CHARACTERIZATION OF MUTANT SMN AND DEVELOPMENT OF MUTANT SMN
TRANSGENIC MICE

DISSertation

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By

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ABSTRACT

Spinal muscular atrophy (SMA) is an autosomal recessive neurodegenerative disease. Loss of the survival motor neuron (SMN1) gene, in the presence of the SMN2 gene causes SMA. Ninety-five percent of SMA patients have a loss of SMN1 and in the majority of the remaining cases, a small mutation in the SMN1 gene occurs. SMN functions in snRNP assembly in all cell types, however, it is unclear how this function results in specifically motor neuron cell death. In this thesis, the properties of various mutations of SMN are investigated in both in vitro studies and in transgenic mouse models to determine the function of SMN important in SMA.

GST-fusion binding assays were used to determine the ability of each mutant SMN to bind itself, full-length SMN or Sm proteins, which form the core of snRNP complexes. All of these properties are necessary for SMN to efficiently perform snRNP biogenesis. The ability of each mutation to rescue axonal defects in zebrafish embryos that occur with knockdown of endogenous smn with morpholinos was investigated. Of the mutations studied, two were striking: SMN(A111G) and SMN(VDQNQKE). SMN(A111G) was able to bind itself and Sm proteins, thus making it able to form snRNP complexes; however, this mutation failed to rescue axonal defects in zebrafish. SMN(VDQNQKE), in contrast, was unable to bind to itself or Sm proteins, but yet was able to rescue axonal defects. These data seemed to indicate that snRNP assembly and the SMA phenotype were independent of each other.
To further investigate these two mutations, transgenic mice were developed. Lack of endogenous mouse SMN (Smn) in mice results in embryonic lethality. Introduction of 2 copies of human SMN2 results in a mouse with severe SMA, while 1 copy of SMN2 is insufficient to overcome embryonic lethality. In this thesis, it is shown that SMN(A111G), an allele capable of snRNP assembly, can rescue mice that lack Smn and contain either one or two copies of SMN2 (SMA mice). In contrast, SMN(VDQNKQKE) did not result in sufficient SMN protein and did not rescue SMA animals. The correction of SMA in these animals was directly correlated with snRNP assembly activity in spinal cord, as was correction of snRNA levels. Also, re-investigation of these mutations in the zebrafish assay revealed that increased amounts of SMN(A111G) could rescue axonal defects; whereas, decreased amounts of SMN(VDQNKQKE) resulted in fish with increased axonal defects. These data support snRNP assembly as being the critical function affected in SMA and suggests that the levels of snRNPs are, indeed, critical to motor neurons.

Furthermore, SMN(A111G) cannot rescue Smn/- mice without SMN2 suggesting that both SMN(A111G) and SMN2 undergo intragenic complementation in vivo to function in heteromeric complexes that have greater function than either allele alone. The oligomer composed of limiting full-length SMN and SMN(A111G) has substantial snRNP assembly activity. Also, the SMN(A2G) and SMN(A111G) alleles in vivo did not complement each other leading to the possibility that these mutations could affect the same function. Lastly, transgenic mice containing the SMN(I116F) missense mutation were developed. SMN(I1116F) is capable of binding itself, but has lowered ability to bind Sm proteins and thus, does not form snRNP complexes efficiently. It is expected that this mutation will not be able to rescue mice and further support the connection of snRNP assembly and SMA.
This dissertation is dedicated in memory of Bill Workman,

April 5, 1951- September 13, 2005.
ACKNOWLEDGMENTS

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Major Field: Ohio State Biochemistry Program
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<th>Definition</th>
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<tbody>
<tr>
<td>BCA</td>
<td>bicinchoninic acid</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>DNA complementary to RNA</td>
</tr>
<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s Modified Eagle Medium</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetate</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-S-transferase</td>
</tr>
<tr>
<td>HPRT</td>
<td>hypoxanthine phosphoribosyl-transferase</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactoside</td>
</tr>
<tr>
<td>µCi</td>
<td>microCurie</td>
</tr>
<tr>
<td>µg</td>
<td>microgram</td>
</tr>
<tr>
<td>µL</td>
<td>microliter</td>
</tr>
<tr>
<td>min(s)</td>
<td>minute(s)</td>
</tr>
<tr>
<td>MN1</td>
<td>motor neuron-like cell line</td>
</tr>
<tr>
<td>MO</td>
<td>morpholino</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NMJ</td>
<td>neuromuscular junction</td>
</tr>
<tr>
<td>PAGE</td>
<td>polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>PBS</td>
<td>phosphate-buffer saline</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDB</td>
<td>protein data bank</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenyl methylsulfonyl fluoride</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>RA</td>
<td>retinoic acid</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecylsulfate</td>
</tr>
<tr>
<td>SMA</td>
<td>Spinal Muscular Atrophy</td>
</tr>
<tr>
<td>SMN</td>
<td>Survival Motor Neuron</td>
</tr>
<tr>
<td>snoRNP</td>
<td>small nucleolar ribonucleoprotein</td>
</tr>
<tr>
<td>SNP</td>
<td>small nucleotide polymorphism</td>
</tr>
<tr>
<td>snRNP</td>
<td>small nuclear ribonucleoprotein</td>
</tr>
<tr>
<td>SSCP</td>
<td>single-strand conformation polymorphism</td>
</tr>
<tr>
<td>3' UTR</td>
<td>3' untranslated region</td>
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CHAPTER 1

INTRODUCTION

1.1 Spinal Muscular Atrophy

1.1.1 History of SMA

Spinal Muscular Atrophy, or SMA, is a leading genetic cause of death of infants (Roberts et al. 1970) with an incidence of 1 in 10,000 (Pearn et al. 1978; McAndrew et al. 1997; Melki 1997). Prior to 1995, SMA was considered an unknown, orphan disease. SMA moved to the forefront only in the early 1990’s with the discovery of the gene affected in SMA. Since then, SMA has attracted a large group of researchers and clinicians who now recognize this orphan disease.

SMA is an autosomal recessive disease that is characterized by neurodegeneration of the anterior horn cells of the spinal cord that causes atrophy of proximal muscles due to denervation. The disease was first described by Werdnig in 1891 and Hoffmann in 1893 (Werdnig 1891; Hoffmann 1893). Both Werdnig and Hoffmann describe (in the original German translated by Victor Dubowitz) children born normal, but with progressive muscle weakness that begins in the first year of life (Dubowitz 1995; Dubowitz 2008). The muscle weakness often began as weakness of the legs
spreading to the arms and back. The children died within 10 months to 6 years of birth. Autopsy of the children revealed degeneration of anterior horn cells of the spinal cord and atrophy of muscle.

While more cases of SMA were described in subsequent years, some more severe and some not, it was not until 1956 when Kugelberg and Welander described a very mild form of the disease, that a re-evaluation of the spectrum of SMA occurred (Kugelberg et al. 1956). These mild patients had weakness of the lower limbs, which was diagnosed from 2 years of age with patient survival reaching into adulthood. These patients retained the ability to stand and walk, whereas more severe patients lose this ability. Up until this time, there had been controversy over whether this disease was actually several different diseases due to the varying degrees of severity of cases. However, it was concluded that these varying phenotypes did, in fact, make up one disease. The evidence lay in that families with more than one affected child often had variable severity of symptoms and furthermore, shared the same underlying histological features (Schmid 1958; Byers et al. 1961; Dubowitz 1964).

This led to speculation that SMA was a broad-spectrum disease with possible patient groupings based on age of onset and severity of disease. But, it would not be until 1971 when Fried and Emery proposed the most promising grouping of SMA patients (Fried et al. 1971). They suggested that there were, in fact, 3 groups of SMA patients. The first group was Type I (Werdnig-Hoffmann, after the first descriptions) and was characterized by onset at birth and death by 2 years of age. The second group, Type II SMA, had a later onset with survival beyond 4 years of age with loss of ambulation. The final group, Type III (Kugelberg-Welander), had the most mild disease
symptoms. Type III patients had an onset of disease by 2 years of age and muscle weakness progresses slowly such that patients reach adulthood and maintain ambulation.

1.1.2 Types of SMA

In 1991 and 1992, an international consortium was formed between SMA clinicians and scientists (Munsat 1991; Munsat et al. 1992). The consortium finally set forth the official clinical classifications of SMA. The three different forms of SMA (Type I, II and III) were to be based on age of onset as well as clinical severity of the disease as suggested by Fried and Emery; however, the ages of onset and phenotypes would be better defined. The most severe form of the disease, Type I, is diagnosed at a very early age, usually before 6 months, and results in death by 2 years of age. These children never gain the ability to sit upright. Type II SMA patients are diagnosed before 18 months of age, but are never able to walk and require assistance even to sit up. Survival is reduced in these patients, but they may live beyond 2 years. The mildest form of childhood SMA is Type III. Type III patients are diagnosed after 18 months of age. They can stand and walk, but may require a wheelchair in later stages of life as they survive into late adulthood.

The SMA disease spectrum was further broadened to include a SMA Type 0 (Dubowitz 1999) and an SMA Type IV (Zerres et al. 1995). Type 0 SMA, or “very severe” SMA, occurs when the developing fetus of a pregnant mother ceases movement during the last trimester and the child is then born with SMA symptoms (Dubowitz 1999). Type IV SMA has a very late onset of symptoms occurring in adulthood past the age of
30 (Zerres et al. 1995). Despite the attempt at grouping patients, a spectrum of disease severity still remains to present day with some patients falling between categories.

1.1.3 Clinical and Laboratory Observations

The three forms of SMA share a range of symptoms. All three types affect the proximal muscles and lower limbs the most. Type I SMA can be considered acute; whereas, Types II and III can be considered chronic diseases (Dubowitz 1995). Infants with Type I SMA have poor head and limb control and difficulty with swallowing and sucking. The most prominent feature of Type I infants is the bell-shaped chest caused by loss of intercostal muscle control and diaphragmatic breathing. They often die due to respiratory complications caused by an enhanced susceptibility to infection. Type II SMA children develop normally early on and are able to reach the sitting up milestone. At this point, they do not progress further to stand unaided. Hallmarks of Type II include fasciculation of the tongue and diaphragmatic breathing. Increased muscle weakness and resultant inability to stand often leads to severe scoliosis, which if left untreated, can cause further respiratory complications and even early death. Type III children also progress normally at an early age and do gain the ability to stand and walk. They often have some difficulty with walking, doing stairs and standing up from a sitting position on the floor. Prognosis for Type III patients is good as long they do not suffer setbacks such as injury or prolonged illness.

A diagnosis of SMA must fit certain clinical criteria. As set forth by the International Consortium in 1991, patients must present with muscle weakness, show evidence of denervation (shown with EMG and muscle biopsy) and there must be
exclusion of other diseases such as muscular dystrophy (Munsat 1991). EMG of SMA patients shows fibrillation (Hausmanowa-Petrusewicz et al. 1986). Nerve conduction velocity studies can also be performed, which are normal for Type II and III, but slow velocities are recorded for Type I SMA (Krajewska et al. 2002). Muscle biopsies of SMA patients show gross atrophy of muscle due to denervation. Hypertrophy may result in some cases of SMA as the muscle becomes reinnervated (Pearn et al. 1978; Bouwsma et al. 1980; Dubowitz 1995). Other clinical findings include normal (Type I) or slightly elevated CK levels (Type II and III), which rules out muscular dystrophy as the diagnosis because elevated CK levels are a hallmark of muscle damage (Dubowitz 1995). Today, SMA is largely diagnosed by non-invasive procedures such as genetic blood tests that have become possible since the discovery of the gene that causes SMA, as will be discussed in section 1.2.2.

1.2 Survival Motor Neuron Genes

1.2.1 SMN Gene

By the 1990’s, the SMA field had finally reached a critical threshold in the clinical and research sectors and progress picked up an accelerated pace. The disease locus was mapped to 5q11.2-q13.3 in 1990 (Brzustowicz et al. 1990; Melki et al. 1990). Markers such as CATT-1 and Ag1-CA were identified within this region as being associated with this gene locus and further refined the map of this area (Burghes et al. 1994; DiDonato et al. 1994). Many different deletions of the region were discovered in these studies. The identification of these markers allowed a series of YAC contigs encompassing this region of chromosome 5 to be isolated and characterized (Carpten et al. 1994). It was
difficult to make a complete physical map of this region of chromosome 5 because the YAC contigs were highly unstable and the region was found to contain repetitive sequence (Carpten et al. 1994). Despite the difficulties, soon after in 1995, the SMA-causing gene, Survival Motor Neuron 1 (SMN1) at 5q13, was identified (Lefebvre et al. 1995). This gene lay in an inverted duplication of chromosome 5 (Lefebvre et al. 1995); which is only one of many configurations of this genomic region (Burghes 1997). Two forms of the SMN gene were found in this region, SMNC and SMNT. Both genes were found in the same genomic region, one telomeric (SMNT, now called SMN1) and one centromeric in location (SMNC, now called SMN2). Both genes form the same protein product; however, 5 single nucleotide polymorphisms were found between the two genes (Lefebvre et al. 1995). SMA patients seemed to lack SMN1 or have a disrupted SMN1 and interestingly, the SMN gene was found to be expressed in all tissues, but a truncated form of the gene lacking the C-terminus was found in SMA patients (Lefebvre et al. 1995). In fact, several variants of SMN were found to be expressed, namely SMN(D7), SMN(D5) and SMN(D5,7), which lacked exon 7, exon 5 or exon 5 and 7 respectively (Gennarelli et al. 1995; Lefebvre et al. 1995). This clouded the results and made it difficult to say how exactly SMN caused SMA and the varying degrees of severity. Further analysis of the SMN genes revealed that the promoters for SMN1 and SMN2 were nearly identical and were quickly ruled out as the reason for the difference between the two genes and the cause for SMA (Monani et al. 1999). It was finally revealed that only one of the SNPs makes the difference between the SMN1 and SMN2 genes. SMN2 has a C to T transition within an exon splice enhancer, which causes most of the transcripts to exclude exon 7 and the resultant truncated protein (SMN(D7) isoform) to be degraded (Lorson et al. 1999; Monani et al. 1999; Cartegni et al. 2002). Figure 1.1 diagrams the two SMN genes. The C to T SNP in exon 7 modulates the
efficiency of recognition of the sequence by the SF2/ASF splicing factors by either disrupting a splice enhancer or by creating a splice silencer, thus causing a reduction in correctly spliced transcripts (Cartegni et al. 2002; Kashima et al. 2003). SMN(D7) is the primary product of SMN2; however, it can still produce full-length SMN (Lorson et al. 1999; Monani et al. 1999; Cartegni et al. 2002). The disease was found to be caused by a two-fold process: loss of SMN1 by mutation or deletion such that it no longer produces functional SMN or conversion of SMN1 to SMN2 and retention of SMN2 (Burghes 1997; Campbell et al. 1997). SMN2 cannot produce sufficient full-length SMN protein for motor neurons (Lorson et al. 1999; Monani et al. 1999).

Figure 1.1 Diagram of the C to T transition of SMN1 and SMN2. The C to T change in SMN2 affects the splicing of the gene and results in most transcripts lacking exon 7 (SMN(D7)); however it still produces a small amount of full-length SMN (SMN(wt)). Adapted from (Butchbach et al. 2004).

A majority (more than 90%) of cases of SMA involve either deletion of the SMN1 gene locus or conversion of the SMN1 gene to SMN2 (McAndrew et al. 1997; Wirth et al. 1999). Some patients harbor rare missense mutations within SMN1 (Wirth et al. 1999). These rare mutations, along with the SMN2 splicing mutation, decrease the self-
association and protein binding potential of SMN resulting in loss of function of the protein (Lorson et al. 1998). While normal SMN1 produces the majority of fully functional protein, SMN2 can provide some full-length protein and thus, can modulate phenotypic severity in humans (Lefebvre et al. 1995). Finally, the continuous spectra of SMA disease could be explained. SMA patients have a loss of SMN1 with retention of varying numbers of SMN2 copies. The number of copies of SMN2 is inversely proportional to the severity of the disease (McAndrew et al. 1997; Parsons et al. 1998) and increased copy number is due to conversion of the SMN1 gene to SMN2 (Campbell et al. 1997). Having more copies of SMN2 lessens the severity of the disease and this concept is diagramed in Figure 1.2.

Figure 1.2 The types of SMA are caused by the loss of SMN1, but retention of varying copy number of SMN2. The severity of the disease is correlated to the number of copies of SMN2 that are present. Loss of SMN1 is a result of deletion or mutation (indicated by the red X) or conversion of SMN1 to SMN2 (indicated by the red number 2). Adapted from (Wirth et al. 2006).
1.2.2 Genetic Testing of SMA

Since the SMA field took off in the early 1990’s with the identification of the SMA-causing gene \( SMN1 \), genetic screening for SMA had become a real possibility. An important outcome of this research was finally being able to diagnose new SMA patients as early as possible, identify carriers and find treatments for these patients. Significant improvements were developed rapidly in the field of screening for SMN mutations and definitively genotyping SMA individuals as well as their family members to determine risk. In 1995, one of the first available tests used SSCP (single-strand conformation polymorphism) and mismatched PCR to distinguish between \( SMN1 \) and \( SMN2 \) (Van der Steege et al. 1995). This method involved PCR amplification using primers within exon 8 followed by digestion with \( Ddel \) (Van der Steege et al. 1995). \( SMN2 \) has an SNP within exon 8 that creates a restriction site that \( SMN1 \) does not have. It is also possible to distinguish between the two genes by using a SNP in exon 7, amplifying with a mismatched primer to introduce a restriction site and then digesting with \( DraI \) (Van der Steege et al. 1995). This test was confirmed by SSCP analysis, whereby the exons were amplified by PCR, strands separated and loaded onto a gel (Lefebvre et al. 1995). The single strands of DNA containing differences in sequence migrate differently than the normal sequence single strands. The restriction digest method allowed for quick, nonradioactive analysis of patients with homozygous deletions.

