO.

2.3 Drug Treatments of Fibroblast Cell Lines

GM03813, GM22592, and AIDHC-SP22 fibroblast cell lines were seeded 24 hours prior to the first drug treatment day at concentrations dependent on the assay being conducted. Treated with drug compounds took place for five days following the initial plating. Cells were then harvested 24 hours after last drug compound treatment. Medium was changed daily and fresh drug compounds or DMSO was added at a 1:1000 dilution approximately every 24 hours during the five-day treatment period. GM03814 fibroblasts were also seeded and grown in the same manner as the GM03813 except, the GM03814 cell line did not receive and drug compound or DMSO treatment and media changes were done every other day during the duration of the treatment.

2.4 Cell Viability Assay

SMA patient derived cell line GM03813 were maintained to 80-90% confluency. Fibroblast cells were washed two times with 10mL of 1X PBS before being trypsinized and centrifuged at 1000rpm for 5 minutes. For the 24-hour treatment the cells were seeded in a 96-well plate at a density of $3x10^4$ while the 96-well plate was seeded at a density of $3x10^3$ in quadruplicates each treatment condition. The following day the media was removed from the plated cells and they were washed twice with 100µl of DMEM with 0.5% BSA. Cells were then treated with drugs compounds as previously described for a duration of 24 hours or 5 days. 20 hours post final treatment time 20µl Cell Titer-Blue (CTB, Promega) and incubated at 37°C for 4 hours. Plates were then read on a plate reader (Victor X4, Perkin Elmer). For each sample, fluorescence was measured at an excitation of 530-570nm and an emission of 580-620 nm. The emissions readings for each compound were analyze by subtracting the reading for the negative control, which contained only DMEM and 0.5% BSA. The average treatment group concentration was then of each sample was the generated and resulting values were used to generate a dose response curve for cell viability.

2.5 Quantitative Reverse Transcription Polymerase Chain Reaction (qRT-PCR)

Patient derived fibroblast cell line GM03813 were plated onto 6-well plates at a density of 3.2 X 104 cells per well and treated with drug compounds as described above. Treated cells were harvested and the total RNA content was extracted using

RNAeasy Mini columns (Qiagen), following the manufacturer's recommendations. Isolated RNA was then used to obtain first-strand complementary DNA with iScript cDNA synthesis kit (Bio-Rad) according to the guidelines provided by the manufacturer. Quantitative Reverse Transcript PCR (QPCR) was performed in a 384 well plate on a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA). Target transcripts plated in triplicates were amplified by real time polymerase chain reaction using the SYBR Green PCR Master Mix (QIAGEN) in 10μL total volume. PCR cycling conditions were an initial denaturing step at 95°C for 10 minutes followed by 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The following primers (Integrated DNA Technologies) were used: GAPDH (F) 5'-aatcccatcaccatcttcca-3'; GAPDH (R) 5'-aaatgagccccagcctcc-3'; ACTB (F) 5'-gaagtcccttgccatcctaaa-3'; ACTB (R) 5'-gctatcacctcccctgtgtg-3' SMNex6Fq 5'-ccatatgtccagattctcttgatga-3'; SMNex78Rq 5'-atgccagcatttctccttaattta-3'; SMNex68R 5'-atgccagcatttccatataatagc-3'; RPLP0 (F) 5'-tcatcaacgggtacaaacga-3'; RPLP0 (R) 5'-agatggatcagccaagaagg-3'; FL-STRN3 (F) 5'-ggaagaaaggggtgaagagg-3', FL-STRN3 (R) 5'-tgattcctgaagggatgtgg-3'; STRN3D89 (F) 5'-cagaatgggctgaaccaataa-3'; STRN3D89 5'-accgtcaagtctgcaaggtc-3'. QPCR data was analyzed for each transcript (FL-SMN, SMNΔ7, FL-STRN3, and STRN3D89) and normalized to the geometric mean of the three reference transcripts beta-actin (ACTB), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and ribosomal protein L0 (RPLP0) (Vandesompele et al., 2002). Relative changes transcript levels were calculated using the 2-ΔΔCt method (Schmittgen and Livak, 2008) by calculating the difference

between the ΔCT for the treated SMA fibroblast in regard to the ΔCT of the control sample (SMA fibroblast treated with DMSO). ΔCT values were the adjusted based on the efficiently of the primer for the fibroblast cell line used to report accurate changes in SMN expression (Livak and Schmittgen, 2001; Pfaffl et al., 2001).

qPCR Primer Efficiencies		
Cell Line	**Primer**	**Efficiency**
Patient Derived Fibroblast	*ACTB*	1.016
	GAPDH	0.946
	RPLP0	1.000
	FL-SMN	0.922
	SMNΔ7	0.998
	FL-STRN3	0.477
	STRNΔ89	0.726
NSC-34	*Smn*	0.855
	Rpll3a	1.018
	Pgk	1.007
	Gusb	0.833

Table 2.1 qPCR Primer Efficiencies. Primer efficiency of target and reference transcripts was determined by qPCR using a 1:2 serial dilution of cDNA from each respective cell line. The CT values of each dilution was then calculated and used to obtain the primer efficiency (Livak and Schmittgen, 2001).

2.6 *FL-SMN/SMNΔ7* Ratio PCR

GM03813 and GM03814 fibroblast cell lines were seeded at a density of 3.2 x 104 plated onto 6-well plates. GM03813 cells was treated with compounds or DMSO while the GM03814 cells only received media changes for the five treatment days. Cells were harvested by scraping 24 hours after final drug treatment. RNA isolation and first strand complementary DNA synthesis were performed as already described. The *FL-SMN* and *SMNΔ7* ratio thermocycling profile was 50°C for 2 minutes, 95°C for 10 minutes, followed by 30 cycles at 95°C for 15 seconds and 60°C for 1 minute, and a final cycle at 72°C for 5 minutes. PCR primers used are specific for sequences within SMN exons 6 and 8. As a control, primers for CollagenIIIA were used since it is highly expressed in fibroblast (Heier et al., 2007). PCR products were electrophoresed through a 2% agarose gel. Data were recorded on AlphaImager (Cell Biosciences) and band intensities were quantified on Image J (Schneider et al., 2012). Primer sets used: SMNex6(F) 5'-cccatatgtccagattctcttgat-3'; SMNex8(R) 5'-ctacaacacccttctcacag-3'; COL3A(F) 5'-gctctgcttcatcccactatt-3'; COL3A(R) 5'-ggaataccagggtcaccattt-3'.

2.7 Beta-Lactamase Assay

NSC-34 cells containing 5.3 clone were utilized for the *SMN2* splicing assay. [NSC-34 5.5 kb clone cells maintained at a confluency of 80-90% (seeded at 5 x 10^4 cells per well) into a black-walled, clear bottom 96-well tissue culture plates (Santa Cruz Biotechnology)]. Drug compounds added to serum free media at a dilution of 2:500 µL. 100 µL of media contain drug compounds was then added to the 100µL seeded NSC-34 cells (plated in quadruplicates) making the final drug dilution of each well 1:1000 µL. After incubation for 19 hours, media containing drug compounds was aspirated and 100 µL of fresh serum media was added to each well. 20µL of 6X CCF2-AM dye (GeneBlazer In Vivo Detection Kit, Life Technologies) was added to each well and plates were incubated at room temperature for 2 hours. Plates were then read on a plate reader (Victor X4, Perkin Elmer). For each sample, the CCF2-AM substrate was measured at 530 nm (green channel) and 460 nm (blue channel). The readings from the 460 and 530nm CCF2-AM substrate for each compound were analyze by subtracting the reading for the negative control, which contained only serum free media, blue and green signal for each sample. The 460/535 ratio of each sample was then generated for each compound concentration. The resulting values were used to generate a dose response curve for the *SMN2* exon 7 inclusion.

2.8 NHE Isoform PCR

To perform human NHE isoform PCR, GM03813 cells were seeded at a density of 3.2 x 104 plated onto 6-well and treated with amiloride compounds or DMSO for five treatment days. Cells were harvested by scraping 24 hours after final drug treatment. Total RNA content was extracted using RNAeasy Mini columns (Qiagen), following the manufacturer's recommendations. Isolated RNA was then used to obtain first-strand complementary DNA with iScript cDNA synthesis kit (Bio-Rad) according to the guidelines provided by the manufacturer. NHE isoform thermocycling program consisted of 2 minutes at 50°C, 10 minutes at 95°C, followed by 40 cycles of 15 secs at 95°C and 1 minute at 60°C. The final extension step was for 5 minutes at 72C. hNHE1-F 5'-ccagctcattgccttctacc-3'; hNHE1-R 5'-tgtgtctgtaggaccgc-3'; hNHE2-F 5'-gaagatgtttgtggacattgggg-3'; hNHE2-R 5'-cgtctgagtcgctgctattgc-3'; hNHE3F 5'-gcagacctggcttctgaacc-3'; hNHE3R 5'-ggaacttcctgtcgaagtgg-3'; hNHE4F 5'-aagaatatccgctacctctccta-3'; hNHE4R 5'-ctgtgtaggctcttcattggtat-3'; hNHE5F 5'-tgctgggcctggtgcta-3'; hNHE5R 5'-actcgccaaagacgatgat-3'. PCR product was ran through a 1% TBE agarose gel and imaged on AlphaImager (BioSciences).

Mouse motor neuron like cell line, NSC-34, were plated onto 6-well plates at a density of 3.2 X 104. After 5 days, ccells were harvested, and RNA isolation and first strand complementary DNA synthesis were performed as previously describe. mSlc9a 1-4 primers sets were generated using mouse NHE reference sequenced and OglioPerfect, Invitrogen (Siyanov, 2015). mSlc9a5 primers generated using NHE5 mouse reference sequence. Slc9a isoform PCR thermocycling profile was an initial denaturing step at 95°C for 15 minutes followed by 35 cycles of 94°C for 1 min, a 1

min annealing step with a temperature gradient from 55°C-65°C (based on idea Tm for each primer set) and 72°C for 1 minute with a final 10 min extension step at 72°C. Mouse NHE primers used: mSlc9a1-F 5'-caccagtggaactggacctt-3'; mSlc9a1-R 5'-agggtggtccaggaactgtg -3(Tm 61.8°C); mSlc9a2-F 5'-atcacggctgctattgtcgtt -3; mSlc9a2-R 5'-gtgaccccagtgtccacacaca -3(Tm 65°C); mSlc9a3-F 5'-tatcttcgccttcctgctgt -3'; mSlc9a3-R 5'-gctctgagatgttggccttc -3'(Tm 56.7); mSlc9a4-F 5'-tgtgtgtgggcagtggagtcac -3; mSlc9a4-R 5'-gactgatagggtgtgggagaagcc -3'(Tm 65°C);mSlc9a5-F 5'-taacttgtggggtcagccac-3;mSlc9a5-R 5'-tggctctgcctaagctatcc-3'.

2.9 *SMN2* Dual Reporter Construct Generation

Mini construct, pCI-neo *SMN2*, containing exon 6-8 (Zhang et al., 2001) and the dual reporter construct containing click beetle green luciferase (CBGr68) and click beetle red luciferase (CBRed) under the control of an internal ribosome entry site (IRES) (previously generated by Dr. Butchbach, personal communication) were used as templates to create the *SMN2* dual reporter construct with high fidelity taq polymerase(Thermo Fisher), using the overlapping PCR extension strategy (Zhang et al., 2001). Thermocycling profile used was per manufactures protocol for high fidelity taq polymerase. Following Primer sets were used: SMNint6F(P1) 5'-cctccgcctcccaaagtt-3'; SMNex7+GR(P2) 5'-gaattactccttagtttaagg-3'; SMNex7+CF(P3) 5'-cattccttaaatctaaggagtaagtc-3'; SMNex8-275RS(P4) 5'-ccccacccagtcttttac-3'; SMNex8-21-CBG68(P5)5'gaaatgctggcatagagcagct

ggtgaccgcgaaa-3'; CBG68-1049R(P6) 5'-gccacttctttggccaaaggagcagcgcc-3'; CBG68-1049F(P7) 5'-ggcgctgctcctttggccaaagagtggc-3'; CBR-1720NotIR(P8) 5'-aatgcggccgcttactaaccggccttc-3'

The four products were generated from this were gel purified using gel extraction kit (Qiagen) and cloned into the TA cloning vector using the TA cloning kit (Thermo Fisher).

2.10 Sanger Sequencing

Sequencing for overlapping PCR was performed by the Biomolecular Core Laboratory (Nemours/Alfred I. duPont Hospital for Children) Isolated plasmid DNA sample was amplified by PCR, using three sequencing primers M13-20, M13R, and T7. The PCR thermocycling profile was 5 min at 96°C followed by 40 cycles of 30 sec 96°C, 30 sec 56°C, and 1 min 72°C followed by a final extension step for 7 min at 72°C. Wizard SV Gel and PCR Cleanup System (Promega, Madison, WI) was used to purify per manufacturer's directions and sequenced with the ABI 3130xl Genetic Analyzer (Life Technologies) automated sequencer, using the BigDye Terminator v3.1 Cycle Sequencing kit (Life Technologies) The overlapping PCR sequences were then checked to make sure they amplified the correct portion of the SMN2 gene and contained no mutations. Sanger sequencing primers: M13-20 5'-gtaaaacgacggccagt-3'; M13R 5'-catggtcatagctgtttc-3'; T7 5'-aatacgactcactaatag-3'

2.11 Subcloning *SMN2* into an Inducible Bacterial Artificial Chromosome (BAC) Vector

P1 artificial chromosome (PAC) containing the complete 35.5 kb *SMN2* genomic sequence (PAC1215P15; BACPAC Resources Center Children's Hospital Oakland Research Institute), were streaked on to 50 μg/mL kanamycin LB agar plates and incubated overnight at 37°C. Single colonies from plates were picked and inoculated in 15ml falcon tubes (BD Biosciences) containing 3 mL of LB broth and 50mg/mL kanamycin added at a 1:1000 μL for 8 hours at 37°C shaking at a speed of 300 rpm. Inoculations were then transferred to 2800 mL flask containing 500 mL of LB broth with 50mg/ml kanamycin added at 1:1000 μL a grown overnight at shaking 37°C at a speed of 300 rpm. PAC1215P15 DNA was then isolated using the Large Construct kit (QIAGEN) per the manufacturer's instructions. Isolated DNA was then used to excise the 35.5kb *SMN*2 fragment via a restriction digest using *Bam*HI and *Cla*I. Restriction digest was performed for 6 hours with the restriction enzymes being added at time 0 and 3 hours and then an aliquot of the product was ran out on a 1% TAE agarose gel at 4.5V/cm. The remainder of the aliquot was used to ligate into a CopyControl BAC vector (pCC1-BAC; Epicentre) and transformed using epi300 bacterial cells (Epicentre). Colonies were grown on LB plates contain 12.5 μg/μl chloramphenicol, 40 μg/ml X-gal, and 100 μM IPTP for blue/white screening. Colonies were then pick and inoculated in 15 mL falcon tubes contain 5mL LB broth and chloramphenicol at 1:000 μL and DNA was isolation with miniprep kit (QIAGEN).

SMN2 presence was confirmed through PCR using the three *SMN2* specific primer sets as well as two primer sets generated for regions in the PAC1215P15 outside of the *SMN2* gene. CopyControl induction solution (Epicentre) was used to confirm that *SMN2* was contained in the CopyControl BAC. Primer sets use: SMN2 intron 1(F) 5'-caaacacctggtatggtcagtc-3'; SMN2 intron 1 (R) 5-gcaccactgcacaacagcctg-3'; SMN2int7.3(F) 5'-cagttgttgtagtataaccttgg-3'; SMN2int7.3(R) 5-ccaatgttagccaggatggtctc-3'; RP1215P15(P1) 5'-actgttacccagagagggtca-3'; RP1215P15(P2) 5'-gtgcttaatatgactctgtgtcc-3'; RP1215P15(P3) 5'-accctaacagccccccttgag -3'; RP1215P15(P4)5'-aagcagtcttgtccaagtggt-3'.

2.12 Retrofitting pCC1-SMN2 with Neomycin Resistance Cassette

Cre enzyme containing bacteria SW106 (National Cancer Institute, Fredrick Maryland), which express Cre recombinase under conditions of high arabinose were made electrocompetent. To make the SW106 cells electrocompetent, 5ml of low NaCl LB media was inoculated with 5µl of SW106 glycerol stock and grown overnight at 32°C. Overnight culture was diluted 1:50 and transferred into a 50 ml Erlenmeyer baffled flask and incubated in a shaking water bath for 4 hours. Flask containing bacteria were then cooled down in an ice bath for 2 minutes before being transferred into pre-cooled 15 mL falcon tubes. 15 ml falcon tubes were spun down at 5000rpm for 5 minutes at 0°C. Pellets were resuspended with 1ml of ice cold 10% glycerol

followed by a wash with 9ml of ice cold 10% glycerol and spun again for 5 minutes. Pellet resuspension, wash, and spin were repeated. Residual glycerol left in falcon tube was used to resuspend pellets and 50ul aliquots per 1.5ml tubes were made of the freshly made electrocompetent cells and stored at -80°C or used directly.

