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Modeling Spinal Muscular Atrophy in Mouse: A Disease of Splicing, Stability, and Timing

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1. Introduction

Proximal spinal muscular atrophy (SMA) is a progressive neurodegenerative disease associated with the loss of alpha motor neurons in the lumbar spinal cord. The loss of these motor neurons leads to the progressive atrophy of the associated proximal muscles, eventual respiratory distress, and death. SMA occurs in about 1 in 6,000 to 10,000 live births and is a leading genetic cause of infant mortality (Burnett et al. 2009). SMA is subdivided into several groupings based on disease onset, severity, and outcome. The most severe form of SMA is referred to as type 0, or embryonic SMA, wherein patients are born with severe muscle atrophy and have expected lifespans of less than 6 months. The most common type of SMA is type I, which is characterized by disease onset within 6 months and mortality by age 2. Children with SMA type I often fail to sit unaided or walk, have difficulty breathing, and often require respiratory assistance. SMA type II children experience disease onset between 6 to 18 months and will not gain the ability to walk. Two forms of SMA are characterized by disease onset later in life and have unaltered life expectancies: juvenile and adult onset SMA, or type III and IV, respectively. Juvenile onset SMA type III is distinguished by disease onset after the age of 2, but there is variability of onset that can extend to early teen years. Type III children are often able to walk, and the ability to remain ambulatory throughout life can further subdivide this juvenile onset SMA. The least severe form of SMA is adult onset SMA type IV. This form of SMA does not emerge until the adult years and often presents with difficulty in performing previously attainable activities, such as climbing stairs (Zerres et al. 1995; Zerres et al. 1997; Zerres et al. 1997).

In SMA disease progression, the inability to achieve normal motor function is often the primary indication of disease. The level of motor function decline in SMA disease progression can be measured by the loss of functional motor units in SMA patients. In severe forms of SMA, there is a rapid loss of motor neurons innervating proximal muscles. This measure of reduced motor units can be observed by evaluating the Compound Motor Action Potential (CMAP) and Motor Unit Number Estimation (MUNE). The total number of innervated motor units is reduced in SMA disease as measured by these two tests and is correlated with age, severity of disease, and disease progression (Swoboda et al. 2005). The loss of motor neurons early in the onset of disease argues for the significance of motor neurons in disease, although additional components of the disease may provide for the

rapid degeneration of SMA patients in late disease. Respiratory weakness is a common feature of both severe and milder forms of SMA. Children with type I or II SMA will often require respiratory assistance by way of Bi-level Positive Airway Pressure (BiPAP) (Bach et al. 2003), just as less severe forms of SMA also exhibit respiratory weakness that may require assistive breathing and respiratory therapy. While SMA is most directly associated with motor neuron loss, neuronal defects in sensory neurons are also observed in severe SMA patients (Rudnik-Schoneborn et al. 2003). A new emerging component of SMA is cardiac involvement in a subset of severe SMA patients, such as congenital cardiac defects and bradycardia (Bach et al. 2003; Rudnik-Schoneborn et al. 2008).

2. Genetic cause of SMA, reduced levels of SMN protein

The decreased level of the crucial Survival Motor Neuron (SMN) protein is the cause of SMA disease. The low levels of SMN protein are due to the loss of the essential Survival Motor Neuron-1 (*SMN1*) gene located on chromosome 5q13. SMA patients have lost *SMN1* by mutation or deletion, and the functional loss of *SMN1* is the disease locus of SMA (Lefebvre et al. 1995). In mice, the loss of the homologous *Smn* gene results in embryonic lethality (Schrank et al. 1997; Hsieh-Li et al. 2000); however, humans carry a duplication of the *SMN1* gene, *SMN2*, which encodes the same SMN protein. All SMA patients are genetically comprised of *SMN1* gene loss (mutation or deletion) with variable copy numbers of *SMN2* (Lefebvre et al. 1995; Lefebvre et al. 1997; McAndrew et al. 1997). The copy number of *SMN2* inversely correlates with the severity of SMA, where higher copy numbers of *SMN2* increase SMN protein and, therefore, decrease the severity of disease (Lefebvre et al. 1997; McAndrew et al. 1997). SMA patients that have 1-2 copies of *SMN2* are often associated with severe SMA type I, and SMA patients with higher *SMN2* copy number exhibit SMA type II-IV (Lefebvre et al. 1997).

The incomplete compensation for the loss of *SMN1* by *SMN2* is due to the inefficient splicing of the *SMN2* pre-mRNA (Lefebvre et al. 1995; Lorson et al. 1999). The altered splicing is due primarily to a single silent C>T point mutation in exon 7 that reduces recognition of exon 7 by the splicing machinery (Lorson et al. 1999). The functional role of the C>T mutation in exon 7 skipping can be explained by two models. The first model describes the loss of a putative exon splicing enhancer for SF2/ASF (SRSF1), where reduced binding of SF2/ASF (SRSF1) is associated with *SMN2* exon 7 skipping (Lorson et al. 2000; Cartegni et al. 2002; Cartegni et al. 2006). The second model is explained by the generation of an exonic splicing silencer for hnRNP A1 (Kashima et al. 2003; Kashima et al. 2007). Both of these models functionally explain the alteration in the recognition of exon 7 via the C>T point mutation that generates transcripts lacking exon 7 ($\Delta X7$) from *SMN2*. The complex splicing regulation of *SMN* exon 7 beyond the critical C>T mutation has been recently reviewed (Beebe et al. 2010; Singh et al. 2011).

SMA is a disease associated with reduced splicing efficiency of SMN encoding transcripts. Furthermore, the significance of reduced SMN protein in normal cellular maintenance is underscored by the importance of *SMN* in splicing. SMN is crucial for splicing, as the SMN protein is the primary scaffolding protein involved in the maturation of the core splicing factors, or U snRNPs (Fischer et al. 1997; Liu et al. 1997; Pellizzoni et al. 1999; Meister et al. 2001; Pellizzoni et al. 2002). The SMN protein, in complex with several other components, comprises the SMN complex (Paushkin et al. 2002). The SMN complex loads the heptameric Sm ring (Raker et al. 1996) onto the Sm site of U snRNPs, completing the processing of the

splicing core components for function in canonical splicing of nascent transcripts (Fischer et al. 1997; Liu et al. 1997). In SMA, the SMN protein levels are reduced due to loss of *SMN1*, and the primary product of *SMN2* is the SMN Δ 7 protein produced by the transcripts lacking exon 7 (Lefebvre et al. 1995; Lefebvre et al. 1997). The SMN Δ 7 protein is unstable and exhibits reduced protein function (Wang et al. 2001; Cho et al. 2010). However, a portion of *SMN2* transcripts encode full-length SMN protein sufficient for birth, but insufficient for motor neuron maintenance. The ability of *SMN2* to generate full-length transcripts provides for potential therapeutic intervention by increasing transcription or correcting the splicing of *SMN2* to increase the pools of SMN to levels consistent with normal development.