These first tests devised, however, could only distinguish between \( SMN1 \) and \( SMN2 \). Most SMA patients have a homozygous deletion of \( SMN1 \) with retention of \( SMN2 \) and so this test would be appropriate, but some SMA patients still have \( SMN1 \). The \( SMN1 \) that they retain has a point mutation, which cannot be distinguished in the original screen. SMA patients that are compound heterozygous (having one exon
deleted SMN1 and one point mutated SMN1) and carriers that are heterozygous for SMN1 cannot be separated. Furthermore, this test was not quantitative and the copy number of SMN1 and SMN2 could not be determined.

Between 1997 and 2000, several screening tests were devised to address these problems also using PCR as the method of choice. One of the first methods used radioactive PCR of SMN1 and SMN2 along with an internal control, CFTR (McAndrew et al. 1997). The PCR products were then quantitated and gene dosage of SMN1 and SMN2 could be determined by comparing the relative intensities of each PCR product. This quantitative PCR method was further improved upon with the addition of the use of fluorescent primers, which allowed for automated detection of PCR products (Scheffer et al. 2000). In another example, researchers used a method called nonradioactive quantitative multiplex PCR that was based on the earlier method by McAndrew et al, 1997, but also used restriction digests as in Van der Steege et al, 1995 (Wirth et al. 1999). In this method, a forward mismatch primer was used that overlapped a SNP of exon 7 that created a HinfI site and a reverse primer in intron 7. The uncut PCR product is 135 base pairs long. Digestion of the product with HinfI results in 2 distinct bands for SMN2 of sizes 101 and 34 base pairs because it has just one restriction site. SMN1, on the other hand, now has two restriction sites resulting in 3 bands of distinct sizes 78, 34 and 23 base pairs long. These products are run on native polyacrylamide gels, silver stained and scanned with a densitometer. Nondeleted SMA patients can then be screened for point mutations in their SMN1 genes. To do so, lymphoblastoid cells are taken from patients and RNA is isolated from these cells. The RNA is reverse-transcribed to DNA and then sequenced to find mutations.

While quantitative PCR methods did have its advantages over previous methods, it still had the drawback that numbers of copies of each gene could be ambiguous due to
an overlap in signal intensities, so in 2002 another more efficient and reliable screening method took shape. The same German group that modified quantitative multiplex PCR, now devised a method using real-time Light-Cycler PCR (Feldkotter et al. 2002). The real-time PCR method enables the detection of \textit{SMN1} deletion as well as confirms the number of \textit{SMN1} or \textit{SMN2} gene copies. It is sensitive enough and specific enough to tell the difference between \textit{SMN1} and \textit{SMN2} even when present in the same genome.

The real-time PCR method begins with collection of blood samples from SMA patients, carriers and controls. DNA is isolated and subjected to PCR. The forward primer used is near the SNP of exon 7 and the reverse in intron 7. As the sample is amplified it is quantitated in real-time by detection of a fluorescent dye called SYBR Green I, which binds to double-strand DNA. By comparing the amplification plots of controls and standards to the unknown, the researchers can arrive at copy number for each unknown. Each unknown was also subjected to nonradioactive quantitative multiplex PCR, haplotype analysis using the polymorphic Ag1-CA and C212 markers as well as multiplex analysis of the subject’s relatives to confirm the real-time result.

The establishment of this method also verified that the number of \textit{SMN2} gene copies directly correlates with the phenotype of human patients (Feldkotter et al. 2002). It was found that most patients with Type I SMA have 1 or 2 copies of \textit{SMN2}. Type II patients tend to have 3 copies and Type III have 3 or 4 copies. This correlation is strong (greater than 80% for each type), but it is not absolute as some SMA patients will vary in \textit{SMN2} copy number. Furthermore, survival time of patients can be assessed from the copy number of \textit{SMN2} (Feldkotter et al. 2002). One copy of \textit{SMN2} predicts survival to 7 months of age in a Type I patient. Two copies with Type I predicts a lifespan of about 8 months and 3 copies about 37.5 months. Lastly, a risk assessment was done for each type of SMA. A risk of 99% for developing Type I SMA was given to children possessing
one copy of \textit{SMN2} and 97\% for two copies. The risk for developing Type II with three copies of \textit{SMN2} is 82.8\%. Four copies of \textit{SMN2} results in a risk assessment of 83.6\% for developing Type III.

In order to further improve upon detection of homozygous deletions as well as compound heterozygotes, the method called multiplex ligation-dependent probe amplification (MLPA) (Schouten \textit{et al.} 2002) was adapted for SMA (Arkblad \textit{et al.} 2006). In MLPA, sets of probes are allowed to anneal on target sequences. Two probes must hybridize to each target region and they each contain the sequence for a primer that will later amplify that probe. The probes are then ligated together to ensure that both probes and thus, both forward and reverse primer sequences, remain together. The ligated probes are then amplified by PCR using a generic primer set that is fluorescently labeled. The PCR amplicons are then separated by capillary electrophoresis as each probe set has a unique length. It is then possible to determine the dosage of each target. To adapt this protocol to SMA, probes specific to SMN, such as those for exon 7 and 8, were used. In this way, the copy numbers of \textit{SMN1} and \textit{SMN2} could be determined. This method can also detect deletions and mutations as probes encompassing all exons of SMN could be used and any probe that fails to amplify would indicate a deletion or mutation that requires sequencing for confirmation.

All of these methods have helped to diagnose SMA patients and carriers. The type of SMA (severity of disease) and probable lifespan of affected patients can be predicted. Carriers can be quickly screened and told their associated risk of transmitting the disease to offspring. While these tests seem to work out a few problems, the complex genetics of SMA still complicates matters. Mutations within copies of \textit{SMN1} can go undetected in PCR-based tests. Also, some SMA patients carry two \textit{SMN1} genes on one chromosome rather than both. Any of these methods would show this as
two copies, as they cannot distinguish that they are on separate chromosomes, making that person a carrier. This situation has been confirmed in SMA by somatic cell hybridization (Mailman et al. 2001). In this technique, hybrids of human and mouse cells are created by fusing the two cell types. As the cells grow and divide, the human chromosomes are ejected randomly and in this way it is possible to separate the two SMA chromosomes (chromosome 5). Then, each chromosome 5 can then be subjected to dosage analysis individually to arrive at the conclusion that one chromosome bears two copies of \textit{SMN1}, while the other has zero copies. Despite these complications, the screening of SMA has advanced dramatically and has continued to provide clues to understanding this disease.

1.2.3 SMA Therapeutic Strategies

Also, with the discovery of the \textit{SMN1} gene came hope for treatment of this disease. Many therapeutic strategies have come to light as the root cause of SMA became more and more clear. Every level of SMN gene expression could now be exploited (Sumner 2006). For instance, at the gene level, SMN gene expression could be upregulated to provide more full-length SMN in hopes of restoring function. This could be achieved through the use of HDAC inhibitors or other compounds that would act to increase overall expression of the \textit{SMN2} gene by stimulating the \textit{SMN2} promoter (Kernochan et al. 2005). A few drug compounds found to increase SMN expression were phenylbutyrate (Andreassi et al. 2004) and valproic acid (Brichta et al. 2003; Sumner et al. 2003). Furthermore, the SMN gene could be targeted for gene therapy replacement by viral delivery of the functional \textit{SMN1} gene to the neurons (Azzouz et al. 2004).
Another approach is to target SMN at the mRNA level and promote exon 7 inclusion in the final transcripts. This could be achieved through the use of drug compounds, such as aclarubicin, that enhance inclusion of exon 7 (Andreassi et al. 2001) or through the use of antisense oligonucleotides that bind directly to transcripts to enhance inclusion of exon 7 (Cartegni et al. 2003; Skordis et al. 2003). Further, SMN at the protein level could be targeted for therapeutics. Again, drug compounds could be used that show activity in stabilization of the SMN protein, specifically, the SMN(D7) isoform (Chang et al. 2004; Lunn et al. 2004; Wolstencroft et al. 2005).

In 2005, researchers undertook a high-throughput drug compound screening effort in order to identify drugs that would increase SMN expression (Jarecki et al. 2005). More than 500,000 synthetic compounds were tested in a beta-lactamase (BLA) reporter gene assay in NSC-34 cells, a motor neuron hybrid cell line. A 3.4 kb fragment of the SMN2 gene containing regulatory elements was used to drive expression of the BLA reporter gene. Seventeen drug compounds representing nine different structural scaffolds were identified as having an affect on the BLA reporter activity. These drugs were then tested on fibroblasts from SMA patients. As expected, it was found that some of the compounds acted to directly increase total mRNA from the SMN2 gene without affecting the ratio of SMN(D7) and full-length transcripts produced. However, unexpectedly, some of the drugs, such as the indole and quinazoline drugs, acted on the splicing of the SMN2 gene by shifting the transcript ratio towards more full-length SMN rather than SMN(D7). These drugs were found to be the most promising as they also increased SMN protein levels, gems and Cajal body numbers. Quinazoline and indole drugs became the best candidates for further studies as they were the most potent, least toxic and had highly modifiable structures.
While great progress has been made in SMA therapeutics and in drug screening, all strategies have their associated difficulties and risks to the patient and there remains no clear treatment for the disease to the present day. Further, all of these therapeutic strategies, have been reliant on fibroblasts from SMA patients; however, these cells do not have motor neuron-like properties. Recently, induced pluripotent stem cells (iPS cells) derived from an SMA patient were developed (Ebert et al. 2009). The authors showed that these iPS cells could then be differentiated into motor neurons, thus providing a source of new SMA motor neurons continuously. Researchers can now use the iPS cells for drug screening instead of fibroblasts and this will better mimic SMA conditions.

1.3 Mouse Models of Spinal Muscular Atrophy

1.3.1 Smn Knockout and SMN2; Smn-- Mice

The discovery of the SMN gene also led to development of mouse models in the laboratory that would help researchers to probe many unanswered questions about SMA. The first mouse model developed was the SMN knockout mouse. Two groups published an Smn knockout mouse in quick succession, but each had used a different strategy to knock out the endogenous mouse Smn (Schrank et al. 1997; Hsieh-Li et al. 2000). Mice were found to have only one SMN1 gene, Smn, that was 83% identical to human SMN (Schrank et al. 1997). The first knockout mouse generated by a German team was made by using a HindIII site of exon 2 of mouse Smn to create a targeting vector for exon 2, which contained a lacZ and neo' gene in the knockout cassette (Schrank et al. 1997). After homologous recombination in ES cells, the endogenous
The Smn gene would have a disrupted exon 2 fused in-frame to the marker and selection genes. This would result in production of beta-galactosidase and neomycin resistance with no functional SMN protein. Selected ES cells were then injected into blastocysts and resultant knockout mice were obtained. Animals heterozygous for the knockout were interbred; however, animals homozygous for the knockout could not be obtained. Upon further investigation, it was revealed that homozygous deletion of Smn was embryonic lethal. The embryos developed normally until about 80 hours of development and then started to undergo apoptosis. It was also concluded that mice heterozygous for Smn, thus expressing 50% SMN, did not exhibit motor neuron loss.

Next, a Taiwanese group came out with their own version of the Smn knockout mouse (Hsieh-Li et al. 2000). They used targeted replacement of exon 7 with HPRT as the means of Smn ablation. This method would result in a truncated SMN protein, as exon 7 is deleted. This group also did not obtain viable mice homozygous for the Smn knockout, thus concluding once more that complete loss of Smn was embryonic lethal. The group then asked whether adding human SMN2 would result in an SMA-like mouse. Transgenic mice were made with the region of human chromosome 5 that contained SMN2 in addition to the nearby NAIP and SERF1 genes. The resultant transgenic mice were then bred to the knockout mice to produce SMA-like mice. The mice had lower body weights and exhibited motor neuron loss with muscle atrophy.

At the same time, another group used the Schrank, et al, 1997 knockout mouse model to add a SMN2 transgene to and thus, develop an SMA mouse model (Monani et al. 2000). Here, only the human SMN2 gene was introduced and the resultant mice also exhibited signs of SMA. They obtained 2 different lines of SMN2 mice, one with 1 copy of SMN2 (low copy) and one with 8 copies of SMN2 (high copy). Mice with 2 copies of SMN2 (homozygous) on the Smn-/- background were smaller in size and weakened and
died by postnatal day 4 to 6. These mice had fewer motor neurons and exhibited muscular atrophy consistent with severe, Type I SMA. Mice with high copy SMN2 were also bred to the Smn-/ background, but resultant offspring showed no signs of SMA, thus lending credence to the observation that more copies of SMN2 lessens the severity of the disease and can in fact rescue the disease at very high copy number.

1.3.2 Muscular and Neuronal Mouse Models

A long-standing question in SMA has been whether or not SMA is truly a disease of neurogenic origins, as its name implies, or of myopathic origins. Introducing SMN2 onto the Smn knockout background, gives low expression of full-length SMN and high expression of SMN(D7) in all tissues. To examine whether tissue-specific ablation of SMN would result in the SMA phenotype, several mouse models were developed. To knockout SMN expression in neurons, Frugier et al. used NSE-Cre mediated deletion of SMN exon 7 (Frugier et al. 2000). This resulted in mice that exhibited muscle weakness and atrophy along with signs of denervation. The mice lived for an average of 25 days. This seemed to suggest that only motor neurons are affected in SMA. Then, a muscle-specific deletion of SMN was made to verify that SMA was indeed neurogenic in origin (Cifuentes-Diaz et al. 2001). HSA-Cre was used to delete Smn exon 7 specifically in muscle. The mice developed severe muscle weakness, paralysis and died within 33 days. This led the authors to conclude that muscle also may have a role in SMA pathogenesis. Deletion of SMN within other tissues, such as liver, has also been performed (Vitte et al. 2004). This resulted in severe liver abnormalities that contributed
to embryonic lethality. However, because SMN is needed in all cell types, deletion of SMN from any tissue will logically result in the death of that tissue and thus, the death of the animal.

Another approach to this question is to introduce tissue-specific expression of SMN on the SMN2; Smn-/- background. In this way, endogenous mouse Smn is deleted, while there are still low levels of full-length SMN and SMN(D7) produced by SMN2 in all tissues. Since this results in barely viable animals, it can now be asked whether any specific transgene can rescue the mice and thus, extend the lifespan of these mice. SMN driven by the Prion promoter was used to express SMN in neurons (Gavrilina et al. 2008). This resulted in mice that could live for up to a year with no visible signs of SMA. Introducing SMN driven by the muscle-specific HSA promoter had no effect on the phenotype of SMN2; Smn-/- mice. Further, one line of HSA mice had leaking expression into neurons, which allowed this line to survive longer.

From these studies, it has become increasingly clear that low levels of full-length SMN are necessary in all tissue types for complete functionality. However, it is also clear that motor neurons have an enhanced requirement for SMN and thus, are more susceptible to a reduction of SMN. Complete ablation of SMN from any given tissue will result in death of that tissue regardless. Maintaining a low level of SMN, such as from SMN2, is optimal for examining the effects of other transgenes on motor neurons.

1.3.3 SMN(D7) Mouse

Another key question in SMA had been whether or not expression of the SMN(D7) isoform of SMN is detrimental to the cell. Complete ablation of full-length SMN in all tissues and the replacement with SMN(D7) in all tissues was embryonic lethal
(Cifuentes-Diaz et al. 2001). However, when SMN(D7) is instead overexpressed in all tissues in the presence of SMN2, a mouse with the equivalent Type II SMA phenotype results (Le et al. 2005). These mice can live up to 14 days. Thus, the SMN(D7) isoform was deemed beneficial and further provided evidence that SMN expression must be present in all tissues to obtain viable organisms.