500 ng of pCC1-SMN2 was electroporated into the SW106 bacteria using 0.1cm gap electroporation cuvettes (Bio-Rad) and Gene Pulser Xcell electroporation system (Bio-Rad) at 1.8kv. 0.96 mls of low salt LB media containing was added to cuvettes and then transferred to a falcon tube and incubated at 32°C for 90 minutes shaking at 250rpm. 200 µl of bacteria cells were then plated on an LB plate with 12.5 µg/ml chloramphenicol and incubated at 32°C for 24 hours. Colonies were the picked and inoculated in 5mL of low salt LB media and made electrocompetent using same process previously discussed. Plasmid pRetroNeo (Lachmann et al., 2003) was then electroporated into electrocompetent SW106 cells containing pCC1-SMN2 using same electroporation conditions. 0.96mls of low salt LB containing 0.2% arabinose was added to cuvette and transferred to a falcon tube and incubated 32°C for 90 minutes shaking at 250rpm. 200 µl of bacteria cells were then plated on LB plate with 12.5 µg/ml chloramphenicol and 50ug/ml of spectinomycin. The plates were the incubated at 32°C for 24 hours (Lachmann et al., 2003).

Single colonies from plates were picked and inoculated in 15ml falcon tubes (where is this from) containing 3 mL of LB broth with 12.5 µg/ml chloramphenicol and 50µg/ml of spectinomycin for 8 hours at 37°C shaking at a speed of 300 rpm. Inoculations were then transferred to 2800mL flask containing 500 mL of LB broth

with 12.5 µg/ml chloramphenicol and 50µg/ml of spectinomycin grown overnight at shaking 37°C at a speed of 300 rpm. pCC1-SMN2-Neo DNA was then isolated using Large construct kit (QIAGEN) per the manufacturer's instructions. Isolated DNA was then tested with the three *SMN2* specific primers used during the isolation of RP1215P15 DNA.

2.13 Transfection of pCC1-SMN2-Neo BAC

NSC-34 cells were grown to 80-90% confluency and the seeded in a 6 well cell culture plate at the density of 2 $x10^5$ the day before transfection. Transient tranfection was performed with 6 µL of LipofectAMINE 2000 was diluted in 244µL of OPTIMEM and incubated for 5 minutes. A total of 5.0 µg of pCC1-SMN2-Neo was added to the 250µL of OPTIMEM and Mixed and incubated for 5 minutes. The pCC1-SMN2-Neo solution was then added Lipofectamine/OPTIMEM solution and mixed via pipetting and incubated for 20 minutes at room temperature. During the transfection complex incubation media was remove from the plated cells and 500 µL of maintenance media. After 20 minutes the transfection complex was added to the cells dropwise and incubated at 37°C overnight. The following day the transfection complex was removed and replace with 2mL of fresh media. NSC-34 cells were culture for two additional days before being harvested. Genomic DNA was then isolated and *SMN2* genotyping PCR was performed to verify the presence of *SMN2*.

SMN2 genotyping primer set used: SMN2(F2) 5'-gcgatagagtgagactccatct -3'; SMN2(R1) 5'- gacatagaggtctgatctttagct-3'

Stable line transfection was performed with LipofectAMINE 2000 (Invitrogen Life Technologies). 2µL of LipofectAMINE 2000 was diluted in 250µL of OPTIMEM and incubated for 5 minutes. A total of 0.5µg of pCC1-SMN2-Neo was added to the 250µL of OPTIMEM and Mixed. The pCC1-SMN2-Neo solution was then added Lipofectamine/OPTIMEM solution and mixed via pipetting and incubated for 30 minutes at room temperature. During the transfection complex incubation media was remove from the plated cells and 1.5mL of OPTIMEM. After 30 minutes the transfection complex was added to the cells and incubated at 37°C for 3 hours. The media was then removed and replaced with 2ml of fresh maintenance media. After overnight incubation transfected cells were trypsinized and 1/12 of cells was plated into each well on a 12 well plate. Media was replaced every two days with fresh media containing 800µg/mL G418 (Santa Cruz).

On day 10 cells were trypsinized and plated on 96 well plates following limited dilution protocol to establish monoclonal cell lines. For limited dilution, cells were counted and diluted at a concentration of 5 cells/ml. 100 µL of the 5 cells/mL solution was seeded into each well of a 96-well plate so that on average each well was seeded at a density of 0.5 cells/well reducing the number of wells with more than one cell. After 7 days of growth plates were scanned for monoclonal lines. Monoclonal lines expanded in 96 well until 80% confluent before being trypsinized for expansion. After expansion, stock vials of monoclonal cell lines were stored in liquid nitrogen or plated

for genomic DNA, RNA, or protein analysis. Genomic DNA was then isolated and *SMN2* genotyping PCR was performed to verify the presence of *SMN2*. RNA isolation was also performed, and first strand complementary DNA synthesis were performed (as described in section 2.10) PCR was then performed with SMN primers under the thermocycling condition of 2 minutes at 50°C then 10 minutes at 95°C followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The final extension step was at 72°C for 5 minutes. SMN primers used: SMNex78Rq 5'-atgccagcatttctccttaattta-3'; SMNex68R 5'-atgccagcatttccatataatagc-3'.

2.14 G418 Resistant Cassette PCR

Genomic DNA was isolated from established pCC1-SMN2 monoclonal lines using Blood and Tissue kit (Qiagen) per manufacturer's instructions. The genomic DNA from the six monoclonal cell lines was then used to perform to confirm retrofitting of pCC1-SMN2 with pRetroNeo had occurred. PCR was performed with NeoR junction primers to confirm the presence of the Neo DNA. PCR reactions were thermocycled under the conditions of 4 minutes at 95°C then 1 minute at 95°C followed by 35 cycles of 1 minute at 95°C ,1.5 minutes at 64°C, and 1 minute at 72°C. The final extension step was at 72°C for 4 minutes. NeoR primers used: Neo (F) 5'-gcaggttctccggccgcttgg-3'; Neo (R) 5'-caccatgatattcggcaagca-3'.

2.15 Data and Statistical analysis

All data is expressed as mean ± standard error. Statistical significance was determined using a one-way analysis of variance (ANOVA). All statistical analyses were performed with Sigma Plot version 12.0. Holm-Sidak post hoc statistical test was performed to determine significance between amiloride treated samples and control samples at a p value of <0.05.

Chapter 3

EFFECT OF AMILORIDE DERIVATIVES ON *SMN* EXPRESSION

3.1 Effect of amiloride and its derivatives on *SMN2* splicing activity in reporter cells

The pH of the cellular microenvironment has been shown to regulation pre-mRNA alternative splicing. In mammalian cells, the NHE tightly regulate changes in intracellular pH. Based on a previously study showing NHE inhibitor EIPA as a modifier of exon 7 inclusion in *SMN2* mRNA (Yuo et al., 2008), we hypothesize that other inhibitors of the NHE may be able to also modulate the inclusion of exon 7 in *SMN2*. In this study six NHE inhibitors (amiloride, cariporide, DMA, EIPA, HMA, and zoniporide) will be evaluated for their ability to modulate the inclusion of exon 7 increasing the amount of *FL-SMN* produced.

To assess the ability of the amiloride compounds to promote the inclusion of exon 7, beta-lactamase activity was measured in NSC-34 clonal cell line 5.3 (Andreassi et al., 2001). The NSC-3.4 clonal cell line 5.3 contains a reporter construct which includes the *SMN2* promoter and *SMN2* exon 6–8 genomic sequence. The β-lactamase reporter gene fused to exon 8 and exon 7 is modified by the addition of a single nucleotide disrupting the translational termination signal. If exon 7 is included in the transcript, the β-lactamase sequence is in-frame and translated while if exon 7 is excluded, the β-lactamase translational reading frame will be disrupted, and the reporter gene will not be expressed (Andreassi et al., 2001). NSC-34 cells are a

combination of neuroblastoma cells and mouse motor neurons. The NSC-34 cell line has motor neuron like characteristics such as expression of choline acetyl transferase and the neurofilament triplet proteins making it ideal for studying SMA (Cashman et al., 1992).

In absence of β-lactamase activity, CCF2-AM substrate remains intact and emits a fluorescence signal of 530nm (green channel). In the presence of β-lactamase, the CCF2-AM is cleaved, and the cells emit a fluorescent signal of 460nm (blue channel). A high 460/530 ratio is therefore indicating increased β-lactamase activity which correlates to increase exon 7 inclusion. NSC-34 clonal cell line 5.3 were then treated with vary concentrations of amiloride compounds and the 460/530 fluorescence ratio was determined for each drug concentration. Amiloride, cariporide, and zoniporide showed a significant decrease in 460/530 fluorescence while DMA showed no change in its 460/530 ratio for any concentration tested. Consist with the previous study EIPA showed an increase in its 460/530 fluorescence ratio for the 10μM treatment as well as HMA correlating to an increase in exon7 inclusion ($p<0.005$) (Figure 3.1)

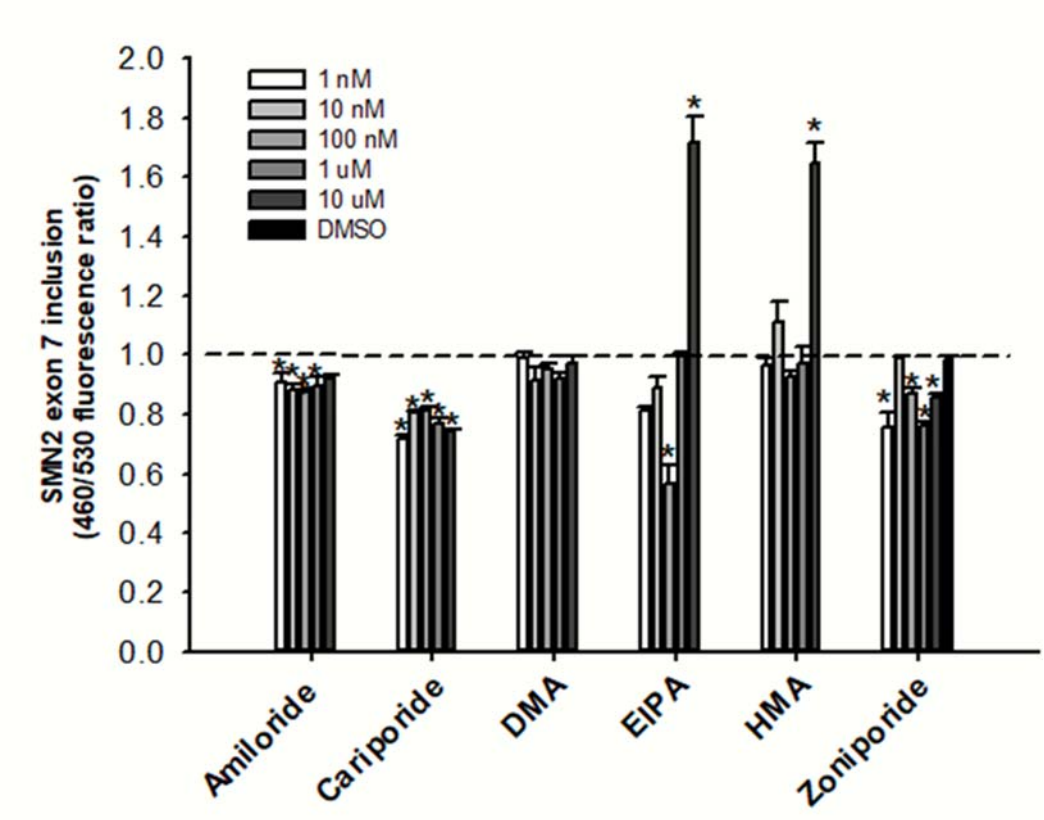


Figure 3.1 Effect of amilorides on exon 7 inclusion in NSC-34. NSC34 clone 5.3 cells were treated with varying concentrations of amiloride (1 nM to 10 μM) or DMSO. Treatment was done in quadruplicate for each drug compound. After 19 hours of treatment BLA substrate, CCF2/AM was added to samples. β-lactamase activity of amiloride treated cells relative to DMSO are plotted to assess exon 7 inclusion. Results are displayed as the ratio of fluorescence intensity of the blue channel (460 nM) to that of the green channel (530 nM). Higher 460/530 ratios indicate an increased in BLA reporter activity relative to DMSO control. (* $p<0.05$, one-way ANOVA)

3.2 Effect of Amiloride and its derivatives on mRNA transcript on SMA patient derived fibroblast

To determine the changes in *SMN2* expression in response to the amiloride compounds, type II SMA patient derived fibroblast line containing three copies of the *SMN2* gene (GM03813) (Stabley et al., 2015) were treated with either 100nM, 1μM, or 10μM of each of the amiloride compounds. Fibroblast cell line GM03814 was used as a comparator for treated GM03813 samples. The GM03814 cell line is derived from the mother of the GM03813 patient cell line (Scudiero et al., 1986; Butchbach, 2016). GM03814 cells contain one copy of the *SMN1* gene and five copies of the *SMN2* gene. From the six amiloride compounds screened, EIPA and HMA significantly increased *FL-SMN* transcript levels with HMA showing effect at 1μM while EIPA showed increased levels at the highest concentration (10μM) ($p<0.005$). No significant increase in *SMNΔ7* transcript level was observed for any of the tested amiloride compounds (Figure 3.2 A and B).

To ensure results seen in the GM03813 fibroblast were not cell line specific, we tested these compounds on two additional type II patient derived fibroblast cell lines with three copies of *SMN2* (GM22592 and AIDHC-SP22). Both cell lines were treated with the highest dose of each compound since at this concentration a significant increase in *FL-SMN* transcript levels was observed for two of the six compounds. Similar to the results seen in GM03813 cells, HMA and EIPA showed a significantly increase *FL-SMN* mRNA levels ($p<0.05$) in the GM22592 and AIDHC-SP22 fibroblast cells (Figure 3.3 A and C). Amiloride, cariporide, and zoniporide

showed a significant increase in *SMNΔ7* mRNA levels ($p<0.005$) (Figure 3.3 B and D).

3.3 EIPA and HMA modify exon 7 inclusion in SMA patient derived fibroblast

SMA patient derived fibroblast GM03813 treated with amiloride compounds mRNA was assayed via RT-PCR to determine whether the NHE inhibitors are capable of increasing the amount of FL-SMN levels produced compared to SMNΔ7 in SMN2 mRNA. Primers targeting SMN exon 6 and 8 were used to amplify the treated fibroblast cDNA. As a control, collagen IIIA (COL3A) transcript levels were used as a comparator since COL3A is highly and constitutively expressed in fibroblast cells (Heier et al.,2007). PCR products were then resolved through a 2% agarose gels via electrophoresis to separate FL-SMN and SMNΔ7 mRNA bands. Relative levels of FL-SMN and SMNΔ7 were quantitative base on the band intensity (Figure 3.4 A-F). EIPA significantly increase the ratio of FL-SMN to SMNΔ7 at a concentration of 10 μM ($p<0.005$). HMA showed an increase in the FL-SMN to SMNΔ7 ratio starting at the concentration of 100 nM ($p<0.05$).

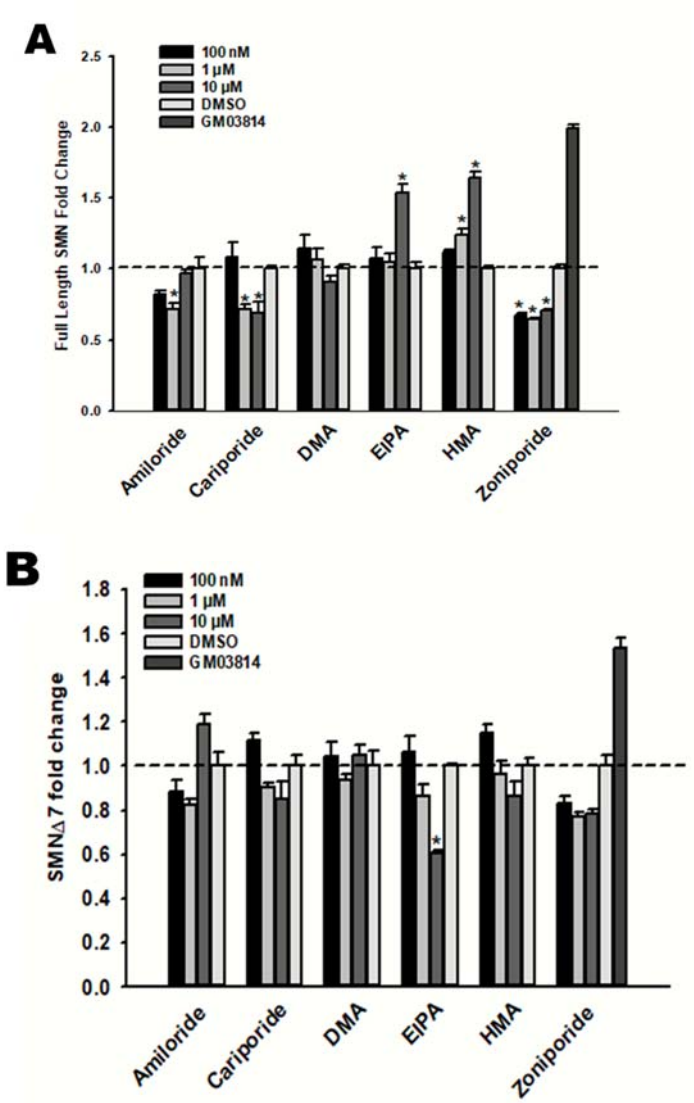


Figure 3.2 Effect of NHE inhibitors on SMA patient derived GM03813 fibroblast *FL-SMN* and *SMNΔ7* mRNA. (**A**) Changes in *FL-SMN* mRNA levels in GM03813 (SMA type II fibroblasts) treated with DMSO or the NHE inhibitors (amiloride, cariporide, DMA, HMA, EIPA and zoniporide) at a concentration of 10µm, 1µm and 100nM for 5 days (n=3/treatment group). (**B**) Changes in *SMNΔ7* mRNA levels in GM03813 treated under the same conditions as (**A**) for 5 days (n=3/treatments group). Data is reported as the fold change in mRNA relative to three reference transcripts mRNAs (*ACTB*, *GAPDH*, and *RPLP0*). Results are expressed as mean ± SEM relative to DMSO control (*p<0.05 one-way ANOVA).