3. Genetic modeling of SMA in mouse

The duplication event of *SMN1* that generated *SMN2* occurred late in evolutionary history, and thus, mice carry only a single *Smn* gene homologous to *SMN1* (Bergin et al. 1997; Rochette et al. 2001). After identification of the gene responsible for SMA disease, efforts to model the human disease in mice began by generating null alleles of the mouse *Smn* gene (Summarized in Table 1). Null alleles of the *Smn* gene were generated by replacing exon 2 or 7 with reporter cassettes, and from these experiments the *Smn* gene was found to be essential as *Smn* $-/-$ mice were embryonic lethal at the eight cell stage post fertilization (Schrank et al. 1997; Hsieh-Li et al. 2000). The requirement of SMN for survival is congruent with the function of SMN in maturation of the splicing machinery as an essential function for viability in eukaryotes. Mice heterozygous for the *Smn* null alleles have normal lifespans and do not exhibit haploinsufficiency; however, they do develop a mild form of SMA with muscle weakness and motor neuron loss later in life (Jablonka et al. 2000; Monani et al. 2000).

SMA is a disease of reduced SMN protein, and although the loss of *Smn* represents the loss of *SMN1* seen in SMA patients, this does not fully model the genetics of SMA. To more closely recapitulate the etiology of SMA, bac transgenes that included *SMN2* were introduced into the *Smn* null mice (Hsieh-Li et al. 2000; Monani et al. 2000). The *SMN2* in the bac transgenes exhibit splicing defects similar to *SMN2*, as exon 7 is primarily skipped and low SMN expression is seen. The level of SMN protein generated from the *SMN2* transgenes was sufficient to correct the embryonic lethality in *Smn* null mice. The rescue by *SMN2* in *Smn* null mice is in accordance with the observation that all SMA patients have *SMN2* present. Moreover, increased copy numbers of *SMN2* led to variable severities of SMA that stratify human SMA disease. Mice with 2 copies of *SMN2* ("Line89") exhibited severe SMA with an average lifespan of 4-6 postnatal days, but an increase of the *SMN2* copy number to 8 rescued the Line89 mice from SMA disease (Monani et al. 2000). Mice generated using a second *Smn* null allele (disruption of *Smn* exon 7) in combination with an *SMN2* bac transgene, termed the Taiwanese or Hsieh-Li mice, generated mice that exhibited severe to mild SMA. Hsieh-Li mice with 2 copies of *SMN2* survived 1 week and mice with 4 copies of *SMN2* had normal survival but developed peripheral necrosis (Hsieh-Li et al. 2000). A simplified crossing scheme of these mice now allows for independent generation of both the severe mice that survive 10 days, as well as the mild mice with normal survival (Riessland et al. 2010).

In severe SMA mouse models, reduced SMN protein, loss of anterior horn lumbar motor neurons, and muscle atrophy lead to the observed reduction in survival of 6-10 days (Hsieh-Li et al. 2000; Monani et al. 2000). Interestingly, the lifespan of the severe Line89 mouse was extended to PND13.5 by transgenic over-expression of the SMN Δ 7 transcript (Le et al. 2005).

Genotype	Phenotype and Notes	Shorthand	References
<i>Smn</i> ^{-/-}	Embryonic lethal at preimplantation (8 cell stage). <i>Smn</i> exon 2 knockout.	<i>Smn</i> knockout	Schrank 1997
<i>Smn</i> ^{+/-}	Grossly normal with normal lifespan in the heterozygous <i>Smn</i> exon 2 knockout mice. Motor neuron loss in spinal cord: 40% at 6 months, 54% at 12 months.	Line89 het carrier	Jablonka 2000
<i>Smn</i> ^{-/-} ; <i>SMN2</i> (2 <i>Hung</i>) ^{+/+}	Bac transgene (115 kb) including human <i>SMN2</i> , <i>SERF1</i> and part of <i>NAIP</i> genes; rescues embryonic lethality of <i>Smn</i> ^{-/-} (HPRT knockout of <i>Smn</i> exon 7). Variable <i>SMN2</i> copy number, with increasing copy number lessening disease severity (Type I-III).	Taiwanese or Hsieh-Li	Hsieh-Li 2000
<i>Smn</i> ^{-/-} ; <i>SMN2</i> (89 <i>Ahmb</i>) ^{+/+}	Transgene containing <i>SMN2</i> (35.5 kb); rescues <i>Smn</i> ^{-/-} (exon 2 null allele) embryonic lethality. 2 copies of <i>SMN2</i> in the mice have severe SMA and die postnatally with mean survival PND4-6. Motor neuron loss PND5. Eight copies of <i>SMN2</i> rescue SMA phenotype.	Line89	Monani 2000
<i>Smn</i> ^{F7/Δ7} ; <i>NSE-Cre</i>	Cre-mediated <i>Smn</i> exon 7 excision in neurons. Mice develop a SMA like pathology with progressive motor neuron loss (cell body number and axon number) in the lumbar spinal cord, muscle atrophy, and have a mean survival of 25 days.	F7 or exon 7 floxed	Frugier 2000, Cifuentes-Diaz 2002
<i>Smn</i> ^{F7/Δ7} ; <i>HSA-Cre</i>	Cre-mediated <i>Smn</i> exon 7 loss in skeletal muscle. Muscle cell death and dystrophy-like pathology, with a mean survival of 33 days.	F7 or exon 7 floxed	Cifuentes-Diaz 2001
<i>Smn</i> ^{-/-} ; <i>SMN2</i> (89 <i>Ahmb</i>) ^{+/+} ; <i>SMN1</i> (A2G) ^{+/-}	Heterozygous expression of <i>SMN1</i> A2G patient mutation transgene increase survival of Line89 mice to ~227 days, but is dependent upon <i>SMN2</i> (2 copies). Reduced motor neuron number in spinal cord (29%) and facial nucleus (19%) at 3.5 months. ~25% reduction in spinal motor axon by 5 months, muscle atrophy, and axonal sprouting in gastrocnemius and triceps. Homozygous A2G are phenotypically normal.	A2G	Monani 2003