1.3.4 SMN(A2G) Mouse

With the discovery of the SMN gene, great progress has been made in being able to identify new deletions and mutations that SMA patients may have. One such mutation discovered was the missense mutation SMN(A2G) (Parsons et al. 1998). This allele causes mild SMA and thus, it was used to create a mouse model that would live longer and exhibit signs of SMA for further study (Monani et al. 2003). This mouse expresses human SMN containing the missense mutation, A2G, in exon 1 using the SMN promoter. The SMN(A2G) transgenic mice were then crossed to the SMN2; Smn-/- background. Mice that were heterozygous for SMN(A2G) and had 1 or 2 copies of SMN2 and no mouse Smn (SMN(A2G)+/−; SMN2+/− or SMN2+/+; Smn−/−) had signs of Type III SMA (mild), such as denervation and axonal sprouting and lived for about 1 year. Mice that were homozygous for the transgene were normal. Interestingly, expression of SMN(A2G) without SMN2 did not result in viable mice, indicating once again that expression of some full-length SMN is crucial. The A2G mouse enables researchers to better study the SMA phenotype because the symptoms are much milder.
1.4 Zebrafish Model of Spinal Muscular Atrophy

Another model of SMA disease currently under investigation is the zebrafish. This animal is amenable to SMA study because of its well-characterized embryonic development and well-defined nervous system. In addition, external fertilization allows for easy observation of development at all time points. Like mice, zebrafish have one SMN gene termed smn. Since knockout strategies used in mice are not yet available for zebrafish, transient knockdown of target genes is necessary. The technique of using antisense morpholinos (Nasevicius et al. 2000) has been used to knockdown the smn gene (McWhorter et al. 2003). By injecting antisense morpholinos into zebrafish embryos at the 1-2 cell stage, it is possible to specifically block translation of smn mRNA, which results in lowered levels of protein. Knockdown of smn can persist for up to two weeks. After injection of embryos with smn morpholino, the fish are allowed to develop for 48 hours. At this time point, the zebrafish exhibit motor neuron truncation and show branching as motor neurons try to reinnervate tissue. This phenotype was shown to be a result of a decrease of SMN protein in motor neurons specifically.

It is also, then, possible to co-inject smn antisense morpholinos with different human mRNAs to probe the ability of any given protein to rescue the zebrafish from abnormal axonal pathology (McWhorter et al. 2003). The authors co-injected the smn morpholino with either human full-length SMN or SMN(D7) mRNA. Knockdown of smn with the smn morpholino alone, results in fish having severe axonal defects such as truncations. Adding back full-length SMN results in no fish with severe axonal defects and thus, this protein rescues the fish as expected. Adding back SMN(D7), however, results in fish with severe defects, but not as many as seen with smn morpholino alone since SMN(D7) does retain some activity, especially in the presence of low levels of full-length smn.
1.5 The SMN Protein

SMN is a 294 amino acid protein encoded by 9 exons (Exons 1, 2a, 2b, 3-8). NMR and X-ray crystallography have revealed that exon 3 of SMN encodes a 5-strand β-barrel structure with homology to proteins with what are known as “Tudor domains” (Selenko et al. 2001). An α-helical region connects β-strand 4 and 5 while the other strands are connected by loops. The amino and carboxyl ends of SMN have unknown structure and are predicted to be flexible and globular in nature. Exon 6 has been shown to be important for self-association of SMN (Lorson et al. 1998). Figure 1.3 shows the determined structure of exon 3 of SMN as well as the domains of the full protein.

SMN localizes to nuclear aggregates termed “gems” and is ubiquitously expressed with the highest levels in the motor neurons of the spinal cord (Liu et al. 1996; Coovert et al. 1997; Lefebvre et al. 1997). Biochemical studies of SMN have shown that SMN not only self-oligomerizes, but also interacts with other proteins in a variety of activities within the cell. SMN has been associated with Sm proteins (core of spliceosomes, U snRNPs), fibrillarin (snoRNPs) and RNA helicase A (transcriptosomes) to name a few (Simic 2008). The function and validity of many of these protein interactions with SMN remain superfluous; however, SMN has been termed the master assembler of RNP macromolecules as it has an undeniable role in the maturation of snRNP complexes (Terns et al. 2001). Sm proteins form the core of the U snRNP complex along with SMN, SIP1 (SMN-interacting partner 1 or Gemin 2), snRNAs and other specific factors (Liu et al. 1997; Will et al. 2001). Table 1.1 summarizes the many interactions of SMN.
Figure 1.3 Domains of SMN.
SMN contains 9 exons. Exon 1 and 2 are involved in association of SMN with SIP1 (Gemin2). Exon 2a/2b associates with RNA. Exon 3 is the Tudor domain of SMN. The structure of this domain has been determined (structure above) and has been shown to be involved in binding Sm proteins. Exon 6 is necessary for self-association of SMN and exon 8 is the 3’ UTR of SMN. PDB accession 1MHN.
<table>
<thead>
<tr>
<th>Protein</th>
<th>Function</th>
<th>Reference</th>
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<td>SMN (Gemin1)</td>
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<td>Liu et al, 1997</td>
</tr>
<tr>
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<td>Core, snRNP assembly, splicing</td>
<td>Liu et al, 1997; Campbell et al, 2000</td>
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<td>tRNA, rRNA maturation</td>
<td>Hua et al, 2004</td>
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**Table 1.1 Summary of SMN protein interactions.**
SMN binds Gems 2-8 and unrip to form the core of snRNP complexes. Adapted from (Wirth et al. 2006).
In snRNP biogenesis, SMN first self-associates and binds to Gemins 2-8 and unrip in the cytoplasm (Wirth et al. 2006; Pellizzoni 2007; Simic 2008). A protein called pICIn binds to Sm proteins at conserved sequences and will not allow snRNP assembly to occur (Pu et al. 1999; Meister et al. 2001). pICIn must associate with PRMT5, which leads to methylation of Sm proteins, of which there are 7 species (B/B’, D3, D2, D1, E, F and G) (Meister et al. 2002). Once the Sm proteins are methylated, they can now form a heptameric ring in association with the snRNA and SMN complex (Kambach et al. 1999; Kambach et al. 1999). The SMN complex facilitates the loading of the Sm ring onto U snRNAs that have been transported out to the cytoplasm with their associated factors (Will et al. 2001). Gemin 5 is responsible for binding of the snRNA and provides specificity of loading (Battle et al. 2006). A protein called TGS1 then comes in to hypermethylate the snRNA cap (Mouaikel et al. 2003). Once the snRNA is hypermethylated, importin-β and snurportin can now associate with the complex to facilitate nuclear localization of the complex (Narayanan et al. 2004). The complexes are targeted to Cajal bodies by coilin (Hebert et al. 2001) and the snRNP complex undergoes further maturation into the spliceosome that will perform mRNA splicing. Figure 1.3 outlines the snRNP biogenesis pathway.

The U snRNAs contain conserved sequences (Sm sites) that dictates which proteins will bind to each snRNA for formation of splicing complexes (Kambach et al. 1999; Achsel et al. 2001; Stark et al. 2001). The U snRNAs also differ by the type of intron that they splice (Patel et al. 2003). There are 8 snRNAs of the Sm class: U1, U2, U4, U4atac, U5, U7, U11 and U12 and they are transcribed by RNA polymerase II. All of these snRNAs bind Sm proteins; however, U7 does not bind SmD1 or SmD2, instead it binds LSm10 and LSm11 along with the other 5 standard Sm proteins (Pillai et al. 2001; Pillai et al. 2003). U7 snRNA only participates in the splicing of histone transcripts (Galli
et al. 1983; Strub et al. 1984). Two other snRNAs, U6 and U6atac, bind to Lsm2-8 proteins (Sm-like proteins) (Mayes et al. 1999; Vidal et al. 1999). These two snRNAs are transcribed by RNA polymerase III and they are also unique in that they never leave the nucleus. Further, U11 and U12 do not associate with the major snRNP complex (Patel et al. 2003). Instead, U11 and U12 make up the minor snRNP assembly pathway, which handles the splicing of a small subset of transcripts that contain different intronic sequences (Patel et al. 2003).
Figure 1.4 Diagram depicting the life cycle of SMN in snRNP assembly.
SMN transcripts are translocated to the cytoplasm where they are translated and undergo self-association (1). SMN also binds Gemins2-8 (designated by numbers) and unrip (unr) to form the core of the snRNP complex (2). The complex binds to Sm proteins, which have been methylated by the PRMT complex in association with pICln (3). Next, snRNAs are transported to the cytoplasm by specific factors. Gemin5 binds to the snRNA and provides the specificity for the SMN complex, which then loads the Sm proteins that form a heptameric ring onto the Sm site of the snRNA (4). Lastly, the snRNA becomes hypermethylated by TGS1 and snurportin (S) and importin-β (I) bind to help the complex translocate to the nucleus (5). In the nucleus, the snRNP complex localizes to Cajal bodies, which are the sites of further maturation of the complex before undergoing splicing activities. Adapted from (Pellizzoni 2007).
1.6 snRNP Assembly Hypothesis vs Axonal Hypothesis

Since SMN has a major role in snRNP biogenesis, but yet has been associated with many more proteins in the cell, two emerging hypothesis have been used to explain the motor neuron specificity of SMA. One hypothesis, the snRNP hypothesis, states that a reduction of SMN results in a reduction of snRNP assembly and thus, causes SMA. But how can a ubiquitous function such as snRNP assembly be attributed to a disease that affects only motor neurons? The other hypothesis, the axonal hypothesis, is that SMN has another, as yet undescribed, function specific to motor neurons.

The axonal hypothesis is particularly appealing in that it avoids the global function of SMN to explain tissue specificity. As SMN is a ubiquitously expressed protein, reduction of SMN primarily affects motor neurons. SMN localizes not only to the nucleus and cytoplasm of motor neurons, but to axons (Pagliardini et al. 2000) and growth cones (Fan et al. 2002). SIP1, snRNPs and Sm proteins have not been found associated with SMN in motor neuron axons (Jablonka et al. 2001; Sharma et al. 2005; Zhang et al. 2006) indicating that SMN may have activities beyond the housekeeping duties of the nucleus. Several other studies suggest that SMN has a role within axons. For example, it was found that motor neuron axons in a zebrafish model of SMA are truncated and exhibit abnormal pathfinding upon knockdown of smn (McWhorter et al. 2003). It was also found that the effect was motor neuron specific. Rossoll et al have shown that a reduction of SMN in motor neurons causes reduced axon growth (Rossoll et al. 2003).

In addition, it has been suggested that mutant SMN causes defects in axonal transport of RNPs, as SMN was found in axons (Zhang et al. 2003). Two proteins, hnRNP Q and hnRNP R, have been identified as interacting with SMN in axons and elsewhere (Mourelatos et al. 2001; Rossoll et al. 2002). Furthermore, SMN, in
conjunction with hnRNP R, has been found to modulate the transport of β-actin mRNA down axons to the growth cone of developing motor neurons. Reduction of SMN or hnRNP R results in disruption of β-actin mRNA transport and axonal truncation (Rossoll et al. 2003). The axonal elongation defects seen in motor neurons with reduced SMN has been attributed to a defect in accumulation of calcium channels in the axon (Jablonka et al. 2007); however mice lacking calcium channels do not display a typical SMA phenotype (Pietrobon 2005; Kaja et al. 2007). Recently, Plastin 3 has been identified as a potential modifier of the SMN gene (Oprea et al. 2008). Patients that were siblings with the same SMN1 and SMN2 alleles, but were SMA-discordant were analyzed. Plastin 3 was found to have different transcript levels in the discordant siblings. Interestingly, only females were found to have modification of the SMA phenotype associated with the increased Plastin 3 levels. Plastin 3 binds to and facilitates bundling of F-actin (Bretscher 1981; Glenney et al. 1981). The bundled actin is used at the growth cone of developing motor neurons for growth, pathfinding and branching (Dent et al. 2003). Knockdown of Plastin 3 in PC12 cells resulted in reduction of neurite length and injection of Plastin 3 mRNA into zebrafish embryos resulted in rescue of axonal defects. Although SMN and Plastin 3 were found to co-localize, a direct interaction between the two proteins was not found (Oprea et al. 2008). It remains unclear whether Plastin 3 is truly a modifier of SMN with a causative role or if it simply has an associated role in SMA as defects of axons are not found in severe SMA mice (McGovern et al. 2008).

All of these studies offer compelling support for the axonal hypothesis of SMA; however, recently the evidence has begun mounting for impairment of SMN’s best known function in cells: snRNP assembly. Gabanella et al. found that a reduction of SMN results in decreased snRNP assembly and it was hypothesized that the cause of
SMA is inefficient snRNP assembly resulting in alteration of splicing of key motor neuron transcripts (Gabanella et al. 2007). They found that the minor snRNP assembly pathway, in particular, was more affected in SMA. If the minor snRNP pathway is disrupted, motor neurons could be more susceptible because this pathway handles motor neuron-specific transcripts. It has been shown that tissue-specific splicing of transcripts can be altered in neuronal tissue resulting in disease (Jensen et al. 2000). In POMA syndrome (paraneoplastic opsoclonus-myoclonus ataxia), loss of the RNA-binding protein, Nova-1, by autoimmune destruction is suspected of causing the disease by allowing misregulation of the splicing of key neuronal transcripts (Jensen et al. 2000). The neuronal-restricted Nova-1 normally binds to the pre-mRNA of the glycine 2 and GABA<sub>A</sub> receptors and regulates their alternative splicing. Loss of this key regulation step results in either gain of function toxicity of the aberrantly spliced transcripts or loss of function that then causes the disease. That defects in splicing can cause disease is not unprecedented and many more examples can be found in the literature. Diseases such as retinitis pigmentosa (RP), Prader-Willi syndrome (PWS) and myotonic dystrophy (DM) can be attributed to disruption of the splicing mechanism (Garcia-Blanco et al. 2004; Wang et al. 2007). Three dominant forms of RP are caused by mutations within pre-mRNA processing proteins (PRPF31, PRPF8 and HPRP3). PWS is caused by a deletion in the SNURF-SNRP locus, which encodes snoRNAs. Finally, DM is caused by CTG trinucleotide expansion within either DMPK or ZNF9 genes. The expanded RNA transcripts generated from these alleles accumulates toxically and disrupts splicing mediated by the RNA-binding proteins MBNL (muscleblind-like) and CUGBP2.

Because it is plausible that SMA is also caused by a defect of splicing, attempts to identify aberrantly spliced transcripts were undertaken. Recently, exon microarrays were used in an effort to identify transcripts that are spliced differently in SMA versus
normal tissue (Zhang et al. 2003). They report a reduction of minor snRNAs in neuronal tissues and hundreds of altered transcripts. However, the tissue used for this study was taken from PND11 SMA mice, which at that time point are near death, making the results possibly artifactual and not necessarily related to the real cause of the disease. The results could reflect a general stress response of the advanced disease state. To date, no specific targets of the minor or major splicing pathway have been identified.

1.7 Significance of Study

Because the function of SMN that is affected in SMA has not yet been determined, this project was designed to study various mutations of SMN and evaluate their utility in determining the function of SMN affected in SMA. Mutations of SMN, in particular missense mutations of SMN occurring in SMA patients as well as synthetic mutations, can be exploited to elucidate the mechanism of dysfunction of SMN. Mutations occurring within different domains of SMN may be used to tease out the various protein-protein interactions of SMN and thus, to point to a function of SMN most affected.
CHAPTER 2

CHARACTERIZATION OF SMN MUTANTS

2.1 Introduction and Objectives

The majority (95%) of SMA patients have a deletion of \textit{SMN1} (Parsons \textit{et al.} 1998). Thus, 5% of patients have missense mutations within \textit{SMN1}, which result in a complete protein with some disruption of function caused by the mutation. Analysis of the SMN protein sequence reveals highly conserved tracts that are hotspots for mutation. The SMN protein sequence is approximately 35% identical across 8 species of vertebrates and humans. In Figure 2.1, the program, ClustalW, was used to align SMN protein sequences (Thompson \textit{et al.} 1994). Alignment of SMN across these species reveals high conservation of exon 2a/2b, the Tudor domain and exon 6. SMN has high variability of the amino- and carboxy-terminal domains.

Exon 2a/2b region is necessary for binding of Gemin2, while exon 3 is necessary for binding of Sm proteins and exon 6 is important for self-association of SMN as discussed in Chapter 1. It is not surprising, then, that most missense mutations cluster within the highly conserved regions of SMN. Mutations within exon 2a/2b or exon 3 could affect the ability of SMN to bind to key proteins in the formation of the snRNP.
complex. The severity of the disruption will correlate with the disease severity.

Mutations occurring within exon 6 of SMN will affect SMN’s ability to self-oligomerize and thus, impede the formation of the entire complex. Table 2.1 summarizes the many missense mutations and the exon in which they occur is indicated.
Figure 2.1 Sequence alignment of SMN across eight species and humans. Key: *, 100% consensus; :, >75% consensus; ., >50% consensus; red text, missense mutations.
<table>
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<th>Domain</th>
<th>SMA Type</th>
<th>SMN2 #</th>
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<td>1</td>
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<td>2 or 3</td>
<td>1</td>
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<td>1</td>
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<td>2</td>
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<td>3</td>
<td>1</td>
<td>Hahnen et al, 1997</td>
</tr>
<tr>
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<td>3</td>
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<td>Clermont et al, 2004</td>
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<td>1</td>
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<td></td>
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</tbody>
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Table 2.1 Missense Mutations of SMN.
These mutations cluster around highly conserved regions of SMN such as the self-association domain. ND, not done.
There are several missense mutations that have been described within the Tudor domain (exon 3, AA #91-145) of SMN. Four of these mutations have been studied in greater detail: SMN(E134K), SMN(Q136E), SMN(I116F) and SMN(A111G). These point mutations result in Type I, severe SMA in humans (1b or 2 for SMN(A111G)). As shown in Figure 2.1, these four Tudor domain residues mutated in SMA patients are rarely substituted. Amino acid A111 and E134 is 100% conserved in these vertebrate species. I116 is replaced with valine in both the rat and the zebrafish. Q136 is replaced with histidine only in the frog.