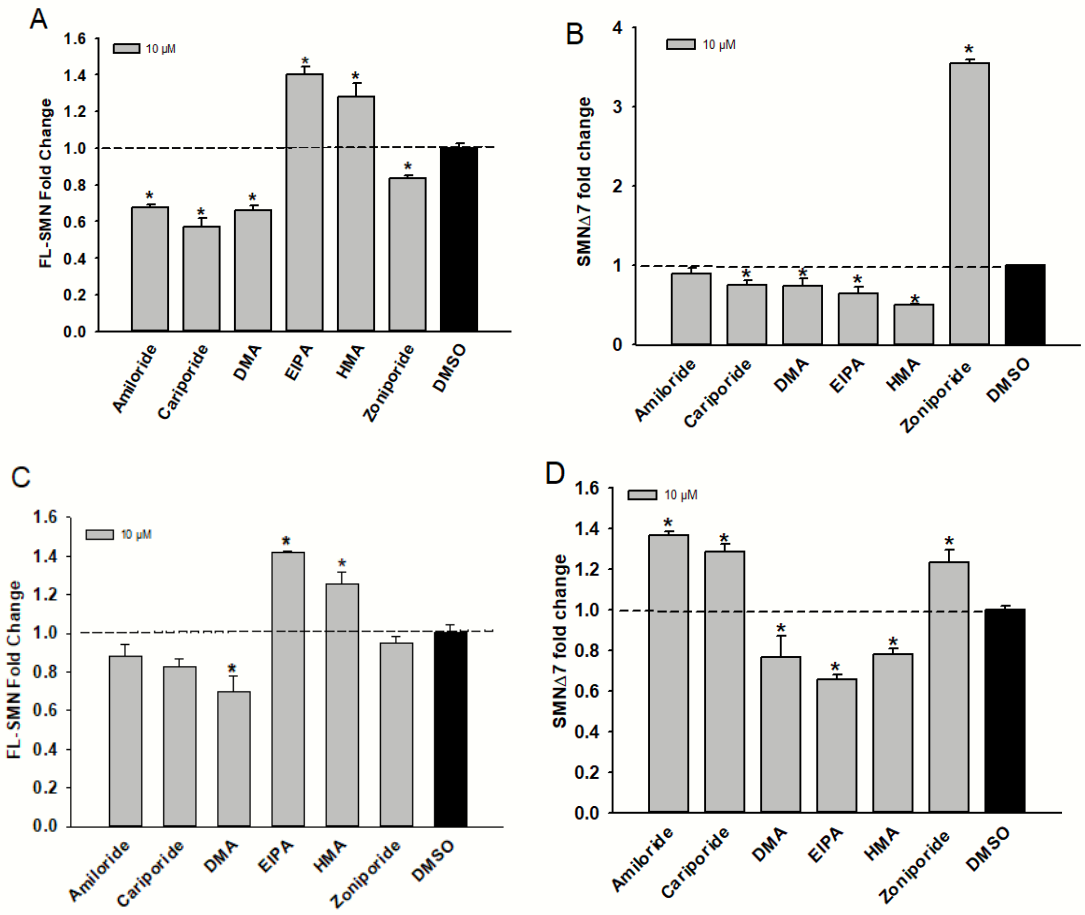


Figure 3.3 Effect of NHE inhibitors on SMA patient derived AIDHC-SP22 and GM22592 fibroblast *FL-SMN* and *SMN*Δ*7* mRNA. Changes in *FL-SMN* and *SMNΔ7* mRNA levels in AIDHC-SP22 (**A** and **B**) GM22592 and (**C** and **D**) fibroblast cell lines (SMA type II) treated with the highest concentration of each NHE inhibitor tested (10 µM) or DMSO) for 5 days (n=3/treatments group). GM03814 (SMA carrier cell line) is included to show normal levels of *FL-SMN* and *SMN*Δ*7*. Data is reported as the fold change in mRNA relative to three reference transcripts mRNAs (*ACTB*, *GAPDH*, and *RPLP0*). Results are expressed as mean ± SEM relative to DMSO control (*$p<0.05$ one-way ANOVA).

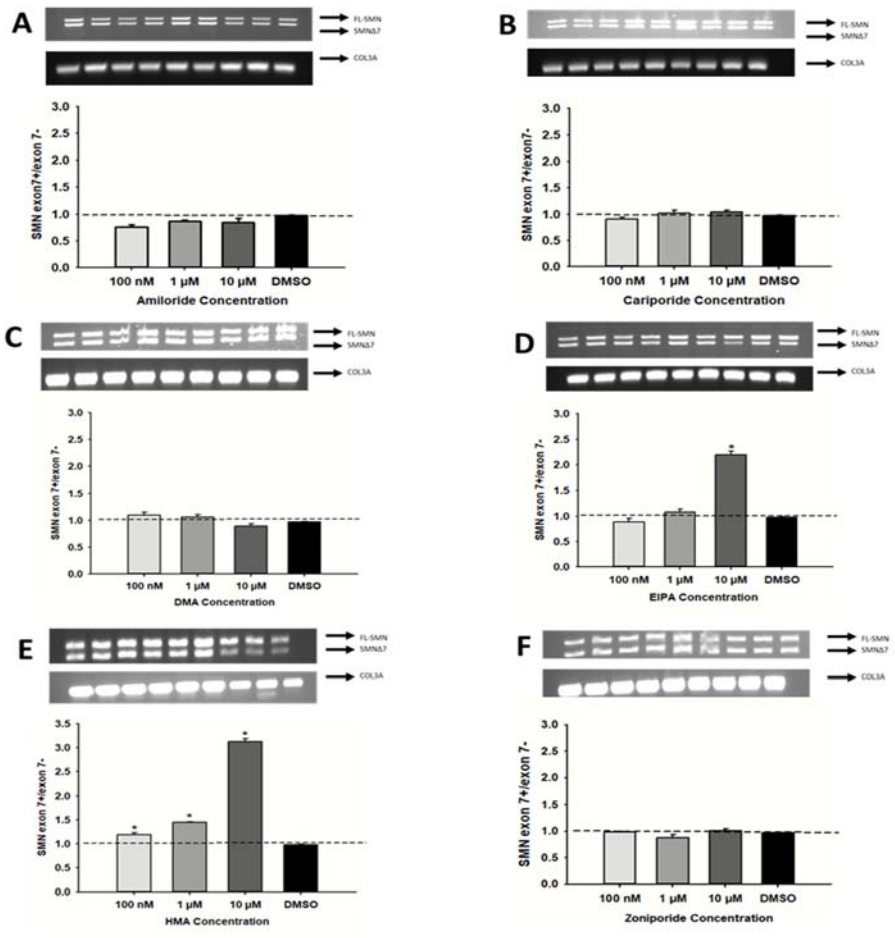


Figure 3.4 SMN exon 7 inclusion in SMA patient derived fibroblast treated with NHE inhibitors. GM03813 fibroblast were treated with DMSO or NHE inhibitors (amiloride, cariporide, DMA, EIPA, HMA, and zoniporide) at the concentration of 100 nm, 1μM, and 10μM for 5 days (n=3/treatment group). RNA was extracted following treatment and cDNA was synthesized. RT-PCR was then performed using SMN specific primers targeting exon 6 and exon 8. PCR products were electrophoresed through a 2% agarose gel. A representative image is shown above graphs (**A-F**) for each drug treatment. Band intensity of *FL-SMN* vs *SMNΔ7* in response to drug compound was quantified using ImageJ. Data is reported as a ratio of FL-SMN/*SMNΔ7* relative to DMSO treated GM03813 (**A-F)**. GM03814 (SMA carrier cell line) is included to show normal ratio of *FL-SMN* and *SMNΔ7*. Results are expressed as mean ± SEM relative to DMSO control (*p<0.05, one-way ANOVA).

3.4 NHE isoforms present in NSC-34, GM03813 and GM03814 cell lines

Although several NHE inhibitors were examined for their capability to modify exon 7 splicing in *SMN2*, only EIPA and HMA were found to significantly increase exon 7 inclusion. To explore the mechanism behind EIPA and HMA effect on modifying the inclusion of exon 7 in *SMN2*, GM03813, GM03814, and NSC-34 were plated for RNA isolation and first-strand complementary DNA was synthesized. Patient derived fibroblast cell lines GM03813 and GM03814, as well as the mouse motor neuron like cell line NSC-34 were screened for the presence of NHE isoforms 1-5. NHE 1-5 were selected for screening because they the SLC9a family members primarily expressed on mammalian cell membranes. Human specific and mouse specific NHE 1-5 primers were then used to identify the NHE isoforms present in each cell line. GM03813, GM03814, and NSC-34 cell lines were found to contain *NHE1* and *NHE5* mRNA (Figure 3.6). Interestingly, GM03813 fibroblast were positive for NHE 4 and GM03814 cells were not. Previous studies on the amiloride compounds ability to inhibit various NHE isoforms show that HMA and EIPA inhibit both NHE1 and NHE5 more effectively than the other compounds tested based on their low Ki (IC50) values (Masereel et al., 2003) (Table 1.2).

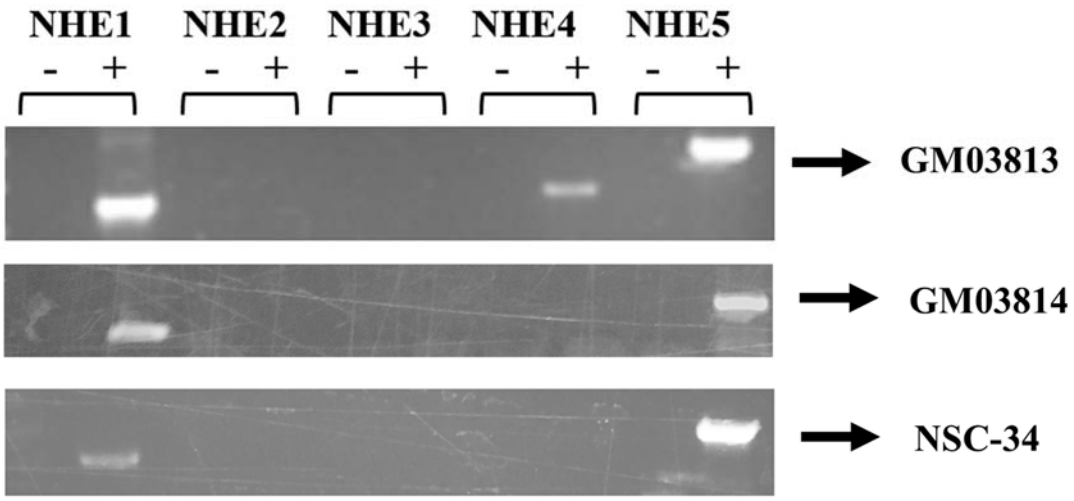


Figure 3.6 NHE isoforms present in GM03813, GM03814, and NSC-34 cell lines. GM03813, GM03814 and NSC-34 cell lines were plated for RNA analysis without the addition of any drug compounds or DMSO and grown for 5 days (n=3/cell line). RNA was extracted, and cDNA was synthesized. RT-PCR was then performed using human or mouse specific NHE 1-5 primers PCR products were electrophoresed through a 1% agarose gel. One set of the triplet tested representative image shown above (Negative control samples contain primer and no cDNA are represent in lane with "-"symbol while samples contain the respective cell line cDNA and NHE primers are represent in lanes with "+" symbol)

3.5 Cytotoxic effects of amiloride and its derivatives on SMA patient derived fibroblasts

Reduction of the cellular pH could potentially lead to off target effects by the NHE inhibitors leading to cytotoxicity. The cytotoxic effects of the amiloride compounds was assessed on SMA patient derived fibroblast, GM03813 after 24 hours and 120 hours of treatment with either no treatment, DMSO, or varying concentrations of the amiloride compounds in quadruplicates. After either 24 hour or 120 hours of treatment, the Cell Titer Blue reagent was added to each sample and incubated for four

hours to measure the number of viable cells. Cell Titer Blue reagent provides an indicator dye with resazurin which allows for the measurement of cellular metabolic activity. Live cells are able to reduce the resazurin to a highly fluorescent substance known as resorufin. If the cells are dead, metabolic capacity will be lost and the indicator dye will not be reduced. The dead cells cannot generate a fluorescent signal while live cells will emit a fluorescence signal of 590 nm. A high 590 nm emission therefore indicates a greater number of viable cells.

After 24 hours of treating the GM03813 fibroblast cells with various concentrations of the NHE inhibitor compounds, the cell viability remained greater than 80% for each of the screened compounds (Figure 3.7 A-F). The time point of 120 hours (5 days) was chosen based on the drug treatment time used for the mRNA analysis. After 120 hours of treatment, NHE inhibitors amiloride, cariporide, DMA, and zoniporide treatments showed similar amounts of viable cells when compared to DMSO treated fibroblast to the no treatment controls. Consistent with the previous study on EIPA, at the 10 μM, the highest concentration tested, EIPA showed about 60% cytotoxicity while HMA treatment at the highest concentration showed about 80% cytotoxicity to the GM03813 cells (Figure 3.8 A-F).

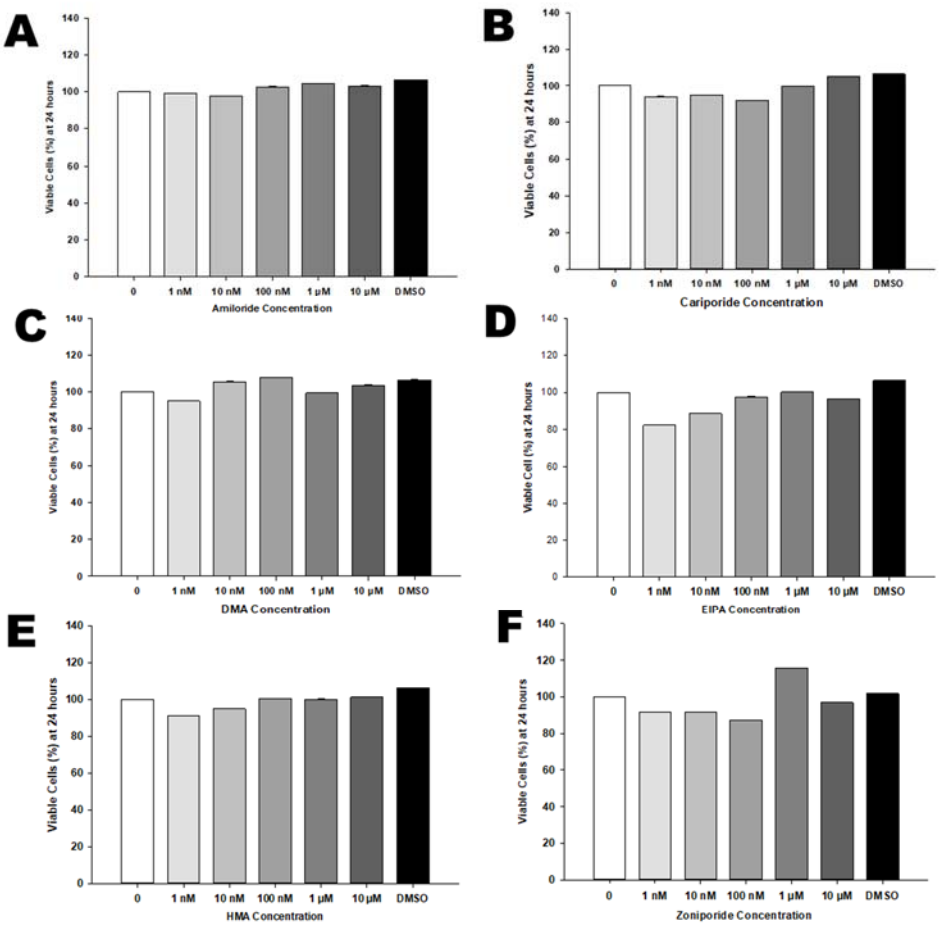


Figure 3.7 Cytotoxic Effects of amiloride and its derivatives after 24 hours of treatment on SMA derived patient GM03813 fibroblast. GM03813 fibroblast were plated for cell viability analysis. 24 hours after plating, the GM03813 were then treated with various concentrations of each NHE inhibitor (1nM to 10 µM) for 24 hours. Treatments were performed in quadruplicates for each drug compound. After 20 hours of treatment, the CTB substrate was added to each treatment group and incubated for 4 hours. The fluorescence of each group was measured at 550 nM. The viability of each treatment group relative to untreated cells are plotted to assess the percent of viable cells for each compound.