Table 1. Mouse models of SMA. (continues on next page)

Genotype	Phenotype and Notes	Shorthand	References
<i>Smn</i> ^{F7/F7} ; <i>Alfp-Cre</i>	Cre-mediated <i>Smn</i> exon 7 excision in hepatocytes is embryonic lethal at E18.5.	F7 or exon 7 floxed	Vitte 2004
<i>Smn</i> ^{-/-} ; <i>SMN2</i> (89 <i>Ahmb</i>) ^{+/+} ; <i>SMNΔ7</i> ^{+/+}	Expression of the SMNΔ7 transgene in Line89 mice extend survival to ~13.5 days. Motor neuron loss by PND9, weight and motor deficits, and muscle atrophy.	SMNΔ7	Le 2005
<i>Smn</i> ^{-/-} ; <i>SMN2</i> (89 <i>Ahmb</i>) ^{+/+} ; <i>SMN1</i> (A111G) ^{+/-}	Transgene of <i>SMN1</i> A111G patient mutation rescues Line89 mice. Survival is normal but requires <i>SMN2</i> (2 copies). Gastrocnemius muscle hypertrophy at 10 months and rescued the deficits in snRNP assembly seen in the Line89 mice.	A111G	Workman 2009
<i>Smn2B</i> ^{-/-}	Mutation of exonic enhancer in <i>Smn</i> exon 7 leads to low <i>Smn</i> protein levels and mean survival of 28 days. Muscle weakness and atrophy, and reduced motor neurons at PND21.	2B	Bowerman 2009, Bowerman 2010
<i>Smn</i> ^{-/-} ; <i>SMN2</i> (N11) ^{+/-} ; <i>SMN2</i> (N46) ^{+/-}	Mice with three copies of <i>SMN2</i> from N11(one copy) and N46 (two copies) alleles have a mean survival of 14-16 days. Muscle atrophy and motor neuron loss by PND15. Respiratory deficits and NMJ defects in diaphragm.	3 copy <i>SMN2</i>	Michaud 2010
<i>Smn</i> ^{F7/-} ; <i>SMN2</i> (89 <i>Ahmb</i>) ^{+/+} ; <i>Olig2-Cre</i>	Cre-mediated <i>Smn</i> exon 7 excision in motor neuron progenitor cells that also express basal SMN from <i>SMN2</i> . 70% survival to 12 months, motor neuron loss in spinal cord, muscle atrophy, and reduced sensory neuron innervation and number in DRG.	Olig2-Cre SMA	Park 2010
<i>SmnC>T</i>	<i>Smn</i> gene carrying the <i>SMN2</i> exon 7 C>T mutation induces exon 7 skipping and reduces <i>Smn</i> protein. Mild SMA disease onset at 60 days associated with hind limb weakness, reduced activity and rearing, and muscle hypertrophy in gastrocnemius. Normal survival.	<i>Smn</i> C>T	Gladman 2010

Table 1. (continued) Mouse models of SMA

The mechanism of life extension in this severe SMA mouse model is not easily understood but begs the question of whether the SMNΔ7 protein has a functional role in normal development and/or neuroprotection. Another transgene that modified the survival of the severe Line89 mice is the *SMN1* A2G mutation. The A2G transgene alone was unable to

rescue the *Smn* null mice; however, survival extension through expression of the A2G transgene was contingent upon expression of SMN from the *SMN2* transgene. The transgenic A2G mouse develops mild SMA early in life, though survival is near 1 year and the mice with SMA pathology can appear normal until disease progression late in life. The A2G mice are associated with motor neuron defects such as motor neuron loss, branching, neurofilament accumulation, and failure to respond to repeated stimuli (Monani et al. 2003; Kariya et al. 2008). The milder SMA phenotype may be explained by the partial ability of the A2G mutant SMN protein to bind to normal full-length SMN. This binding is greater than that of the *SMN Δ 7* protein, arguing the potential for partial rescue of SMN complex formation in the A2G SMA mouse model (Monani et al. 2003).

SMA mouse models that carry the *SMN2* gene can be used to evaluate the expression and splicing of *SMN2* in tissues otherwise inaccessible in SMA patients, such as the central nervous system (CNS). Furthermore, the effect of disease progression and therapies targeting correction of *SMN2* can also be evaluated in mice that have the *SMN2* bac transgenes. The short lifespan of the severe SMA mouse models also allows for rapid evaluation of treatments aimed at increasing SMN, as changes in survival can readily be addressed in the short-lived mice. However, the short lifespan of these SMA models does not allow for therapeutic intervention at later time-points in development and SMA disease. Further attempts to modulate the lifespan of the severe SMA by simply titrating the copy number of *SMN2* in mice have proven difficult. One group generated two *SMN2* transgenic lines that harbor 1 or 2 copies of *SMN2* per allele (lines N11 and N46). When combined to generate a 3 copy *SMN2* mouse, the survival was extended to PND14-16 with some outlier longer lived mice (Michaud et al. 2010). This average survival is similar to the *SMN Δ 7* mice and is accompanied by severe muscle weakness and atrophy late in disease, motor neuron loss by PND13, abnormal EMG, and NMJ defects. These mice also exhibit respiratory deficiencies associated with reduced volume and frequency. Additional allelic series of mice have been generated harboring alterations in the *Smn* gene and *SMN* hybrid alleles to titrate SMN protein levels in mice (These mice can be found on the Jackson Laboratories web site <http://jaxmice.jax.org/list/ra1733.html>).

