GENE THERAPY IN MOUSE MODELS OF SPINAL MUSCULAR ATROPHY:
USING SMN GENE REPLACEMENT TO ADDRESS BIOLOGICAL QUESTIONS

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**GENE THERAPY IN MOUSE MODELS OF SPINAL MUSCULAR ATROPHY:
USING SMN GENE REPLACEMENT TO ADDRESS BIOLOGICAL QUESTIONS**

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Gene Therapy in Mouse Models of Spinal Muscular Atrophy: Using SMN Gene Replacement to Address Biological Questions

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ABSTRACT

Spinal Muscular Atrophy (SMA), an autosomal recessive neuromuscular disorder, is the leading genetic cause of infant mortality. SMA is caused by the functional, homozygous loss of the *Survival Motor Neuron-1* (SMN1) gene which encodes for the ubiquitously expressed Survival Motor Neuron (SMN) protein.

Here we utilize SMA mouse models to demonstrate that self-complementary Adeno-associated virus encoding SMN1 (scAAV-SMN) is a promising therapeutic for the treatment of SMA. We show that pre-symptomatic, ubiquitous restoration of SMN via scAAV is able to dramatically improve the SMA phenotype extending lifespan from ~14 to 200+ days. We show that direct delivery of scAAV-SMN into the central nervous system is able to more robustly rescue the phenotype of a mouse model of SMA compared to systemic, intravascular delivery. After determining the most efficacious route of injection, we seek to determine the therapeutic window and investigate the effectiveness of viral delivery after the onset of disease symptoms. We report that early, pre-symptomatic scAAV-SMN delivery produces a better phenotypic rescue than treatment after the onset of symptoms, emphasizing the need for early therapeutic intervention. We assess the need for SMN in a specific cell population, upper cortical motor neurons, reporting that restoration of SMN to solely to these cells is not sufficient to modify the SMA phenotype. Lastly, we use the scAAV vector to delivery mutant forms of SMN and other genes which may be able to modify the SMA phenotype. In this experiment, we identify several modifying genes which are able to improve the phenotype of an intermediate mouse model. Collectively, these studies help to provide insight into the use of scAAV-SMN as a therapeutic

and help to address clinically relevant questions about the temporal and spatial requirements of SMN protein during disease progression.

CHAPTER ONE: INTRODUCTION

Spinal Muscular Atrophy Overview

Spinal Muscular Atrophy (SMA) is the second most common autosomal recessive disorder and the leading genetic killer of infants [1]. SMA has a carrier frequency of 1:40, affecting approximately 1:6000 live births [2,3]. SMA affects all ethnic groups similarly, with the exception of the Hutterites, a small religious group located in South Dakota. The Hutterites have a carrier frequency of 1:8, representing the highest carrier frequency reported to date for SMA [4]. The main pathological feature of SMA, present in all human patients, is the specific loss of lower alpha motor neurons in the ventral horn of the spinal cord [5]. Loss of these cells results in muscle atrophy which progresses in a proximal to distal fashion. Frequently, muscle weakness is so severe that affected infants die before two years of age. However, in milder forms of the disease, muscle weakness may decline more slowly or be static for many years. All SMA patients share a common set of symptoms including, but not limited to: absence of deep tendon reflexes, tremor of hands and fingers, hypotonia, and atrophy of the voluntary muscle groups. The majority of premature deaths in SMA patients can be attributed to respiratory failure [5].

SMA is classified into five types based upon disease severity and age at onset of symptoms [6] (for review of types see [7]). Type 0 is defined as being embryonically lethal. Type I, also known as Werdnig-Hoffmann disease, is the most severe form affecting infants and accounts for approximately 50% of all newly diagnosed cases of SMA [8]. These patients have symptoms at birth or present with symptoms within the

first few months of life. Affected infants are often described as “floppy” as they lack muscular tone and are never able to sit without support. The vast majority of SMA Type I infants die during the first two years of life due to respiratory failure [6]. SMA Type II, also known as juvenile SMA, presents clinically between 6 and 18 months. Generally, these infants are diagnosed when failing to crawl or walk within a normal developmental timeframe. Life expectancy for type II patients ranges from early childhood to middle adulthood. Most type II patients become wheelchair bound due to significant muscle weakness. Type III, also called Wohlfart-Kugelberg-Welander disease, is usually diagnosed in late childhood. These children are able to stand unaided and may be able to walk unassisted for a time. However, they eventually require a wheelchair or walking assistance as muscle atrophy progresses [5]. Type IV is the rarest form of SMA. These patients present with symptoms in adulthood. Like type III patients, they often must rely on wheelchair or walking assistance. Unlike type III patients, however, they do not experience a shortened lifespan.

Genetics of SMA and Survival Motor Neuron

Despite its broad clinical spectrum, all forms of SMA are caused by the homozygous, functional loss of a single gene, *Survival Motor Neuron 1* (*SMN1*) located on chromosome 5q13 [1,9]. *SMN1* is a 20 kb gene encoding a 294 amino acid protein from 9 exons, only 8 of which are expressed. Most cases of SMA are caused by deletions or large rearrangements of *SMN1*. Far fewer cases can be attributed to missense mutations [9]. In humans an inversion and duplication event has occurred such that a nearly identical copy gene is present, *SMN2* (**Figure 1.1**) [10]. *SMN2* is retained in all

Figure 1.1 Schematic representation of the SMN-containing duplicated region of chromosome 5 (upper row), genomic *SMN1* and *SMN2* (middle row), and SMN mRNA splicing (bottom row). Upper row based upon [14]. The middle row highlights nucleotide changes from *SMN1* to *SMN2*. The C to T transition is highlighted as this nucleotide change is responsible for the splicing patterns seen in the bottom row.

SMA patients, and encodes the same protein as *SMN1*. However, *SMN1* and *SMN2* differ by a translationally silent, C to T transition at the 5' end of exon 7 [11-13]. This single nucleotide difference attenuates an exonic splice enhancer and at the same time creates an exonic splice silencer, causing the alternative splicing of *SMN2* transcripts such that the primary *SMN2* product lacks exon 7, *SMN Δ 7* [15-17]. Approximately 90% of the transcripts from *SMN2* are alternatively spliced resulting in the *SMN Δ 7* product, while the other 10% of transcripts produce full-length SMN [11]. *SMN2* does not produce enough full-length SMN to provide protection from SMA development when *SMN1* is not present [18]. It is the low levels of full length SMN that result in the development of SMA. However, because *SMN2* does produce some full length SMN transcripts, it has a critical disease modifying function [2,19].

Due to the duplication of the 500 kb region containing the human *SMN* locus, the gene copy number of *SMN1* and *SMN2* is unstable. Therefore, approximately 76% of the general population has fewer or more than two copies of *SMN2* [20]. For that reason, *SMN2* copy number also varies greatly amongst SMA patients. *SMN2* copy number has been shown to inversely correlate with disease severity in that less severe SMA patients often have more copies of *SMN2* than more severely affected patients (**Figure 1.2**) [2] Type I patients generally have 1-2 copies, type II patients 2-3 copies, and type III patients often have 3-4 copies of *SMN2* [2,21]. Also there has been documented cases of individuals lacking a functional copy of *SMN1*, but having 5 copies of *SMN2*, who were clinically normal [22].

Pathophysiology of SMA

Figure 1.2 SMN2 copy number inversely correlates with disease severity. Graph of the frequency of patients with type I, type II, and type III SMA versus SMN2 copy number.

The hallmark of SMA is the loss of alpha motor neurons in the spinal cord. This leads to defects at the neuromuscular junction (NMJ), including denervation, accumulation of neurofilaments at the synapse, and reduction of the quantal content of vesicular neurotransmitter release [23,24]. These NMJ defects lead to reduced neuronal input to muscle cells, resulting in muscle wasting [25]. Interestingly, restoration of SMN solely to motor neurons was only moderately able to rescue the phenotype of a severe mouse model of SMA [26]. This study and others indicate that other CNS cell types play an important, albeit lesser, role in the pathogenesis of SMA.

The requirement of SMN for sensory-motor circuit function has recently been explored. SMA motor neurons show reduced proprioceptive reflexes that correlate with decreased number and function of synapses on motor neuron somata and proximal dendrites [27]. Likewise, restoration of SMN in proprioceptive neurons and interneurons in the motor circuit is able to nonautonomously correct defects in motor neurons and muscles in *D. melanogaster* models of SMA, whereas, restoration of SMN in either muscles or motor neurons alone is not [28]. Additionally, microglia, the first line of immune defense in the CNS, have been reported to be activated in SMA mice. Microglia physically interact with SMA motor neurons suggesting microglia-mediated synaptic stripping may contribute to the denervation seen in SMA [29].

While the most profound defects are seen in the CNS, there are number of peripheral defects which arise from low levels of SMN. Defects in muscle, heart, vasculature, bone, pancreas, liver, lung, and intestine have all been reported in patients or animal models of SMA (for review see [30]). The identification of these defects

suggests that SMA is a multi-system disorder, not the motor neuron autonomous disorder once thought. Further elucidation of the complex pathology underlying SMA will aid not only our understanding of the disease, but also aid in the development of therapeutics.

Functions of SMN Protein: Why Do Low Levels of SMN Cause Motor Neuron Death?

SMN is an evolutionarily conserved 38kDa protein which is ubiquitously expressed, consistent with its essential role in survival [31]. Early studies into SMN function revealed that genetic deletion of *SMN* in the mouse results in pre-implantation lethality [32]. SMN is expressed at high levels in the brain, spinal cord, kidneys, and liver. SMN is also less abundantly expressed in cardiac and skeletal muscles. When the spinal cord from an SMA type I patient was examined for SMN protein there was a 100-fold reduction compared to age-matched standards [12].