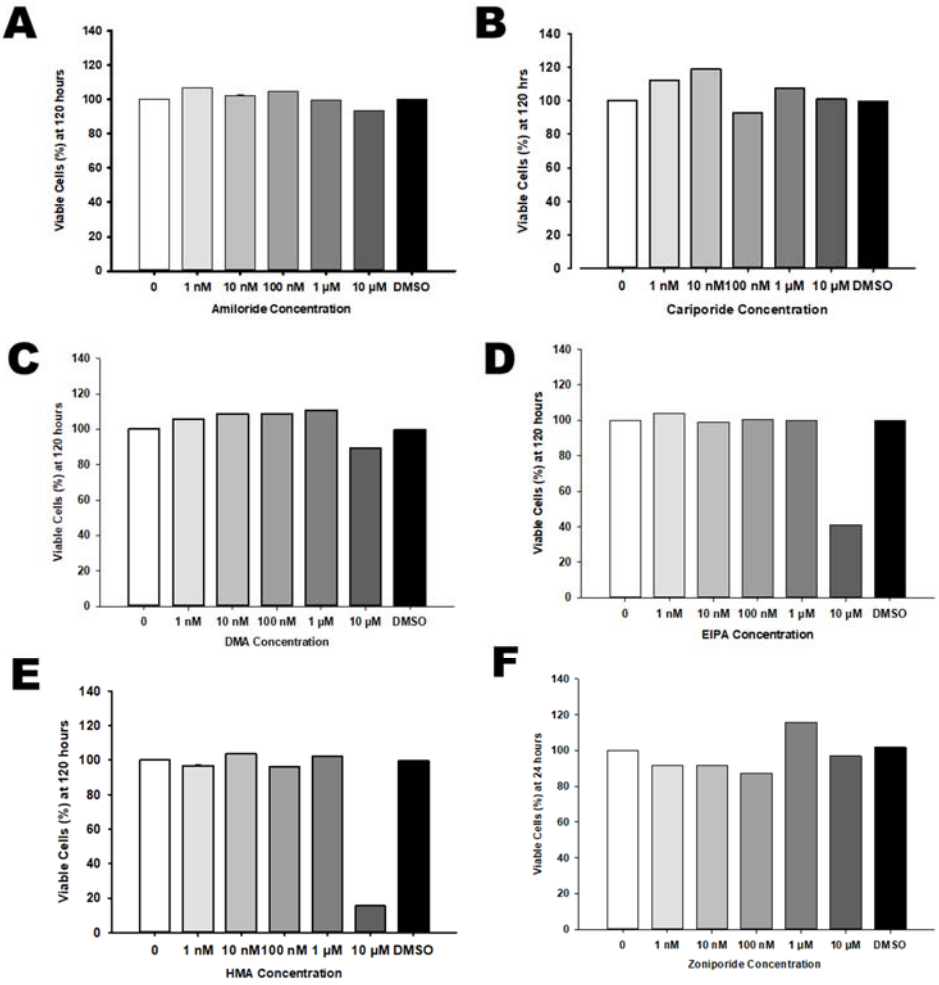


Figure 3.8 Cytotoxic Effects of amiloride and its derivatives after 120 hours of treatment on SMA derived patient GM03813 fibroblast. GM03813 fibroblast were plated for cell viability analysis. 24 hours after plating, the GM03813 were then treated with various concentrations of each NHE inhibitor (1nM to 10 µM) for 120 hours (5 days). Treatments were performed in quadruplicates for each drug compound. After 116 hours of treatment, the CTB substrate was added to each treatment group and incubated for 4 hours. The fluorescence of each group was measured at 550 nM. The viability of each treatment group relative to untreated cells are plotted to assess the percent of viable cells for each compound.

3.6 Effect of Amiloride and its derivatives on *striatin 3* mRNA transcripts on SMA patient derived fibroblast

To determine the changes in *striatin 3* (*STRN3*) expression in response to the amiloride compounds. Like *SMN2*, *striatin 3* pre-mRNA undergoes alterative splicing. Screening of small molecules found to specifically bind to specific sites on the *SMN2* gene stabilizing the gene for the production of *FL-SMN* mRNA were also found to stabilize the *STRN3* pre-mRNA allowing for the inclusion of exon 8 and 9 of the gene (Sivaramakrishnan et al., 2017). Based on these finding, the amiloride compounds were screened for their effects on exon 8 and 9 inclusion in *STRN3* mRNA. Type II SMA patient derived fibroblast, GM03813 were treated with 10μM of each of the amiloride compounds. Fibroblast cell line GM03813 treated with 1uM of RG7800, a known *SMN2* splice modifying compound used as a comparator for amiloride treated GM03813 samples. Amiloride, cariporide, DMA, as well as RG7800 were all shown to increase *FL-STRN3* mRNA ($p<0.05$) while no change was seen in samples treated with EIPA, HMA, or zoniporide when compared to the DMSO control. *STRN3Δ89* mRNA was shown to be increased in samples treated with amiloride, cariporide, DMA, and EIPA ($p<0.05$). HMA and zoniporide were not shown to have a significant change in *STRN3Δ89* mRNA.

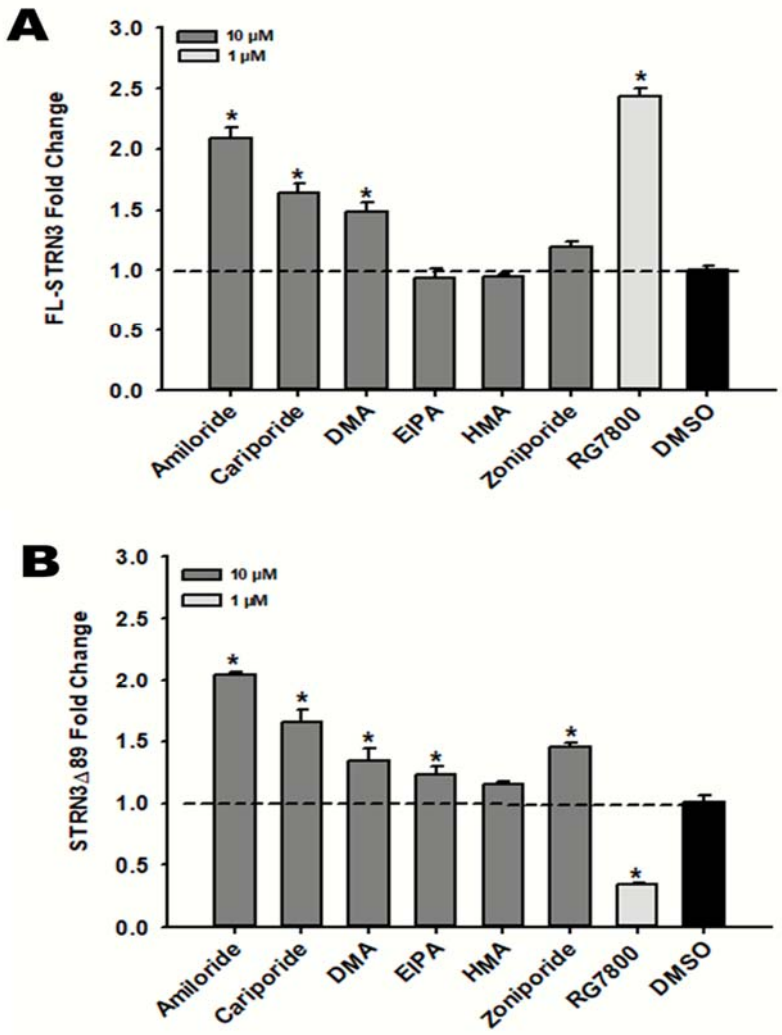


Figure 3.9 Effect of the NHE inhibitors on SMA patient derived GM03813 fibroblast FL-STRN3 and STRN3Δ89 mRNA. (**A**) Changes in FL-STRN3 mRNA levels in GM03813 (SMA type II fibroblast) treated with 1uM of RG7800, DMSO, or 10uM of the NHE inhibitors for 5 days (n=3/treatment group). (**B**) Changes in STRN389 mRNA levels under same conditions as (**A**). Data is reported as the fold change in mRNA relative to three reference transcripts mRNAs (*Rpl13a*, *Gusb*, and *Pgk*). Results are expressed as mean ± SEM relative to DMSO control (*p<0.05, one-way ANOVA).

Chapter 4

DEVELOPMENT OF NOVEL SCREENING ASSAYS TO IDENTIFY *SMN2* INDUCERS

4.1 *SMN2* Dual Reporter Construct

Currently developed reporter assays behave as surrogates of *SMN2* that can be correlated to changes that would be endogenous expression of *SMN2* to identify small molecules that can modify *SMN2*. These reporter assays cover a wide range of approaches in targeting *SMN2* such as changes in the promoter, exon 7 inclusion, protein stability, and multifunction reporter assays that can monitor more than one type of change in *SMN2* simultaneously. The main benefit of reporter assays is that these surrogates of *SMN2* can be subcloned into immortalized cell lines allowing for the use of high-throughput screening of hundreds of thousands of compounds. Although the currently designed reporter assays have identified many compounds that can modulate *SMN2* expression, since these reporters only contain small fragments of the *SMN2* promoter or mini-gene cassettes of the *SMN2* sequence, changes in *SMN2* expression can be caused by reporter assay artifacts leading to the identification of false positives (Cheng et al., 2012; Thorne et al., 2012; Cherry et al., 2014). The novel dual reporter construct developed in this project will contain the entire *SMN2* genomic locus allowing for changes in *SMN2* seen in the dual reporter to more closely recapitulate *SMN2* gene regulation seen *in vivo*. By using the entire *SMN2* genomic locus, this novel reporter line should more accurately identify new small molecules with a reduce number of false positives.

A dual reporter construct monitor changes in the *SMN2* promoter and/or exon 7 inclusion was created with *SMN2* mini-gene cassette and dual reporter plasmid using overlapping PCR (OLP) (Zhang et al., 2001) and Gibson assembly (Figure 4.1 A). Primers were designed to insert CBGr68 and CBRed click beetle luciferase reporter genes to the 3′ end of the exon 8 reading frame allowing readthrough. Exon 7 termination codon was inactivated with the addition of a single nucleotide (guanine) which was inserted after the 48th nucleotide in exon 7. Previous studies of this region have shown that this portion of exon 7 is less sensitive to mutations (Zhang et al., 2001). The addition of a single nucleotide insertion allows for CBGr68 expression of dual reporter, which is inserted in exon 8 in frame with exon 7. The addition of the guanine in this position does not interfere with exon 7 splicing of the *SMN2* mini-gene constructs (Dye et al., 1999; Lorson et al.,2000; Zhang et al., 2001). Because the dual reporter will be in frame with exon 7, CBGr68 will be transcribed when exon 7 is included while CBRed, which is regulated by an internal ribosome entry site, can simultaneously be transcribed due to changes in promoter activity allowing for luciferase expression to be monitored for the identification of *SMN2* inducers (Figure 4.1 B). OLP products were resolved through a 1% agarose to identified correct band size (Figure 4.2). These products were then sequenced to verify the correct portion of *SMN2* had been amplified and contained no mutations. OLP 3-4 and OLP 5-6 were confirmed by Sanger sequencing. OLP 1-2 and OLP 7-8 are in the process of being sequenced.

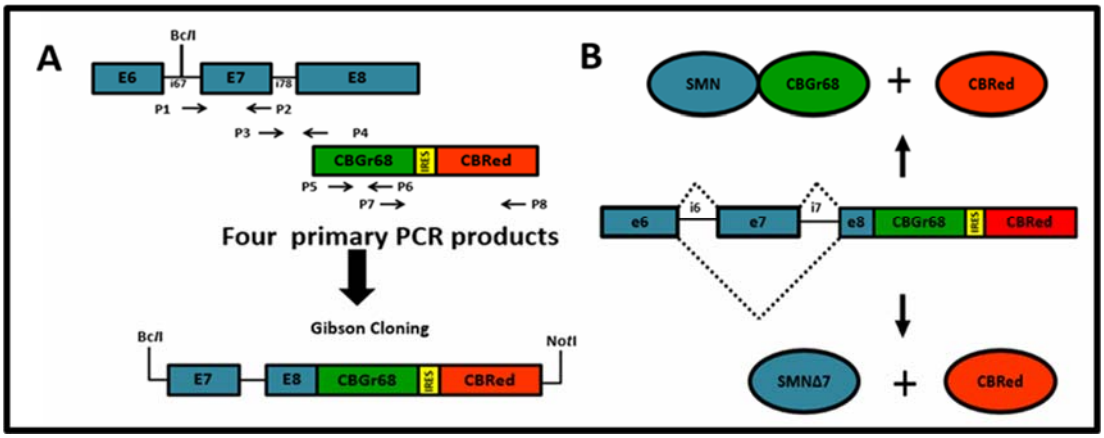


Figure 4.1 *SMN2* dual reporter construct strategy. Figure 4.1A shows OLP strategy (Adapted from Zhang et al., 2001). Four sets of primers were used spanning the *SMN2* mini-gene cassette which covers exon 6-8 and the CBGr68: IRES: CBRed templates. PCR products were cloned into TA cloning vectors for sanger sequencing. Once assembled click beetle luciferase CBGr68 will be in frame with exon 7 with full-length SMN transcripts while CBRed monitors the promoter activity (Figure 4.1B).

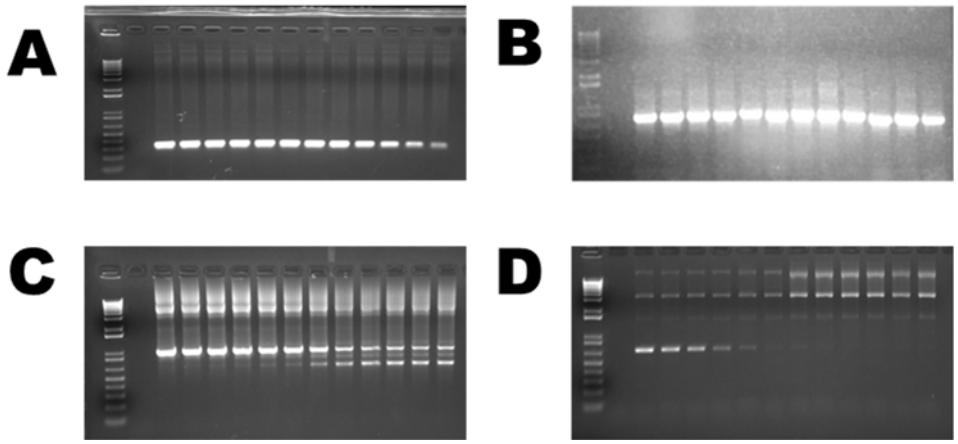


Figure 4.2 Overlapping PCR Products. Four primer sets spanning the SMN2 mini-gene cassatte and the dual reporter (CBGr68:IRES:CRed) were used to amplify regions of SMN2 and the dual reporter construct with overlapping 5' and 3' seqeunces for construct assembly. (**A**) OLP product for OLP 1-2 primers with the predicted size of 300bp. (**B**) OLP product for OLP primers 3-4 with the predicted size of 500bp. (**C**) OLP product for OLP 5-6 primers with the predicted size of 1-1.1 kb. (**D**) OLP product for OLP 7-8 primers with the predicted size of about 5 kb.

4.2 Humanizing NSC-34 motor neuron like cells with *SMN2* gene

Small molecules identified as *SMN2* expression modifiers through high throughput screenings utilizing the reporter based cell lines are validated with primarily culture SMA patient derived cells. SMA patient derived cells are used to ensure the positive effects exhibited by these compounds have similar effects on endogenous *SMN2* expression (Jarecki et al., 2005; Thurmond et al., 2008; Cheng et al., 2012; Thorne et al., 2012). Generation of NSC-34 cell lines that express human *SMN2* will allow for a cell line with high throughput screening capability that can monitor changes in the full human *SMN2* gene in a cell line that exhibits characteristics of motor neurons.

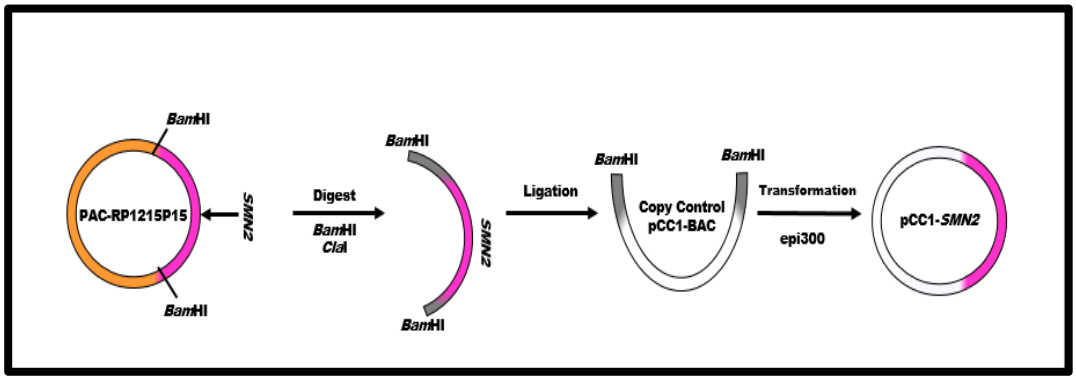


Figure 4.3 *SMN2* Subcloning Strategy. Schematic representation of the removal of RP1215P15 PAC was digested with *Bam*HI and *Cla*I. *Bam*HI isolates the *SMN2* genomic DNA while *Cla*I was used to further separate PAC DNA from *SMN2* since the PAC DNA and *SMN2* band are close in size. *SMN2* was then ligated into *Bam*HI linearized pCC1-BAC vector and transformed with epi300 E. coli cells.