Successful generation of SMA mouse models was achieved through the loss of *Smn* protein using *Smn* null alleles in combination with transgenic expression of SMN from *SMN2* and other SMN transgenes. However, to simplify the genetics of modeling SMA in mice, it would be desirable to place both the gene loss and modifier expression in the same locus. The cosegregation of these two genetic events would allow for regulation of transcription and splicing in the endogenous genetic locus. The *Smn* C>T allele was engineered by introducing the *SMN2* exon 7 C>T mutation in the murine *Smn* gene. The *Smn* C>T mouse mimics the genetics of SMA patients, and recapitulates the splicing defect of *SMN2*, wherein exon 7 is skipped due to the C>T mutation. These mice exhibit reduced total *Smn* protein and a mild SMA phenotype characterized by hind limb weakness and inactivity consistent with type III SMA (Gladman et al. 2010). A second *Smn* C>T allele has been engineered (*Smn* C-T-Neo); however, this allele harbors the exon 7 C>T mutation with the neo cassette in intron 7 and additional sequences at the 5' end of exon 8. The splicing pattern of this mouse allele is primarily exon 7 skipped transcripts, and as such, produces very low *Smn* protein levels leading to embryonic lethality. The *Smn* C-T-Neo mouse has been used as an inducible *Smn* allele as the low *Smn* protein levels can be increased by the removal of the neo cassette; however, this must be done early in embryonic development (E7.5) or the embryo will be resorbed (Hammond et al. 2010).

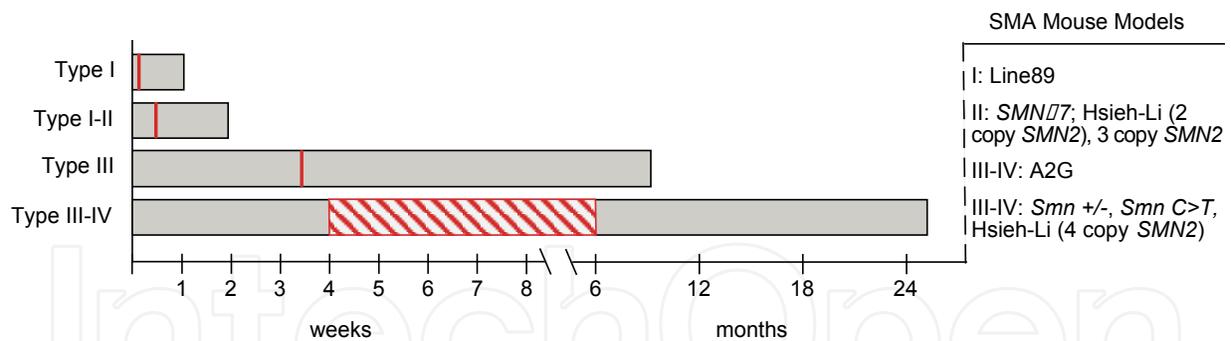


Fig. 1. SMA mouse models: survival and disease onset. Graphical representations of mean survivals of SMA mouse models types I-IV are shown by grey bars. Time scale is in weeks 0-8 and in months 6-24. A red line indicates the disease onset of SMA phenotype. For the mild SMA mouse models, a red-hashed box indicates variable onset in the listed SMA mouse models (i.e.: *Smn* +/- is 6 months, *Smn* C>T is 8 weeks, and Hsieh-Li (4 copy *SMN2*) has tail necrosis starting at 4 weeks)

Mutations in the mouse exon splicing enhancer for Tra2beta (2B) in exon 7 produces high levels of skipped transcripts in the absence of the C>T mutation (DiDonato et al. 2001). The mutation in the Tra2Beta enhancer is not associated with SMA mutations, and though it does induce exon 7 skipping, it does not recapitulate the splicing defect of *SMN2*. A mouse with the Tra2beta mutation in *Smn* (*Smn2B*-Neo) has low *Smn* protein levels that are associated with reduced size at two weeks, a lifespan of one month, and motor neuron loss late in disease progression (Bowerman et al. 2009; Bowerman et al. 2010). *Smn* alleles that genetically simplify and model SMA disease provide for evaluation of *Smn* protein reduction in SMA disease progression and can be used in evaluating therapeutic regimes for SMA. In choosing the appropriate mouse model to test therapeutics the survival and disease onset will be crucial considerations (graphically represented in Fig. 1).

4. Addressing the tissue specificity and timing of SMN expression in mouse models of SMA

The requirement for SMN expression in survival and motor neuron maintenance was evident in the genetic modelling of SMA in mice. However, the spatial and temporal requirement for SMN expression in tissues affected by SMA was not initially evaluated. To evaluate the requirement of SMN in specific tissues for SMA disease pathology, the deletion (Table 1) and expression of SMN in specific tissues (Table 2) has been tested in mice. An inducible *Smn* allele in which exon 7 is flanked by loxP sites (F7 allele) can replicate the loss of exon 7 (Frugier et al. 2000). This model has been utilized to knockout *Smn* exon 7 in a variety of tissues such as neurons, muscles, and liver by tissue specific Cre expression. The removal of exon 7 by Cre recombinase is irreversible and is associated with cell death in the target tissue being tested (Frugier et al. 2000; Cifuentes-Diaz et al. 2001; Vitte et al. 2004). The loss of full-length SMN through use of the inducible F7 *Smn* allele in neurons lead to neuronal cell death and mice exhibiting SMA pathology associated with motor neuron loss, whereas loss in muscle leads to a muscular dystrophy-like phenotype in the absence of motor neuron loss (Frugier et al. 2000; Cifuentes-Diaz et al. 2001). In an inverse experiment, increased expression of SMN was isolated to either neurons or muscle by tissue specific promoters in the severe Line89 mice. Rescue of the SMA phenotype was observed in

neuronal expression of SMN whereas muscle expression did not rescue the SMA disease (Gavrilina et al. 2008). Together, these studies argue that the expression of SMN is a requirement for basic cellular maintenance and that SMN expression in neurons can rescue the SMA phenotype.

The targeted excision of *Smn* exon 7 (F7) can remove Smn protein rather than reduce the levels, as is seen in SMA patients. To recapitulate the reduced levels of SMN protein, the F7 targeted deletion was used in motor neuron progenitor cells in conjunction with low levels of SMN generated by two copies of the *SMN2* transgene. SMN reduction restricted to motor neuron precursors resulted in a mild SMA pathology, survival rate of 70% at one year, reduced weight and muscle mass, and central and peripheral nerve dysfunction. This study reports a direct effect of reduced SMN protein levels in motor neurons leading to SMA pathology in mice. The reduced severity of SMA may be explained by the presence of normal levels of SMN in other cell types, which would also have reduced SMN protein levels in SMA disease (Park et al. 2010).