The E134K mutation has been characterized and was shown to affect the association of Sm proteins, but not self-oligomerization (Buhler et al. 1999). The Q136E and I116F mutations were described in the Spanish population (Cusco et al. 2004), while A111G was described more recently (Sun, 2005). All mutations occur within the β-strands of the β-barrel secondary structure formed by the Tudor domain. The I116 residue is described as being part of a highly conserved hydrophobic core (Selenko et al. 2001). It was shown that I116 is oriented toward the interior of SMN in NMR and X-ray crystal structures of the Tudor domain. Mutation of this residue to a bulky phenylalanine residue, as in SMN(I116F), could cause problems in packing of the hydrophobic core of the Tudor domain. The amino acid E134 is part of a conserved negatively charged binding pocket for Sm proteins. This residue makes two hydrogen bonds with Serine 103. The Q136 amino acid is positioned at the end of the fourth β-strand going into the α-helical turn. Sm protein binding can thus be compromised by mutations at this site, such as in SMN(E134K) and SMN(Q136E). The effect is caused either by directly blocking protein binding or by a shift in local protein structure. Selenko and Sprangers showed that in the case of E134K, the structure is not shifted by the
mutation and must be a direct repulsion of the positively charged Sm protein tails, which bind the Tudor domain (Selenko et al. 2001; Sprangers et al. 2003). The A111 amino acid is also oriented towards the interior of the hydrophobic core. Mutation of this residue, SMN(A111G), could result in a local shift of the packing of the hydrophobic interior of the Tudor domain, thus causing disruption of the Sm binding site. All mutations described here are outlined in Figure 2.2.

Figure 2.2 Selected missense mutations of the Tudor domain of SMN. PDB accession 1MHN.

Along with these four Tudor domain mutations, several other mutations of SMN were also studied. These mutations include: SMN(RT), SMN(VDQNQKE), SMN(D7), and SMN(Q282A). These mutations are not missense mutations found in SMA patients; however, they serve to manipulate key regions of the SMN protein so that the binding
properties and behavior of SMN can be analyzed. SMN(D7) is the SMN protein with exon 7 completely removed so as to create an allele only capable of creating SMN(D7) protein. This mutation was used in mouse studies to assess the benefits of upregulation of SMN(D7) as discussed in Chapter 1 (Le et al. 2005). SMN(RT), RT for read-through, is a synthetic mutation created by removing the correct stop codon from SMN(D7) and allowing a read-through to the next stop codon which results in an addition of 9 amino acids from exon 8 (3' UTR) (Wolstencroft et al. 2005). This was done in an attempt to study the possible beneficial nature of using drugs to create a read-through product that would be more stable and help lessen or cure the disease. SMN(Q282A) is a (non-patient) mutation of the highly conserved QNQKE motif in exon 7 of SMN (See Figure 2.1) (Carrel et al. 2006). Finally, SMN(VDQNQKE) is a synthetic mutation comprised of SMN exons 1-6 with a VD linker to the QNQKE motif of exon 7 (Zhang et al. 2003). Because the SMN(D7) isoform did not localize to axons, this conserved sequence was used to stabilize the truncated protein and resulted in restoration of localization of SMN to axons (Zhang et al. 2003). It is also important to note that the VD linker used in SMN(VDQNQKE) introduces a mutation seen in SMA patients, SMN(G279V). SMN(wt) is used as a control and henceforth, will refer only to full-length SMN. These mutations are summarized in Figure 2.3.
In this chapter, the examination of the various mutations of SMN for their protein binding capacities and ability to rescue axonal defects in zebrafish is described. Protein binding capabilities were determined in GST-fusion protein binding assays. The various mutants of SMN were made by site-directed mutagenesis and cloned into appropriate vectors for expression as a GST-fusion protein or for in vitro translation of the protein. The mutants were then injected into zebrafish embryos along with smn morpholinos to assess whether each mutant can rescue the disease phenotype. Insights into the function of SMN in motor neurons that were gained from this data will be discussed.

Portions of this chapter have been previously published (Carrel et al. 2006).

2.2 Results

2.2.1 Binding Assays

Each of the SMN mutants described above were tested for their ability to bind in GST-fusion binding assays. In the assay, the proteins were tested for the ability to self-associate, associate with wildtype SMN and associate with Sm proteins. All 3
capabilities are necessary for snRNP complex formation. Site-directed mutagenesis was performed on an SMN cDNA construct to create the E134K, I116F and Q136E mutants. They were subcloned into pGEX (GST-fusion protein vector) and pcDNA3. The pGEX vector was used to express and purify GST-tagged mutant proteins and the pcDNA3 vector was used for in vitro transcription and translation of mutant protein with $^{35}$S as a label. Luciferase was the positive control for in vitro transcription and translation and non-specific control for the binding assay. SMN(D7) was used as a control as it has been shown to have decreased self-association as well as decreased association with wildtype Smn and Sm protein (Monani et al. 2003). Glutathione-S-transferase (GST) alone was used as a control to verify that the proteins were not binding the tag alone. The binding assay was done with varying concentrations (0.1, 0.5, 1, 2, 4, 6 µg) of GST-fusion protein and the amount of $^{35}$S-labelled protein recovered was measured.

Of the mutations occurring in the Tudor domain, only SMN(I116F) and SMN(A111G) performed well in binding assays (Figure 2.4 and 2.5). SMN(I116F) bound a maximum of $50.4 \pm 7.2\%$ protein in self-association assays, $35.2 \pm 2.3\%$ wildtype SMN was bound and $38.3 \pm 6.9\%$ Sm protein was bound by this mutant, which is comparable to SMN(D7). This is also compared to wildtype SMN, which bound $40.2 \pm 2.9\%$ of wildtype SMN and $80.4 \pm 9.2\%$ Sm proteins in these assays. The control protein SMN(D7) bound $1.8 \pm 0.1\%$ protein in self-association studies, $2.0 \pm 0.5\%$ in wildtype SMN and $33.7 \pm 3.9\%$ Sm protein. SMN(A111G) bound $24.7 \pm 0.8\%$ protein in self-association assays, $32.3 \pm 1.2\%$ wildtype SMN and $42.7 \pm 7.5\%$ Sm proteins (Figure 2.5). The other Tudor mutants, SMN(Q136E) and SMN(E134K), performed poorly in these assays (Figure 2.4). SMN(Q136E) bound $18.8 \pm 0.9\%$ in self-association assays, $15.4 \pm 2.6\%$ and $10.8 \pm 0.6\%$ Sm proteins. SMN(E134K) had a self-association
recovery rate of 15.8 ± 1.9%, 17.6 ± 3.4% recovery of wildtype SMN and only 4.4 ± 0.1% recovery of Sm proteins. Both SMN(Q136E) and SMN(E134K) bound less Sm protein than the SMN(D7) protein did, but more than SMN(D7) in self-association and wildtype SMN assays, not approaching the levels of wildtype SMN.
Figure 2.4  Protein binding analysis of three Tudor domain mutant SMN proteins. FL-SMN, full-length wildtype SMN; I116F, SMN(I116F); E134K, SMN(E134K); Q136E, SMN(Q136E); Δ7, SMN(D7). Self-association (top), full-length SMN association (middle), and Sm association (bottom).
Figure 2.5 Protein binding analysis of mutations of the SMN protein. FL-SMN, full-length wildtype SMN; Q282A, SMN(Q282A); A111G, SMN(A111G); Δ7, SMN(D7); RT, SMN(RT); QNQKE, SMN(VDQNQKE). Self-association (top), full-length SMN association (middle), and Sm association (bottom).
Another mutation, SMN(Q282A) fared well in all 3 assays, but SMN(RT) and SMN(VDQNQKE) did not (Figure 2.5). SMN(Q282A) bound 30.6 ± 0.7% protein in self-association assays, 32.5 ± 0.6% in wildtype SMN and 60.7 ± 1.1% of Sm proteins. The latter 2 mutations only bound protein at or below the level of SMN(D7). SMN(RT) bound 4.2 ± 0.1% of itself in self-association assays, 5.4 ± 0.7% of wildtype SMN protein and 22.9 ± 4.8% Sm proteins. SMN(VDQNQKE) bound 2.7 ± 0.1% in self-association studies, 3.2 ± 0.1% in association with wildtype SMN and 16.9 ± 0.1% of Sm proteins were bound.

Mutations that failed to self-associate (SMN(D7), SMN(RT), SMN(VDQNQKE), SMN(Q136E) and SMN(E134K)), were then predicted to not be able to assemble snRNP complexes. Indeed, the mutations SMN(Q136E) and SMN(E134K) were found to be not capable of snRNP assembly in an in vitro assay (Shpargel et al. 2005). Mutations that still retained the ability to self-associate, associate with wildtype SMN and Sm proteins (SMN(A111G) and SMN(Q282A)) were predicted to be able to form snRNP complexes. SMN(A111G) was found to be capable of snRNP assembly in an in vitro assay; however, SMN(I116F) was still deficient in assembly activity despite retaining ability to self-associate (Shpargel et al. 2005). This is not surprising, though, since SMN(I116F) bound Sm proteins at the level of SMN(D7) and SMN(D7) is clearly not capable of snRNP assembly (Shpargel et al. 2005). These mutations were then tested in an in vivo zebrafish model of SMA for rescue of phenotype. The hypothesis being that mutants still capable of protein association and complex assembly would rescue the phenotype.
2.2.2 Zebrafish Assays

When morpholino oligonucleotides (MOs) are injected into zebrafish embryos, they block translation of SMN by binding to \textit{smn} mRNA (Summerton \textit{et al.} 1997; Summerton 1999). This results in reduction of SMN protein and causes motor neuron truncation and branching in the zebrafish (McWhorter \textit{et al.} 2003). This motor neuron pathology can be reversed by co-injecting full-length human SMN mRNA with the \textit{smn} MO. The pathology is not reversed by co-injection of SMN(D7) mRNA with the \textit{smn} MO. Transgenic zebrafish containing gata2-GFP (green fluorescent protein) are used because the gata2 promoter expresses in the central nervous system and allows easy visualization of embryonic motor neurons (Meng \textit{et al.} 1997).

Zebrafish embryos were injected at the 2-4 cell stage and then allowed to develop for 36 to 52 hours. The embryos were injected with a control MO for antisense MO specificity, \textit{smn} MO as a negative control for rescue, \textit{smn} MO and Tudor mutant mRNA as experimental, \textit{smn} MO and human SMN mRNA as a positive control for rescue, and finally \textit{smn} MO with SMN(D7) mRNA as negative control as described previously (McWhorter \textit{et al.} 2003). The expression levels were measured by western blot and the pathology of the fish was observed in whole-mounts under a fluorescent microscope. The fish were scored as being mild, moderate or severe based on the number of axonal defects as well as severity of the defects observed. Only fish with normal body morphology were scored. Truncation of an axon was classified as being the most severe defect. Excessive branching and innervation of neighboring myotomes

\* Zebrafish assays were performed by T. Carrel in Carrel \textit{et al.} 2006.
were classified as moderate defects while ectopic branching, ventral roots, and defasciculation were classified as being mild defects (Carrel et al. 2006).

The control injections are depicted in Figure 2.6. Injection of a non-specific control MO resulted in the majority of fish having no defects of axons. Injection of smn MO on its own resulted in a decrease in fish with normal axons and an increase in fish with severe axons. This shows that the smn MO is specific and the result is not caused by injection trauma. Injection of rescuing human SMN RNA without smn MO showed that the RNA levels injected are not toxic and result in normal fish axons. As expected, co-injection of smn MO with SMN results in rescue; whereas, co-injection of smn MO with SMN(D7) RNA does not result in rescue of axons.

**Figure 2.6 Control injections of MO and rescuing RNAs in a zebrafish assay.**
Ct MO, non-specific control morpholino; smn MO, smn morpholino; hSMN, human SMN mRNA; hSMNΔ7, human SMN(D7) mRNA. From (Carrel et al. 2006).
Next, the various mutations of SMN were co-injected with *smn* MO (Figure 2.7). All of the Tudor mutations, SMN(E134K) (unpublished), SMN(Q136E) (unpublished), SMN(I116F) (unpublished) or SMN(A111G) (Carrel *et al*. 2006) failed to rescue the zebrafish. SMN(A111G) failed to rescue even though it performed well in binding assays and is capable of snRNP assembly. Large numbers of fish with severe axonal defects were recovered. SMN(Q282A) and SMN(RT) also failed to rescue the axonal defects seen in these fish. SMN(Q282A), interestingly, had a dominant negative effect as the number of zebrafish with severe defects exceeded the number of severe fish obtained when injecting *smn* MO alone. To further determine the specificity of the QNQKE motif, SMN(E286A) was made and injected. This mutates the last residue in the QNQKE motif. This mutant rescued axonal defects. Also, two patient mutations, SMN(G279V) and SMN(Y272C), occurring in the self-association domain of SMN were tested (not shown) and they failed to rescue the zebrafish. The only mutant mRNA that did rescue was SMN(VDQNQKE). SMN(VDQNQKE) rescued while not able to bind protein well and thus, was predicted to not be able to assemble complexes.
Figure 2.7  Co-injection of mutations of SMN with smn MO in a zebrafish assay.  

*smn* MO, *smn* morpholino; hSMN, human SMN mRNA; hSMNΔ7, human SMN(D7) mRNA; hSMN A111G, SMN(A111G) mRNA; hSMNΔ7 read-through, SMN(RT) mRNA; hSMNΔ7 VDQNQKE, SMN(VDQNQKE) mRNA; hSMN Q282A, SMN(Q282A) mRNA; hSMN E286A, SMN(E286A) mRNA. From (Carrel et al. 2006).
2.2.3 Generation of Mutant SMN Stable Cell Lines

Two of the point mutations were chosen for further analysis in tissue culture, SMN(A111G) and SMN(Q282A). SMN(A111G) was chosen because it was capable of self-association and capable of binding Sm proteins, but failed to rescue axonal defects in zebrafish. SMN(Q282A) was chosen because it also maintained self-association, Sm binding, but failed to rescue fish. This suggested that the two were forming an snRNP-independent complex in motor neurons, specifically in the axons. These two mutations, along with SMN(wt) were cloned to contain the mutant cDNA under the control of the CMV promoter and were fused to a carboxy-terminal FLAG tag. Constructs were cloned as shown in Figure 2.8. Through the use of the FLAG-tag on each mutant SMN protein, complexes formed in the cells could be pulled down from differentiated motor neurons and compared to the complex pulled down with full-length wildtype SMN. Differences between the protein profiles could be further analyzed, as this could indicate the protein(s) most affected by each mutation and lead to the possible identification of the complex important in SMA.

Figure 2.8 Diagram of FLAG-tagged mutant SMN constructs used for transfection of MN1 cells.
MN1 cells were transfected with each of these constructs. MN1 cells are motor neuron-like hybrid cells made from a fusion of embryonic mouse spinal cord motor neurons and mouse neuroblastoma cells (Salazar-Grueso et al. 1991). The cells were then selected with G418 (neomycin) for 2-3 weeks and individual clones were isolated by limiting dilution. Sixteen clones were screened by RT-PCR. Three clones were obtained for SMN(wt), 3 for SMN(A111G) and 5 for SMN(Q282A). The three highest expressing clones for each construct are shown in Figure 2.9.

In order to demonstrate that the FLAG-tag system was functional, pull-downs of the SMN(wt)-FLAG MN1 cells were then performed using anti-FLAG beads and the captured complex was probed with known constituents of the more abundant snRNP complex. Gemin 5, SMN and Sm (not shown) proteins were recovered (Figure 2.10). This demonstrated that the FLAG-tag system would work and that complexes could be obtained; however, the snRNP-independent complex would likely be in lower abundance compared to the snRNP complex.

Figure 2.9 Expression of mutant SMN in MN1 clones.
A1, SMN(A111G) clone 1; Q9, SMN(Q282A) clone 9; W4, SMN(wt) clone 4.
2.3 Discussion

Summarized in Table 2.2 are the combined results of the binding assays and zebrafish rescue assays. From this data, it was concluded that mutations of exon 7, SMN(D7), SMN(G279V), and SMN(Y272C), affect the self-association of the SMN complex, thus resulting in SMA. Additions to the truncated SMN(D7) isoform as in SMN(VDQNQKE) and SMN(RT) resulted in increased stability of the protein; however, SMN(RT) was not able to rescue axonal defects. SMN(RT) has been considered as a possible therapeutic target to increase the stability of the transcripts made from the SMN2 gene. However, these results indicate that this form of SMN is not capable of self-association or binding Sm proteins and fails to rescue axons. Surprisingly, SMN(VDQNQKE) was able to rescue axonal defects in zebrafish despite its inability to self-associate and bind Sm proteins, which are properties necessary for efficient snRNP assembly. Further, mutations of the conserved QNQKE sequence, as in SMN(Q282A) and SMN(E286A) showed that the leading Q of this sequence is necessary for rescue of axonal defects.