The 35.5kb *Bam*HI fragment containing the full *SMN2* genomic sequence was isolated from P1 artificial chromosome (PAC, RP1215P15). via a restriction digest using *Bam*HI and *Cla*I. RP1215P15 contains a 130 kb insert that includes the *SMN2* gene, *neural apoptosis inhibitory protein* (*NAIP*) gene, the *H4F5* gene, and numerous expressed sequence tags (ESTs) like the brain cadherin pseudogene (Roy et al., 1995; Thompson et al., 1995; Scharf et al., 1998; Selig et al., 1995). Southern blot analysis and DNA sequencing was performed to make sure inserted *SMN2* was intact and present in the PAC (Monani et al., 1999) RP1215P15 was then resolved through a 1% agarose gel to confirm the 35.5 kb *SMN2*. Isolated *SMN2* was then subcloned into the high copy inducible pCC1-BAC vector. pCC1-BAC containing *SMN2*, here after referred to as pCC1-SMN2, was then transformed into epi300 *E. coli* and colonies of pCC1-SMN2 were picked for BAC DNA isolation (Figure 4.3). The presence of *SMN2* was confirmed through PCR using the three *SMN2* specific primer (Figure 4.4). To confirm the removal of RP1215P15 genomic DNA, two primer sets generated for amplification of regions in the PAC1215P15 outside of the *SMN2* gene. The removal of RP1215P15 genomic was confirmed by no amplification of the RP215P15 primers (Figure 4.5).

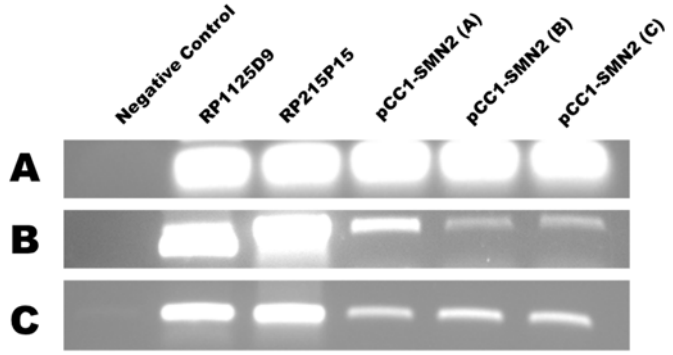


Figure 4.4 pCC1-BAC contains *SMN2* genomic DNA. Isolated BAC DNA, pCC1-SMN2 was tested for the presence of *SMN2* using *SMN2* intron 1 primers (A), *SMN2* intron 2 primers (B), and *SMN2* intron 7 primers (C). Mini gene cassette, Isolated PAC DNA containing *SMN1* RP1125D9 and RP215P15 were used as positive controls while the primers with no template DNA were ran as a control for each primer set. pCC1-SMN2 was ran in triplets for each primer set. pCC1-SMN2 was found positive for each *SMN2* primer.

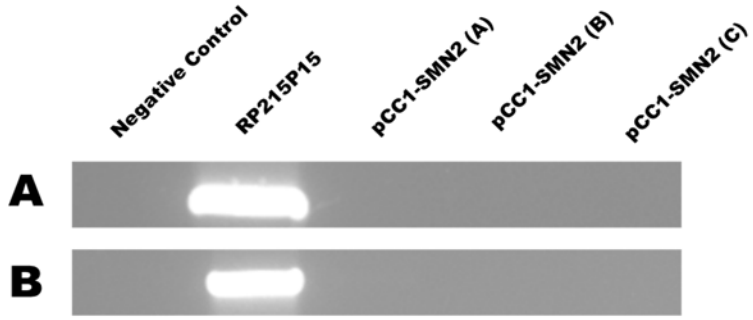


Figure 4.5 Removal of RP215P15 genomic DNA. Isolated BAC DNA, pCC1-SMN2 was tested for the presence of PAC genomic DNA using primers designed to target regions of the RP215P15 PAC outside of the SMN2 region. RP215P15 was used as a positive control while the primers with no template DNA were ran as a control for each primer set. pCC1-SMN2 was ran in triplets for each primer set. pCC1-SMN2 was found to be negative for both RP215P15 primer sets [Image "A" is primer set: RP1215P15 (P1) and RP1215P15 (P2) and image "B" is primer set: RP1215P15(P3) and RP1215P15(P4)].

When establishing cell lines containing BACs for therapeutic studies, giving the BACs selectable genes such as G418 resistance allows for selection of transfected cells containing the BAC and long-term maintenance of the BAC in the particular cell line of interest (Lachmann et al., 2003). The pCC1-SMN2 BAC was retrofitted with pRetro-Neo to introduce the Neo cassette, which encodes for the G418-resistance gene (Figure 4.6). The pCC1-SMN2 BAC and pRetroNeo were transfected into SW106 Cre-expressing bacteria. In SW106 *Cre* expression is induced through the addition of arabinose (Warming et al., 2005). Transformed bacteria were grown on plates containing both chloramphenicol and spectinomycin allows selection for the retrofitted pCC1-SMN2 to determine whether retrofitting of pCC1-SMN2 had occurred. Colonies were then picked for BAC DNA isolation. Arabinose was added to inoculations of pCC1-SMN2 NEO to induce the high copy promoter of the BAC to increase the yield of the BAC since BACs maintain genomic DNA at single copies making the yield fairly low under normal conditions (Choi and Wang, 2000). *SMN2* PCR was performed on isolated BAC DNA to confirm the presence of *SMN2* using the three *SMN2* specific primers (Figure 4.7).

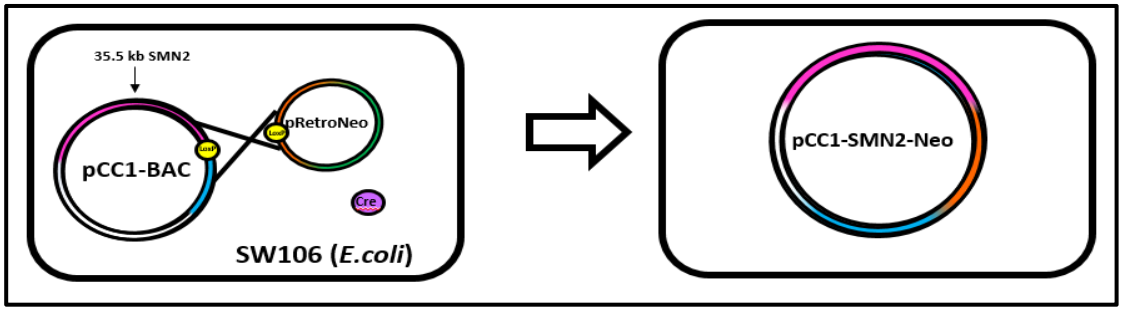


Figure 4.6 Retrofitting pCC1-SMN2. pCC1-SMN2 and pRetro plasmids are transfected into *Cre*-expressing E. coli, SW106. In SW106 *Cre* gene is induced by arabinose. Recombination between the pCC1-SMN2 and pRetroNeo loxP sites (LoxP) results in a retrofitted BAC containing SMN2 and Neo genes as well as chloramphenicol and spectinomycin resistance.

To perform transient transfection, 5.0 µg of pCC1-SMN2-Neo was transfected into NSC-34 using lipofectAmine2000 and the transient transfected cells were grown for four days. Genomic DNA was then isolated from transient transfection and *SMN2* PCR was performed on samples. The experiment was performed triplicate with untransformed NSC-34 cells as a negative control. Transient transfect cells were positive for SMN2 (Figure 4.8).

Stable NSC-34 pCC1-SMN2-Neo cells lines were generated via transfection with lipofectamine 2000 using 0.5µg of DNA. Transfected cells were selected for 7 days with G418 for establishing monoclonal cell lines via limited dilutions. 96 well plates used for screening on average contained 15% of wells that contained single cells, 25% that contained more than one cell with the rest of the wells were empty. 6 monoclonal cell lines were chosen for further characterization. Genomic DNA (Figure 4.9 and 4.10) and RNA (Figure 4.11 and 4.12) was then extracted from the 6

monoclonal cell lines. Isolated genomic DNA was used to confirm the presence of the neomycin gene cassette (Figure 4.9) and the presence of *SMN2* (Figure 4.10). Isolated RNA was used to synthesize cDNA to measure *FL-SMN* and *SMNΔ7* mRNA expression.

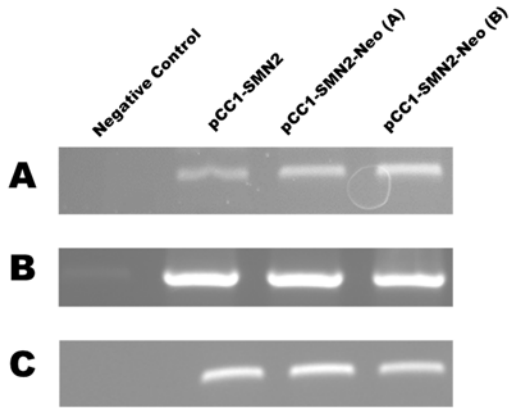


Figure 4.7 pCC1-SMN2-Neo contains *SMN2*.Isolated BAC DNA, pCC1-SMN2 was tested for the presence of *SMN2* using *SMN2* intron 1 primers (A), *SMN2* intron 2 primers (B), and *SMN2* intron 7 primers (C). Mini gene cassette, pci-neo *SMN2* and RP215P15 were used as positive controls while the primers with no template DNA was ran as a control for each primer set. pCC1-SMN2 was ran in triplets for each primer set. pCC1-SMN2 was found positive for each *SMN2* primer.

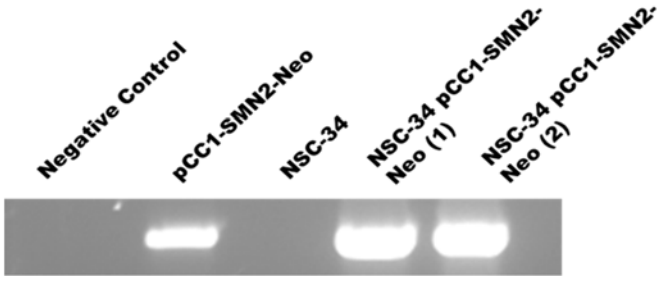


Figure 4.8 Transient transfection of pCC1-SMN2 into NSC-34 cells. NSC-34 cells were transfected with pCC1-SMN2-Neo and grown for 2 days. The NSC-34 cells were then harvested, and genomic DNA was isolated. NSC-34 pCC1-SMN2-Neo was then tested for the presence of *SMN2* using *SMN2* specific genotyping primers. pCC1-SMN2-Neo genomic DNA was used as a positive control while NSC-34 cells that were not transfected were used as a negative control, as well as the *SMN2* primers with no template DNA. pCC1-SMN2-Neo was found to be positive the presence of *SMN2*.

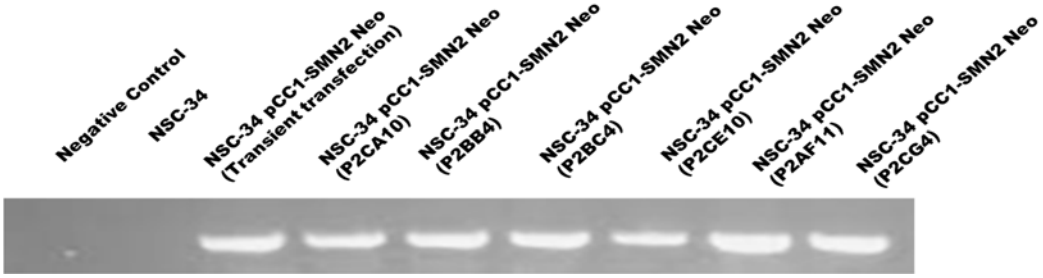


Figure 4.9 Monoclonal NSC-34 pCC1-SMN2 Neo genomic DNA contains Neo cassette. NSC-34 cells were transfected with pCC1-SMN2-Neo and treated with G418 to remove any NSC-34 cells that were not transfected. The transfected NSC-34 cells were then used to establish monoclonal cell lines via limited dilutions. After expansion, 6 monoclonal cell lines were harvested, and genomic DNA was isolated. NSC-34 pCC1-SMN2-Neo was then tested for the presence of the Neo cassette using Neo junction specific primers. Transient transfected NSC-34 genomic DNA was used as positive control while the Neo primers with no template DNA and NSC-34 that were not transfected were used a negative control. pCC1-SMN2-Neo monoclonal cell lines were found to be positive the presence of *SMN2*. Cell lines are name based on the original transfected plate they were derived from (Plate 1 (P1) or Plate 2(P2) followed by the 96 well plate the monoclonal line was expanded from (A, B, or C) and the row and number of the well on the 96 well plate

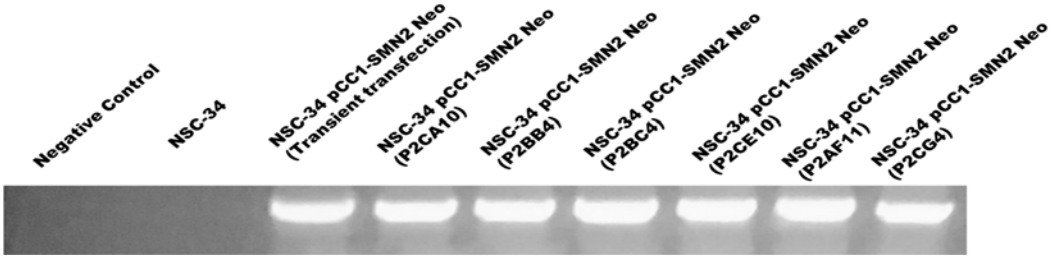


Figure 4.10 Monoclonal NSC-34 pCC1-SMN2 Neo genomic DNA contains *SMN2*. NSC-34 cells were transfected with pCC1-SMN2-Neo and treated with G418 to remove any NSC-34 cells that were not transfected. The transfected NSC-34 cells were then used to establish monoclonal cell lines via limited dilutions. After expansion, 6 monoclonal cell lines were harvested, and genomic DNA was isolated. NSC-34 pCC1-SMN2-Neo was then tested for the presence of SMN2 using *SMN2* specific genotyping primers. Transient transfected NSC-34 genomic DNA was used as positive negative control while the *SMN2* primers with no template DNA and NSC-34 cells that were not transfected genomic DNA was used a negative control. pCC1-SMN2-Neo monoclonal cell lines was found to be positive the presence of *SMN2*.

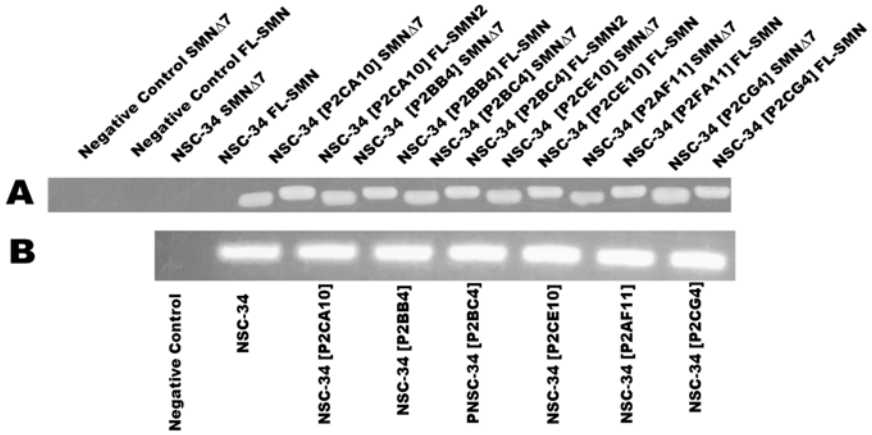


Figure 4.11 Presence of *FL-SMN* and *SMNΔ7* mRNA in monoclonal NSC-34 pCC1-SMN2 cell lines. (**A**) RNA isolated from NSC-34 pCC1-SMN2-Neo monoclonal cell lines were tested for the presence of *FL-SMN2* and *SMNΔ7* primers. Both primers set with no template DNA and NSC-34 cells that were not transfected genomic DNA was used a negative control. (**B**) *Rpl13a* was also ran on each same as an internal control since *Rpl13a* is constitutively express in murine cells. pCC1-SMN2-Neo monoclonal cell lines were found to be positive the presence of *FL-SMN* and *SMN*

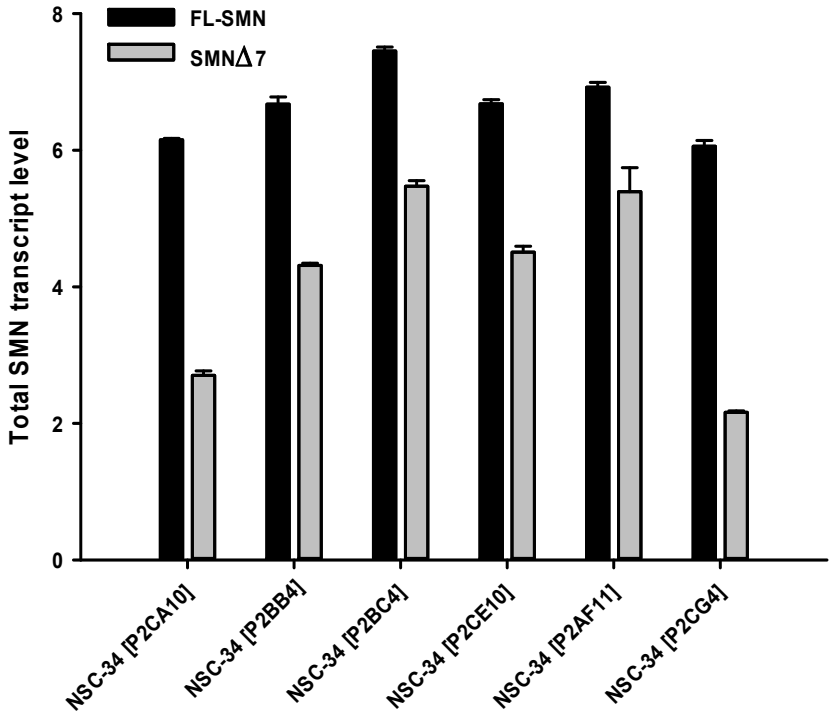


Figure 4.12 Relative levels of *FL-SMN* and *SMNΔ7* mRNA transcripts in monoclonal NSC-34 pCC1-SMN2-Neo. RNA isolated from NSC-34 pCC1-SMN2-Neo monoclonal cell lines was used to synthesis cDNA to test the relative amount of *FL-SMN* and *SMNΔ7* transcript in each monoclonal cell line using *FL-SMN* and *SMNΔ7* primers. The ΔCT for each monoclonal cell lines was the calculated for *FL-SMN* and *SMNΔ7* after adjusting to the murine reference transcripts *Rpl13a* and *Pgk.*