SMN is present within the cytoplasm and the nucleus. In the nucleus, it is often localized into discrete nuclear foci called “gems” which are closely related to, and often located near, Cajal bodies [33,34]. Cajal bodies are major sites of nuclear RNA transcription and processing. Biochemical analyses have shown that SMN forms a complex with proteins called Gemins [35]. SMN together with Gemins 2-8 and unrip regulates the assembly of small nuclear ribonuclear proteins (snRNPs)(for review see [35,36]). snRNPs are RNA-protein complexes that combine with unmodified pre-mRNA and various other proteins to form a spliceosome, a large RNA-protein molecular complex upon which splicing of pre-mRNA occurs. snRNPs are assembled in the

cytoplasm and then transported into the nucleus and contain a single snRNA and Smith Core (Sm) proteins which form a heptameric ring [35].

The exact mechanism behind the specific loss of motor neurons seen in SMA remains unclear. Currently, there are two primary schools of thought outlined below. The first argues that low levels of SMN cause the missplicing of genes specific to motor neurons, leading to their demise. The second suggests that SMN has a specific axonal function that when disrupted causes motor neuron death.

SMN has known functions in snRNP assembly for all cell types. It has been suggested that SMN deficiency may in turn cause deficiency of U11 and U12 snRNPs, components of the minor spliceosome [37-39]. The minor spliceosome is responsible for splicing U12-type introns which make up less than 1% of all introns in human cells. However, U12-type introns are enriched in some of the genes in the central nervous system, such as voltage-gated ion channels and synaptic components. In this way, low levels of SMN could lead to the missplicing of genes specific to motor neurons, leading to their subsequent degeneration. Recently, the identification of a U12 intron containing gene misspliced in SMA, Stasimon, directly linked defective splicing of a neuronal gene induced by SMN deficiency to motor neuron dysfunction and degeneration [40]. However, the identification of other U12-intron containing genes specifically misspliced in SMA would lead further credibility to this hypothesis.

The other theory suggests that SMN has a neuron-specific function which is responsible for the specific susceptibility of motor neurons to SMN protein deficiency [41]. SMN is actively transported in discrete granules within axons and it has been

hypothesized to be a chaperone to mRNAs that are transported to the neuronal growth cone [42]. SMN has been shown to interact and/or co-localize with several mRNA binding proteins such as hnRNP-R [43], hnRNP-Q, HuD [44-46], COP1 [43] and with candidate-related plasticity gene 15 [44] and actin mRNA [47]. The local accumulation of these proteins in the nerve terminal has been shown to be impaired in cultured SMN-deficient neurons. These data have led some to hypothesize that SMN has a role in mRNA axonal transport and processing in the axon and nerve terminals. For example, it is known that proper localization of β -actin mRNA and protein is required for growth cone guidance and axon elongation in neurons. Furthermore, cultured motor neurons from a mouse model of SMA have shown decreased levels of β -actin mRNA and protein in the growth cones. Together these data suggest that low levels of β -actin at the growth cone or pre-synaptic nerve terminal contribute to SMA pathogenesis. However, the generation of motor neuron specific β -actin knockout mice has shown that β -actin is not required for motor neuron viability or the maintenance of neuromuscular junctions in vivo [48]. Investigation into SMN's neuronal function is still ongoing. It has also been suggested that SMN may be involved in cytoskeletal disruptions in the nerve terminal. There are several reports that support this. For example, plastin 3, which encodes an actin modifying protein, has been shown to be a protective disease modifier in discordant siblings [49]. It has also been shown that overexpression of plastin 3 is able to rescue the axon length and outgrowth defects associated with SMN down-regulation in motor neurons of zebrafish [49]. Furthermore, compounds which modulate actin

dynamics via the inhibition of RhoA/ROCK signaling have been shown to prolong the lifespan of an intermediate mouse model of SMA [50,51].

Much debate still remains as to the mechanism behind the specific loss of motor neurons resulting from low levels of SMN. Most likely, SMN has several molecular functions and the exact mechanism is complex and multi-faceted. The elucidation of this mechanism may become more clear as assays screening SMN functionality (i.e. SMN-dependent mRNA transport and snRNP assembly) become more prevalent. Most therapeutic assays to date have focused on screening for SMN induction rather than improvements in SMN functionality.

Animal Models of SMA

A number of animal models have been created that aim to mimic aspects of human SMA. SMN has homologs in every known eukaryote, making the generation of a variety of animal models of SMA, from lower organisms to mice feasible. Currently, the development of a large animal model of SMA (porcine) is underway via genetic disruption of *SMN1* [52] or alternatively, knockdown of *SMN1* with viral vectors [53].

The observation that complete knockout of SMN is embryonically lethal in any organism confirms the need for SMN protein for survival of all cells [32,54,55] .

Generally, SMA models are created by deletion or disruption of *SMN1* and addition of transgenic, human *SMN2* to rescue from lethality. One of the simplest organisms used to model SMA has been yeast. *S. pombe* has a *SMN* ortholog termed *Yab8*, that when deleted causes lethality. This lethality can be rescued by addition of human *SMN* [56]. Yeast-two-hybrid screens have revealed many of SMN's interacting partners.

Invertebrate models of SMA include the fruit fly (*D. melanogaster*) and nematode worm (*C. elegans*). Located on chromosome 3, *Drosophila* has a single *SMN* gene (*smn*) encoding a 226 amino acid protein [57]. An array of *Drosophila smn* mutants have been generated, the severity of which correlates to the reduction in SMN levels. Mutants range from those which die in the larval stages to those which demonstrate a motor phenotype, being unable to fly or jump [58,59]. The *C. elegans SMN* ortholog is known as *C41g7.1* or *CeSMN*. It encodes a 209 amino acid protein. Knockdown of *CeSMN* by RNA interference in the germline is lethal to all progeny, while hypomorphic progeny have locomotor defects and are sterile [60].

Zebrafish (*D. rerio*) have also been used to model SMA either transgenically or via knockdown of the zebrafish *SMN* ortholog [61]. Knockdown is performed using RNA interference or morpholinos. Using antisense morpholinos to reduce *Smn* levels throughout the entire embryo results motor axon-specific pathfinding defects, but has no effect on the muscle phenotype [62]. This axonal pathfinding defect can be rescued by the expression of human *SMN* [63].

The first mouse model of SMA, known as the “severe” model, was generated in the Burghes laboratory [64]. This mouse has a deletion of mouse *Smn* (*mSmn*), which by itself is pre-implantation embryonic lethal. To rescue from lethality, one copy of human *SMN2* was inserted. The genotype of this animal, when bred to homozygosity, is *mSmn*^{-/-}, *SMN2* ^{+/+}. These animals have normal numbers of motor neurons at birth, but vastly reduced numbers by day 5, around which time the animals die (**Figure 1.3**). These mice are symptomatic at birth and are significantly weaker than their unaffected littermates.

Figure 1.3 Graphical characterization of the severe and SMN Δ 7 mouse models. Overt phenotype of the severe (**A**) and SMN Δ 7 (**B**) mouse models compared to heterozygous, unaffected littermates. Weight (**C**) and survival (**D**) of severe and SMN Δ 7 mouse models in comparison to each other and unaffected animals. This data was obtained in the Lorson laboratory.

When fully backcrossed onto the FVB background, these mice have a more severe phenotype than on their original mixed (C57/BL6 and FVB) background, dying around embryonic day E13, suggesting the presence of modifier genes.

The workhorse of the SMA models, known as the SMN Δ 7 mouse model, was also generated in the Burghes lab [65]. This model, like the severe model, lacks *mSmn* and has two copies of *SMN2*. Additionally, these mice have approximately 15 copies of a SMN Δ 7 cDNA transgene, making their genotype *mSmn*^{-/-}, *SMN2* ^{+/+}, SMN Δ 7. The animals were originally made to test if the primary product of *SMN2*, SMN Δ 7, was toxic. However, instead of being toxic, it was found that the SMN Δ 7 protein was beneficial to the phenotype of these animals (**Figure 1.3**). As originally reported, these animals have an average lifespan of 13.3 days and are on a mixed genetic background (C57BL/6 and FVB). These mice begin to display symptoms around one week of age and then begin to decline in weight and motor ability. Like in the severe model, genetic background dramatically influences the phenotype of the SMN Δ 7 model.

When bred to be fully congenic, either pure FVB or pure C57BL/6, the average lifespan is severely attenuated [66]. This model closely mimics human SMA patients in that there is a loss of ventral horn cells and the voluntary muscle groups are affected while the diaphragm is spared.

Recently, the Ko lab performed a systematic examination of NMJ innervation patterns in more than 20 muscles in the SMN Δ 7 mouse [23]. They found that severe denervation (<50% fully innervated endplates) occurs selectively in many vulnerable axial muscles and several appendicular muscles at end stage disease. Because the

affected muscles were located throughout the body and are made up of varying muscle fiber types, it is unlikely that fiber type or location of the muscle is determining its susceptibility to denervation. Additionally, they demonstrated that neurofilament accumulation at the NMJ occurs in both vulnerable and resistant muscles prior to the onset of denervation, indicating that neurofilament accumulation is not a predictor of NMJ denervation. In the severe mouse model, axons will often overshoot their muscle targets. However, no defects in axonal outgrowth have been seen in the SMN Δ 7 mouse [67].