Figure 2.10  FLAG-tag pull-down of SMN and Gemin 5 from stable SMN-FLAG MN1 cell lines.
Additionally, SMN(VDQNQKE) could be considered an intragenic suppressor mutant. The correct sequence at the end of exon 6 into the QNQKE region of exon 7 is \textit{GFRQNQKE}; however, mutation of this sequence to \textit{VFRQNQKE}, as in SMN(G279V), does not result in rescue. Further mutation of the sequence to \textit{VDQNQKE}, led to rescue of severe axonal defects. Replacement of the VFR sequence with VD could cause a shift in protein structure that now allows protein binding that was not possible before. This provides further evidence of the importance of this exon 7 sequence.

Analysis of the mutations of the Tudor domain also led to some surprising results. Two mutations, SMN(E134K) and SMN(Q136E), had no ability to self-associate, bind Sm proteins or function to rescue axonal defects in zebrafish assays. However, SMN(I116F) was able to self-associate and bind Sm proteins, but failed to rescue axons. This further showed a disconnect between ability to form snRNPs and the ability to rescue axonal defects. The last piece of data, SMN(A111G), led to the conclusion that the ability of a mutant SMN to form snRNP complexes was not tied to its ability to rescue the SMA phenotype. SMN(A111G) retains its ability to self-associate, bind Sm proteins and perform snRNP assembly, but it fails to rescue the axonal defects in zebrafish. This mutation along with the SMN(VDQNQKE) mutation provided the most evidence for the independence of snRNP assembly and SMN function in motor neurons. Thus, the function of SMN required is not snRNP assembly at all, but rather another as yet unknown motor-neuron specific function of SMN. This hypothesis also invoked the existence of another presumably axonal-specific complex with SMN.

Stable cell lines were also generated to further analyze the SMN complex in motor neurons. The mutations that retained properties necessary for snRNP assembly, but lacked the ability to rescue axonal defects in zebrafish could provide clues to the snRNP-independent life of SMN. However, this complex, if present, would most likely be
low affinity and in lower abundance in motor neurons. Use of moderate expressing lines would help to decrease non-specific interactions. In addition, low salt conditions to recover low affinity complexes would also be beneficial. Also, use of RNAi to knockdown endogenous mouse Smn in the MN1 cell lines or the use of SMA stem cells would help to eliminate normal complexes formed with homomeric wildtype Smn and increase the heteromeric complexes of transfected mutant SMN. Although the recovery of SMN complexes is possible, identification of an snRNP-independent complex remains elusive.

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Table 2.2 Summary of properties of mutant SMN species.
* snRNP assembly results as reported in an in vitro assembly assay (Shpargel et al. 2005); ^ unpublished results, Christine Beattie, personal communication.
2.4 Materials and Methods

2.4.1 Site-Directed Mutagenesis

Site directed mutagenesis was done essentially as described (Le et al. 2000). SMN (Exon 1-8) cDNA in the pcDNA3 vector was used as a template. To create the mutations, oligos containing the mutant codon were designed. For the E134K mutation the codon GAG was replaced with AAG. For the I116F mutation the codon ATT was replaced with TTT and for Q136E the codon CAA was changed to GAA. The template was amplified with these oligos using PfuTurbo polymerase. The PCR conditions were 95 °C for 30 seconds, 55 °C for 1 minute and 68 °C for 10 minutes for extension. This was repeated for 15 cycles. The product was digested with DpnI to remove the methylated parental template. The remaining mutagenized plasmid was transformed into competent DH5α cells according to the manufacturer's specifications (Invitrogen). The resultant colonies were grown in liquid culture, plasmid isolated by QIAGEN mini-prep and screened by restriction digest. All clones were confirmed by direct sequencing.

2.4.2 GST-Fusion Protein Expression and Purification

The expression and purification was done essentially as described (Lorson et al. 1998). 100 ml of BL21 cells containing each cDNA in the pGEX-3X vector was cultured. The cells were induced with 1 mM IPTG for 3 hours at 30°C. The cells were pelleted and resuspended in 10 ml of NETN buffer. The cells were chilled on ice and then sonicated 4 times with a pulse of 30 second duration. Cells were then centrifuged at 12,000 rpm for 20 minutes. Supernatant was removed and incubated with glutathione sepharose beads (Amersham) for 2 hours at 4°C. The beads were washed 4 times with
NETN and then resuspended in 500 µl NETN. Protein concentration was determined by BCA assay and then proteins visualized by 12% SDS-PAGE and staining with Coomassie Blue.

### 2.4.3 *In vitro* Transcription and Translation

The pcDNA3 vectors was used for *in vitro* transcription and translation of each insert to produce $^{35}$S-labelled protein. This was done as per Promega kit specifications. The plasmid DNA was prepared by QIAGEN mini-prep kit. A reaction containing rabbit reticulocyte lysate, buffer, T7 DNA polymerase, amino acid mix without methionine, radiolabeled methionine, RNase inhibitor, and DNA template was set up and incubated for 90 min at 30°C. Labeled protein was quantified on SDS-PAGE using the PhosphoImager. Luciferase protein was produced and labeled as a control.

### 2.4.4 GST-fusion Protein Binding Assays

The GST-tagged proteins (0.1, 0.5, 1, 2, 4, 6 µg) and $^{35}$S labeled proteins (1-3 µl) were co-incubated with glutathione beads for 1 hour at 4°C and then washed 5 times with buffer. The bound protein was eluted with 1X SDS loading buffer, boiled and then run on 12% SDS-PAGE gel. The gel was exposed to a phosphor screen overnight and then quantitated with the PhosphoImager.

### 2.4.5 Generation of Mutant SMN FLAG-tagged Stable MN1 Cell Lines

MN1 cells were transfected with each FLAG-tagged construct using the Invitrogen transient transfection protocol. 6 µL Invitrogen Lipofectamine was added to
244 µL of DMEM without serum and 1 µg DNA was added to 250 µL DMEM without serum. DNA and lipofectamine aliquots were mixed and incubated for 5 min at room temperature. The DNA aliquot was then added to the Lipofectamine aliquot, mixed and incubated at room temperature for a further 20 min. The mixture was added to one well of a 6-well plate containing MN1 cells. The plate was mixed by rocking and then 2 mL of media (10% FBS (Atlas Biologicals), 1X Penicillin/Streptomycin (Invitrogen), 2 mM Glutamine (Invitrogen) in DMEM (Invitrogen)) was added. Transfected cells were incubated for 48 hours at 37°C and 5% CO₂ with one media change at 24 hours. Cells were then split, allowed to adhere for 24 hours, and then selected with 400 µg/mL G418, which was added directly to each well. Media and G418 was renewed every 2 to 3 days for 2 weeks. After 2 weeks, surviving cells were split and selected by the limiting dilution method. Cells were trypsinized and counted using 0.4% Trypan blue and a hemocytometer. Cells were diluted accordingly down to 100 cells/mL and plated the final dilution 1 mL per well of a 96-well plate to get ~1 cell/well. Cells were then incubated for less than 24 hours and then single colonies were marked for expansion. Cells were allowed to expand up to 48-well, then 24-well, etc until enough quantities were obtained.

2.4.6 RT-PCR of Stable Cell Lines

Total RNA was isolated using a combined TRIZol (Invitrogen) and RNeasy Mini Kit protocol (QIAGEN). MN1 cells were scraped off plates into PBS. Cells were spun down and the PBS was aspirated. Them 1 mL of TRIZol reagent was added. The cells were homogenized and incubated at room temperature for 5 minutes. 200 µL of chloroform was added, shaken vigorously and incubated for 5 minutes at room
temperature. The samples were then centrifuged for 15 minutes at 4 °C at 12,000 X g. The top layer was removed to a new tube and then 1 volume 70% ethanol was added. The QIAGEN manufacturer’s protocol was then resumed at this step. Total RNA was treated for DNA contamination using the DNA-free Kit (Ambion) as per manufacturer’s instructions. First strand cDNA synthesis was carried out using 6 µg of total DNA-free RNA. This was added to 250 ng random hexamer in nuclease-free water. This mixture was heated at 70 °C for 10 minutes and then cooled on ice for 10 minutes to allow primers to anneal. A master mix was added to the tube, which contained 1 mM dNTPs, 4 units of RNase-OUT (Invitrogen), 1X Reverse Transcriptase enzyme buffer and 7 units of RT (Life Sciences, Inc.). The samples were then incubated at 42 °C for 1 hour. PCR was carried out on the cDNA using forward primer 4F gtgagaacctccagtctcttg and reverse primer BGH-R tagaagcagctcag. PCR conditions were: 95 °C for 3 min for denaturation, followed by 35 cycles of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min, then extension. Control primers used were cyclophilin forward agacgcgcttgctctttc and cyclophilin reverse primer ccacagtggagatggt.

2.4.7 FLAG-tag Pull-downs of Stable Cell Lines

Stable SMN mutant cells were harvested in 1X PBS. The cells were then spun down and resuspended in RSB-100 (0.1%) (10mM Tris HCl, pH 7.4; 2.5 mM MgCl₂; 100mM NaCl; 0.1% Nonidet P-40; Roche protease inhibitor). Cells were passed through a 27-gauge needle 5 times and sonicated briefly. The lysate was then centrifuged at 10,000 rpm for 15 minutes at 4 °C. Anti-flag beads (Sigma) were prepared by pre-washing in RSB-100 (0.1%). The lysate was passed through a 0.2 µm filter and then the anti-FLAG beads were added to the cleared lysate. The beads were
then incubated for 2 hours at 4 °C with rotation. The beads were pelleted by centrifugation. The supernatant was removed and the beads were washed 3 times in RSB-100 (0.02%) (10mM Tris HCl, pH 7.4; 2.5 mM MgCl$_2$; 100mM NaCl; 0.02% Nonidet P-40; Roche protease inhibitor). Beads were washed 3 times with 10 bed volumes of RSB-500 (0.02%) (10mM Tris HCl, pH 7.4; 2.5 mM MgCl$_2$; 500mM NaCl; 0.02% Nonidet P-40; Roche protease inhibitor). Finally, beads were washed 3 times with RSB-100 (0.02%). Complexes were eluted with 10 bed volumes of RSB-100 (0.02%) with 0.5 mg/mL FLAG peptide for 1 hour at 4 °C with rotation.

Western blot analysis was carried out by mixing eluted proteins with 6X SDS Loading Buffer (350 mM Tris, pH 6.8, 10.28% SDS, 36% glycerol, 600 mM DTT, 0.012% bromophenol blue). The eluant was boiled and then loaded onto a 12% SDS polyacrylamide gel. The gel was transferred onto Hybond P PVDF membrane (GE Healthcare). The membrane was blocked for 1 hour at room temperature in 5% milk, 1% BSA, 0.1% Tween-20 in 1X PBS. The blot was then probed with primary antibody 8F7 against SMN at 1: 1,000 or with 4G7 anti-Gemin5 at 1:50 for 1 hour. Secondary antibody conjugated to horseradish peroxidase (Rockland) was added at 1: 5,000 and incubated for one hour. The blot was then exposed to ECL substrate (GE Healthcare) and developed. The blot was stripped and re-probed with a loading control β-actin primary antibody (Sigma-Aldrich) at 1:10,000 for one hour followed by the same Rockland secondary.
3.1 Introduction and Objectives

SMN has a well-characterized function essential to all eukaryotic cells (snRNP assembly), yet SMA is a motor neuron-specific disorder. Why, then, does the disruption of a ubiquitous protein preferentially affect motor neurons? In SMA, there is reduction of SMN as opposed to complete loss of SMN, which has been shown to result in disruption of snRNP assembly in fibroblasts from severe, type 1, SMA patients (Wan et al. 2005). Analysis of snRNP assembly in tissue extracts shows that this activity is developmentally regulated in normal mice (Gabanella et al. 2005) and that the degree of its impairment in SMA mice correlates with phenotypic severity as it is strongly reduced in spinal cords of severe mice (Kolossova et al. 1997; Gabanella et al. 2007; Zhang et al. 2008). Importantly, SMN deficiency unevenly alters the snRNP profile of tissues and appears to preferentially decrease the levels of snRNP of the minor splicing pathway in severe SMA mice (Kolossova et al. 1997). Although array analysis has been performed and
various splicing changes identified, it is still not clear which changes are due to reduced SMN levels as opposed to stress or secondary changes (Zhang et al. 2008). Thus, no specific targets of SMN reduction have been identified to date.

SMN has also been found in axons and growth cones of neurons, but not associated with Sm proteins (Rossoll et al. 2002; Sharma et al. 2005; Zhang et al. 2006). Reduction of SMN in zebrafish results in motor axon defects (McWhorter et al. 2003) and motor neurons cultured from SMA mice have truncated axons and smaller growth cones (Rossoll et al. 2003). In addition, these growth cones have lower amounts of beta-actin mRNA, which results in an altered distribution of calcium channels (Jablonka et al. 2007). This has led to the suggestion that SMN functions in axonal transport of mRNA and it is this function that is disrupted in SMA (Rossoll et al. 2003). In Chapter 2, two SMN mutations, SMN(A111G) and SMN(VDQNQKE) were examined. The SMN(A111G) allele, which occurs in a type 1b/II SMA patient with 2 copies of SMN2 (Sun et al. 2005), has been shown in culture to perform snRNP assembly when SMN levels are knocked down by siRNA (Shpargel et al. 2005). SMN(VDQNQKE) is a truncated form of SMN (exons 1-6) with the added motif VDQNQKE (Zhang et al. 2007). SMN(VDQNQKE) does not efficiently associate with itself, full-length SMN or Sm proteins and thus, is predicted to be inefficient in snRNP assembly (Carrel et al. 2006). However, when assayed for its ability to correct axonal defects in zebrafish where endogenous SMN had been knocked down, SMN(VDQNQKE) rescued and SMN(A111G) did not. This indicated that snRNP assembly was not critical for correction of axonal defects in zebrafish. However, it has not been possible to measure snRNP assembly in zebrafish. In SMA mice, no defects of axonal growth or patterning have been detected (McGovern et al. 2008). To further understand these SMN alleles, they have been investigated in SMA mice.
In the current chapter, it is shown that, when expressed at high levels, the SMN(A111G) allele rescues mice that lack mouse *Smn* and contain one or two copies of *SMN2* (Monani *et al.* 2000), but does not rescue the embryonic lethality of *Smn*-/- mice (Schrank *et al.* 1997) that do not have *SMN2*. Thus, the SMN(A111G) allele is complemented by *SMN2* and performs snRNP assembly. In contrast, the SMN(VDQNQKE) transgene does not produce sufficient RNA or protein and thus, does not rescue either snRNP assembly or SMA mice. A reinvestigation of SMN(A111G) in zebrafish reveals it is capable of rescuing axonal defects when present at higher concentrations. Lastly, the SMN(A111G) allele and SMN(A2G) allele do not complement each other to rescue embryonic lethality of *Smn*-/- mice, indicating that these two mutations could affect the same functional domain of SMN. This data shows a direct correlation of the ability of a *SMN* allele to perform snRNP assembly and correct the SMA phenotype. Furthermore, the data suggests that the majority of milder SMA missense alleles work by interacting with small amounts of full-length SMN from the *SMN2* gene. Full-length SMN, in a hetero-oligomer with mutant SMN, compensates for the mutant to restore function; whereas, the homo-oligomer composed of just mutant SMN lacks function. Indeed, mild alleles of SMA are those that can complement full-length SMN produced by *SMN2*.

### 3.2 Results

#### 3.2.1 Generation of Transgenic Mouse Lines

Transgenes containing SMN(A111G) or SMN(VDQNQKE) under the control of a 4.1 kb SMN promoter were microinjected into FVB/N oocytes. Ten founder lines were
obtained for SMN(VDQNQKE) and 11 founder lines for SMN(A111G). The construct used for generation of the transgenic lines is diagramed in Figure 3.1. Of the 10 SMN(VDQNQKE) lines, 8 transmitted the transgene to offspring and 9 of 11 SMN(A111G) lines successfully transmitted the transgene. Three lines of SMN(VDQNQKE) were chosen by RT-PCR for further analysis as they had detectable SMN RNA transcripts; lines 1946, 1947 and 1951. Line 1951 was found by real-time PCR to have 14 copies of the transgene. Three lines of SMN(A111G) were also chosen by RT-PCR for further analysis; lines 588, 591, and 2117. Line 588 was found to have 4 copies of the transgene, line 591 had 5 copies and line 2117 had 17 copies. The mice were backcrossed onto the SMA mouse background (SMN2+/−; Smn+/−) until mice that were heterozygous for the transgene, homozygous for SMN2 and homozygous for the mouse Smn knockout allele (SMN(VDQNQKE)+/−; SMN2+/−; Smn−/−) or (SMN(A111G)+/−; SMN2+/−; Smn−/−) were obtained.

Figure 3.1  Diagram of transgene constructs.
SMNp stands for the SMN promoter 4.1 kb fragment. BGH pA is the poly-adenylation sequence of bovine growth hormone.
3.2.2 Expression of Transgenes

RT-PCR analysis revealed that the 3 SMN(VDQNQKE) lines had SMN RNA transcript expression in spinal cord, forebrain and liver (Figure 3.2). Line 1951 appeared to have the highest mRNA transcript levels (Figure 3.2). Western blot analysis of postnatal day 3 (PND03) SMN(VDQNQKE) SMA mice revealed low SMN levels in forebrain and spinal cord (Figure 3.3) of all lines. Levels of SMN were quantitated for line 1951 (Figure 3.3). This line had higher levels of expression than SMA mice, but the levels were not statistically different from SMA mice in forebrain or spinal cord. This indicates that this transgenic mouse line is not producing enough SMN for rescue.