The evaluation of SMA mouse models has elucidated changes that occur early and late in disease progression. Thus, the timing of SMN replacement in disease may alter the therapeutic benefit of SMN replacement for disease correction. This consideration has been addressed in severe SMA mouse models in both genetic and therapy based experiments. Most drug treatment regimes require early administration to allow for survival extension and improvement of phenotype in severe mouse models of SMA, including prenatal treatment for Line89 mice (Riessland et al. 2010). Gene therapy using scAAV9 SMN expression argues that early postnatal expression of SMN in motor neurons is required for survival extension, with the greatest success at PND1. However, the rescue observed by systemic delivery of the viral vector required transduction of motor neurons in the CNS (Foust et al. 2010; Valori et al. 2010; Dominguez et al. 2011). Accordingly, the inability to rescue survival at later time-points may be a result of limited access to the CNS by the maturation of the blood brain barrier. The use of RNA molecules that mediate splicing correction can extend survival when administered early postnatally as well (Baughan et al. 2009; Coady et al. 2010; Passini et al. 2010). The ability of each of these therapeutic modalities requires access to the CNS and motor neurons for effective therapeutic benefit in SMA mouse models.

The use of genetic models of induced SMN expression can bypass the limitation of other therapeutic strategies to determine the therapeutic window in SMA mouse models. Genetic induction of *Smn* from embryonic lethal *Smn* hypomorphic alleles can prevent early embryonic lethality when induced early in utero (E7.5) (Hammond et al. 2010). Recently, genetic induction of SMN expression using a high expressing doxycycline inducible SMN transgene showed similar survival extension in the severe *SMN Δ 7* mice, comparable to that of scAAV9 SMN treatment (Foust et al. 2010; Le et al. 2011). In the doxycycline inducible SMN model, induction in embryos (E13.5) had the greatest survival extension to over 200 days, whereas neonatally treated (PND0-3) *SMN Δ 7* mice survived 86 days. Interestingly, the removal of doxycycline (i.e.: SMN over-expression ceased) at weaning (28 days) did not lend to rapid decline or death, arguing that lower levels of SMN expression may be sufficient later in life (Le et al. 2011). Together, these data argue for the requirement of early postnatal expression of SMN for therapeutic benefit in severe SMA mouse models, and SMN expression in motor neurons is required for considerable increase in mean lifespan. A still unresolved question is whether the same timing of SMN replacement will be required for treatment in milder SMA mouse models, or if a later therapeutic window exists. Some evidence offers that late treatment in mild SMA may be beneficial as the use of ASOs that

correct *SMN2* splicing in the Hsieh-Li (4 copy *SMN2*) mouse can reduce tail necrosis seen in these mice (Hua et al. 2010).

Genotype	Phenotype and Notes	Shorthand	References
<i>Smn</i> ^{-/-} ; <i>SMN2(89Ahmb)</i> ^{+/+} ; <i>PrP92-SMN</i> ^{+/+}	SMN expression in neurons under the prion promoter (PrP) recues disease in Line89 mice.	Neuronal SMN	Gavrillina 2008
<i>Smn</i> ^{-/-} ; <i>SMN2(89Ahmb)</i> ^{+/+} ; <i>HAS63-SMN</i> ^{+/+}	SMN expression in muscle under the human skeletal actin (HAS) promoter does not rescue SMA disease in Line89 mice. Leaky expression in neurons can afford moderate rescue in Line89 mice.	Muscle SMN	Gavrillina 2008
<i>Smn</i> ^{-/-} ; <i>SMN2(89Ahmb)</i> ^{+/+} ; <i>SMNΔ7</i> ^{+/+} ; <i>ROSA26rtTA</i> ^{+/+} ; <i>Luci-TRE-SMN</i> ^{+/+}	Induction of SMN expression by doxycycline within 3 days. Embryonic (E13) and PND0 treatment extend survival, mean 100 days but can extend to 200 days, and corrects NMJ defects. Removal of doxycycline at 28 days exhibits continued survival and rescue of NMJ defects.	Doxycycline Inducible SMN	Le 2011
<i>SmnC-T-Neo/C-T-Neo</i> ; <i>CreEsr1</i> and <i>Smn2B-Neo/2B-Neo</i> ; <i>CreEsr1</i>	Tamoxifen induced Cre-mediated excision of the neomycin cassette alters the hypomorphic <i>C-T-Neo</i> and <i>2B-Neo</i> alleles from ~5% SMN to ~30% and 16%, respectively. Early (E7.5) tamoxifen treatment can rescue to embryonic lethality and resorption of the <i>C-T-Neo</i> mouse early in embryonic development, though the ability to rescue animals to birth has not been shown.	Inducible <i>Smn</i> hypomorphic alleles	Hammond 2011
<i>Smn</i> ^{+/+} ; <i>Tg SMN2</i> ; <i>CreERT2</i>	Inducible SMN expression is under control of tamoxifen inducible Cre (CreERT2). Tamoxifen administration in embryonic (E13.5), neonatal (PND1.5), and weanling (4 week old) mice induces SMN expression. Ability to rescue survival has not yet assessed in SMA mouse models.	Tamoxifen Inducible SMN	Bebbe 2011

Table 2. Inducible SMN mouse models

5. SMN snRNP function and splicing in SMA mouse models

The best-characterized function of SMN is the maturation of the core snRNPs required for splicing (Fig. 2). To test the effect of reduced SMN levels on snRNP assembly in SMA, the maturation of both major (U2) and minor (U12) snRNPs were assessed in severe Line 89 mice. In these severe mice, the reduction of SMN protein correlated with reduced maturation of a subset of snRNPs, especially the minor spliceosomal pathway U11 and U12 snRNPs (Gabanella et al. 2007). In the $SMN\Delta 7$ mice, reduced U4 snRNP maturation was also reduced in the brain and kidney. The relative levels of snRNAs in the $SMN\Delta 7$ mice were altered in disease progression, with the minor spliceosome again showing the greatest reduction at PND11 in the SMA mouse models (Zhang et al. 2008). To genetically evaluate perturbations of snRNP assembly in SMA disease, a transgene of the mild SMA mutation in *SMN1* (A111G) was expressed in Line89 mice. Expression of the A111G transgene extended survival to a normal lifespan with no observable phenotype, though the A111G mice showed signs of muscle hypertrophy in the gastrocnemius at 10 months of age. The survival extension was *SMN2* dependent as A111G failed to rescue the *Smn* $-/-$ mice alone, arguing