Chapter 5

DISCUSSION

Spinal muscular atrophy is caused by the mutation or deletion of the SMN1. Unique to the human genome is a duplicate of the *SMN1* gene known as *SMN2*. *SMN2* is an almost identical copy of the *SMN1* gene except for a C-to-T nucleotide transition that occurs in exon 7 (Rochette et al., 2001; Tisdale and Pellizzoni, 2015) resulting in a splicing defect such that the majority of *SMN2* transcripts (80-90%) lack exon 7. This *SMN*Δ*7* mRNA produces a truncated non-functional protein. The remainder of the time (10%) *SMN2* produces full length mRNA and functional protein (Monami et al., 1999; Lorson et al., 1999; Butchbach, 2016). Although *SMN2* has not been shown to cause the development of SMA, the copy number of *SMN2* plays a role in the severity of the disease (Taylor et al., 1998). The inverse relationship between SMA severity and *SMN2* copy number has made *SMN2* the primary target of SMA treatment and drug discovery through multiple mechanisms including promoter activation and modifications promoting exon 7 inclusion in *SMN2* transcripts (Cherry et al., 2014). Alterations in pH have been shown to affect the splicing of multiple mRNA transcripts including *SMN2* (Borsi et al., 1996; Chen et al., 2008). Low extracellular pH was shown to increase the splicing of exon 7 in SMN2 pre-mRNA while a high extracellular pH was shown to promote exon 7 inclusion. Splicing enhancer hnRNP A1, which promotes exon 7 exclusion through an enhancer element located in the middle of exon 7, was found to be increased in the nucleus suggesting that hnRNP A1 may mediate exon 7 skipping under conditions of low pH (Chen et al., 2008). Cellular

pH in mammalian cells is specifically regulated by the NHEs (Putney et al., 2002; Masereel et al., 2003). In this study 6 NHE inhibitors were examine for their potential to modulate the inclusion of exon 7.

From the six different NHE inhibitors tested, only HMA and the previously tested compound EIPA were found to increase exon 7 inclusion in mRNA transcripts and *SMN2* β-lactamase assay (Figure 3.1-Figure 3.4). A cell viability test was performed on the NHE inhibitors to assess if the changes in the cellular pH caused by the inhibitors also had cytotoxic effects on the SMA fibroblast cells. All of the compounds showed high cell viability after 24 hours of treatment (Figure 3.7), but after 120 hours of treatment, the number of viable cells for HMA was reduced to 15% at the highest concentration tested and the cell viability of EIPA treated cells was reduced to about 40% at the highest concentration tested (Figure 3.8). The 120-hour treatment with EIPA was similar to previous toxicity test performed using this compound (Yuo et al., 2007). Many studies have shown that HMA has strong cytotoxic properties and is being looked at as a potential cancer therapeutic (Park et al., 2009; Rowson et al., 2016).

Striatin 3(*STRN3*) belongs to the striatin subfamily of WD-40 repeat proteins (Castetset al., 1996; Benoist et al., 2006). *STRN3* was initially identified as a nuclear autoantigen whose expression is augmented during the S to G_2 phase (Castets et al., 2000). Like striatin, *STRN3* has scaffolding functions related to vesicular trafficking and cell signaling and has been found to have functions in dendritic spines and estrogen receptors (Moreno et al., 2000). Drug studies on small molecule (SMN-C

class compounds) that specifically increase the inclusion of exon 7 in *SMN2* transcripts were also found to increase exon 8-9 in *STRN3* transcripts (Naryshkin et al., 2014). It was determined that *STRN3* has an identical weak 5' splicing site as *SMN2,* as well as a purine rich region similar to the ESE2 motif of *SMN2*. Treatments done with the SMN-C class compounds were shown to stabilize the RNP complexes unique to *STRN3* and *SMN2* (Manaswini, et al., 2017). Interestingly, no significant increase in exon 8 and 9 inclusion of *STRN3* was seen for EIPA or HMA. NHE compounds amiloride, cariporide, and DMA were able to modulate exon 8 and 9 inclusion similar to the positive control compound RG7800 (Figure 3.9). This data suggests that EIPA and HMA modulation may be SMN specific or that the HMA and EIPA mechanism of action is not through stabilizing the RNP complex stabilized by RG7800. To determine the mechanism in which EIPA and HMA is modulating exon 7 inclusion, NHE isoform 1-5 was screened in each cell line used for the amiloride treatment. NHE1 and NHE5 were both found to be present in GM03813, GM03814, and NSC-34 cells (Figure 3.6). NHE1 is ubiquitously expressed in most mammalian cell types (Donowitz et al., 2013), while NHE5 is primarily expressed in neurons and skeletal muscle which are the regions that seem to be most affected by the loss of *SMN1*. Unlike the other amiloride compounds screened, HMA and EIPA have the capacity to inhibit NHE5 at fairly low concentrations supporting the finding of NHE5 possibly being the mechanism in which these two NHE inhibitors are working through.

NHE 1 is ubiquitously expressed in most cell types. In the brain, NHE 1 plays a role in microglial and astrocyte activation. Loss of the NHE1 in the brain has been shown to lead to ataxia, neurodegeneration, and epileptic seizures (Donowitz et al., 2031; Verma et al., 2015). Although NHE5 is highly expressed in the central nervous system, its role in brain is poorly understood. NHE5 has been shown to be involved in neuronal excitation and long-term potentiation. NHE inhibitor EIPA also has been shown to modulate neuronal plasticity and LTP in mice (Ronicke et al.,2009). When synaptic activity is enhanced, NHE5 is actively recruited to the synapses where it initiates local pH changes. Many components of the synaptic cleft are highly sensitive to changes in synaptic pH such as the voltage gated calcium channels (VGCC), N-methyl-D-aspartate (NMDA) receptors, and GABA receptors (Banke et al., 2005; Levinthal et al., 2009; Diering et al., 2011). The most characterized function of NHE5 in the CNS is as a negative regulator of activity-induced dendritic spine growth. During most of development, NHE5 suppresses spontaneous spine growth. Over expression of NHE has been shown to inhibit spinal growth while blockage of NHE5 has been shown to lead to spontaneous spine outgrowth (Diering et al.2011).

Screening of compounds that modulate *SMN2* expression is done via *SMN2* reporter assays. Reporter assays behave as surrogates of *SMN2*, which are correlated to changes that would be endogenous expression of *SMN2*. These SMN reporter assay target the *SMN2* gene through mechanisms such as changes in the promoter activity, exon 7 inclusion, protein stability, and multifunction reporter assays that can monitor more than one type of change in *SMN2* simultaneously (Cherry et al., 2014). Since the

currently developed reporter assays only contain small fragments of the *SMN2* promoter or mini-gene cassettes of the *SMN2* sequence, changes in *SMN2* expression can be caused by reporter assay artifacts leading to the identification of false positives (Cheng et al., 2012; Thorne et al., 2012; Cherry et al., 2014). In this study, two novel *SMN2* cell lines were developed containing the entire *SMN2* genomic locus. Using the entire *SMN2* gene allows for changes in *SMN2* to more closely recapitulate gene regulation seen *in vivo* reducing the error rate of the reporter cell lines.

The first cell line being developed will contain a dual reporter constructs to monitor changes in the *SMN2* promoter and or exon 7 inclusion using the *SMN2* mini-gene cassette and dual reporter plasmid via OLP (Zhang et al., 2001). Primers used to amplify regions of the *SMN2* mini-cassette CBGr68-IRES-CBRed luciferase dual reporter have overlapping 5' and 3' ends that correspond to next portion of the sequence for proper assembly off the reporter gene. Primers were design to inactivate the exon 7 termination codon by addition of a single nucleotide (guanine) after the 48th nucleotide in exon 7. By adding a single nucleotide to this region of exon 7, CBGr68 of dual reporter which is inserted in exon 8 will now be in frame with exon 7. CBGr68 will be transcribed when exon 7 is included while CBRed which is regulated by an internal ribosome entry site can simultaneously be transcribed due to changes promoter activity independently of exon 7 inclusion allowing for luciferase expression to be monitored for the identification of *SMN2* inducers. Before assembling the four OLP products, each product must be sequenced to verify the correct portion of *SMN2*

had been amplified and contained no mutations. OLP 3-4 and OLP 5-6 have been confirmed thus far by Sanger sequencing.

The second cell line generated was NSC-34 cells that express human *SMN2*. The NSC-34 cell line was generated by fusing together neuroblastoma cells and primary cultured mouse motor neurons. NSC-34 cell possesses similar characteristics to those observed of motor neurons including their ability to generate action potentials, as well as the synthesizing, storage, and release acetylcholine (Cashman et al.,1992; Durham et al., 1993) making them useful in assays since SMA preferentially affects motor neurons. Inserting *SMN2* into the NSC-34 cell line will allow for a cell line with high throughput screening capability that can be used to monitor changes in the full human *SMN2* gene in a cell line that exhibits characteristics of motor neurons. Since the *SMN2* gene is unique to the human genome, it can be inserted into the NSC-34 cell allowing for changes in *SMN2* to be monitored without being concealed by the *SMN1* gene which occurs sometimes in human derived primary culture cells (Cherry et al., 2014). The humanized NSC-34 cell line was created by using the 35.5kb *Bam*HI *SMN2* genomic sequence and subcloning it into the inducible pCC1-BAC vector. The pCC1-SMN2 BAC was then retrofitted with the plasmid pRetroNeo giving the BAC a selectable gene (G418 resistance). NSC-34 cells were then transfected with pCC1-SMN2-NEO and monoclonal cell lines were established by limited dilution. Genomic DNA and RNA analysis showed the presence of the *SMN2* gene (Figure 4.10-Figure 4.12) in the transfected NSC-34 cells but, copy number variation between the cell lines needs to be determined before they can be utilized for therapeutic screenings.

Chapter 6

SUMMARY AND FUTURE DIRECTIONS

Copy number variation of *SMN2* has been shown to modulate the disease severity of SMA patients making *SMN2* the primary target of SMA treatment and drug discovery. NHE inhibitor EIPA was previously found to increase the inclusion of exon 7 in SMN mRNA transcripts in a dose dependent and time dependent manner (Yuo et al., 2008). In this study six NHE inhibitors were assessed at various concentrations for their ability to modulate the inclusion of exon 7 through drug treatments utilizing three different type II SMA patient derived cell lines to evaluate changes in mRNA. The motor neuron like cell line, NSC-34, containing the *SMN2* β-lactamase report cell line was also used to examine the direct effect of these compounds in regard to their ability to promote exon 7 inclusion. EIPA and HMA were found to promote the inclusion of exon 7. NHE inhibitors EIPA and HMA may exude their effects in modifying the splicing of *SMN2* through NHE5, the NHE isoform present in neurons and skeletal muscles.

Further studies on the mechanism in which the NHE inhibitors exude their effects in modifying the splicing of *SMN2* transcripts by assessing the different isoforms of the sodium hydrogen exchangers. The toxicity of each drug was also monitored using various concentrations during the treatment of patient derived fibroblast to asses if compounds identified as modifiers of exon 7 inclusion can be further developed for SMA patient treatment.

Novel tools to assess changes in *SMN2* through promoter activity and or by inducing the inclusion of exon 7 in SMN pre-mRNA were also developed for the screening of small molecular compounds. Currently developed *SMN2* reporter assays contain short regions of the *SMN2* gene in order to examine the effects of small molecules on the *SMN2* promoter or splicing. Rather than using a small fragment of the *SMN2* promoter or a mini-gene cassette containing exon 7, the reporter cell lines generated will contain the entire *SMN2* locus allowing for the two cell lines to show changes in *SMN2* gene regulation in context to the entire locus of the gene in a motor neuron intracellular environment.

Here we described the development of our reporter cell line that can simultaneously monitor the activation of the *SMN2* promoter and inclusion of exon 7 using a sensitive enzyme assay. Sanger sequencing results have shown two of the overlapping PCR products (OLP 3-4 and OLP 5-6) contain the correct *SMN2* sequence. OLP 1-2 and OLP 7-8 will need to be repeated to obtain the correct sequence without any errors. Once all four products have been obtained, Gibson assembly will be performed to ligate the four PCR products. Gibson assembly is a single reaction that allows for the seamless assembly of multiple overlapping DNA products. Gibson assembly utilizes a 5′ exonuclease creating single stranded 3′ overhangs slowing for overlapping segments of the PCR product to anneal while the polymerase fills in gaps within each annealed fragment. The DNA ligase then seals any nicks in as the assembled DNA (Gibson et al., 2009). Recombineering of the *SMN2* gene with the dual reporter

construct will then be performed integrating the dual reporter construct into *SMN2* replacing its endogenous exon 6-8 (Figure 6.1) region followed by transfection into the NSC-34 cell line. Recombineering is a technique that utilizes homologous sequences between the target gene of interest and the gene and or construct allowing for the insertion, deletion, or alteration the sequence. Because recombineering is dependent on the homologies of the two sequences, it allows for precise targeting of the gene of interest. Unlike traditional cloning methods, recombineering is not dependent on restriction sites. Recombineering is catalyzed by bacteriophage-encoded homologous recombination function between target site and construct sequences with at least 50 bp of homology (Sharan et al., 2008).

A second cell line was also developed by transfecting the complete SMN2 genomic sequence into NSC-34 motor neuron like cells. The presence of SMN2 was confirmed in the cell lines through genomic DNA and mRNA analysis. Once the monoclonal cell lines are analyzed for the presence of SMN protein via immunoblots, array digital PCR will then be performed. Array digital PCR will allow for the assessment of the SMN2 copy number of each cell line using genomic DNA isolated from each of the developed monoclonal cell lines to assess (Stabley et al., 2015; Butchbach, 2016). Once these novel tools are developed, of these reporter cell lines will allow for a more accurate evaluation of previously discovered regulators like the NHE inhibitors as well as allowing for the development of new therapeutics for SMA patients.

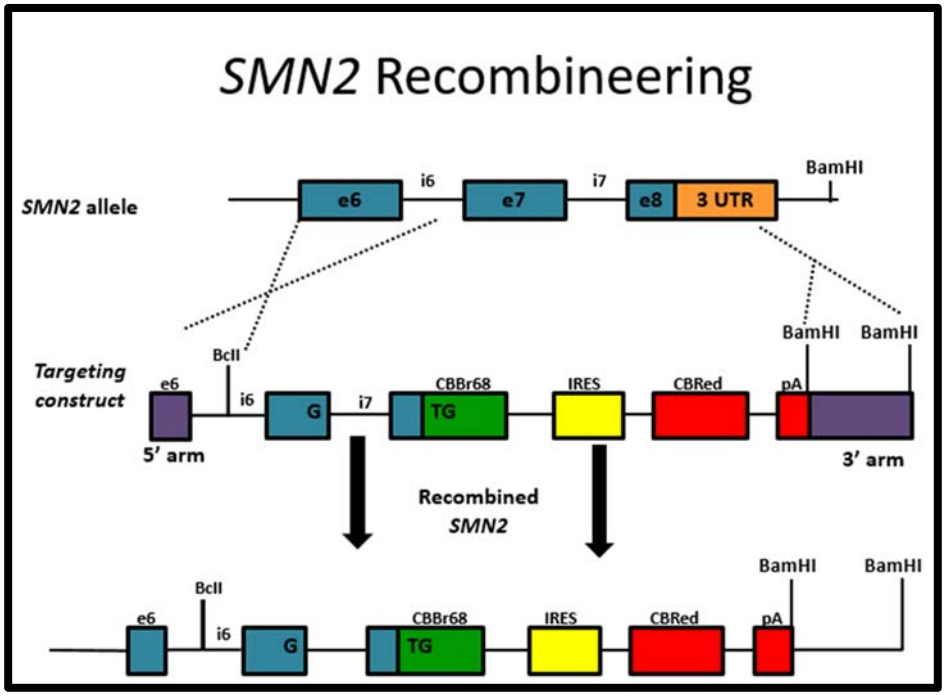


Figure 6.1 *SMN2* recombineering with dual reporter construct. Schematic representation of the insertion of the dual reporter construct into the *SMN2* gene via recombineering. Recombineering is based on the homologous ends between the construct and gene of interest. The homologous 5' and 3' ends shared by the dual reporter construct and the *SMN2* genomic sequence allow for the dual reporter construct to be swapped in for the endogenous SMN2 exon 6-8 region. Once recombination occurs, the *SMN2* gene will contain the reporter gene.