There has long been a need for more “intermediate” mouse models of SMA. The SMN Δ 7 mouse model is a very valuable model, but its severe phenotype and short lifespan make it difficult to test therapeutics which may be more applicable to less severe forms of disease. When looking at the phenotypes of previously generated mouse models, the animals tend toward one of three phenotypes: those dying shortly after birth [64,68] , those that die prior to weaning age (P30) [65], and those with a normal lifespan and extremely mild SMA-like pathologies [69-71] . In an attempt to create SMA mice with a spectrum of phenotypes ranging from severe to unaffected, the Jackson Laboratory utilized a genetic engineering approach. In this approach variable numbers of the *SMN2* sequence were incorporated directly into the mouse *Smn1* locus [68]. By combining these alleles, they were able to generate an allelic series of SMA mice having 0-8 copies of *SMN2*. This allelic series produced one of two effects: embryonic lethality or viable animals with normal life spans, albeit some mild phenotypic differences. These outcomes show that there is likely a threshold of SMN

needed for longevity, rather than it having a more titratable effect (i.e. improving phenotype with increasing amounts of SMN).

Recently, there have been some successful attempts to create an intermediate model. Cobb *et al.* have developed a model based the human genomic *SMN2* gene [72]. Genetically, this model is similar to the well-characterized *SMNΔ7* model; however, the *SMNΔ7* transgene has been manipulated to encode a modestly more functional protein referred to as SMN read-through (SMN^{RT}). By introducing the SMN^{RT} transgene onto the background of a severe mouse model of SMA ($SMN2^{+/+}; Smn^{-/-}$), disease severity was significantly decreased and lifespan was extended from an average of 7 to 34 days. The longest lived mice survived more than 70 days. Importantly, there is not a full phenotypic correction, which allows for the examination of a broad range of therapeutics, including *SMN2*-dependent and SMN-independent pathways.

Another intermediate model, known as the $SMN^{2B/-}$ model, is based upon a knock-in mutation within the endogenous *mSmn* exon7 sequence [50]. The mutation is designed to disrupt a highly conserved splice enhancer sequence required for the proper splicing of *Smn* exon 7. The SMN^{2B} mutation causes *SMN1* to mimic *SMN2* pre-mRNA splicing, resulting in reduced levels of full-length SMN and primarily generating the *SMNΔ7* product. It is important to note, however, that the SMN^{2B} mutation is not equivalent to the *SMN2* gene. Therefore, therapies designed to modulate SMN2 splicing, such as splice-site switching oligonucleotides, cannot be tested in this model. The SMN^{2B} allele is combined with a knockout allele to create the experimental $SMN^{2B/-}$ animals. SMA disease progression is slower than in the *SMNΔ7* model and the majority

of mice die by one month of age (**Figure 1.4**). However, much like the SMN^{RT} model, some animals are longer lived reaching 70 or more days of age.

AAV Therapeutics for SMA Applications:

Adeno-Associated Virus (AAV) Biology

AAV is a small (20-25 nm), single-stranded DNA (4680 nucleotides), non-enveloped, replication-defective virus that has recently become the primary vector used in gene therapy applications [73]. Wild type AAV is known to infect humans and some other primate species where it transduces both dividing and nondividing cells and then becomes latent for the life of the cell. As a dependovirus, AAV cannot replicate and form viral capsids in its host cell without the cell being coinfecting by a helper virus such as adenovirus [74]. In fact, AAV derives its name from the discovery that new AAV particles can be generated in the presence of adenovirus. Later, the specific proteins from adenovirus which facilitate AAV replication were identified [75,76]. These proteins are now used when generating AAV in the lab (see vector production section below). Wild type AAV has the ability to stably integrate into the host cell genome at a specific site (designated AAVS1) in the human chromosome 19 [77,78]. However, development of AAV as a gene therapy vector has eliminated its ability to integrate into the genome. AAV-based gene therapy vectors form episomal concatemers in the host cell nucleus (**Figure 1.5**) [79]. In non-dividing cells, these concatemers remain intact for the life of the host cell. In mice, AAV expression has been shown to be persistent over long periods of time eliminating the need for readministration after the initial dosing [81,82].

Figure 1.4 Phenotype of SMN^{2B/-} mice compared to a wild type control mice. Overt phenotype (**A**), weight (**B**), and survival (**C**) of SMN^{2B/-} and unaffected, SMN^{2B/+}. Average lifespan of the SMN^{2B/-} animals is approximately 30 days. This data was obtained in the Lorson laboratory.

Figure 1.5 Wild type AAV integrates into the host cell genome. When rAAV was engineered to be a gene therapy vector, its integration ability was removed. rAAV forms episomal concatemers in the host cell nucleus. In non-dividing cells, these concatemers remain intact for the life of the cell. When cells divide, the concatemers are not retained in daughter cells. Figure adapted from [80].

Self-Complementary AAV

It is important to note that AAV comes in two varieties, traditional AAV (ssAAV) and self-complementary AAV (scAAV). AAV vectors package single-stranded genomes and require host-cell synthesis of the complementary strand for transduction. The inverted terminal repeats (ITRs) form hairpins at the end of the sequence which serve as primers to initiate synthesis of the second strand before expression can begin [83]. This process can take several days (~5 to 7 days). In SMA applications this delay in expression is detrimental as the most commonly used mouse model, the SMN Δ 7 model, displays symptoms around one week of age and generally dies around two weeks. In order to effectively utilize this model, one needs a viral vector which will express its genome more quickly after neonatal delivery. scAAV has been engineered to form an intra-molecular double-stranded DNA template. After transduction, rather than waiting for cell mediated synthesis of the second strand, the two complementary halves of scAAV associate to form a double stranded DNA unit, ready for immediate expression (**Figure 1.6**). In addition to rapid expression, scAAV vectors have more robust expression in mouse liver, brain, and muscle when compared to ssAAV [84-86]. Improved expression in these tissues is especially beneficial in the treatment of SMA, as SMN has proposed functions in both the brain and muscle (see SMN protein function). However, one disadvantage of the use of scAAV is its limited carrying capacity. While AAV can carry approximately 4.7-6 kb, scAAV can only carry 2.5-3.3 kb [87].

	Pros	Cons
ssAAV	Larger packaging capacity	Rate limiting second strand synthesis
scAAV	Rapid gene expression More robust expression in liver, brain, and muscle	Reduced packaging capacity

Figure 1.6 Pros and cons of the use of ssAAV and scAAV vectors for gene therapy applications.

AAV Serotypes and Tropism

There are 11 serotypes of AAV: 1-11. The most commonly used serotype for SMA applications is serotype 9. When compared to serotypes 1-8, AAV9 results in the best viral genome distribution and highest protein levels [81]. AAV9 has a broad tissue tropism; when injected intravenously, it is known to transduce the liver, heart, brain, lungs, pancreas, kidney, and central nervous system (CNS) [88]. Importantly, AAV9 is able to cross the blood-brain barrier in neonatal mice following a systemic injection [89,90]. Recent publications have shown that ~60% of total motor neurons are transduced 20 days following an IV injection in mice given at P2 [90]. As further validation of the ability of scAAV9 to cross the blood brain barrier, Duque et al. intravenously injected scAAV9-GFP into two-day-old LIX1 kittens [89]. At 15 days of age, robust GFP expression was observed in the spinal cord with approximately 35% of motor neurons expressing GFP. Furthermore, when scAAV9-GFP was administered via systemic injection to cynomolgus macaques at 1 day of age, four weeks later GFP transduction in the spinal motor neurons was similar to that seen in mice [90]. This demonstration of scAAV9's ability to cross the blood brain barrier in non-human primates argues well for human applications. Taken together, these studies show that early injection of AAV9 sufficiently targets cells in the CNS making it a viable vector for gene replacement in SMA.

AAV Vector Production and Purification

Significant progress has been made regarding the production of AAV since the first report of its use as a transducing vector [91]. However, the basic requirements for

production remain largely unchanged. All AAV production methods require certain components to be offered *in trans* with respect to the vector genome (vg). These components are the AAV non-structural proteins, the AAV structural proteins, the adenovirus helper factors, and cellular factors needed for assembly and vg replication. The only *cis* elements required for the generation of AAV vectors are two 145 nucleotide ITRs. The ITRs are necessary for viral replication, packaging and stabilization of the vg in the transduced cell [92-95].

There are two main strategies used to generate AAV particles in cells, characterized based upon how the adenovirus helper factors are provided. One method depends on wild-type adenovirus infection into cell lines that stably harbor AAV replication (rep) and encapsidation (cap) genes in addition to the AAV vg. The other method, known as the helper-free method, uses adenovirus-free transient transfection of all the components required for AAV production into host cells such as HEK293 cells [96]. The virus produced for the work described here was produced using the latter method. In this method three plasmids are co-transfected into HEK293 cells: 1) an AAV2 ITR-containing plasmid carrying the gene of interest, 2) a plasmid with AAV9 rep and cap genes, and 3) a plasmid which provides necessary helper genes (E2A, E4ORF6, VA RNAs) isolated from adenovirus (**Figure 1.7**). The HEK293 cells provide the E1A and E1B genes, the other adenovirus helper genes needed [97]. The plasmids are transfected into the cells using linear polyethylenimine [98]. Forty-eight hours after transfection, the cells are harvested and viral purification is initiated.

Figure 1.7 Triple transfection of HEK293 cells to produce rAAV. The three plasmids are: 1) an AAV2 ITR-containing plasmid carrying the gene of interest, 2) a plasmid with AAV rep and cap genes, and 3) a plasmid providing the needed adenovirus helper genes. Together with the HEK293 cells, these plasmids provide the gene necessary for AAV production. Adopted from [100].

Viral particles can be purified from cells and concentrated for in vivo use in a number of ways (for review see [99]). One of the oldest and most widely used methods for viral particle purification uses isopycnic cesium chloride (CsCl) ultracentrifugation to generate a density gradient [100]. In this method, ultracentrifugation is used to remove impurities and concentrate the viral particles. After the last ultracentrifugation step, the concentrated virus is titered using quantitative real-time PCR [101].