Figure 3.2 RT-PCR analysis of SMN expression in forebrain and spinal cord of SMN(VDQNQKE) lines 1946, 1947 and 1951. Fbr, forebrain; sc, spinal cord.
Figure 3.3 Western blot analysis in spinal cord and forebrain of 3-day-old SMN(VDQNYKE) mice.
All mice are homozygous for SMN2 and lack mouse Smn unless otherwise noted. +/- refers to the presence of 1 copy of mouse Smn and -/- being absence of mouse Smn.

Three lines of SMN(A111G) were found by RT-PCR to express SMN mRNA.
Line 588 had high RNA expression in spinal cord, forebrain and liver (Figure 3.4). Line 591 had high levels of transcript in spinal cord, but low in forebrain and liver (not shown). Line 2117 had medium levels of transcript in spinal cord, high levels in the forebrain, but no detectable transcript in the liver (not shown). Western blot analysis of PND03 tissue from SMA mice with the transgene showed that SMN(A111G) line 588 and 591 had SMN expression lower than a carrier mouse (\(\text{SMN}^{2/+}; \text{Smn}^{+/+}\)) and considerably higher (6-fold) than severe SMA mice (\(\text{SMN}^{2/-}; \text{Smn}^{-/-}\)) (Figure 3.5A and B). As the two lines behaved in a similar manner for both expression and survival analysis most subsequent analysis is done with SMN(A111G) line 588. The SMN(A111G) line 2117 expressed
considerably less SMN protein (approximately one-fifth level) than either 588 or 591 in all tissues analyzed (Figure 2C and 2D). It does, however, express slightly more SMN than a typical SMA mouse (SMN2+/+; Smn−/−).

Figure 3.4. RT-PCR analysis of SMN expression in forebrain, spinal cord and liver of SMN(A111G) line 588.
Fbr, forebrain; sc, spinal cord; Li, liver.
Figure 3.5 Western blot analysis of SMN expression in spinal cord, forebrain and liver of SMN(A111G) mice.

(A) 3-day-old tissue from lines 588 and 2117 and (B) adult tissue from lines 588 and 591. All mice are homozygous for SMN2 and lack mouse Smn unless otherwise noted. +/- refers to the presence of 1 copy of mouse Smn and -/- being absence of mouse Smn.
3.2.3 Survival of SMN2; Smn/- Mice with SMN(A111G) or SMN(VDQNGKE)

To determine the effect of SMN(A111G) and SMN(VDQNGKE) on SMA mice, we bred the mice to obtain those that lacked mouse Smn, had one or two copies of SMN2 and contained the SMN(A111G) or SMN(VDQNGKE) transgene. It has been previously shown that mice lacking mouse Smn and containing two copies of SMN2 die at approximately 5 days (Monani et al. 2000), whereas mice with 1 copy of SMN2 show embryonic lethality at or before embryonic day 10.5 (McGovern et al. 2008). Severe SMA mice (SMN2+/−; Smn−/−) have a mean life span of 4.4 ± 0.3 days (n = 90), in these experiments (Figure 3.5), consistent with previous reports (Monani et al. 2000). The presence of SMN(VDQNGKE) transgene had a minimal effect on the survival of SMA animals with line 1946, having a mean life span of 2.4 ± 0.5 days (n = 26), line 1947 having a mean life span of 4.7 ± 0.8 days (n=15); and the highest expressing line, 1951, had a mean life span of 6.5 ± 0.8 days (n=18) (p=0.03) (Figure 3.6). Making the SMN(VDQNGKE) homozygous did not result in significant enhancement of survival of SMA animals. SMA pups that contained SMN(VDQNGKE) showed a typical SMA phenotype (Monani et al. 2000) with no noticeable difference to SMA pups lacking the transgene. Thus, SMN(VDQNGKE) has a minor impact on survival, most likely due to the low expression of this transgene.
Figure 3.6 Survival analysis of SMN(VDQNQKE) transgenic mice.
Kaplan-Meier survival curve of SMN(VDQNQKE) SMA mice showing minimal or no correction of lifespan. Blue line: SMN2+/−; Smn−/− control SMA mice have a mean survival of 4.4 ± 0.3 days (n= 90). Green line: Line 1946 SMN(VDQNQKE)+/− or SMN(VDQNQKE)+/−; SMN2+/−; Smn−/− mice have a mean lifespan of 2.4 ± 0.5 days (n= 26). This is significantly fewer days (p<0.001) than the control SMA mice. Red line: Line 1947 SMN(VDQNQKE)+/− or SMN(VDQNQKE)+/−; SMN2+/−; Smn−/− mice survive for 4.7 ± 0.8 days (n= 15). Purple line: Line 1951 SMN(VDQNQKE)+/− or SMN(VDQNQKE)+/−; SMN2+/−; Smn−/− mice survive for 6.5 ± 0.8 days (n= 18), which is a minimal improvement over control (p= 0.03).

In contrast, the SMN(A111G) transgene had a major impact on survival of SMA mice with high expressing animals (lines 588 and 591) surviving for more than a year. SMN(A111G) line 2117, with low expression of SMN, when present in SMA animals, resulted in a mean survival of 8.2 ± 1.1 days (n=24) (p<0.001) (Figure 3.7A). In two cases in line 2117, mice have lived considerably longer than 8 days (11 months and 1 year), however, in general, this is not the case and we consider these mice to be escapers. All mice analyzed had two copies of SMN2. The presence of SMN(A111G)
from lines 588 or 591 with high expression of SMN caused a marked impact on survival with the mean censored survival times being over a year and animals living for at least 1.5 years before being removed from the study (Figure 3.7A). This was the same as control animals that had mouse Smn. In addition, there was no obvious phenotype in these animals and they were comparable to animals that possessed mouse Smn (Figure 3.7B).
Figure 3.7 Survival and phenotype analysis of SMN(A111G) transgenic mice.
Kaplan-Meier survival curves of SMN(A111G) SMA mice showing complete correction of lifespan for lines 588 and 591 and line 2117 having a minimal, but significant increase in lifespan (A). Blue line: SMN2+/+; Smn−/− control SMA mice have a mean survival of 4.4 ± 0.3 days (n= 90). Purple line: Line 2117 SMN(A111G)+/−; SMN2+/− or SMN2+/+; Smn−/− mice live for 8.2 ± 1.1 days (n=24). This is a significant (p<0.001) increase in lifespan over the control SMA mice. Red line: Line 591 SMN(A111G)+/−; SMN2+/− or SMN2+/+; Smn−/− mice survive a normal lifespan (p<0.001). The mice can live for up to 1.5 years at which time they were removed from the study. Green line: Line 588 SMN(A111G)+/−; SMN2+/− or SMN2+/+; Smn−/− mice also survive for up to 1.5 years at which time they were removed from the study. One mouse died at 10 months of age from this cohort from an unknown cause. (B) Weight curves for SMN(A111G) line 588 mice. All mice are of the genotype SMN(A111G)+/−; SMN2+/−; Smn−/− or control SMN2+/+; Smn−/−. The SMN(A111G) mice have an identical weight to control animals. Weight data is represented in mean weight in grams each day with standard deviation.
Due to the nature of the crosses used to obtain these animals, the mice could be either homozygous for *SMN2* (2 copies of *SMN2*) or heterozygous for *SMN2* (1 copy of *SMN2*). Again, when mice have one copy of *SMN2*, it is embryonic lethal on or before embryonic day 10.5 (McGovern *et al.* 2008). However, for both SMN(A111G) lines 588 and 591 animals of the genotype SMN(A111G); *SMN2*+/−; *Smn*−/− are frequently obtained and survive for over a year with no noticeable difference to mice that have 2 copies of *SMN2*. Thus, the SMN(A111G) allele complements *SMN2* to give a SMN complex that functions to rescue embryonic lethality of one copy-*SMN2* (*SMN2*+/−; *Smn*−/−) mice, as well as SMA in two-copy *SMN2* mice. In addition, this indicates that the SMN(A111G) transgene is expressed in all cell types in sufficient amounts to perform SMN’s essential function. This led to the question of whether SMN(A111G) by itself could rescue *Smn*−/− mice. Therefore, *SMN2* was removed and crosses were set up to address whether SMN(A111G) could rescue *Smn*−/− mice in the absence of *SMN2*. Analysis of these crosses (Table 3.1) revealed that SMN(A111G) never rescued embryonic lethality. Of 55 progeny obtained, approximately 7 animals of this genotype were expected, but this never occurred. This indicates that SMN(A111G) has an absolute requirement for full-length SMN in order to form functional heteromeric complexes.

Intragenic complementation occurs when a multimeric protein is formed from subunits produced by different mutant alleles of a gene. Thus, SMN produced by *SMN2* and SMN(A111G) undergo intragenic complementation. SMN(A111G) and SMN(A2G) were also tested for their ability to perform this type of complementation, as both are functional in the presence of *SMN2*. SMN(A2G)*+/−; *Smn*+/− mice were crossed with SMN(A111G); *Smn*+/− mice so as to obtain SMN(A111G)+/−; SMN(A2G)+/−; *Smn*−/− mice.
However, no animals of this genotype were observed out of 199 progeny obtained (Table 3.1). This indicates that the SMN(A2G) and SMN(A111G) alleles do not complement each other to form a functional SMN complex.
Cross type: SMN(A111G)\(^{+/-}\); Smn\(^{-/-}\) X Smn\(^{-/-}\)

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Cross type: SMN(A111G)\(^{+/-}\); Smn\(^{-/-}\) X SMN(A2G)\(^{+/-}\); Smn\(^{-/-}\)

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**Table 3.1. Survival of SMN(A111G) animals without SMN2.**

(Top) Survival of SMN(A111G) animals in crosses in the absence of the SMN2 transgene. All possible genotypes of offspring are noted. Mice of the genotype Smn\(^{-/-}\) have been previously reported to be embryonic lethal (Schrank et al. 1997). No mice of the genotype SMN(A111G)\(^{+/-}\); Smn\(^{-/-}\) were obtained (\(X^2=6.9, p<0.01\)) indicating that this transgene cannot function without SMN2. (Bottom) Survival of SMN(A111G) and SMN(A2G) animals when crossed together. Three genotypes Smn\(^{-/-}\), SMN(A111G)\(^{+/-}\); Smn\(^{-/-}\) and SMN(A2G)\(^{+/-}\); Smn\(^{-/-}\) are omitted as they have been previously determined to be embryonic lethal and skew the Chi-square results. No mice of the genotype SMN(A111G)\(^{+/-}\); SMN(A2G)\(^{+/-}\); Smn\(^{-/-}\) were obtained (\(X^2=15.3, p<0.001\)) indicating that these two transgenes do not complement each other. Fewer wildtype (Smn\(^{+/+}\)) mice were observed than expected, but this is just at the level of significance (\(X^2=4.5, p<0.05\)). All expected values are rounded to the nearest whole mouse.

* statistically significant (\(p<0.05\))
3.2.4 snRNP Assembly Correlates with SMA Phenotype and Survival*  

Previously, in Chapter 2, it was shown that SMN(VDQNQKE) performs poorly in association assays with either full-length SMN or itself and does not bind Sm proteins efficiently in vitro (Carrel et al. 2006). In contrast, SMN(A111G) does self-associate efficiently and does bind Sm proteins. In addition, assays in transiently transfected cells showed that SMN(A111G) expression is capable of increasing in vitro snRNP assembly activity of cells with reduced levels of SMN (Sun et al. 2005). Therefore, snRNP assembly activity was assayed in the spinal cord extracts of SMN(A111G) and SMN(VDQNQKE) SMA mice using in vitro transcribed radioactive U1 snRNA and immunoprecipitation with anti-Sm antibodies (Figure 3.8). Normal control animals (SMN2+/+; Smn+/+) were used to set the 100% normal snRNP assembly level in spinal cord. Animals of the carrier genotype (SMN2+/+; Smn+/−) had 65.95 ± 16.4% snRNP assembly in spinal cord and severe mice (SMN2+/+; Smn−/−) had 3.06 ± 1.49% assembly. SMN(VDQNQKE) line 1951 showed very low snRNP assembly activity at 9.40 ± 0.89%; whereas SMN(A111G) line 2117 (low expressing) had 9.57 ± 2.85% assembly and line 588 (high expressing) had 43.37 ± 5.31% assembly. The level of snRNP assembly correlated well with the survival and phenotype of each genotype. Both normal (SMN2+/+; Smn+/+) and carrier (SMN2+/+; Smn+/−) lived a normal lifespan with as low as 66% assembly, as does the high expressing SMN(A111G) line 588 which lives normally with 43% the normal snRNP assembly. Both the SMN(VDQNQKE) line 1951 and the low SMN(A111G) line 2117 had about 10% snRNP capacity and lifespans of 6.2 days.

* snRNP assembly assays were performed by L. Saieva. Tissue was provided by E. Workman.
and 8.0 days respectively. $SMN2^{+/+}$; $Smn^{-/-}$ mice lived 4.3 days and had very low
snRNP capacity at about 3%. Thus, mouse survival showed marked correlation to the
snRNP activity present in spinal cord.
Figure 3.8 Analysis of snRNP assembly activity and snRNA levels in the spinal cord of SMN(A111G) and SMN(VDQNQKE) mice.

(A) Representative snRNP assembly reactions were carried out using in vitro transcribed radioactive U1 snRNA and 25 mg of whole spinal cord extracts from the indicated mice at postnatal day 3, followed by immunoprecipitation with anti-Sm antibodies. Immunoprecipitated U1 snRNAs were analyzed by electrophoresis on denaturing polyacrylamide gels and autoradiography. Western blot analysis of the spinal cord extracts that were probed with antibodies against SMN and tubulin is shown. Genotypes represented are: normal (SMN2+/+; Smn+/+), carrier (SMN2+/+; Smn−/+), SMA (SMN2−/−; Smn−/−), line 588 SMN(A111G)+/-; SMN2+/+; Smn−/−, line 2117 (low) SMN(A111G)+/-; SMN2+/+; Smn−/− and line 1951 SMN(VDQNQKE)+/-; SMN2+/+; Smn−/−. (B) Quantitation of snRNP assembly activity. The amount of immunoprecipitated U1 snRNA from in vitro snRNP assembly experiments as in (A) was quantified using a STORM 860 Phosphorimager (Molecular Dynamics). The normal control animals (SMN2+/+; Smn+/+) were set as the 100% normal snRNP assembly level and values are presented as mean ±SEM. Animals of the carrier genotype (SMN2+/+; Smn−/+) had 65.95 ± 16.4% snRNP assembly and severe mice (SMN2−/−; Smn−/−) had 3.06 ± 1.49% assembly. SMN(VDQNQKE) line 1951 showed very low snRNP assembly activity at 9.40 ± 0.89%; whereas SMN(A111G) line 2117 (low expressing) had 9.57 ± 2.85% assembly and line 588 (high expressing) had 43.37 ± 5.31% assembly.
We then measured the levels of major and minor snRNAs in the spinal cord of normal and SMA animals to determine which snRNAs were reduced (Figure 3.9). The levels of U4, U11, U12 and U4atac were significantly reduced in SMA mice. The levels of U1 and U2 major snRNAs were not affected in SMA animals and this is consistent with previously reported real-time PCR results (Zhang et al. 2008). The 4 reduced snRNAs were then assayed in SMN(VDQNQKE), SMN(A111G) line 2117 and SMN(A111G) line 588 mice and compared to normal, carrier and SMA mice (Figure 3.9). The levels of these snRNAs are restored in the high expressing SMN(A111G) line 588, but not in SMN(VDQNQKE) line 1951 and SMN(A111G) line 2117 mice. Thus, restoration of a normal snRNA profile in the spinal cord correlates with correction of the SMA phenotype in mice.
Figure 3.9 Analysis of snRNA levels of SMN(A111G) and SMN(VDQNQKE) mice. (Top) Quantitation of major and minor snRNAs in normal (SMN2+/+; Smn+/+) and severe SMA (SMN2−/−; Smn−/−) mice. Total RNA from the spinal cord of postnatal day 3 normal and SMA mice were analyzed by real-time RT-PCR for the levels of specific snRNAs. Data are from three independent biological replicates. The levels of U4, U11, U12 and U4atac were significantly reduced in SMA mice (p<0.05). (Bottom) Analysis of snRNA levels in transgenic mice. Total RNA from the same spinal cord extracts as in (top) were used for real-time RT-PCR quantitation of snRNA levels. Compared to normal mice, the levels of U11, U12, U4atac and U4 were significantly reduced in SMN(VDQNQKE) and SMN(A111G) low expressing lines, but were restored in the SMN(A111G) high expressing line (p<0.05).
3.2.5 Rescue of Axonal Defects in Zebrafish by SMN(A111G)

In the previous chapter, it was reported that in transient assays, SMN(A111G) did not rescue motor neuron axonal defects caused by low Smn in zebrafish (Carrel et al. 2006). In contrast, SMN(VDQNQKE) rescued these axonal defects (Carrel et al. 2006). The rescue of axonal defects by SMN(A111G) and SMN(VDQNQKE) was re-examined in zebrafish using varied concentrations of RNA for rescue because SMN(A111G) expression (at high levels) rescues transgenic SMA mice and SMN(VDQNQKE) fails to do so at low levels. As shown in Figure 3.10, it was found that at high concentration the SMN(A111G) construct was capable of rescue. In the case of SMN(VDQNQKE), rescue did still occur, but at reduced concentration of RNA this form of SMN was less effective at rescue than full-length SMN.