REFERENCES

Aharonovitz, Orit, and Yosef Granot. "Stimulation of Mitogen-Activated Protein Kinase and Na+/H+ Exchanger in Human Platelets Differential Effect of Phorbol Ester and Vasopressin." *Journal of Biological Chemistry* 271.28 (1996): 16494-99.

Ahmad, Saif, et al. "Molecular mechanisms of neurodegeneration in Spinal Muscular Atrophy." *Journal of experimental neuroscience* 10 (2016): 39.

Aihara, Kazuyuki, et al. "Cardioprotective effect of TY-12533, a novel Na+/H+ exchange inhibitor, on ischemia/reperfusion injury." *European journal of pharmacology* 404.1 (2000): 221-229.

Alexander, Stephen, et al. "The Concise Guide to Pharmacology 2017/18: G Protein Coupled Receptors." *British journal of pharmacology* 174.S1 (2017).

Andreassi, Catia, et al. "Aclarubicin treatment restores SMN levels to cells derived from type I spinal muscular atrophy patients." *Human Molecular Genetics* 10.24 (2001): 2841-2849.

Aronson, Peter. "Kinetic properties of the plasma membrane Na+-H+ exchanger." *Annual review of physiology* 47.1 (1985): 545-60.

Banke, Tue, Shashank Dravid, and Stephen Traynelis. "Protons trap NR1/NR2B NMDA receptors in a nonconducting state." *Journal of Neuroscience* 25.1 (2005): 42-51.

Banno, Hitoshi, et al. "Effects of MS-31-038, a novel Na+-H+ exchange inhibitor, on the myocardial infarct size in rats after postischemic administration." *Arzneimittelforschung* 49.04 (1999): 304-310.

Baumgarth, Manfred, Norbert Beier, and Rolf Gericke. "(2-Methyl-5-(methylsulfonyl) benzoyl) guanidine Na+/H+ antiporter inhibitors." *Journal of medicinal chemistry* 40.13 (1997): 2017-2034.

Benos, Dale. "Amiloride: a molecular probe of sodium transport in tissues and cells." *American Journal of Physiology-Cell Physiology* 242.3 (1982): C131-C145.

Benoist, Marion, Stéphane Gaillard, and Francis Castets. "The striatin family: a new signaling platform in dendritic spines." *Journal of Physiology-Paris* 99.2 (2006): 146-153.

Bergman, Naomi, et al. "Lsm proteins bind and stabilize RNAs containing 5′ poly (A) tracts." *Nature structural & molecular biology* 14.9 (2007): 824-831.

Bertrand, Benedicte, et al. "The Na+/H+ exchanger isoform 1 (NHE1) is a novel member of the calmodulin-binding proteins. Identification and characterization of calmodulin-binding sites." *Journal of Biological Chemistry* 269.18 (1994): 13703-13709.

Bianchini, Laurence, Gilles L'Allemain, and Jacques Pouysségur. "The p42/p44 mitogen-activated protein kinase cascade is determinant in mediating activation of the Na+/H+ exchanger (NHE1 isoform) in response to growth factors." *Journal of Biological Chemistry* 272.1 (1997): 271-279.

Borsi, Laura, et al. "Extracellular pH controls pre-mRNA alternative splicing of tenascin-C in normal, but not in malignantly transformed, cells." *International journal of cancer* 66.5 (1996): 632-635.

Brahms, Hero, et al. "Symmetrical dimethylation of arginine residues in spliceosomal Sm protein B/B′ and the Sm-like protein LSm4, and their interaction with the SMN protein." *RNA* 7.11 (2001): 1531-1542.

Branscombe, Tina, et al. "PRMT5 (Janus kinase-binding protein 1) catalyzes the formation of symmetric dimethylarginine residues in proteins." *Journal of Biological Chemistry* 276.35 (2001): 32971-32976.

Brett, Christopher, Mark Donowitz, and Rajini Rao. "Evolutionary Origins of Eukaryotic Sodium/Proton Exchangers." *American Journal of Physiology-Cell Physiology* 288.2 (2005): C223-C39.

Briese, Michael, Behrooz Esmaeili, and David Sattelle. "Is spinal muscular atrophy the result of defects in motor neuron processes?" *Bioessays* 27.9 (2005): 946-957.

Briese, Michael, et al. "SMN, the product of the spinal muscular atrophy-determining gene, is expressed widely but selectively in the developing human forebrain." *Journal of Comparative Neurology* 497.5 (2006): 808-816.

Burghes, Arthur, and Christine Beattie. "Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick?" *Nature Reviews Neuroscience* 10.8 (2009): 597-609.

Burnett, Barrington, et al. "Regulation of SMN protein stability." *Molecular and cellular biology* 29.5 (2009): 1107-1115.

Butchbach, Matthew. "Applicability of digital PCR to the investigation of pediatric-onset genetic disorders." *Biomolecular detection and quantification* 10 (2016): 9-14.

---. " Copy number variations in the survival motor neuron genes: implications for spinal muscular atrophy and other neurodegenerative diseases." *Frontiers in molecular biosciences* 3 (2016).

Bühler, Dirk, et al. "Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy." *Human molecular genetics* 8.13 (1999): 2351-2357.

Carissimi, Claudia, et al. "Unrip is a component of SMN complexes active in SnRNP assembly." *FEBS letters* 579.11 (2005): 2348-54.

---. " Gemin8 is a novel component of the survival motor neuron complex and functions in small nuclear ribonucleoprotein assembly." *Journal of biological chemistry* 281.12 (2006): 8126-8134.

Carrel, Tessa, et al. "Survival motor neuron function in motor axons is independent of functions required for small nuclear ribonucleoprotein biogenesis." *Journal of Neuroscience* 26.43 (2006): 11014-11022.

Cartegni, Luca, and Adrian Krainer. "Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1." *Nature genetics* 30.4 (2002): 377-384.

Carter, Todd, et al. "A multicopy transcription-repair gene, BTF2p44, maps to the SMA region and demonstrates SMA associated deletions." *Human molecular genetics* 6.2 (1997): 229-236.

Carvalho, Teresa, et al. "The spinal muscular atrophy disease gene product, SMN." *The Journal of cell biology* 147.4 (1999): 715-728.

Cashman, Neil, et al. "Neuroblastoma× spinal cord (NSC) hybrid cell lines resemble developing motor neurons." *Developmental dynamics* 194.3 (1992): 209-221.

Castets, Francis, et al. "A novel calmodulin-binding protein, belonging to the WD-repeat family, is localized in dendrites of a subset of CNS neurons." *The Journal of cell biology* 134.4 (1996): 1051-1062.

Castets, Francis, et al. "Zinedin, SG2NA, and striatin are calmodulin-binding, WD repeat proteins principally expressed in the brain." *Journal of Biological Chemistry* 275.26 (2000): 19970-19977.

Chambrey, Regine, Jean-Michel Achard, and David Warnock. "Heterologous expression of Rat NHE4: a highly amiloride-resistant Na+/H+ exchanger isoform." *American Journal of Physiology-Cell Physiology* 272.1 (1997): C90-C98.

Chen, Yi-Ching, et al. "Extracellular pH change modulates the exon 7 splicing in SMN2 mRNA." *Molecular and Cellular Neuroscience* 39.2 (2008): 268-72.

Cheng, Ken, and James Inglese. "A coincidence reporter-gene system for high-throughput screening." *Nature methods* 9.10 (2012): 937-37.

Cherry, Jonathan, et al. "Identification of novel compounds that increase SMN protein levels using an improved SMN2 reporter cell assay." *Journal of biomolecular screening* 17.4 (2012): 481-495.

---. "Assays for the identification and prioritization of drug candidates for spinal muscular atrophy." *Assay and drug development technologies* 12.6 (2014): 315-341.

Cho, Sungchan, and Gideon Dreyfuss. "A degron created by SMN2 exon 7 skipping is a principal contributor to Spinal Muscular Atrophy severity." *Genes & development* 24.5 (2010): 438-42.

D'Amico, Adele, et al. "Spinal Muscular Atrophy." *Orphanet journal of rare diseases* 6.1 (2011): 71.

d'Ydewalle, Constantin, and Charlotte J. Sumner. "Spinal muscular atrophy therapeutics: where do we stand?" *Neurotherapeutics* 12.2 (2015): 303-316.

Dhanasekaran, N., et al. "Protein kinase C-dependent and-independent activation of Na+/H+ exchanger by G alpha 12 class of G proteins." *Journal of Biological Chemistry* 269.16 (1994): 11802-11806.

di Penta, Alessandra, et al. "Dendritic LSm1/CBP80-mRNPs mark the early steps of transport commitment and translational control." *The Journal of cell biology* 184.3 (2009): 423-435.

DiDonato, Christine, et al. "Cloning, characterization, and copy number of the murine survival motor neuron gene: homolog of the spinal muscular atrophy-determining gene." *Genome research* 7.4 (1997): 339-352.

Diering, Graham, et al. "Regulation of dendritic spine growth through activity-dependent recruitment of the brain-enriched Na+/H+ exchanger NHE5." Molecular biology of the cell 22.13 (2011): 2246-2257.

Donowitz, Mark, Ming Tse, and Daniel Fuster. "SLC9/NHE gene family, a plasma membrane and organellar family of Na+/H+ exchangers." *Molecular aspects of medicine* 34.2 (2013): 236-251.

Durham, H. D., S. Dahrouge, and N. R. Cashman. "Evaluation of the spinal cord neuron X neuroblastoma hybrid cell line NSC-34 as a model for neurotoxicity testing." *Neurotoxicology* 14.4 (1993): 387-395.

Dye, Michael, and Nick Proudfoot. "Terminal exon definition occurs cotranscriptionally and promotes termination of RNA polymerase II." *Molecular cell* 3.3 (1999): 371-378.

Eggert, Christian, et al. "Spinal muscular atrophy: the RNP connection." *Trends in molecular medicine* 12.3 (2006): 113-121.

Fan, Li, and Louise Simard. "Survival motor neuron (SMN) protein: role in neurite outgrowth and neuromuscular maturation during neuronal differentiation and development." *Human Molecular Genetics* 11.14 (2002): 1605-1614.

Fischer, Utz, Qing Liu, and Gideon Dreyfuss. "The SMN–SIP1 complex has an essential role in spliceosomal snRNP biogenesis." *Cell* 90.6 (1997): 1023-1029.

Fischer, Utz, et al. "Nucleo-cytoplasmic transport of U snRNPs: definition of a nuclear location signal in the Sm core domain that binds a transport receptor independently of the m3G cap." *The EMBO journal* 12.2 (1993): 573.

Fliegel, L., and O. Fröhlich. "The Na+/H+ exchanger: an update on structure, regulation and cardiac physiology." *Biochemical Journal* 296.Pt 2 (1993): 273.

Frankel, Adam, and Steven Clarke. "PRMT3 is a Distinct Member of the Protein Arginine N-Methyltransferase Family Conferral of Substrate Specificity by a Zinc-Finger Domain." *Journal of Biological Chemistry* 275.42 (2000): 32974-82.

Friesen, Westley, et al. "SMN, the product of the spinal muscular atrophy gene, binds preferentially to dimethylarginine-containing protein targets." *Molecular cell* 7.5 (2001): 1111-1117.

---. "The methylosome, a 20S complex containing JBP1 and pICln, produces dimethylarginine-modified Sm proteins." *Molecular and cellular biology* 21.24 (2001): 8289-8300

Fukumoto, Shoji, et al. "Novel, non-acylguanidine-type Na+/H+ exchanger inhibitors: synthesis and pharmacology of 5-tetrahydroquinolinylidene aminoguanidine derivatives." *Journal of medicinal chemistry* 45.14 (2002): 3009-3021.

Gabanella, Francesca, et al. "Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs." *PloS one* 2.9 (2007): e921.

---."The activity of the spinal muscular atrophy protein is regulated during development and cellular differentiation." *Human molecular genetics* 14.23 (2005): 3629-3642.

Gandini, Rosaria, et al. "LSm4 associates with the plasma membrane and acts as a co-factor in cell volume regulation." *Cellular Physiology and Biochemistry* 22.5-6 (2008): 579-590.

Gangwani, Laxman, et al. "Spinal muscular atrophy disrupts the interaction of ZPR1 with the SMN protein." *Nature cell biology* 3.4 (2001): 376-383.

Gibson, Daniel, et al. "Enzymatic assembly of DNA molecules up to several hundred kilobases." *Nature methods* 6.5 (2009): 343-345.

Giesemann, Torsten, et al. "A role for polyproline motifs in the spinal muscular atrophy protein SMN Profilins bind to and colocalize with smn in nuclear gems." *Journal of Biological Chemistry* 274.53 (1999): 37908-37914.

Golembe, Tracey, Jeongsik Yong, and Gideon Dreyfuss. "Specific sequence features, recognized by the SMN complex, identify snRNAs and determine their fate as snRNPs." *Molecular and cellular biology* 25.24 (2005): 10989-11004.

Grice, Stuart, and Ji-Long Liu. "Survival motor neuron protein regulates stem cell division, proliferation, and differentiation in Drosophila." *PLoS genetics* 7.4 (2011): e1002030.

Gubitz, Amelie K, Wenqin Feng, and Gideon Dreyfuss. "The SMN Complex." *Experimental cell research* 296.1 (2004): 51-56.

Gumina, Richard, et al. "Inhibition of the Na+/H+ exchanger confers greater cardioprotection against 90 minutes of myocardial ischemia than ischemic preconditioning in dogs." *Circulation* 100.25 (1999): 2519-2526.

Gumina, Richard, Juergen Daemmgen, and Garrett Gross. "Inhibition of the Na+/H+ exchanger attenuates phase 1b ischemic arrhythmias and reperfusion-induced ventricular fibrillation." *European journal of pharmacology* 396.2 (2000): 119-124.

Guzman-Perez, Angel, et al. "Discovery of zoniporide: A potent and selective sodium–hydrogen exchanger type 1 (NHE-1) inhibitor with high aqueous solubility." *Bioorganic & medicinal chemistry letters* 11.6 (2001): 803-807.

Hamm, Jörg, et al. "The trimethylguanosine cap structure of U1 snRNA is a component of a bipartite nuclear targeting signal." Cell 62.3 (1990): 569-577.

Harada, T., et al. "Pace-mapping conduction delay at reentry circuit sites of ventricular tachycardia after myocardial infarction." *Heart and vessels* (1997): 232-234.

Heier, Christopher, Rocky Gogliotti, and Christine DiDonato. "SMN transcript stability: could modulation of messenger RNA degradation provide a novel therapy for spinal muscular atrophy?" *Journal of child neurology* 22.8 (2007): 1013-1018.

Heier, Christopher, and Christine DiDonato. "Translational readthrough by the aminoglycoside geneticin (G418) modulates SMN stability in vitro and improves motor function in SMA mice in vivo." *Human molecular genetics* 18.7 (2009): 1310-22.

Hofmann, Yvonne, and Brunhilde Wirth. "hnRNP-G promotes exon 7 inclusion of Survival Motor Neuron (SMN) via direct interaction with Htra2-β1." *Human molecular genetics* 11.17 (2002): 2037-49.

Hooley, Rebecca, et al. "G13 stimulates Na-H exchange through distinct Cdc42-dependent and RhoA-dependent pathways." *Journal of Biological Chemistry* 271.11 (1996): 6152-58.

Hsieh-Li, Hsiu Mei, et al. "A mouse model for spinal muscular atrophy." *Nature genetics* 24.1 (2000): 66-70.

Jablonka, Sibylle, et al. "Defective Ca2+ channel clustering in axon terminals disturbs excitability in motoneurons in spinal muscular atrophy." *J Cell Biol* 179.1 (2007): 139-149.

Jarecki, Jill, et al. "Diverse small-molecule modulators of SMN expression found by high-throughput compound screening: early leads towards a therapeutic for spinal muscular atrophy." *Human molecular genetics* 14.14 (2005): 2003-2018.

Kapus, Andras, et al. "Functional characterization of three isoforms of the Na+/H+ exchanger stably expressed in Chinese hamster ovary cells. ATP dependence, osmotic sensitivity, and role in cell proliferation." *Journal of Biological Chemistry* 269.38 (1994): 23544-23552.

Kiss, Tamás. "Biogenesis of small nuclear RNPs." *Journal of cell science* 117.25 (2004): 5949-5951.

Kolb, Stephen, and John Kissel. "Spinal muscular atrophy." *Neurologic clinics* 33.4 (2015): 831-846.

Krapivinsky, Grigory, et al. "Molecular characterization of a swelling-induced chloride conductance regulatory protein, plCIn." *Cell* 76.3 (1994): 439-448.

Kuribayashi, Yoshikazu, et al. "Cerebroprotective properties of SM-20220, a potent Na+/H+ exchange inhibitor, in transient cerebral ischemia in rats." *European journal of pharmacology* 383.2 (1999): 163-168.

Laeckmann, Didier, et al. "Synthesis and biological evaluation of aroylguanidines related to amiloride as inhibitors of the human platelet Na+/H+ exchanger." *Bioorganic & medicinal chemistry* 10.6 (2002): 1793-1804.

Le, Thanh, et al. "The survival motor neuron (SMN) protein: effect of exon loss and mutation on protein localization." *Neurogenetics* 3.1 (2000): 7-16.

Le, Thanh, et al. "SMNΔ7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN." *Human molecular genetics* 14.6 (2005): 845-857.