Studies Utilizing scAAV for SMN1 Gene Replacement

Because SMA is monogenic, it is well suited to gene replacement via gene therapy. The first study demonstrating that delivery of ubiquitously driven SMN1 cDNA via scAAV is able to rescue the phenotype of the SMN Δ 7 mouse model was published in 2010 [102]. In this study SMN Δ 7 mice were given a one-time intravenous injection of scAAV9-hSMN at P1. Since this initial report, several groups have explored using scAAV as a vector to deliver SMN. For a detailed summary of these studies see **Table 1.1**. Taken together, these studies demonstrate that scAAV carrying *SMN1* is able completely rescue the lifespan of the SMN Δ 7 mouse model (**Figure 1.3**). Furthermore, they provide a platform from which optimization of variable parameters, such as route of delivery and time of viral administration, can be examined.

scAAV9-SMN Vector Design

Studies thus far utilizing scAAV for ubiquitous expression of SMN have all used a very similar vector design. This vector design consists of a ubiquitous promoter, a chimeric intron, the SMN cassette, and a poly A tail inserted between the viral ITRs. For the work described here, the CAG promoter was used (**Figure 1.8**). The CAG promoter is

Vector	SMA Mouse Model	Route of Delivery	Site of Injection	Total Dose	Median Survival Benefit (day)	Refs
scAAV9-hSMN	SMNΔ7	IV	Facial Vein	5x10 ¹¹ gc	Complete lifespan rescue	[102]
scAAV9-hSMN (co)	SMNΔ7	IV	Facial Vein	1x10 ¹¹ gc	69	[109]
scAAV9-hSMN (co)	SMNΔ7	IV	Facial Vein	4.5x10 ¹⁰ gc	242	[110]
scAAV8-hSMN	SMNΔ7	CNS	ICV Lumbar IP	1.7x10 ¹⁰ gc	157	[111]
scAAV8-hSMN	SMNΔ7	CNS	ICV Lumbar IP	5x10 ¹⁰ gc	50	[111]
scAAV9-hSMN	SMNΔ7	CNS	ICV	1x10 ¹⁰ gc	169	[112]
scAAV9-hSMN	SMNΔ7	IV	Facial Vein	1x10 ¹⁰ gc	34	[112]
scAAV9-hSMN	Severe	CNS	ICV	1x10 ¹¹ gc	17	[113]
scAAV9-hSMN	Severe	IV	Facial Vein	1x10 ¹¹ gc	8	[113]
scAAV9-hSMN (co)	SMNΔ7	IM	Bilateral gastrocnemius	7 × 10 ¹⁰ vg	26	[114]
scAAV9-hSMN (co)	SMNΔ7	IM	Bilateral gastrocnemius and triceps	7 × 10 ¹⁰ vg	163	[114]

Table 1.1 SMN gene therapy studies conducted in SMA mice. Genotypes of mouse models used: SMNΔ7 (mSMN^{+/-} SMN2^{+/+} SMNΔ7^{+/+}) and Severe (mSMN^{+/-} SMN2^{+/+}).

Abbreviations used: co=codon optimized, gc=genome copies (vector genomes),

ICV=intracerebroventricular, IP=intraparenchymal.

Figure 1.8 Schematic representation of the scAAV vector used in the studies represented here. The CAG promoter contains a chimeric intron to enhance expression of the SMN gene. The expression cassette is flanked by the ITRs (inverted terminal repeats) which serve as replication and encapsidation signals and must be in cis with SMN in order to produce viral particles.

a strong hybrid promoter frequently used to drive high levels of gene expression in mammalian expression vectors [103,104]. It consists of the following sequences; (C) the cytomegalovirus (CMV) early enhancer element, (A) the promoter, the first exon, the first introns of the chicken beta-actin gene, and (G) the splice acceptor of the rabbit beta-globin gene [105,106]. The presence of the chimeric intron within the promoter serves to increase SMN expression [107,108]. The SMN cassette used here is strictly the human SMN cDNA. However, some labs have used a codon optimized *SMN* sequence in an effort to maximize expression [109,110].

Clinical Trials Using AAV

In September 2013, the FDA granted approval to physician-scientists at Nationwide Children's hospital in Columbus, Ohio to begin a phase I clinical trial using AAV9 carrying the human *SMN1* gene. The trial is slated to begin in early 2014 and will be limited to Type I SMA patients ranging in age from birth to nine months. Previous, pre-clinical research from Nationwide Children's principal investigator, Brian Kaspar, PhD, has demonstrated that AAV9 is able to cross the blood brain barrier in non-human primates following systemic injection [115]. This work has set the stage for the upcoming clinical trial, in which AAV9-SMN will be injected to patients intravenously. An additional program also using the AAV9-SMN will examine a different route of delivery by injecting the virus into the cerebrospinal fluid. This program is currently moving toward clinical testing.

More broadly, the United States National Institutes of Health reports 59 clinical trials in various phases of completion which utilize AAV. Among the conditions being

addressed are muscular dystrophies, heart failure, hemophilia, retinal degeneration, Pompe disease, and Parkinson's disease. Several studies have shown AAV to be safe. However, there have yet to be reports of disease improvement with AAV vectors. However, clinical use of AAV as a therapeutic is new and there remains tremendous potential for it in the future.

Other Therapeutic Approaches for the Treatment of SMA:

In addition to viral gene replacement, various other therapeutic interventions, with various targets, are currently being examined for SMA. There are many approaches that have been used when designing a therapeutic, many of which will be highlighted below:

SMN Upregulation

Because all patients retain at least one copy of *SMN2* and because *SMN2* is capable of producing full-length SMN protein, this gene is a common therapeutic target. One strategy for upregulating the production of full length *SMN* is histone deacetylase inhibition. This has been achieved with a class of compounds known as Histone Deacetylase inhibitors (HDACi). HDACi relieve the transcriptional repression of HDACs which allow histones to wrap DNA more tightly thereby creating a tightly coiled, transcriptionally repressed region of chromatin. Sodium butyrate was the first drug used to show that the *SMN2* gene could be activated by HDACi [116]. Several other HDACi such as valporic acid (VPA), phenylbutyrate, trichostatin A (TSA), and suberoylanilide hydroxamic acid (SAHA) have since been shown to increase *SMN2* expression in both cell lines and animal models [117-122]. These drugs decrease

acetylation levels on HDAC2, which surrounds the transcriptional start site of *SMN*. VPA has been reported to improve motor function in a type III mouse model [121] and TSA has been shown to increase the median lifespan of the *SMN* Δ 7 model by 19% [122].

SMN can also be upregulated by targeting the STAT5 pathway [123] [124]. STAT5 proteins are involved in cytosolic signaling and in mediating the expression of specific genes, such as *SMN*. Stat5 activation has been shown to enhance *SMN2* promoter activity with increases in both full-length and *SMN* Δ 7 transcripts in *SMN2*-NSC34 cells [123]. Most recently, it was shown that prolactin (PRL) is able to increase *SMN* levels by activating the STAT5 pathway. Systemic administration of PRL to *SMN* Δ 7 mice resulted in improved motor function and enhanced survival (median survival increased by 7 days) [124]. Prolactin is FDA approved and thus, would be available for use in SMA clinical trials. However, it is unknown if the dosages commonly used in clinical practice for other applications would be sufficient to upregulate *SMN2* expression.

Splicing Modulation

The splicing of *SMN2* can be modulated by a number of approaches, the shared goal of which is to increase exon 7 inclusion. One approach for increasing *SMN2* exon 7 inclusion is the use of nucleic acid-based therapeutics. Antisense oligonucleotides (ASOs) are short sequences of nucleic acid able to recognize a target sequence with a high degree of specificity. ASOs for SMA are designed to be splice-switching, altering *SMN2* exon 7 pre-mRNA splicing in an effort to generate more full-length, exon 7 containing transcripts. Various sequences of *SMN2* mRNAs have been targeted with varying results. The most successful ASOs are able to extend the lifespan of the *SMN* Δ 7

mouse model to greater than 100 days [125]. Different ASOs utilizing various chemistries [126-128] as well as a transsplicing molecule [129] have been studied in cell and animal models. In 2009 ISIS pharmaceuticals, Inc. began working on a 2'-O-2-methoxyethyl-modified ASO (ISIS SMNRx). Due to its success in animal studies, ISIS SMNRx entered clinical trials in 2011. These trials are progressing and ongoing and have shown efficacy in human patients thus far [130].

In addition to ASOs, several small molecule compounds have been identified that modulate *SMN2* exon 7 splicing. Most recently, PTC Therapeutics, a company focused on developing drugs that target post-transcriptional control of gene expression, has identified compounds which are able to increase *SMN2* exon 7 inclusion. They have reported three compounds, one of which is able to extend the median survival of *SMNΔ7* mice from 14 to 132 days [131]. These compounds are able to cross the blood-brain barrier after oral delivery, but their chemical structures and mechanism of action have not yet been released.

SMN Stabilization

Glycogen synthase kinase 3 (GSK-3) inhibitors have been explored as a therapeutic target for SMA. GSK-3 is a serine/threonine protein kinase which mediates the addition of phosphates onto serine and threonine amino acid residues. Inhibition of GSK-3 has been shown to stabilize the SMN protein, increasing its intracellular levels [132]. A GSK-3 inhibitor, BIP-135, has been shown to modestly prolong survival of the *SMNΔ7* mouse model.

An alternative approach to SMN protein stabilization is proteasome inhibition. The FDA approved ubiquitin proteasome inhibitor, Bortezomib, while able to increase SMN protein levels, was unable to improve the survival of SMA mice. However, when administered with TSA (see SMN upregulation), Bortezomib produced an improvement in survival better than that seen when TSA was administered alone indicating that inhibition of SMN degradation can reduce disease severity [133].