* Zebrafish assays were performed by T. Carrel.
Figure 3.10 Analysis of SMN mutations in zebrafish.

(A) Increasing the dose of RNA containing the SMA allele SMN(A111G) allows for rescue of motor axon defects caused by the Smn MO. Zebrafish embryos injected with 120 pg SMN(A111G) mRNA together with 9 ng of smn MO (n=92 embryos) did not exhibit a rescue of motoneuron defects in comparison to smn MO only injections (p=0.117). At this dose, wildtype SMN mRNA demonstrates the ability to rescue (n=152; p<0.001 versus smn MO). Co-injection of smn MO with 240 pg SMN(A111G) mRNA (n=96) however leads to a significant rescue of these motoneuron defects (p<0.001). Control MO (n=86) and smn MO (n=478) are shown as controls. Data were obtained from at least 3 independent experiments for each condition.

(B) SMN(VDQNQKE) mRNA rescues motor axon defects caused by the smn MO in a dose dependent manner. Zebrafish embryos co-injected with smn MO and 120 pg SMN(VDQNQKE) mRNA (n=117 embryos) displayed a significant rescue in the motoneuron defects in comparison to smn MO alone (p<0.001). This rescue is similar to wild type SMN mRNA (p< 0.001 smn MO vs. Smn MO + SMN mRNA; p=0.539 Smn MO + SMN mRNA vs. 120 pg Smn MO + SMN(VDQNQKE)). Injecting 60 pg of SMN(VDQNQKE) mRNA still exhibits the ability to rescue the MO effects (p=0.003), but does not do so in a manner comparable to SMN mRNA (p=0.012). Control MO (n=86), smn MO (n=478) and smn MO + SMN mRNA (n=152) are shown as controls. Data were obtained from at least 3 independent experiments for each condition.
3.2.6 Muscle Analysis of SMN(A111G) SMA Animals

Gastrocnemius muscle was examined from 10-month-old adult SMN(A111G) transgenic mice (line 588) (Figure 3.11). The SMN(A111G) mice show hypertrophy of gastrocnemius fibers. They also have patches of smaller atrophic fibers, but the majority of fibers at this stage are larger with an average size of 2870.2±28.5 µm² versus the control with a mean size of 2456.1±28.8 µm². The distribution of fiber sizes was shifted in the SMN(A111G) mice to a larger range of 2800-3900 µm² (Figure 3.11C and D). While the survival and phenotype of SMN(A111G) mice is rescued, there is still a change in muscle morphology suggesting that the muscle has experienced denervation followed by re-innervation. This could be due to the fact that these mice express less SMN than normal and carrier mice and have an overall reduction in snRNP assembly, which could contribute to this change. Furthermore, calf hypertrophy is occasionally seen in mild SMA patients (Pearn et al. 1978; Bouwsma et al. 1980).
Figure 3.11 Muscle morphology of SMN of SMN(A111G) line 588.

(A and B) Hematoxylin and eosin staining of gastrocnemius muscle from 10-month-old adult mice reveal larger muscle fibers in SMN(A111G) animals. (A) Control is of the genotype SMN2+/−; Smn+/- and (B) is of the genotype SMN(A111G)+/-; SMN2+/−; Smn−/−. (C and D) represent the distribution of fiber types in control and SMN(A111G) animals. The mean fiber size for the control was 2456.1±28.8 µm² (SE) and the mean fiber size for SMN(A111G) was 2870.2±28.5 µm². This is a mean difference of 414.0 µm². The median fiber size for the control was 2480.5 µm² and 2962.0 µm² for SMN(A111G). The median fiber sizes are statistically different by Mann-Whitney and two-sample Kolmogorov-Smirnov tests (p< 0.001).
3.2.7 SMN Expression and Rescue in Motor Neurons of SMN(A111G) Animals

The expression of SMN in the spinal cords of SMN(A111G) line 588 mice was examined (Figure 3.12). Cross-sections of adult spinal cord were immunostained for SMN. Both nuclear (Gems) and cytoplasmic staining of SMN was visible in spinal cord motor neurons. Further, axons of the L4 ventral root of 6-month-old SMN(A111G)$^{+/−}$; $SMN2^{+/−}$; $Smn^{+/−}$ (line 588) spinal cords were counted (Figure 3.13). The SMN(A111G) spinal cords had normal root counts as compared to age-matched carrier controls ($SMN2^{+/+}$; $Smn^{+/+}$). Normal root counts are consistent with a model of denervation, followed by re-innervation of muscle resulting in hypertrophy.

![Image](image1.png)

**Figure 3.12 Immunostaining of SMN(A111G) spinal cord.**
8 $\mu$m sections of spinal cord from 10-month-old control (A) and SMN(A111G) (B) mice. Nuclear and cytoplasmic staining of SMN (red) is clearly present in SMN(A111G) animals. DAPI (blue) was used as a nuclear stain. Scalebar is 20 $\mu$m.
Figure 3.13  L4 ventral root count in 6-month-old SMN(A111G) mice. The SMN(A111G) mice do not differ from carrier mice. Group means are represented with standard errors.

3.3 Discussion

3.3.1 Mutations of SMN

A number of studies have examined in vitro how missense mutations found in SMA patients affect SMN’s ability to bind either to itself or members of the SMN complex (Lorson et al. 1998; Buhler et al. 1999; Pellizzoni et al. 1999; Young et al. 2000; Monani et al. 2003; Carrel et al. 2006; Otter et al. 2007). A large number of mutants have been described in exon 6 (Hahnen et al. 1997; Alias et al. 2008) that disrupt the ability of SMN to oligomerize and this reduces the capacity of the SMN complex to bind Sm proteins (Lorson et al. 1998; Pellizzoni et al. 1999). The loss of SMN exon 7 (SMN(D7)) has a
similar effect on these SMN interactions (Lorson et al. 1998; Pellizzoni et al. 1999). SMN protein levels have been examined in lymphoblasts from a type 1 patient with a missense mutation that disrupts oligomerization (Lefebvre et al. 1997). In this case, it was found that SMN protein levels were reduced to similar levels that occur in classic type 1 SMA patients with loss of SMN1. Furthermore, it is known that loss of SMN exon 7 impairs its ability to associate with itself (Lorson et al. 1998) or full-length SMN (Carrel et al. 2006) and results in rapid degradation of SMN (Burnett et al. 2009).

Furthermore, it was shown that severe SMA missense mutations that disrupt SMN oligomerization such as SMN(Y272C) and SMN(G279V) undergo rapid degradation in a similar manner to SMN(D7) (Burnett et al. 2009). In contrast to SMN(D7) and SMN(G279V), the addition of amino acids to the C-terminus of SMN(exons 1-6) stabilizes SMN and gives some functionality at least in transient assays in cultured cells (Mattis et al. 2008; Heier et al. 2009). One suggested strategy for therapeutics in SMA is to induce read-through of the translational stop codon in exon 8 of SMN transcripts lacking exon 7 (Mattis et al. 2008). It will be important to determine whether transgenes with these read-through constructs can correct SMA. Certainly one type of severe SMA allele, as indicated in Figure 3.14, are those that disrupt SMN’s ability to oligomerize and result in rapid degradation. We have previously shown that SMN(VDQNQKE) could rescue axonal defects in zebrafish caused by knockdown of endogenous Smn (Carrel et al. 2006). In this case, SMN protein was produced at sufficient levels to mediate this correction and was comparable to full-length SMN and SMN(A111G). In the current paper, SMN(VDQNQKE) did not rescue SMA mice. However, the SMN levels obtained from this transgene were low. The difference in protein levels accounts for the different results between mice and zebrafish. Further studies with stronger expression will be required to resolve this issue.
The missense mutation, SMN(A111G), can associate with full-length SMN to form an oligomer \textit{in vitro} (Sun \textit{et al.} 2005; Carrel \textit{et al.} 2006). In addition, \textit{in vitro} binding studies demonstrate that SMN(A111G) does bind Sm proteins, but with reduced affinity (Carrel \textit{et al.} 2006). Furthermore, when endogenous SMN is knocked down by siRNA and SMN(A111G) introduced, this construct does produce SMN that can perform assembly of Sm proteins onto snRNA much like other milder SMA missense mutations (Shpargel \textit{et al.} 2005). This contrasts with other Tudor domain mutations, which severely disrupt the binding of Sm proteins and do not perform snRNP assembly (Shpargel \textit{et al.} 2005). In this study, it is shown that SMN(A111G) can rescue SMA when expressed at high levels in the presence of 1 copy of SMN2. This results in mice that have no obvious phenotype and live for one year or more. Furthermore, SMN(A111G) SMA mice with 2 copies of SMN2 have substantial snRNP assembly and the levels of snRNAs are restored to normal as compared to SMA mice.

3.3.2 The Heteromer Theory of Mild SMA Mutations

In the current study, it is shown that SMN(A111G) does not rescue the SMN knockout allele on its own and we suggest that on its own, this allele has no activity in snRNP assembly. This is similar to the SMN(A2G) allele that does not correct lethality in the absence of Smn (Monani \textit{et al.} 2003). One copy of SMN2 in mice lacking mouse Smn results in an embryonic lethal phenotype, presumably due to insufficient SMN. However, when 1 or 2 copies of SMN2 and SMN(A111G) are on a Smn knockout background, then there is rescue of lethality with animals living for more than a year with no signs of SMA. Thus, SMN2 and SMN(A111G) transgenes undergo allelic complementation so as to give substantially greater function than either allele on its own.
Intragenic or allelic complementation occurs when proteins oligomerize to form a functional unit from two different alleles. The resulting heteromer has greater activity than either homomeric allele (Howell *et al.* 1998; Yu *et al.* 2000). Allelic complementation has been reported in a number of recessive conditions, particularly certain enzyme deficiencies such as argininosuccinate lyase (Yu *et al.* 2000) and propionyl-CoA carboxylase (Rodriguez-Pombo *et al.* 2005). In these cases, the interaction of two mutant proteins creates an active site in the heteromer, which does not exist in either of the homomeric mutants. The SMN complex can be viewed as an enzyme that acts to place Sm proteins onto snRNA (Fischer *et al.* 1997). The demonstration that the SMN(A111G) allele can undergo allelic complementation with limited SMN from a single copy of *SMN2* indicates that the critical functional unit of SMN in SMA and snRNP assembly is a heteromer between full-length SMN from *SMN2* and mutant SMN (Figure 3.14). While a SMN complex comprised solely of SMN(A111G) must lack function, the addition of at least a single full-length SMN to SMN(A111G) yields a heteromeric SMN complex, where activity is created. Moreover, that high levels of SMN(A111G) by themselves have no effect and that high levels of SMN(A111G) rescue phenotype more than does additional SMN from a second copy of *SMN2* suggests that the SMN complex is a heteromer of multiple subunits. High levels of SMN(A111G) in the presence of low levels of full-length SMN should result in complexes that contain, on average, one molecule of full-length SMN. As full-length SMN from low copy numbers of *SMN2* is limiting, this would maximize the number of SMN complexes capable of functioning. Low levels of SMN(A111G) in the presence of low levels of full-length SMN results in complexes that, on average, contain more than one molecule of full-length SMN and thus, there are fewer competent complexes in total. This leads to low function and limited rescue with a slight extension of lifespan (as in line 2117).
Further, it can be hypothesized that in the heteromer the mutant SMN(A111G) functions
to bind the Sm heptamer, while the full-length SMN functions to allow a single snRNA
per complex to be loaded with Sm proteins.

Severe missense mutations in SMA are those that disrupt a critical function and
cannot complement with full-length SMN produced by SMN2, whereas mild mutations
are those that can complement with SMN produced by SMN2. Alleles that cannot
interact efficiently with full-length SMN are severe, as are those that have severe
disruption of Sm binding (i.e. E134K). In the case of mild alleles, it was found that
SMN(A2G) and SMN(A111G) together cannot rescue the loss of Smn and thus, these
alleles do not complement each other. One interpretation of this result is that the
SMN(A2G) and SMN(A111G) mutations affect the same function. It would be interesting
to determine whether any point mutations, such as SMN(A2G) or SMN(A111G), can
complement mild C-terminal mutations. C-terminal mutations have been shown to
reduce, but not to eliminate the ability to bind SMN. If self-association is the only
function affected by C-terminal mutations, then at high expression levels, these alleles
are predicted to still retain function in the absence of full-length SMN.
Figure 3.14 The heteromer theory of SMA to explain why retention of SMN2 is critical for phenotypic rescue.

Expression of mutant SMN transgenes in the absence of full-length (wildtype) SMN results in lethality due to the inability of the mutant to function on its own (top). Addition of SMN2 results in functional SMN complexes, wherein full-length SMN provides a missing property necessary for the activity of the complex. Thus, any SMN complex that contains a molecule of full-length SMN will be functional and any complex that does not contain full-length SMN will not be functional. In other words, there is no dominant negative effect of SMN(A111G) and the SMN from SMN2 is limiting. The maximum number of functional SMN complexes will be obtained when SMN(A111G) levels are high and each complex contains one full-length SMN (middle), while lower levels of SMN(A111G) will result in complexes containing more than one full-length SMN, thus limiting the number of functional complexes (bottom). The SMN ring is shown as an octamer based on (Man et al. 2008) and our data suggest that the complex is more than a dimer because much greater amounts of SMN(A111G) than SMN from SMN2 are necessary to obtain full rescue (in other words the ratio is not 1:1).
3.3.3 The Connection of SMA to snRNP Assembly

Even though SMN is a ubiquitous protein, reduction of SMN primarily affects motor neurons. Two theories exist to explain why this specificity occurs: the axonal theory and the snRNP theory. Evidence for a role of SMN in axons has come from studies in zebrafish (McWhorter et al. 2003) and cultured motor neurons (Rossoll et al. 2003) where SMA motor axons are truncated in development. Also, reduced amounts of β-actin mRNA in the growth cones of motor neurons (Rossoll et al. 2003) leads to altered distribution of Ca^{2+} channels (Jablonka et al. 2007). However, no alteration of axon growth is detected in vivo in SMA mice (McGovern et al. 2008). Winkler et al., 2005 reported that knockdown of SMN or Gemin 2 resulted in motor axon defects in zebrafish (Winkler et al. 2005). In contrast, we have reported that knockdown of SMN, but not Gemin 2 results in axon defects (McWhorter et al. 2008). One notable difference between these studies is whether or not the fish scored had altered morphology. In the case of Winkler et al, 2005, as indicated in supplemental tables, over 50% of the fish scored were morphologically altered; whereas, in the study by McWhorter et al., 2003, no morphologically abnormal fish were scored (McWhorter et al. 2003; Winkler et al. 2005). In addition, Plastin 3 has been identified as a potential modifier of SMA (Oprea et al. 2008); however, whether overexpression of Plastin 3 acts to alter splicing or simply to encourage axon growth in general is currently unclear.

To further investigate SMN’s function and the correction of axon defects in zebrafish, various SMN mutants were examined for their ability to correct (Carrel et al.). It was found that co-injection of morpholino with SMN(A111G) or the synthetic mutant, SMN(Q282A), did not result in rescue even though the mutant SMN (A111G) is capable of snRNP assembly in the presence of low amounts of full-length SMN (Shpargel et al. 2008).
The mutant SMN(Q282A) has not been assayed for its ability to perform snRNP assembly, although it does retain its ability to self-associate and bind Sm proteins in vitro (Carrel et al. 2006). In contrast, SMN(VDQNQKE) did rescue in this assay, even though it is predicted not to be capable of snRNP assembly. These experiments lent support to an axonal function of SMN separate from that of snRNP assembly; however, it has proved difficult to measure snRNP assembly in zebrafish. Given the finding that SMN(A111G) rescues SMA mice, the ability of this allele to correct axonal defects in zebrafish was reinvestigated. It was found that at higher concentrations SMN(A111G) did indeed correct the axonal defects indicating that this mutant cannot be used to distinguish an axonal function from a snRNP function.

A disruption of snRNP function, however, could be the likely cause of SMA as it could also produce an axonal phenotype if abnormal splicing of axonal-specific transcripts occurs. Previously, it has been shown that reduction in snRNP assembly results in altered levels of snRNPs. In particular, in spinal cord, there is a reduction in the levels of assembled U11 snRNP (Gabanella et al. 2007). This leads to the prediction that the minor splicing pathway could be specifically affected in SMA, perhaps affecting a gene(s) of importance for motor neurons. Microarray studies revealed a large number of expression and splicing changes in SMA animals (Zhang et al. 2008). However, the splicing changes cannot be explained by the observation that only snRNAs of the minor splicing pathway were decreased. Possible secondary alterations due to the state of the animals could occur and a series of changes that are detected, such as the increase in cytochrome P450 (Zhang et al. 2008), suggest non-specific stress responses. It is currently not clear what constitutes the primary targets and secondary effects of reduced snRNPs. The availability of mice with various levels of snRNP assembly activity, as well
as different levels of snRNPs, would allow changes observed in the arrays to be correlated with these factors enhancing the ability to identify true targets of reduced snRNPs.