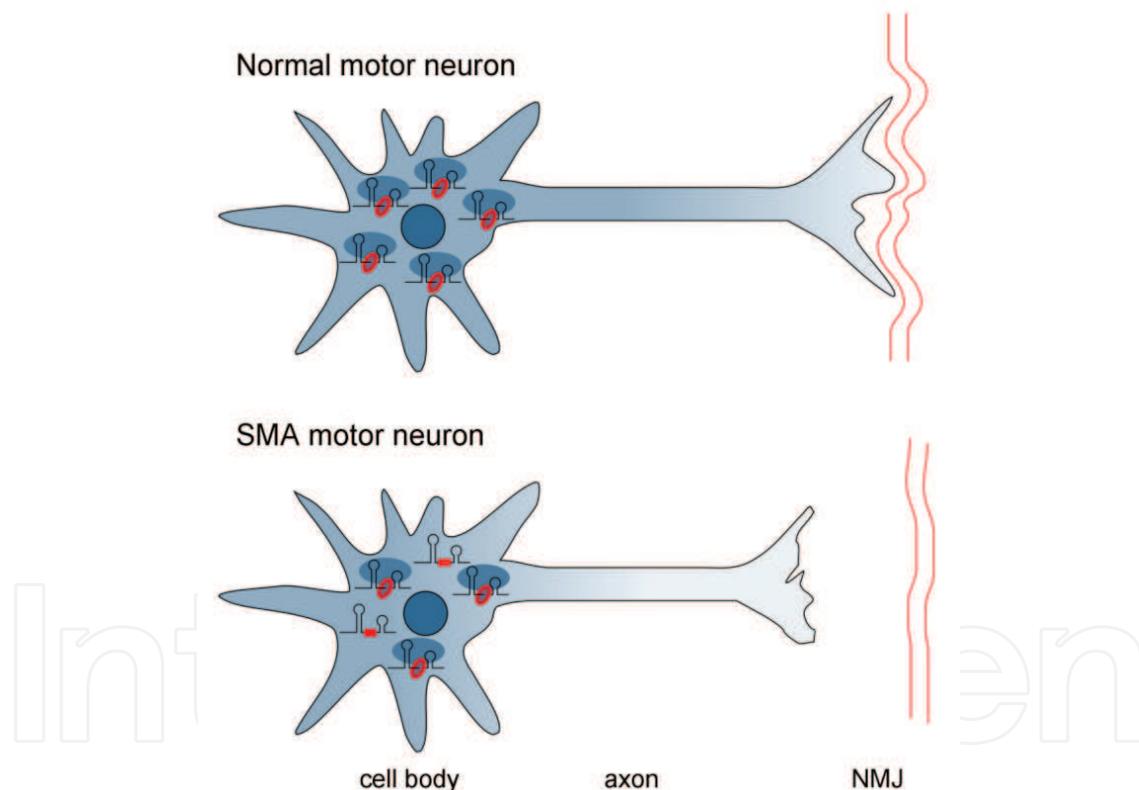


Fig. 2. SMN function in motor neurons. Normal and SMA motor neurons are depicted with blue shading corresponding to SMN protein levels. The SMN complex (blue oval) loads the Sm core (red circle) onto the Sm site (red box) of U snRNAs. In normal motor neurons sufficient SMN is present to form the mature U snRNPs, whereas SMA motor neurons lack sufficient SMN to mature all U snRNAs. Reduced SMN leads to motor neuron loss and dysfunction, which can be seen by immaturity of the NMJ and eventual loss of motor neurons by retraction of the axonal processes and cell death. While U snRNP maturation is the primary function of SMN, other functions of SMN in the axon or at the NMJ may still contribute to SMA pathology

for intragenic complementation between the SMN from *SMN2* and the mutant A111G SMN protein. Analysis of snRNP assembly in the severe Line89 mice also showed reduced U4 and minor spliceosomal snRNP maturation that was subsequently rescued by the expression of the A111G transgene (Workman et al. 2009).

The impact of SMN reduction in mice leads to reduced maturation and relative levels of snRNAs during SMA disease progression. The impact of the alteration in the maturation of the core splicing machinery on splicing in SMA disease was addressed in the severe mouse models of SMA at early and late stage disease. Evaluation of *SMN Δ 7* mice prior to disease (PND1) and early in disease (PND7) did not show differences in splicing, but later in disease progression (PND11 and 13), global splicing changes were observed using a splicing sensitive microarray (Zhang et al. 2008; Baumer et al. 2009). Major changes in splicing of neuronal and cellular damage genes were observed (Baumer et al. 2009). In the more severe Line89 mice, gene expression analysis in PND1 mice did not show major expression changes in the presymptomatic mice. However, genes involved in extracellular matrix, myelination, and cellular growth factors showed major changes in late disease progression at PND5 (Murray et al. 2010). These observations set SMA as a global splicing disease late in disease progression, which may account either for the effect of cellular stress on global splicing or a global alteration in snRNPs that contribute to disease in mouse models of SMA. While the importance of SMN protein in other non-snRNP associated neuronal functions is still under evaluation, it is clear that the reduction of SMN directly correlates with reduced maturation of snRNPs. As *SMN2* is itself improperly spliced, the potential for reduced exon 7 inclusion when SMN is low was evaluated using in vitro splicing assays. Extracts prepared from cells expressing low levels of SMN reduced inclusion of *SMN* exon 7, implicating a negative feedback loop effect of low SMN protein levels on exon 7 inclusion. This could be associated with the weak definition of exon 7, making the *SMN* transcript more susceptible to low levels of mature snRNPs required for canonical splicing (Jodelka et al. 2010).