Yet another strategy for SMN stabilization is the induction of a readthrough event. Aminoglycosides are a class of drugs that have been shown to induce readthrough events by suppressing codon recognition. These drugs have been shown to increase the length of the SMN Δ 7 C-terminus, reading through the first stop codon in exon 8 and incorporating an additional five amino acids [134-137]. This C-terminal extension is believed to confer increased stability to the SMN Δ 7 mRNA and subsequently generated protein. Treating primary fibroblasts from SMA patients or SMA iPS-derived neuronal cultures with aminoglycosides has been shown to increase SMN protein levels [134,137,138]. In vivo, the aminoglycosides G418 and TC007 have been shown to improve gross motor function and improve some molecular pathologies [134,139]. Additionally, when delivered into the central nervous system, TC007 extends survival of the SMN Δ 7 mouse model by approximately 30% [140].

SMN Independent Therapies

In addition to therapeutics designed to target SMN, many SMN-independent targets have been explored for their use as therapeutics. One of the most promising therapeutics studied, Y-27632, targets the signaling pathway of active RhoA by inhibiting

Rho-Kinase (ROCK), a direct downstream effector of RhoA-GTP [141,142]. Y-27632 is known to alter actin dynamics without altering SMN levels. In vitro, Y-27632 partially rescued neuronal differentiation defects seen in SMN-depleted PC12 cells [143]. In vivo, when the Y-27632 compound was administered to the SMN^{2B/-} mouse model, survival was extended from 3-4 weeks to 14-33 weeks [51]. While their survival was dramatically improved, these animals failed to gain weight compared to untreated animals. However, they did display improvements in the cellular phenotypes of the neuromuscular junction (NMJ) and muscle. Another ROCK inhibitor, Fasudil, was able to significantly extend the survival of SMN^{2B/-} mice with 57% of treated animals surviving more than 300 days. Significant improvements in NMJ and muscle phenotypes were also observed [144].

Other SMN-independent approaches have focused on muscle enhancement. It is unlikely that muscle enhancement alone will be able to improve the SMA phenotype as transgenic mouse work expressing SMN solely in skeletal muscle causes no phenotypic benefit [145]. However, it remains possible that muscle enhancement could serve as a synergistic therapy, enhancing the phenotypic improvement seen with other therapeutics. The myostatin and follistatin pathway, involved in regulation of muscle growth, has been examined for its utility as therapeutic targets. Delivery of recombinant follistatin has been shown to modestly extend survival in the SMN Δ 7 mouse [146]. However, transgenic overexpression of follistatin had no effect on the same model [147]. Transgenic overexpression of Insulin-like growth factor 1 (IGF-1), which stimulates myoblast proliferation, induces myogenic differentiation, and generates myocyte hypertrophy, increased the survival of SMN Δ 7 mice by 40%. [148].

Questions Addressed in the Following Chapters

The work outlined in the following chapters was done with the intent of answering outstanding questions regarding the development, delivery, and administration of scAAV9-SMN. Additionally, this work seeks to answer clinically relevant questions about the temporal and spatial requirements of SMN protein. In an attempt to orientate the reader, the primary question each chapter addresses is outlined below:

Chapter 2

How can AAV and other therapeutics be delivered to neonatal mice? This chapter describes the development and optimization of two injection techniques.

Chapter 3

When using the same dose of scAAV9-SMN, what is the most efficacious route of delivery? This chapter compares the two routes of injection described in chapter 2.

Chapter 4

Can scAAV9-SMN be delivered to pups after the onset of disease symptoms and still provide a phenotypic benefit? Previous work has shown that pre-symptomatic, day 2 delivery is efficacious. This chapter utilizes the severe model, in which pups are born with disease symptoms to investigate viral delivery after the onset of symptoms.

Chapter 5

What is the therapeutic window of the SMN Δ 7 model? This chapter examines the temporal requirements of SMN protein by delivering scAAV9-SMN at varying time points in neonatal development.

Chapter 6

Is restoration of SMN to EMX-1 expressing cortical neurons sufficient to improve the phenotype of the SMN Δ 7 model? This chapter focuses on the spatial requirements of SMN in upper motor neurons (Emx-1 expressing cortical) which synapse onto lower motor neurons, the primary cell type affected in SMA.

Chapter 7

What genes/mutant forms of SMN are able to modify disease progression? In this chapter, scAAV9 is used to deliver potential modifying genes and mutant forms of SMN to the SMN^{2B/-} mouse model, an intermediate SMA model.

Chapter 8

The concluding chapter outlines some of the potential challenges of moving AAV-mediated *SMN1* gene replacement from the laboratory to the clinic. As well as addressing these challenges, this chapter summarizes and contextualizes the work presented in the previous chapters.

CHAPTER TWO: DELIVERY OF THERAPEUTIC AGENTS THROUGH INTRACEREBROVENTRICULAR (ICV) AND INTRAVENOUS (IV) INJECTION IN MICE

This work has been published in the Journal of Visualized Experiments [149] and reprinted with permission.

Abstract

Despite the protective role that blood brain barrier plays in shielding the brain, it limits the access to the central nervous system (CNS) which most often results in failure of potential therapeutics designed for neurodegenerative disorders. Neurodegenerative diseases such as Spinal Muscular Atrophy (SMA), in which the lower motor neurons are affected, can benefit greatly from introducing the therapeutic agents into the CNS. The purpose of this video is to demonstrate two different injection paradigms to deliver therapeutic materials into neonatal mice soon after birth. One of these methods is injecting directly into cerebral lateral ventricles (intracerebroventricular) which results in delivery of materials into the CNS through the cerebrospinal fluid. The second method is a temporal vein injection (intravenous) that can introduce different therapeutics into the circulatory system, leading to systemic delivery including the CNS. Widespread transduction of the CNS is achievable if an appropriate viral vector and viral serotype is utilized. Visualization and utilization of the temporal vein for injection is feasible up to postnatal day 6 (P6). However, if the delivered material is intended to reach the CNS, these injections should take place while the blood brain barrier is more permeable due to its immature status, preferably prior to P2. The fully developed blood brain barrier greatly limits the effectiveness of intravenous delivery. Both delivery

systems are simple and effective once the surgical aptitude is achieved. They do not require any extensive surgical devices and can be performed by a single person.

However, these techniques are not without challenges. The small size of postnatal day 2 pups and the subsequent small target areas can make the injections difficult to perform and initially challenging to replicate.

Materials

See table 2.1

Protocol

Intracerebroventricular Injection

1. Prepare the injection stock solutions (i.e. viral vector, plasmid DNA, drug) under sterile conditions in a small tube.
2. Mix the desired volume of injectate (5-7 μL total) with 0.05% w/v trypan blue in PBS in order to visualize the injection.
3. Immobilize the P2 neonates via cryo-anesthesia for 1-2 minutes.
4. The needle used for this injection is a micro-liter calibrated sterilized glass micropipette attached to a 3 mL Hamilton syringe via long tubing.
5. Break the tip of the needle (with spatula) to adjust for 2 mm penetration into the skull. Place the needle diagonally in the small tube containing the injection solution. Load the injectate solution into the needle by carefully pulling the plunger of the syringe.
6. After uptake of the solution, disassociate the syringe from the tube. Then, pull the plunger up further and reattach the needle.

7. Hold the needle in the dominant hand between the thumb and index fingers and place the syringe between the middle and ring fingers with the plunger touching the palm of the right hand.
8. Grasp the immobilized mouse firmly by the skin behind the head using the non-dominant hand. Place mouse on a fiber-optic light to illuminate relevant anatomical structures that can be used as a guide.
9. Insert the needle 2 mm deep, perpendicular to the skull surface, at a location approximately 0.25 mm lateral to the sagittal suture and 0.50–0.75 mm rostral to the neonatal coronal suture (**Figure 2.1A**). Push the plunger using the palm of the right hand very slowly and carefully. Monitor for ruptured vessels or facial swelling.
10. Remove the needle 15 seconds after expulsion of injectate and discontinuation of plunger movement to prevent backflow.
11. Keep the mice in a warmed container for 5–10 minutes until movement and general responsiveness is restored.

Intravenous Injection via the Temporal/Facial vein

1. The solution to be injected should be prepared and supplemented with filtered green food dye at 1:100 dilution and injected under sterile conditions.
2. Attach a small plastic luer to the end of a 100 μ L glass Hamilton syringe. To the luer, attach a 33 gauge, 0.25 inch hypodermic needle. Insure that all parts are securely connected.

3. Load the syringe with the volume to be injected, ensuring that there is no air in the syringe as injection of air into the vessel is lethal. It is often helpful to pipet the volume to be injected onto a piece of parafilm. This allows for the clear visualization of uptake of the solution into the syringe.
4. A Wee Sight Transilluminator is used to easily visualize the superficial temporal vein, or facial vein, in the neonate (**Figure 2.2A**). Before injection, secure the neonate to the transilluminator using surgical tape. Gauze should be placed between the neonate and the tape to prevent the tape from damaging the skin of the animal. Secure the animal to the transilluminator on its side, ensuring that the tape does not prevent respiration. The neonate's neck should be gently turned so that the facial vein is easily visible and the nose should be taped to stabilize the head.
5. Using a 2.25X headband magnifier to allow for easier visualization, slowly insert the needle into the vein. The vein is very superficial so the needle should remain visible beneath the skin.
6. Slowly infuse the volume of solution into the vein. The green dye allows for easy visualization of the injection.
7. Wait 15 seconds before removing the needle as there is a lag between fully depressing the plunger of the syringe and the expulsion of the remaining solution due to the extremely narrow needle bore.
8. After removing the needle, use gauze to apply pressure to the injection site until the bleeding ceases.

9. Monitor the neonate for signs of distress. After a proper injection, no overt distress should be observed. The neonate should be given approximately 5 minutes to recover before it is returned to the cage.
10. Flush the needle/syringe unit with PBS in between injections as blood in the needle will quickly clog it due to the small bore.