In the current chapter, it is shown that SMN(A111G) rescues SMA mice and provides substantial snRNP assembly activity. The levels of snRNP assembly activity in spinal cord correlate with the level of rescue obtained with each line of mice. The high expressing line 588 lives the longest with the highest snRNP assembly; whereas, the low expressing line (2117), which lives 8 days, has minimal snRNP assembly levels. Investigation of the levels of major and minor snRNAs in the spinal cord confirm that SMA animals experience a preferential reduction of minor snRNAs (U11, U12 and U4atac) compared to major snRNAs (U4). Importantly, analysis of snRNA levels in transgenic SMA mice expressing mutant SMN alleles showed correlation of the rescue of SMA to the restoration of snRNAs in SMN(A111G) mice. Thus, both snRNP assembly activity and snRNA levels correlate with severity of SMA animals and correction of SMA.

In summary, SMN alleles that are capable of snRNP assembly correct SMA mice if expressed at sufficient levels and this also results in correction of snRNP profiles. Thus, snRNP assembly activity is highly correlated with the ability to correct SMA and no alleles analyzed to date separate SMA correction from snRNP assembly. However, whether SMN is critical for assembly of protein complexes onto mRNA for transport in axons remains unknown. Currently, it is possible that reduced snRNP levels results in altered splicing of a gene that functions in axons, thus, uniting the axon and snRNP hypothesis. Alternatively, SMN-dependent RNP assembly reactions critical for axons could be disrupted in SMA. It, thus, becomes important to determine what the targets of reduced snRNPs are and what RNP assembly reactions are affected by reduced SMN.
The SMN missense alleles examined to date do not rescue Smn-/- mice, presumably because they lack snRNP assembly activity by themselves. The missense alleles examined, in particular SMN(A111G), can complement 1 copy of SMN2, indicating that small amounts of full-length SMN in a heteromeric SMN complex with mutant SMN restore function. In addition, this indicates that an oligomer is the functional unit in vivo, perhaps with the active site being formed by the interaction of subunits, as in other cases of intragenic complementation.

3.4 Materials and Methods

3.4.1 Generation of Transgenic Mice and Breeding

Both the SMN(A111G) and SMN(VDQNQKE) transgenes were made similarly. A 4.1 kb SMN promoter (SMNp) fragment in the pcDNA3 vector was isolated using BglII and BamHI. The SMN cDNA was isolated with BglII and BamHI. The SMN cDNA was ligated to the pcDNA3 vector containing SMNp using T4 DNA ligase (USB). The SMNp was then screened by PCR for directionality using the forward primer SMNPF tggagttcgagacgaggcctaagc and the reverse primer 3.2R agtagatcgagacagattttgct. Also, EcoRI and SacII digests to confirm direction of promoter. Subsequently, mutant SMN cDNAs were then inserted by BamHI and EcoRI restriction digest and ligation. The construct was confirmed by sequencing. Seventy-five micrograms of SMNp:SMN(A111G) pcDNA3 or SMNp:SMN(VDQNQKE) pcDNA3 plasmid was then linearized using PvuI and DraIII. The constructs were then given to the Ohio State Transgenic Animal Facility (TAF) and were microinjected into FVB/N oocytes. Animals born at TAF were screened for potential founders by PCR using the same SMNPF and 3.2R primers.
Founders were then backcrossed to SMA carrier mice (SMN2\(^{+/+}\); Smn\(^{+-}\)) to add a low level of human SMN while eliminating endogenous mouse Smn. Mice resulting from these crosses were genotyped for the mutant cDNA with SMNPF and 3.2 R primers. The presence of SMN2 and mouse Smn was screened as previously described (Le et al. 2005).

3.4.2 RT-PCR and Western Analysis

Total RNA was isolated using a combined TRIzol (Invitrogen) and RNeasy Mini Kit protocol (QIAGEN). 30 mg of tissue was used and 1 mL of TRIzol reagent was added. Tissue was homogenized, incubated at room temperature for 5 minutes. 200 \(\mu\)L of chloroform was added, shaken vigorously and incubated for 5 minutes at room temperature. The samples were then centrifuged for 15 minutes at 4 °C at 12,000 X g. The top layer was removed to a new tube and then 1 volume 70% ethanol was added. The QIAGEN manufacturer’s protocol was then resumed at this step. Total RNA was treated for DNA contamination using the DNA-free Kit (Ambion) as per manufacturer’s instructions. First strand cDNA synthesis was carried out using 6 \(\mu\)g of total DNA-free RNA. This was added to 250 ng random hexamer in nuclease-free water. This mixture was heated at 70 °C for 10 minutes and then cooled on ice for 10 minutes to allow primers to anneal. A master mix was added to the tube, which contained 1 mM dNTPs, 4 units of RNase-OUT (Invitrogen), 1X Reverse Transcriptase enzyme buffer and 7 units of RT (Life Sciences, Inc.). The samples were then incubated at 42 °C for 1 hour. PCR was carried out on the cDNA using forward primer 4F gtgagaactccaggtctcctgg and reverse primer BGH-R tagaaggcacagtctgga. PCR conditions were: 95 °C for 3 min for denaturation, followed by 35 cycles of 95 °C for 1 min, 59 °C for 1 min, 72 °C for 1 min,
then extension. Control primers used were cyclophilin forward agacgccgtgtctttttcg and cyclophilin reverse primer ccacagtggagatgggatc.

Western blot analysis was carried out by homogenizing tissue in 200 µL of blending buffer (62.5 mM Tris, pH 6.8, 5 mM EDTA, 10% SDS, Mini Complete Protease Inhibitor Cocktail (Roche)). The samples were sonicated briefly, mixed with 6X SDS Loading Buffer (350 mM Tris, pH 6.8, 10.28% SDS, 36% glycerol, 600 mM DTT, 0.012% bromophenol blue) and loaded onto a 12% SDS polyacrylamide gel. The gel was transferred onto Hybond P PVDF membrane (GE Healthcare). The membrane was blocked for 1 hour at room temperature in 5% milk, 1% BSA, 0.1% Tween-20 in 1X PBS. The blot was then probed with primary antibody 8F7 against SMN at 1:1,000 for 1 hour. Secondary antibody conjugated to horseradish peroxidase (Rockland) was added at 1:5,000 and incubated for one hour. The blot was then exposed to ECL substrate (GE Healthcare) and developed. The blot was stripped and re-probed with a loading control β-actin primary antibody (Sigma-Aldrich) at 1:10,000 for one hour followed by the same Rockland secondary. Quantitation of Western blots was carried out using NIH Image software and comparison of protein expression from Western blots was carried out using two-sample T-tests.

3.4.3 Histology and Immunofluorescence

Gastrocnemius muscles from 6-month-old animals were prepared as described (Le et al. 2005). The muscles were dissected, mounted on wooden blocks and flash-frozen in liquid nitrogen-cooled isopentane. The muscles were sectioned at 12 µm and mounted on Superfrost slides (Fisher Scientific). Sections were fixed with 100% alcohol, rinsed in tap water and stained in Harris’s hematoxylin (Sigma-Aldrich). This sections
were rinsed in tap water, stained with eosin Y (Sigma-Aldrich), rinsed in tap water again, dehydrated with alcohol and cleared in xylene. Sections were then mounted with coverslips using Permount (Sigma-Aldrich). Fiber areas were calculated using a SPOT-RT digital camera and SPOT software (Diagnostic Instruments, Inc.).

Root counts were done on spinal cords of 6-month-old mice as previously described (Gavrilina et al. 2008). The L4 ventral root of animals perfused with 4% paraformaldehyde and 1% glutaraldehyde was dissected. The roots were post-fixed in glutaraldehyde, epon embedded and then sectioned. The 1 μm sections were stained with toluidine blue. Axonal counts were obtained with BIOQUANT software (BIOQUANT Image Analysis Corp.) Groups were compared by two-sample t-tests.

Immunofluorescence was performed on 8 μm spinal cord sections of 6-month-old adult mice as described (Gavrilina et al. 2008). Animals were perfused with 4% paraformaldehyde and spinal cords were dissected from the lumbo-sacral region. Spinal cords were then fixed in 4% paraformaldehyde overnight, then floated in 20% sucrose. After the spinal cords sank to the bottom of the tube, they were mounted on wooden blocks and flash-frozen in liquid nitrogen-cooled isopentane. Spinal cords were sectioned onto Superfrost slides (Fisher Scientific) and allowed to dry. Sections were stained in a humidified chamber. The sections were washed in 1X PBS, blocked with 0.5% Tween-20, 4% goat serum in 1X PBS, washed 3 times with 0.5% Tween-20, 0.4% goat serum in 1X PBS (wash buffer), incubated with 1:500 rabbit polyclonal SMN antibody (Sharma et al. 2005) in wash buffer. Sections were then washed 7 times and incubated with 1:1,000 goat anti-rabbit Alexa-Fluor 594 (Molecular Probes). Samples were washed 7 times and mounted in Vectashield containing DAPI (Vector Labs). Images were obtained with an Eclipse E800 fluorescent microscope (Nikon) and MagnaFIRE software (Optronics).
3.4.4 Statistical Analysis

Survival curves (Kaplan-Meier) and statistical analysis was carried out with SPSS v16 (SPSS, Inc.). Kaplan-Meier analysis was done using the log rank method. Muscle fiber size medians were tested using the Wilcoxon Mann-Whitney and two-sample Kolmogorov-Smirnov test. Survival statistics of mouse crosses were obtained with Chi-square distributions.
CHAPTER 4

GENERATION OF SMN(I116F) TRANSGENIC MICE

4.1 Introduction and Objectives

In Chapter 3, two mutations, SMN(A111G) and SMN(VDQNKQE), were analyzed in transgenic mice. In this chapter, another missense mutation of SMN found in Chapter 2 is also analyzed in transgenic mice. The missense mutation SMN(I116F) was found to be capable of self-association, association with wildtype, but deficient in binding Sm proteins. All three of these properties are necessary for efficient snRNP assembly. However, SMN(I116F) was not able to rescue axonal defects in zebrafish assays. Furthermore, this mutant was shown to be incapable of snRNP assembly in an in vitro assay, despite its retention of protein binding (Shpargel et al. 2005). This could result since SMN(I116F) has reduced affinity for Sm proteins, equivalent to that of SMN(D7), which also does not efficiently form snRNP complexes. In contrast to SMN(A111G), then, SMN(I116F) would be expected to fail to rescue SMA in mice. This would show that snRNP assembly and the SMA phenotype are very much correlated.
4.2 Results

The SMN(I116F) transgene was cloned the same way that SMN(A111G) and SMN(VDQNQKE). The transgene contains the same SMN promoter 4.1 kb fragment attached to exon 1-8 SMN(I116F) cDNA with the BGH poly A sequence as shown in Figure 4.1. This transgene was injected into FVB/N mouse oocytes by the OSU Transgenic Animal Facility and 40 resultant offspring were obtained. The offspring were screened by PCR for founders containing the SMN(I116F) transgene. Eight founders were identified: lines 7700, 7701, 7702, 7703, 7704, 7705, 7706, and 7707. One founder (7702) has since died, leaving 7 founder lines.

Figure 4.1 Diagram of the SMN(I116F) transgene.
SMNp stands for the SMN promoter 4.1 kb fragment. BGH pA is the poly-adenylation sequence of bovine growth hormone.

4.3 Discussion

With the generation of the SMN(I116F) transgenic mouse, it will now be possible to assay this mutant allele for rescue of SMA. If it does not rescue, as expected, then it will support the correlation of snRNP assembly with the SMA phenotype, as it cannot
form snRNP complexes. Even at high expression, it is expected that the I116F mutation disrupts the Tudor domain enough that assembly of the snRNP complex will still not be possible. This is because the I116F mutation disrupts Sm binding, so even though a heteromer is formed between I116F and wildtype SMN, the snRNP complex will not be complete as the I116F proteins will not be able to bind to the full snRNP complex. All members of the oligomer must retain some function in some domain in order for complementation to occur. To date, there is no indication from the 7 founder lines of SMN(I116F) that rescue will occur as at least one dead SMA pup with the transgene has been recovered, although this is not enough to be significant as yet.

4.4 Materials and Methods

4.4.1 Generation of SMN(I116F) Transgenic Mice

A 4.1 kb SMN promoter (SMNp) fragment in the pcDNA3 vector was isolated using BglII and BamHI. The SMN cDNA was isolated with BglII and BamHI. The SMN cDNA was ligated to the pcDNA3 vector containing SMNp using T4 DNA ligase (USB). The SMNp was then screened by PCR for directionality using the forward primer SMNPF tggagtctgagagggcttaagc and the reverse primer 3.2R agtagatatcggagcagttttgct. Also, EcoRI and SacII digests to confirm direction of promoter. Subsequently, mutant SMN cDNAs were then inserted by BamHI and EcoRI restriction digest and ligation. The construct was confirmed by sequencing. Seventy-five micrograms of SMNp: SMN(A111G) pcDNA3 or SMNp:SMN(VDQNQKE) pcDNA3 plasmid was then linearized using PvuI and Drall. The constructs were then given to the Ohio State Transgenic Animal Facility (TAF) and were microinjected into FVB/N oocytes.
4.4.2 Screening and Breeding of SMN(I116F) Transgenic Founders

Animals born at TAF were screened for potential founders by PCR using the same SMNPF and 3.2R primers. Founders were then backcrossed to SMA carrier mice ($SMN2^{+/+}; Smn^{+/−}$) to add a low level of human SMN while eliminating endogenous mouse Smn. Mice resulting from these crosses were genotyped for the mutant cDNA with SMNPF and 3.2 R primers. The presence of $SMN2$ and mouse $Smn$ was screened as previously described (Le et al. 2005).
CHAPTER 5

CONCLUSIONS AND PERSPECTIVES

In this project, several lines of transgenic mice were made based on mutations of SMN. Most mutations of SMN cluster within hotspots. One of these hotspots, exon 6, falls within the self-association domain of SMN. These mutations affect the ability of SMN to oligomerize and to perform snRNP assembly. Mutations outside this domain, such as the Tudor domain mutations, do not affect self-association of SMN. Rather, these mutations affect the binding of Sm proteins. If a mutation still retains enough activity, it will self-associate, associate with wildtype SMN, bind Sm proteins, form snRNP complexes and full-length SMN can then compensate for the mutant SMN by perhaps allowing the loading of the Sm ring onto the snRNA. This seems to be the case for SMN(A111G). A mutation that cannot self-associate or bind Sm proteins will not be capable of snRNP assembly and cannot rescue, such is the case for SMN(VDQNQKE).

In the case of SMN(I1116F), it remains to be seen whether this allele will rescue in the transgenic mouse model. It is expected that it will not, due to the fact that it has severely reduced binding to Sm proteins and thus, will be unable to form the snRNP complex at all. Even a heteromer of SMN(I1116F) and full-length SMN would not be functional, as the mutant would not be able to bind the full complement of proteins necessary to form
the snRNP complex. This is supported by *in vitro* snRNP assays that showed that this mutant is deficient in forming snRNP complexes (Shpargel *et al.* 2005).

Studying mutations of SMN has proved to be very useful in trying to elucidate the function of SMN most affected in SMA. However, as has been shown in this thesis, it is very important to consider the behavior of the mutant under various experimental conditions. For instance, it is necessary to titrate the amount of mutant protein in each assay, such as in the binding assays and the zebrafish assays. It was shown that varying the amount of rescuing mRNA in zebrafish can result in different outcomes, which can affect the interpretation of the result. It is also important to consider how much normal endogenous protein is present in each assay. In the snRNP assembly reactions, small amounts of wildtype SMN are still present, thus leading to functional complexes for some mutations. If, however, the assay were to be done by somehow depleting the extracts of normal SMN or re-constituting the complex in the absence of normal SMN, then the assembly activity measured may be very different. In fact, it would be expected that the SMN(A111G) mutant would then fail to assemble snRNP complexes. Thus, in the future, a combination of *in vitro* assays that measure assembly and *in vivo* genetic models can be used to further delineate the forms of SMN capable of rescuing SMA and snRNP assembly.

Further, the SMN(A111G) mutation was found to be complemented by wildtype SMN, but not by another mutant, SMN(A2G). This indicated that perhaps these two mutations are affecting the same function, namely snRNP assembly. It would be interesting to investigate the other mutations of SMN, occurring in separate domains, to determine whether any two mutants could complement each other and restore function. If no two existing mutations can restore function, it is feasible to logically mutate SMN to create synthetic mutations that will complement a known mutation and thus, restore its
function. These protein-protein interactions should be first tested in *in vitro* assays and then can be tested *in vivo* if the mutants show promising results.

Also, if SMN(I116F) cannot bind Sm proteins and cannot be complemented by full-length SMN, perhaps by mutating the Sm protein instead of SMN, would enhance binding and thus, would restore its ability to form snRNP complexes. As Sm proteins form the core of snRNP complexes, a suppressor mutation in an Sm protein could be used to formally prove that snRNP assembly is the critical function affected in SMA. It is clear now that a variety of different strategies must be undertaken to investigate the mutations of SMN, which will hopefully lead to more evidence for the function of SMN affected in SMA.
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