Lefebvre, Suzie, et al. "Identification and characterization of a spinal muscular atrophy-determining gene." *Cell* 80.1 (1995): 155-165.

Levine, Susan, et al. "Kinetics and regulation of three cloned mammalian Na+/H+ exchangers stably expressed in a fibroblast cell line." *Journal of Biological Chemistry* 268.34 (1993): 25527-25535.

Levinthal, Cynthia, et al. "Modulation of group III metabotropic glutamate receptors by hydrogen ions." *Pharmacology* 83.2 (2009): 88-94.

Li, Darrick, et al. "A cell system for phenotypic screening of modifiers of SMN2 gene expression and function." *PloS one* 8.8 (2013): e71965.

Lin, Xia, et al. "Gα12 differentially regulates Na+-H+ exchanger isoforms." *Journal of Biological Chemistry* 271.37 (1996): 22604-22610.

Liu, Qing, and Gideon Dreyfuss. "A novel nuclear structure containing the survival of motor neurons protein." *The EMBO journal* 15.14 (1996): 3555.

Liu, Qing, et al. "The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins." *Cell* 90.6 (1997): 1013-1021.

Livak, Kenneth, and Thomas Schmittgen. "Analysis of relative gene expression data using real-time quantitative PCR and the 2− ΔΔCT method." *methods* 25.4 (2001): 402-408.

Lorson, Christian, et al. "A single nucleotide in the SMN gene regulates splicing and is responsible for Spinal Muscular Atrophy." *Proceedings of the National Academy of Sciences* 96.11 (1999): 6307-11.

Lorson, Christian, and Elliot Androphy. "An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN." *Human molecular genetics* 9.2 (2000): 259-65.

Lorson, Christian, et al. "A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy." *Proceedings of the National Academy of Sciences* 96.11 (1999): 6307-6311.

---."SMN oligomerization defect correlates with spinal muscular atrophy severity." *Nature genetics* 19.1 (1998): 63-66.

Lotti, Francesco, et al. "An SMN-dependent U12 splicing event essential for motor circuit function." *Cell* 151.2 (2012): 440-454.

Lunn, Mitchell, et al. "Indoprofen upregulates the survival motor neuron protein through a cyclooxygenase-independent mechanism." *Chemistry & biology* 11.11 (2004): 1489-1493.

Lunn, Mitchell, and Ching Wang. "Spinal Muscular Atrophy." *The Lancet* 371.9630 (2008): 2120-33.

Lührmann, Reinhard, Berthold Kastner, and Montserrat Bach. "Structure of spliceosomal snRNPs and their role in pre-mRNA splicing." *Biochimica et Biophysica Acta (BBA)-Gene Structure and Expression* 1087.3 (1990): 265-292.

Magin-Lachmann, Christine, et al. "Retrofitting BACs with G418 resistance, luciferase, and oriP and EBNA-1–new vectors for in vitro and in vivo delivery." *BMC biotechnology* 3.1 (2003): 2.

Mahnensmith, Rex, and Peter Aronson. "The plasma membrane sodium-hydrogen exchanger and its role in physiological and pathophysiological processes." *Circulation research 56.6* (1985): 773-788.

Massenet, Séverine, et al. "The SMN complex is associated with SnRNPs throughout their cytoplasmic assembly pathway." *Molecular and cellular biology* 22.18 (2002): 6533-41.

Masereel, B., L. Pochet, and D. Laeckmann. "An overview of inhibitors of Na+/H+ exchanger." *European journal of medicinal chemistry* 38.6 (2003): 547-554.

McWhorter, Michelle, et al. "Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding." *The Journal of cell biology* 162.5 (2003): 919-932.

Meister, Gunter, Christian Eggert, and Utz Fischer. "SMN-mediated assembly of RNPs: a complex story." *Trends in cell biology* 12.10 (2002): 472-478.

Meister, Gunter, et al. "Methylation of Sm proteins by a complex containing PRMT5 and the putative U snRNP assembly factor pICln." *Current Biology* 11.24 (2001): 1990-1994.

Meister, Gunter, Christian Eggert, and Utz Fischer. "SMN-mediated assembly of RNPs: a complex story." *Trends in cell biology* 12.10 (2002): 472-478.

Meister, Gunter, and Utz Fischer. "Assisted RNP assembly: SMN and PRMT5 complexes cooperate in the formation of spliceosomal UsnRNPs." *The EMBO journal* 21.21 (2002): 5853-5863.

Michaud, Magali, et al. "Neuromuscular defects and breathing disorders in a new mouse model of spinal muscular atrophy." *Neurobiology of disease* 38.1 (2010): 125-135.

Monani, Umrao, et al. "A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2." *Human molecular genetics* 8.7 (1999): 1177-1183.

---. " The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in Smn–/–mice and results in a mouse with spinal muscular atrophy." *Human molecular genetics* 9.3 (2000): 333-339.

Monani, Umrao. "Spinal muscular atrophy: a deficiency in a ubiquitous protein; a motor neuron-specific disease." *Neuron* 48.6 (2005): 885-895.

Monani, Umrao, et al. "A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2." *Human molecular genetics* 8.7 (1999): 1177-1183.

Moreno, Carlos, et al. "WD40 repeat proteins striatin and S/G2 nuclear autoantigen are members of a novel family of calmodulin-binding proteins that associate with protein phosphatase 2A." *Journal of Biological Chemistry* 275.8 (2000): 5257-5263.

Nakamura, Norihiro, et al. "Four Na+/H+ exchanger isoforms are distributed to Golgi and post-Golgi compartments and are involved in organelle pH regulation." *Journal of Biological Chemistry* 280.2 (2005): 1561-1572.

Nakamura, Tomoe, et al. "Activation of Na+/H+ exchanger 1 is sufficient to generate Ca2+ signals that induce cardiac hypertrophy and heart failure." *Circulation research* 103.8 (2008): 891-899.

Narayanan, Usha, et al. "SMN, the spinal muscular atrophy protein, forms a pre-import snRNP complex with snurportin1 and importin β." *Human molecular genetics* 11.15 (2002): 1785-1795.

Naryshkin, Nikolai, et al. "SMN2 splicing modifiers improve motor function and longevity in mice with spinal muscular atrophy." *Science* 345.6197 (2014): 688-693.

Nesic, Dobrila, Goranka Tanackovic, and Angela Krämer. "A role for Cajal Bodies in the final steps of U2 snRNP biogenesis." *Journal of cell science* 117.19 (2004): 4423-33.

Nishimune, Hiroshi, Joshua Sanes, and Steven Carlson. "A synaptic laminin–calcium channel interaction organizes active zones in motor nerve terminals." *Nature* 432.7017 (2004): 580-587.

Numata, Masayuki, and John Orlowski. "Molecular cloning and characterization of a novel (Na+, K+)/H+ exchanger localized to the trans-Golgi network." *Journal of Biological Chemistry* 276.20 (2001): 17387-17394.

Ohno, Mutsuhito, et al. "PHAX, a mediator of U snRNA nuclear export whose activity is regulated by phosphorylation." *Cell* 101.2 (2000): 187-198.

Orlowski, John, and Sergio Grinstein. "Na+/H+ exchangers of mammalian cells." *Journal of Biological Chemistry* 272.36 (1997): 22373-76.

Park, Kyu-Sang, et al. "Amiloride derivatives induce apoptosis by depleting ER Ca2+ stores in vascular endothelial cells." *British journal of pharmacology* 156.8 (2009): 1296-1304.

Paushkin, Sergey, et al. "The SMN Complex, an assemblyosome of ribonucleoproteins." *Current opinion in cell biology* 14.3 (2002): 305-12.

Pellizzoni, Livio. "Chaperoning ribonucleoprotein biogenesis in health and disease." *EMBO reports* 8.4 (2007): 340-45.

Pellizzoni, Livio, et al. "The Survival of Motor Neurons (Smn) Protein Interacts with the snoRNP Proteins fibrillarin and GAR1." *Current Biology* 11.14 (2001): 1079-88.

---. " A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing." *Cell* 95.5 (1998): 615-624.

Pellizzoni, Livio, Jeongsik Yong, and Gideon Dreyfuss. "Essential Role for the SMN complex in the specificity of snRNP assembly." *Science* 298.5599 (2002): 1775-79.

Pfaffl, Michael. "A new mathematical model for relative quantification in real-time RT–PCR." *Nucleic acids research* 29.9 (2001): e45-e45.

Pillai, Ramesh, et al. "Unique Sm core structure of U7 snRNPs: assembly by a specialized SMN complex and the role of a new component, Lsm11, in histone RNA processing." *Genes & development* 17.18 (2003): 2321-2333.

Prior, Thomas, et al. "A positive modifier of spinal muscular atrophy in the SMN2 gene." *The American Journal of Human Genetics* 85.3 (2009): 408-413.

Michaud, Magali, et al. "Neuromuscular defects and breathing disorders in a new mouse model of spinal muscular atrophy." *Neurobiology of disease* 38.1 (2010): 125-135.

Pu, William, et al. "pICln inhibits snRNP biogenesis by binding core spliceosomal proteins." *Molecular and Cellular Biology* 19.6 (1999): 4113-4120.

Putney, L. K., S. P. Denker, and D. L. Barber. "The changing face of the Na+/H+ exchanger, NHE1: structure, regulation, and cellular actions." *Annual review of pharmacology and toxicology* 42.1 (2002): 527-552.

Raker, Veronica, et al. "Spliceosomal U snRNP core assembly: Sm proteins assemble onto an Sm site RNA nonanucleotide in a specific and thermodynamically stable manner." *Molecular and Cellular Biology* 19.10 (1999): 6554-6565.

Rochette, C., N. Gilbert, and L. Simard. "SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to Homo sapiens." *Human genetics* 108.3 (2001): 255-266.

Rönicke, Raik, et al. "The Na+/H+ exchanger modulates long-term potentiation in rat hippocampal slices." *Naunyn-Schmiedeberg's archives of pharmacology* 379.3 (2009): 233.

Rosskopf, Dieter, Klaus-Jan Schröder, and Winfried Siffert. "Role of sodium-hydrogen exchange in the proliferation of immortalised lymphoblasts from patients with essential hypertension and normotensive subjects." *Cardiovascular research* 29.2 (1995): 254-259.

Rossoll, Wilfried, et al. "Smn, the spinal muscular atrophy–determining gene product, modulates axon growth and localization of β-actin mRNA in growth cones of motoneurons." *The Journal of cell biology* 163.4 (2003): 801-812.

Rowson-Hodel, Ashley, et al. "Hexamethylene amiloride engages a novel reactive oxygen species-and lysosome-dependent programmed necrotic mechanism to selectively target breast cancer cells." *Cancer letters* 375.1 (2016): 62-72.

Russman, Barry. "Spinal muscular atrophy: clinical classification and disease heterogeneity." *Journal of child neurology* 22.8 (2007): 946-951.

Scholz, Wolfgang, et al. "Protective effects of HOE642, a selective sodium-hydrogen exchange subtype 1 inhibitor, on cardiac ischaemia and reperfusion." *Cardiovascular Research* 29.2 (1995): 260-268.

Schrank, Bertold, et al. "Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos." *Proceedings of the National Academy of Sciences* 94.18 (1997): 9920-9925.

Sharan, Shyam, Lynn Thomason, and Sergey Kuznetsov. "Recombineering: a homologous recombination-based method of genetic engineering." *Nature protocols* 4.2 (2009): 206-223.

Simone, Chiara, et al. "Is spinal muscular atrophy a disease of the motor neurons only: pathogenesis and therapeutic implications?" *Cellular and molecular life sciences* 73.5 (2016): 1003-1020.

Sivaramakrishnan,Manaswini, et al. "Binding to SMN2 pre-mRNA-protein complex elicits specificity for small molecule splicing modifiers." *Nature Communications* 8.1 (2017): 1476.

Sleeman, Judith, and Angus Lamond. "Newly assembled snRNPs associate with coiled bodies before speckles, suggesting a nuclear snRNP maturation pathway." *Current Biology* 9.19 (1999): 1065-1074.

Stabley, Deborah, et al. "SMN1 and SMN2 copy numbers in cell lines derived from patients with spinal muscular atrophy as measured by array digital PCR." *Molecular genetics & genomic medicine* 3.4 (2015): 248-257.

Staněk, David, and Karla Neugebauer. "The Cajal body: a meeting place for spliceosomal snRNPs in the nuclear maze." *Chromosoma* 115.5 (2006): 343-354.

Sugarman, Elaine, et al. "Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of> 72 400 specimens." *European journal of human genetics* 20.1 (2012): 27-32.

Szabó, Elöd, et al. "Kinetic and pharmacological properties of human brain Na+/H+ exchanger isoform 5 stably expressed in Chinese hamster ovary cells." *Journal of Biological Chemistry* 275.9 (2000): 6302-6307.

Taylor, Joanne, et al. "Correlation of SMNt and SMNc gene copy number with age of onset and survival in spinal muscular atrophy." *European Journal of Human Genetics* 6.5 (1998).

Terns, Michael, and Rebecca Terns. "Macromolecular complexes: SMN—the master assembler." *Current biology* 11.21 (2001): R862-R864.

Tharun, Sundaresan, et al. "Mutations in the Saccharomyces cerevisiae LSM1 gene that affect mRNA decapping and 3′ end protection." *Genetics* 170.1 (2005): 33-46.

Thorne, Natasha, et al. "Firefly luciferase in chemical biology: a compendium of inhibitors, mechanistic evaluation of chemotypes, and suggested use as a reporter." *Chemistry & biology* 19.8 (2012): 1060-1072.

Thurmond, John, et al. "Synthesis and biological evaluation of novel 2, 4-diaminoquinazoline derivatives as SMN2 promoter activators for the potential treatment of spinal muscular atrophy." *Journal of medicinal chemistry* 51.3 (2008): 449-469.

Tisdale, Sarah, and Livio Pellizzoni. "Disease mechanisms and therapeutic approaches in spinal muscular atrophy." *Journal of Neuroscience* 35.23 (2015): 8691-8700.

Tizzano, Eduardo, Carmen Cabot, and Montserrat Baiget. "Cell-specific survival motor neuron gene expression during human development of the central nervous system: implications for the pathogenesis of spinal muscular atrophy." *The American journal of pathology* 153.2 (1998): 355-361.

Verma, Vivek, et al. "Implications of sodium hydrogen exchangers in various brain diseases." *Journal of basic and clinical physiology and pharmacology* 26.5 (2015): 417-426.

Viollet, Louis, et al. "cDNA isolation, expression, and chromosomal localization of the mouse survival motor neuron gene (Smn)." *Genomics* 40.1 (1997): 185-188.

Wakabayashi, Shigeo, Munekazu Shigekawa, and Jacques Pouyssegur. "Molecular physiology of vertebrate Na+/H+ exchangers." *Physiological reviews* 77.1 (1997): 51-74.

Wan, Lili, et al. "The survival of motor neurons protein determines the capacity for snRNP assembly: biochemical deficiency in spinal muscular atrophy." *Molecular and cellular biology* 25.13 (2005): 5543-5551.

Wang, Jin, and Gideon Dreyfuss. "A cell system with targeted disruption of the SMN gene functional conservation of the SMN protein and dependence of Gemin2 on SMN." *Journal of Biological Chemistry* 276.13 (2001): 9599-605.

Warming, Søren, et al. "Simple and highly efficient BAC recombineering using Galk selection." *Nucleic acids research* 33.4 (2005): e36-e36.

Will, Cindy, and Reinhard Lührmann. "Spliceosomal UsnRNP biogenesis, structure and function." *Current opinion in cell biology* 13.3 (2001): 290-301.

Williams, Barbara, Susan Hamilton, and Hemanta Sarkar. "The survival motor neuron protein interacts with the transactivator FUSE binding protein from human fetal brain." *FEBS letters* 470.2 (2000): 207-210.

Xiao, Jingbo, et al. "Discovery, synthesis, and biological evaluation of novel SMN protein modulators." *Journal of medicinal chemistry* 54.18 (2011): 6215-6233.

Yong, Jeongsik, et al. "snRNAs contain specific SMN-binding domains that are essential for snRNP assembly." *Molecular and cellular biology* 24.7 (2004): 2747-2756.

Young, Philip, et al. "A direct interaction between the survival motor neuron protein and p53 and its relationship to spinal muscular atrophy." *Journal of Biological Chemistry* 277.4 (2002): 2852-2859.

---. "Srp30c-dependent stimulation of Survival Motor Neuron (Smn) exon 7 inclusion is facilitated by a direct interaction with Htra2β1." *Human molecular genetics* 11.5 (2002): 577-87.

Yu, F. H., G. E. Shull, and John Orlowski. "Functional properties of the rat Na/H exchanger NHE-2 isoform expressed in Na/H exchanger-deficient Chinese hamster ovary cells." *Journal of Biological Chemistry* 268.34 (1993): 25536-25541.

Yuo, Chung-Yee, et al. "5-(N-ethyl-N-isopropyl) -amiloride enhances SMN2 exon 7 inclusion and protein expression in spinal muscular atrophy cells." *Annals of neurology* 63.1 (2008): 26-34.

Zhang, Honglai, et al. "Multiprotein complexes of the survival of motor neuron protein SMN with Gemins traffic to neuronal processes and growth cones of motor neurons." *Journal of Neuroscience* 26.33 (2006): 8622-8632.

Zhang, Min-Ling, et al. "An in vivo reporter system for measuring increased inclusion of exon 7 in SMN2 mRNA: potential therapy of SMA." *Gene therapy* 8.20 (2001): 1532.