6. Neurological manifestations in SMA mouse models

SMA is a disease of motor neurons, and as such mouse models of SMA have been evaluated for both the loss of motor neurons in the development of SMA disease, as well as other neurological manifestations in the SMA mouse models. Neuronal evaluation of the SMA mouse models has uncovered the timing of motor neuron loss and populations of neurons affected in the SMA mice. The neurological manifestations and major findings in the SMA mouse models have been outlined below.

The loss of motor neurons in SMA as observed in SMA patients was the first neuronal cell types evaluated in models of SMA mice. Histological analyses of motor neuron populations in the spinal cord of SMA mouse models have demonstrated that the loss of motor neurons is also seen during the progression of the disease in mice. Surprisingly, the loss of motor neurons in the spinal cord is a late marker of disease, occurring after a motor deficit is already observed and late in disease progression (Hsieh-Li et al. 2000; Jablonka et al. 2000; Monani et al. 2000; Monani et al. 2003; Le et al. 2005; Avila et al. 2007; Kariya et al. 2008; Bowerman et al. 2009; Michaud et al. 2010; Park et al. 2010; Riessland et al. 2010). The development of motor neurons was thus further analysed in the most severe mice (Line 89) during embryonic and neonatal development. In the Line89 mice the neuronal pathfinding and development of motor neurons appears normal in this SMA mouse model, although some abnormal axonal swelling were more pronounced in the SMA mouse model compared

to control littermates (McGovern et al. 2008). The presence of motor function deficits in the SMA mouse models prior to motor neuron loss raised the question of whether motor neuron function was compromised. To evaluate the function of the motor neurons, both anatomical and electrophysiological analyses have been performed in severe and mild SMA mouse models. In severe and mild models of SMA, accumulation of neurofilament in the presynaptic termini of motor neurons, disorganization of the postsynaptic NMJ, reduced synaptic vesicle release, and motor neuron sprouting have been reported (Cifuentes-Diaz et al. 2001; Monani et al. 2003; Kariya et al. 2008; McGovern et al. 2008; Murray et al. 2008; Kong et al. 2009; Ling et al. 2010; Michaud et al. 2010; Park et al. 2010; Lee et al. 2011). The changes at the NMJ are also associated with expression of immature isoforms of both the acetylcholine receptor (AChR) and myosin heavy chain (MyHC) (Kariya et al. 2008; Lee et al. 2011). The loss of motor neurons and changes in expression of genes involved in myelination, growth factor signalling, and extracellular matrix are associated with late stage of disease in SMA mouse models (Murray et al. 2010). The susceptibility of motor neurons holds true from mouse to human, in addition the susceptibility can also differ in subpopulations of motor neurons in a single muscle such as the levator auris longus (LAL) muscle (Murray et al. 2008; Murray et al. 2010; Ruiz et al. 2010).

The defects observed in motor neuron function followed by loss of motor neurons late in disease raised the question of whether other neuronal populations were affected in the SMA mouse models. Defects in sensory neurons have been reported in SMA patients (Rudnik-Schoneborn et al. 2003), and the 3 copy *SMN2* mouse also exhibited latency in response to heat (Michaud et al. 2010). The potential for sensory neuron malfunction in SMA was further evaluated in the *SMN Δ 7* mouse model. A reduction in the input of sensory neurons to the motor neurons of the spinal cord as well as perturbation of the neuronal circuitry was evident as early as PND4 in the *SMN Δ 7* mice (Ling et al. 2010; Mentis et al. 2011). Furthermore, the reduction of sensory input into lumbar motor neurons was also observed in the *Olig2-Cre* mouse even when the sensory neurons did not have reduced SMN protein (Park et al. 2010). The reduction of SMN protein also has consequences for neuronal growth and development as evidenced by reduced neuronal numbers in the brain and eye of severe SMA mouse models Line89 and *Smn* 2B (Liu et al. 2010; Wishart et al. 2010). The modelling of SMA in mice have recapitulated the motor neuron loss as is seen in SMA patients, as well as identified neuronal populations affected by reduced SMN levels in the SMA mouse models.

7. Cardiac and pulmonary involvement

Reports of cardiac defects including congenital heart defects, bradycardia, left ventricular cardiomyopathy, and sympathetic-vagal imbalance have recently been reported in severe SMA mouse models (Bevan et al. 2010; Heier et al. 2010; Shababi et al. 2010). Evaluation of 63 severe SMA type I patients reported that 24% presented with severe bradycardia (Bach 2007). In the severe *SMN Δ 7* mouse model bradycardia was also present due to reduced autonomic innervation, and in turn increased vagal tone. Anatomical analysis of *SMN Δ 7* mice hearts was positive for dilated cardiomyopathy and reduced contractile function (Bevan et al. 2010; Heier et al. 2010). Therapies aimed at increasing SMN protein by treatment with scAAV9 SMN or TSA were able to improve heart function and survival in the treated *SMN Δ 7* mice (Bevan et al. 2010; Heier et al. 2010). A third group reported congenital defects present in the severe Line89 mice in the left ventricular septum and wall,

but these abnormalities were not observed in the SMN Δ 7 mice. However, fibrotic deposition and oxidative stress markers accumulated in the SMN Δ 7 heart as disease progressed (Shababi et al. 2010). The collective effect of changes in the heart of SMA models decreases the functional output and implicates additional neuronal targets by way of autonomic neuronal dysfunction in SMA mouse models. As cardiac involvement has not been reported in most severe SMA patients, further analysis of patients to identify cardiac defects will be required to determine if cardiac treatment is a consideration for SMA patients.

Respiratory complications are common during SMA disease progression and are often associated with mortality in severe SMA. Long-term respiratory treatment is often requirement in both severe and mild SMA patients. The 3 copy *SMN2* mice appear normal at PND1 but have reduced numbers of innervated NMJ in the diaphragm at PND9, associated with reduced respiratory capacity starting at PND7 (Michaud et al. 2010). Similar structural defects at the NMJ in the diaphragm of SMA type I patients have also been reported (Kariya et al. 2008). Analysis of the most severe SMA mouse model (Line89) from E10.5 to PND2 for motor neuron developmental defects showed the diaphragm was not affected; however, intercostal muscle denervation was observed (Kariya et al. 2008; McGovern et al. 2008). The defects in motor neurons of the diaphragm and intercostal muscles may account for respiratory deficiency in mouse models of SMA, and may be a point of therapeutic intervention in SMA patients.