Results

ICV Injection

The brain can be harvested at different time points post-injection and visualized to validate successful injection technique. If the dye is not added to the injection solution, it will be difficult to check for the accuracy of the injection. Proper injection into one of the ventricles will result in distribution of the trypan blue on the injected side of the brain approximately 10-15 minutes post-injection (**Figure 2.1B**). A uniform distribution of trypan blue in both right and left cerebral hemispheres and olfactory bulbs will be visible approximately 60 minutes post injection (**Figure 2.1C**). This is due to connectivity of the cerebral ventricles that will permit the dissemination of the blue dye to the adjacent ventricle. Accurate injections can also be visualized by dispersal of the blue dye in the rostral central spinal canal approximately 12 hours post-injection (**Figure 2.1D**). Inaccurate injections can be distinguished by lack of blue color in cerebral hemispheres. In this case, the depth of the needle was not sufficient and the injection solution has probably distributed under the skin. An alternative possibility is that the needle penetrated too deep in the brain, thereby passing beyond the ventricle and the solution was emptied underneath the cerebral ventricles.

Figure 2.1 Demonstration of successful ICV injections in P2 mice. **(A)** Schematic of injection sites utilized in ICV injections **(B)** Brain from pup injected ICV with trypan blue in PBS into left ventricle, photo taken 15 minutes post injection. **(C)** Brain from pup injected ICV with trypan blue in PBS into left ventricle, photo taken 60 minutes post injection. **(D)** Rostral central spinal canal from pup injected ICV with trypan blue in PBS, photo taken 12 hours post injection.

Figure 2.2 Demonstration of successful IV injection in P2 mice. **(A)** Schematic of facial vein utilized for IV injections. **(B)** Pup injected IV with green dye in PBS compared to non-injected littermate, photo taken immediately post injection.

IV Injection

Accurate and efficient temporal/facial vein injection can be visualized immediately following the injection. As the solution is injected, the green dye allows for the visualization of the solution entering and flowing through the facial vein (**Figure 2.2A**). Additionally, altered skin color of the mouse will be observed following injection. The neonate's normal pink color is converted to green, due to presence of green food dye in the injection solution (**Figure 2.2B**). The extent of the green color change observed in the neonate is dependent upon the concentration of dye used. For simple visualization of injection and during a practice period, a 1:50 dilution is recommended. However, the dramatically altered skin tone often results in the pup being rejected by the mother and therefore, removal of the dye or a significantly reduced concentration should be considered during experimental procedures. An inaccurate injection will result in the accumulation of green dye under the skin at the site of the injection. Incomplete injection, which occurs when the opening of the needle is not fully inserted within the vein, is seen when some solution flows in the vein and some accumulates under the skin. If this is observed, the needle should be inserted further into the vessel.

Discussion

Research using mouse models of disease often requires the administration of drugs or other substances to neonates. In this video, we demonstrate the step-by-step procedures involving two types of injection strategies that can be used to target the CNS: 1) direct injection into the CNS utilizing intracerebroventricular (ICV) injection; or 2) IV injection targeting the temporal/facial vein. The timing of these injections is of

great importance. Since the ICV injections are freehand, the skull must be relatively malleable. This type of injection is only possible through the first week of life and becomes increasingly more challenging and detrimental to the animals as they age. However, the primary benefit is that direct delivery to the CNS is achieved, effectively bypassing the blood brain barrier. Therefore, if an investigator is developing CNS therapeutics, it is possible to analyze compounds that are not fully optimized for drug-like properties such as blood brain barrier permeability while allowing for compound activity to be measured in disease-relevant tissues. Use of the temporal vein during the first few days of life has proven valuable as this is a relatively non-invasive technique for general systemic administration. At this developmental stage, no additional veins are easily accessible, such as the tail vein which becomes more accessible during the second week of life [150]. Both injection techniques are safe and can be performed freehand, with mice recovering shortly after injection without any detrimental side effects. Both techniques are suitable for rapid injection of a group of animals in a short period of time. They require proper visualization of the targeted area to ensure a successful injection. This is provided by the transilluminator and magnifying headband in IV injection and a fiber-optic light in ICV injection (**Table 2.1**).

One of the advantages of IV injection is that a larger volume (100 μ L) can be injected with ~90% success rate [151]. Common problems encountered while learning the IV technique include needle misplacement resulting in subcutaneous injections and/or incomplete injections. These problems can be countered by ensuring the bevel of the needle is thoroughly threaded through the vessel. The needle should be inserted into

the vessel far enough that the bevel of the needle is completely surrounded by the vessel. Additionally, the vessel is extremely superficial, thus the injector must ensure that the needle is in the vessel and not beneath it. To check if the needle is in the vessel, after placement the needle should be gently moved side to side. If the needle has penetrated the vessel, the vessel will move along with the needle. If the vessel does not move, the needle is not inserted properly. Performing this check before injection will increase the number of successful injections. Neonatal delivery of AAV9 vectors has been recently utilized to successfully transduce a high percentage of motor neurons using recombinant AAV vectors in SMA mice [90,102].

The most important consideration during the ICV injection involves the length of the needle tip. The tip must be long enough such that it can penetrate at least 2 mm into the skull. If the tip is not long enough, the needle will not be able to efficiently enter into the ventricles. Alternatively, if the tip is too long, this hinders the uptake of the solution to be injected and also creates a fragile tip that is difficult to guide correctly through the skull. The angle of the needle when injecting is also important. To ensure exact penetration into the ventricle hold the needle perpendicular to the skull with the tip toward the sagittal suture. ICV injections have been utilized in numerous studies to introduce compounds, drugs, therapeutic RNAs, plasmid DNAs, and viral vectors into the CNS of the diseased mice models [111,126,129,140,152-157]. However, the limiting factor in ICV injections is the small capacity of the cerebral ventricles in the neonate. This small capacity may not allow for delivery of the desired volume of therapeutic

agent. Therefore, the ability to reach therapeutic expression levels using a lower dose is particularly important when utilizing ICV injections.

Video

The video portion of this chapter is available at:

<http://www.jove.com/video/2968/delivery-therapeutic-agents-through-intracerebroventricular-icv>

Name	Company	Catalog Number	Comments
Green Food Dye	McCormick & Co.	n/a	Must be filtered
Hamilton Glass Syringe (100 µL)	Sigma-Aldrich	20702	
LuerMxF Thread Style White Nylon	Small Parts, Inc.	VPLF-LC78-1-25	
Fine gauge Hypodermic Needles	Popper & Sons, Inc.	7111	Size: 33(SWG) x ¼" (6.35 MM)
Wee Sight Transilluminator	Respironics	1017920	
2.25X Headband Magnifier	MagEyes	Model No. 5	Select magnification to fit individual preferences

Table 2.1 Materials needed to perform ICV and IV injections.

CHAPTER THREE: DIRECT CENTRAL NERVOUS SYSTEM DELIVERY PROVIDES ENHANCED PROTECTION FOLLOWING VECTOR MEDIATED GENE REPLACEMENT IN A SEVERE MODEL OF SPINAL MUSCULAR ATROPHY

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Abstract

Recently gene replacement strategies have shown tremendous promise in animal models of SMA. In this study, we used self-complementary Adeno Associated Virus (scAAV) expressing full-length SMN cDNA to compare two different routes of viral delivery in a severe SMA mouse model. This was accomplished by injecting scAAV9-SMN vector intravenously (IV) or intracerebroventricularly (ICV) into SMA mice. Both routes of delivery resulted in a significant increase in lifespan and weight compared to untreated mice with a subpopulation of mice surviving more than 200 days. However, the ICV injected mice gained significantly more weight than their IV treated counterparts. Likewise, survival analysis showed that ICV treated mice displayed fewer early deaths than IV treated animals. Collectively, this report demonstrates that route of delivery is a crucial component of gene therapy treatment for SMA.

Introduction

Because SMA is monogenic, vector-based gene replacement of *SMN1* is an attractive option for the treatment of SMA. Encouraging reports have been published using a relatively severe model of SMA called SMN Δ 7. These mice lack endogenous mouse *Smn*, but express the human *SMN2* gene and the cDNA encoding the alternatively spliced isoform produced by *SMN2*, SMN Δ 7 (*Smn*^{-/-}; *SMN2*^{+/+}; SMN Δ 7^{+/+})

[65]. Untreated SMN Δ 7 animals live approximately 14 days with disease symptoms becoming overtly apparent around day 7 [65]. Delivery of full-length SMN cDNA to SMN Δ 7 neonates using scAAV8 or scAAV9 vectors resulted in significant extensions in survival ranging from an average of 60–200+ days [102,109-111], with some treated mice displaying a full rescue in terms of lifespan and motor function. However, it remains unclear whether the different injection paradigms or the vector serotype was the primary cause for the differences in the degree of phenotypic rescue.

In this report we utilized a scAAV9-SMN vector and examined two routes of injection in neonatal SMN Δ 7 mice [149]. Pups received injections of 2×10^{10} viral genomes via the facial vein (IV) or directly into the brain ventricles (ICV) on postnatal day 2 (P2). We demonstrate that at this relatively low viral titer, animals receiving ICV injections gained significantly more weight and lived longer than animals receiving IV injections. As expected, animals receiving ICV injections also had higher SMN protein levels in the brain and lumbar spinal cord as compared to IV injected animals. From these results, we conclude that the route of injection for scAAV9-SMN has a significant impact upon the degree of phenotypic rescue and sheds light upon the development of disease and potential therapeutic implications.