8. Therapeutic approaches in SMA treatment

The severity of SMA is directly associated with low SMN protein levels, thus the reintroduction of SMN would predict correction of the disease. The presence of *SMN2* in all SMA patients provides for a direct therapeutic target to increase SMN levels. Therapeutic approaches for increasing transcriptional activity of *SMN2*, splicing correction of *SMN2* exon 7, or stability of SMN protein are major considerations in development of SMA therapies. Additionally, increasing SMN levels by gene therapy or treating the disease pathology in an SMN independent manner are alternative approaches in SMA therapeutic development. Mouse models of SMA have enabled the evaluation of these therapeutic approaches in pre-clinical therapeutic regimes for treatment of SMA.

Small molecules or drugs were the first therapies evaluated for treatment of SMA in mouse models of SMA. Many of the initial drugs are FDA approved drugs and transcriptional activators, histone deacetylase (HDAC) inhibitors. These drugs increased SMN levels by transcriptional activity, splicing of *SMN2* exon 7, and/or SMN protein stability (Chang et al. 2001; Avila et al. 2007; Mattis et al. 2008; Narver et al. 2008; Tsai et al. 2008; Riessland et al. 2010; Mentis et al. 2011). Additional drugs have been used that target increased stability of SMN, read through of *SMN2* transcripts, incorporation of *SMN2* exon 7, as well as second-generation drugs that work via multiple mechanisms (Garbes et al. 2009; Hastings et al. 2009; Butchbach et al. 2010). These drugs improved SMN protein levels and SMA phenotype, and often the extension of survival observed in treatment of severe SMA mouse models was 30%. However, in current clinical trials in SMA patients, these therapies have proven ineffectual (Chen et al. 2010).

Drug correction of *SMN2* splicing underscores the importance of targeting the correction of *SMN2* splicing as a mode for increased SMN expression. The use of RNA based therapies aimed at increasing exon 7 inclusion can be achieved by sequestering a negatively acting

sequence in the pre-mRNA and preventing its negative function (Singh et al. 2006; Hua et al. 2007; Williams et al. 2009; Hua et al. 2010; Passini et al. 2010), recruiting positively acting splicing factors to exon 7 (Baughan et al. 2009; Meyer et al. 2009; Voigt et al. 2010), blocking exon 8 inclusion (Dickson et al. 2008), or providing an RNA substrate used in the splicing reaction (trans-splicing) (Coady et al. 2010). These techniques have successfully increased exon 7 inclusion and SMN levels, and have improved survival and phenotype in SMA mouse models.

To reintroduce SMN, viral gene therapies that encode SMN can correct disease if SMN is expressed in the appropriate tissues and cell types. Self-complementary adeno-associated virus serotype 9 (scAAV9) can transduce motor neurons in mice when administered early postnatally, and the expression of SMN in motor neurons can reduce disease pathology, as well as extend survival in the SMN Δ 7 mouse model (Foust et al. 2010; Valori et al. 2010; Dominguez et al. 2011). The requirement for early expression of SMN using scAAV9 argues that SMN is required early postnatally for disease correction. However, the ability of the viral treatment to reach the spinal cord and motor neurons was reduced after PND5. The early therapeutic window outlined by scAAV9 treatment likely represents a limitation of viral gene therapy, and does not necessarily reflect the therapeutic window for SMN replacement.

Most SMA patients present with disease after motor neuron loss and motor function has already occurred. Treatment of the disease pathology may afford therapeutic intervention even in the absence of altering SMN levels. Cell based therapies to replace the lost motor neurons have initially been tested by transplanting normal cells that were programmed to mature into motor neurons. These cells were injected in the spinal cord, engrafted in the spinal cord, and can improve function and survival in the SMN Δ 7 mice (Corti et al. 2008). Furthermore, increasing muscle mass by inhibiting myostatin with the antagonist follistatin can reduce muscle loss and improve function and survival, though transgenic overexpression of follistatin in SMN Δ 7 mice failed to improve the survival of the mice (Rose et al. 2009; Sumner et al. 2009). As therapeutic development for SMA continues, the use of SMA mouse models will continue to be instrumental in pre-clinical testing of therapies for SMA.

9. Summary and conclusion

The modeling of SMA in mice has successfully recapitulated many components of SMA pathology associated with reduced levels of SMN protein. The use of these SMA mouse models has proven invaluable in further understanding the etiology of SMA disease, uncovering the neurological manifestations of SMA disease, and the importance of when and where SMN expression is required for correction of SMA pathology in mice. The use of mouse models of SMA to determine the pathological manifestations in SMA and testing therapeutic approaches continues as the need for therapies for this devastating disease remains.

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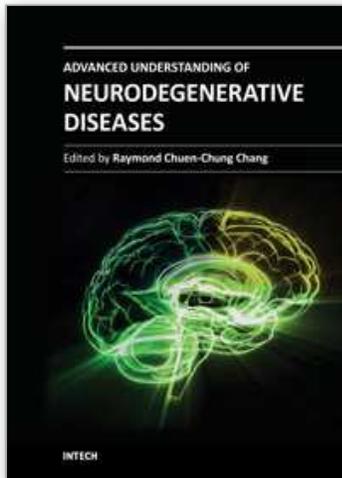
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Advanced Understanding of Neurodegenerative Diseases focuses on different types of diseases, including Alzheimer's disease, frontotemporal dementia, different tauopathies, Parkinson's disease, prion disease, motor neuron diseases such as multiple sclerosis and spinal muscular atrophy. This book provides a clear explanation of different neurodegenerative diseases with new concepts of understand the etiology, pathological mechanisms, drug screening methodology and new therapeutic interventions. Other chapters discuss how hormones and health food supplements affect disease progression of neurodegenerative diseases. From a more technical point of view, some chapters deal with the aggregation of prion proteins in prion diseases. An additional chapter to discuss application of stem cells. This book is suitable for different readers: college students can use it as a textbook; researchers in academic institutions and pharmaceutical companies can take it as updated research information; health care professionals can take it as a reference book, even patients' families, relatives and friends can take it as a good basis to understand neurodegenerative diseases.

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