Materials and Methods

Genotyping and Mouse Handling

Animals were handled according to the University of Missouri Animal Care and Use Committee approved protocols. Mice heterozygous for *mSmn* (*Smn*^{+/-}; *SMN2*^{+/-}; SMN Δ 7^{+/-}) were interbred to generate experimental SMA cohorts (*Smn*^{-/-}; *SMN2*^{+/-};

SMN $\Delta 7^{+/+}$). The day of birth was counted as P1 and the neonates were genotyped within 24 h. Animals were genotyped using PCR conditions as previously described [155]. SMA mice were raised with two heterozygous siblings. Additional heterozygous and wild-type animals were culled at the time of injection in experimental cages to control for litter size.

Tissue Collection

Dissections were done as follows: the vertebral column was separated from the torso, then the spinal cord was removed and divided into the cervical, thoracic, and lumbar (C-T-L) regions. The C-T-L sections were immediately frozen. The brain was removed from the skull and divided into four equal sections and each section was immediately frozen. The hindlimbs of each animal were removed at the highest point possible. The foot was bent at a 90° to control for differences in muscle stretching and the tissue was fixed in 4% paraformaldehyde overnight. After fixation, the gastrocnemius and tibialis anterior were removed from the bone, embedded in paraffin, and cross sectioned. The sections were stained with hematoxylin and eosin stain for muscle fiber size analysis. Quantification of fiber size was done as previously described [121].

Western Blotting

Tissues were harvested at indicated times and analysis was performed as previously described [138,157]. Mouse monoclonal anti-SMN (BDBiolabs), 1:2000, and anti-IP90 polyclonal rabbit antibody, 1:2000, were used for SMN and calnexin detection, respectively.

Production of scAAV-SMN Viral Vector

scAAV9-SMN was produced via triple transfection in HEK293T cells using polyethyleneimine as previously described [73]. The scAAV plasmid expresses the SMN full-length cDNA (NCBI accession number NM_000344) under the control of the chicken beta actin promoter. Forty eight hours post transfection, the cells were collected and the vector was purified by two cesium chloride density gradient ultracentrifugation steps and dialyzed against HEPES buffer (20 mM HEPES, 100 mM NaCl). Viral particles were titered by quantitative realtime PCR using SYBR green.

In Vivo Injections

Beginning on P2 *mSmn*^{-/-}; *hSMN2*^{+/+}; *SMNΔ7*^{+/+} mice were injected with 2×10^{10} viral particles of scAAV9-SMN. Due to the volume restrictions of the ICV technique, a series of three injections were given to obtain a titer of 2×10^{10} . Animals chosen for ICV injections were injected twice on P2 (AM and PM) and once on P3 (AM). Animals chosen for IV injection received a single injection on P2. Injections were visualized for accuracy by the additional of filter sterilized food dye.

Motor Function Analysis

Time to right was measured from P10 to P18. Mice were placed on their backs and given a maximum of 30 s to right themselves onto their paws. Failure to right within 30 s was considered failure. Grip strength and rotarod tests were performed with mice older than 80 days. The rotarod and grip strength assessments were done for 20 consecutive days. The first 10 days served as an initiation and learning period for the

animals and the last 10 days were used for analysis. Each individual mouse was given three trials and the best trial was graphed.

Results

To determine if the route of injection significantly impacts the degree to which the SMA phenotype is corrected in SMA mice following treatment with scAAV9-SMN, we performed a titration experiment to identify a relatively low concentration of vector that still resulted in significant survival (data not shown). This concentration (2×10^{10}) as opposed to a maximal dose was used in subsequent experiments since a lower concentration was more likely to reveal differences regarding the ability to rescue the SMA phenotype. SMN Δ 7 neonatal pups received IV or ICV injections with identical concentrations of the self-complementary AAV9 vector expressing full-length SMN cDNA. SMN protein levels were examined at P7 and P14 in disease relevant tissues, including brain and spinal cord, following scAAV9-SMN delivery (**Figure 3.1**). As anticipated, following the ICV injection directly into the brain, SMN expression was dramatically increased in the brain and spinal cord to levels comparable to unaffected heterozygous animals (**Figure 3.1**). IV injected animals expressed higher levels of SMN as well, although not to the same extent as ICV injected tissues. To monitor the gross phenotypic changes in treated mice, weight and survival were measured in each of the treatment groups (**Figure 3.2**). The ICV and IV treated animals gained significantly more weight than untreated controls (**Figure 3. 2A,B**), gaining nearly 2-fold more weight from P3 to their peak weight. We also observed that IV treated animals experienced more early deaths compared to ICV treated mice (**Figure 3.2C**).

Figure 3.1 Western blot showing protein expression is increased to near normal levels following ICV treatment with scAAV9-SMN, while IV treatment results in a more modest increase. Western blots of **(A)** PND 7 and **(B)** PND14 brain tissue. **(C)** PND7 and **(D)** PND14 spinal cord. All tissues were collected on the respective days from animals injected on PND2 with 2×10^{10} viral particles. Controls were untreated SMA (*Smn*^{-/-}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}) and unaffected, heterozygous (het) animals (*Smn*^{+/-}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}).

Figure 3.2 scAAV-SMN ICV treated animals gain significantly more weight and experience fewer early deaths than IV treated SMA animals while both groups gain significantly more weight and live longer than untreated SMA controls (*Smn*^{-/-}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}). (A) Average weight, in grams, of animals at each day of life: untreated (*n* = 9), IV treated (*n* = 6), ICV (*n* = 4), and Het (*n* = 24). Each treatment group is significantly different from the others by one way ANOVA (*p* < 0.0001). Tukey's multiple comparison test: Untreated vs. ICV *p* < 0.001, Untreated vs. IV *p* < 0.001 and IV vs. ICV *p* < 0.001. (B) Percent body weight gained from PND3 to peak weight of untreated, IV treated, and ICV treated SMA animals: untreated = 130.6%, IV treated = 435.9%, and ICV

treated = 1011.7%. Unpaired two-tailed student's *t* test: untreated vs. ICV *p* < 0.0001, untreated vs. IV *p* = 0.0157, and IV vs. ICV *p* = 0.0085. **(C)** Kaplan–Meier survival curve of untreated, IV treated, and ICV treated SMA animals. Log-rank (Mantel–Cox) test: untreated vs. IV *p* = 0.0004, untreated vs. ICV *p* = 0.0019, and IV vs. ICV *p* = 0.3251. **(D–G)** Representative images of treated SMA animals with both untreated SMA (*Smn*^{−/−}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}) and unaffected controls (*Smn*^{+/-}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}). **(D)** PND14 IV treated with controls, **(E)** PND14 IV treated with controls, **(F)** PND43 IV treated with unaffected littermate, and **(G)** PND63 ICV treated.

Not all phenotypic parameters, however, were different between ICV and IV treatment groups. At early time points both treatment groups were visually indistinguishable from unaffected controls (**Figure 3.2D–F**). Later on despite the differences in body weight and survival, animals in both treatment groups developed mild ear and tail necrosis around 50–60 days of age (**Figure 3.2G**), similar to previous reports [102,121,158]. However, the distal necrosis resolved shortly thereafter and did not progress further.

Time-to-right is an established means to monitor gross mobility for SMA mice [159]. Therefore, SMA mice were subjected to a time-to-right assessment beginning on P10. Interestingly, IV treated animals begin righting themselves at an earlier age than ICV treated animals and a greater percentage of the IV treated animals as compared to the ICV treated animals were able to right throughout P10 to P18 (**Figure 3.3A**). However, by P17 ICV animals that did right, righted faster than IV treated animals (**Figure 3.3B,C**). Later in life, motor function and coordination in surviving animals was measured by rotarod and grip strength tests. Both ICV and IV treated animals performed as well as normal animals in the rotarod test (**Figure 3.3D**) indicating that motor coordination, as measured by this assay, is fully rescued regardless of the route of injection. Grip strength analysis revealed that ICV treated animals have significantly better forearm strength when compared to IV treated animals (**Figure 3.3E,F**). Likewise, ICV treated animals also have slightly larger muscle fibers (**Figure 3.4**), perhaps accounting for the difference in strength observed in the grip strength assays. As expected based on their dramatic improvement in weight and survival, both IV and ICV

treated animals have significantly improved tibialis anterior fiber size when compared to untreated SMA controls (**Figure 3.4A–D**).

Discussion

Recently, four reports have demonstrated that viral-mediated gene replacement in SMA can significantly rescue the SMN Δ 7 mouse model [102,109-111]. These reports differ in the usage of a variety of parameters in their therapeutic approach including: viral serotype, viral promoter, route of injection, and time of injection [102]. Together these studies represent the most profound improvement in phenotype and survival seen in the SMN Δ 7 mouse model to date. However, without a direct comparison, it is difficult to compare these studies in order to determine the most efficient and effective course of treatment and to assess serotype and promoter activity that can profoundly impact gene expression in AAV vectors [160,161].

This study was performed to directly compare the influence of the injection route on the SMA phenotype. Here we chose to compare the two injection techniques that have been used thus far in viral gene therapy treatment of SMA: ICV and IV injections [102,109-111]. In doing so, we have found that route of injection makes a dramatic difference in survival and mouse phenotype. ICV injections have proven to be more effective in rescuing the SMN Δ 7 mouse. We propose that this is due to the direct introduction of virus into the central nervous system (CNS) [149]. Because SMA is primarily a disease of the CNS, using ICV injections to physically overcome the blood–brain barrier is advantageous. Conversely, systemically injected viral particles must cross the blood–brain barrier before they are able to transduce motor neurons, reducing their

Figure 3.3 ICV and IV scAAV9-SMN treatment improves motor function in SMA animals (*Smn*^{-/-}; *SMN2*^{+/-}; *SMNΔ7*^{+/-}). **(A)** Percent of pups able to right themselves on PND10–PND18. Time it took for each individual pup assessed to right on **(B)** PND14 and **(C)** PND17. **(D)** Rotorod performance and **(E)** Grip strength assessed for 10 consecutive days. **(F)** Mean grip strength quantified from the 10 day course of assessment. **(D–F)** Unaffected het controls (*Smn*^{+/-}; *SMN2*^{+/-}; *SMNΔ7*^{+/-}) *n* = 10, ICV treated *n* = 2, and IV treated *n* = 2. All tested mice >80 days of age.

Figure 3.4 Treatment with scAAV9-SMN results in increased muscle fiber size. (A–D) Representative muscle cross sections taken at 10× of an (A) unaffected het control (*Smn*^{+/-}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}), (B) untreated SMA (*Smn*^{-/-}; *SMN2*^{+/+}; *SMNΔ7*^{+/+}), (C) ICV treated SMA animal, and (D) IV treated SMA animal. (E) Average tibialis anterior muscle fiber size (in um²) at P14 following treatment with 2×10^{10} viral particles. Untreated SMA $n = 5$, Unaffected het $n = 8$, IV treated $n = 8$, and ICV treated $n = 5$.

efficiency and requiring more viral particles to achieve results similar to those of the ICV injection. However, it is known that there is a need for SMN protein in the peripheral organ systems, including, but not limited, to the heart [158,162,163]. While IV injections are able to meet this need directly, it is likely that some of the ICV injected vector is able to escape the blood–brain barrier in the same fashion that the IV delivered vector is able to penetrate it, especially within the context of SMA compromised neonatal animals. Thus, ICV injections are able to meet the need for SMN protein in peripheral tissues, while first meeting the primary need for SMN protein in the CNS, resulting in a more complete phenotypic rescue.

When considering the clinical translation of these approaches, ICV injections, while easily executed in mice, are difficult to perform in humans. However, intrathecal injections, while still not as straightforward as IV administration, directly overcome the blood–brain barrier and can be performed safely in humans [164]. Additionally, intrathecal injections have recently been shown to result in efficient motor neuron transduction when using AAV9 [160]. Thus, intrathecal delivery may prove to be a desirable route of administration should AAV gene therapy for SMA reach clinical trials. While the results of this study emphasize the importance of route of delivery when examining gene therapy in the context of SMA, route of delivery is an important component of any therapeutic.

CHAPTER FOUR: DECREASING DISEASE SEVERITY IN SYMPTOMATIC, $SMN^{-/-};SMN2^{+/+}$, SPINAL MUSCULAR ATROPHY MICE FOLLOWING SCAAV9-SMN DELIVERY

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Abstract

Recently, self-complementary adeno-associated virus (scAAV) vectors have been used to deliver the SMN cDNA to an animal model of disease, the SMN Δ 7 mouse. In this study, we examine a severe model of SMA, $Smn^{-/-};SMN2^{+/+}$, to determine whether gene replacement is viable in a model in which disease development begins *in utero*. Using two delivery paradigms, intracerebroventricular injections and intravenous injections, we delivered scAAV9-SMN and demonstrated a two to four fold increase in survival, in addition to improving many of the phenotypic parameters of the model. This represents the longest extension in survival for this severe model for any therapeutic intervention and suggests that postsymptomatic treatment of SMA may lead to significant improvement of disease severity.

Introduction

A number of potential therapeutic strategies have recently emerged in the SMA field, including oligonucleotides that redirect splice decisions, transcription modulating small molecules such as histone deacetylase inhibitors, and gene replacement [165,166]. The most profound correction of the SMA phenotype was observed following neonatal delivery of a self-complementary adeno-associated virus (scAAV) expressing SMN cDNA, in which a single injection of scAAV-SMN dramatically extended survival of SMN Δ 7 mice, and was consistent with an improvement in motor function and increased

numbers of motor neurons [102,109-111]. At birth, SMN Δ 7 mice are pre-symptomatic, with disease symptoms manifesting around P7 [65].

In this report, we use a severe animal model of SMA referred to as the *SMN2* model [64]. In this model, the human *SMN2* gene has been added to a mouse *Smn* null background (*Smn*^{-/-}; *SMN2*^{+/+}). These mice are born symptomatic, gain minimal body weight, and live approximately 4–6 days. Recently, this model has been shown to exhibit nonneuronal defects, including cardiac dysfunction and disrupted hippocampal development [163,167]. Due to the severity of the model, improving its phenotype via therapeutics has proven to be difficult. To date, the best improvement in the *SMN2* model has been modest increases in weight and survival [129,154].

We chose to use the *SMN2* model as a means to determine whether a symptomatic model of disease could be completely rescued using scAAV9-SMN. We delivered scAAV9-SMN at P1 to SMA mice via intracerebroventricular (ICV) injections and intravenous (IV) temporal vein injections. Serotype 9 was selected based on its ability to transduce a variety of neuronal lineages, including motor neurons [89,90]. In both treatment groups, SMN protein levels were significantly increased in the brain and spinal cord, and treated animals survived significantly longer than untreated SMA mice. Additionally, ICV delivery of the vector resulted in a greater extension of life span compared with SMA animals that received the same dose via IV injection. Collectively, this study demonstrates the most profound improvement seen in the *SMN2* model and illustrates that postsymptomatic treatment of SMA can significantly lessen disease severity.

Material and Methods

Genotyping and Mouse Handling

Animals were handled according to the University of Missouri Animal Care and Use Committee approved protocols. Genotyping was performed as previously described [129]. SMA mice, both treated and untreated controls, were raised with two *SMN* heterozygous siblings to control for litter size.

Tissue Collection

Dissections were as done as follows: The vertebral column was separated from the torso, and the spinal cord was excised and immediately frozen in liquid nitrogen. The brain was removed from the skull and divided into four equal sections, and each section was immediately frozen.

Muscle Fiber Size Analysis

The hind limbs of each animal were removed at the highest point possible. The foot was bent at a 90° angle, and the tissue was fixed in 4% paraformaldehyde overnight at 4°C to control for differences in muscle stretching. After sectioning, fibers were stained with hematoxylin and eosin and quantitated as previously described [121].

Western Blotting

Spinal cord or brain tissues were harvested at P5 from heterozygous or SMA pups (either scAAV9-SMN treated or untreated controls) and immediately frozen, as described above. Westerns were quantitated using Fujifilm MultiGauge software. Mouse monoclonal anti-SMN antibody (BD Biolabs; 1:2,000) and anti-IP90 polyclonal

rabbit antibody (1:2,000) were used for SMN and calnexin detection, respectively [138,157].

Production of the scAAV9-SMN Virus

scAAV9-SMN was produced via triple transfection in HEK 293T cells using polyethyleneimine prepared to a working concentration of 1 mg/ml, pH 5.0 [73]. The scAAV plasmid was constructed to express the open reading frame of SMN1 cDNA (NCBI accession number NM_000344) under the control of the chicken β -actin promoter. The vector was purified by two cesium chloride density-gradient ultracentrifugation steps and dialyzed against HEPES buffer. Viral particles were titered by quantitative PCR using SYBR Green.

In Vivo Injections

On P1, SMA ($Smn^{-/-};SMN2^{+/+}$) mice were injected with 2×10^{11} viral particles of scAAV9-SMN. Due to the volume restrictions of the ICV technique, only $\sim 7 \mu\text{l}$ can be injected into the ventricle at one time; therefore, two injections were given to obtain a titer of 2×10^{11} viral particles. Animals chosen for ICV injections were injected twice on P1, with at least 1 hr between injections. Animals chosen for IV injection received a single injection on P1. Injections were visualized for accuracy by the addition of filter-sterilized green food dye.

Results

SMN Protein is Significantly Elevated in the CNS of ICV and IV Treated SMA Mice

To determine whether scAAV9-SMN delivery is able to correct a severe model of disease after the initiation of disease symptoms, we used the *SMN2* model ($Smn^{-/-}$

;SMN2^{+/+}). These severe SMA mice are born symptomatic, having a marked decrease in motor neurons and weighing less than wild-type animals. The disease is so severe that many of the SMA embryos are absorbed *in utero* [163]. We began by administering 2×10^{11} viral particles of scAAV-SMN via ICV and IV temporal vein injections. Injections were administered within 24 hr of birth, and animals were weighed daily until death. Because SMA is primarily a central nervous system (CNS) disease, increasing SMN protein levels in the brain and spinal cord is desirable. To determine if ICV and IV administration of scAAV9-SMN increased SMN levels, we performed Western blots on extracts derived from brain and spinal cord tissue of scAAV9-SMN treated animals, untreated controls, and unaffected animals. Initially, it was important to provide a comparison with the untreated SMA animals; therefore, due to the short life span of this model, we selected a relatively early time point, P5. As expected, SMN levels are very low in untreated animals in both the brain and spinal cord extracts (**Figure 4.1**). In each of the brain and spinal cord extracts from ICV and IV treated animals, significantly higher levels of total SMN protein were detected at P5 compared with untreated controls. In the brain (**Figure 4.1A**), SMN protein expression in the heterozygous animals (*Smn*^{+/-};SMN2^{+/+}) was nearly five fold higher than that in untreated SMA animals (*Smn*^{-/-};SMN2^{+/+}), whereas ICV and IV treated SMA animals displayed a five fold increase over untreated SMA controls. In the spinal cord, ICV and IV treated extracts contained nearly six and four fold more SMN protein over untreated SMA levels, respectively. These results demonstrate that the scAAV9-SMN vector can be efficiently delivered and used

Figure 4.1 SMN protein expression was increased to near-normal levels following treatment with scAAV9-SMN. Western blots of (A) brain tissue and (B) spinal cord collected from P5 animals injected on P1 with 2×10^{11} viral particles were performed. Representative blots from at least three repeat experiments are shown. Controls were untreated SMA ($Smn^{-/-};SMN2^{+/+}$) and unaffected animals ($Smn^{+/-};SMN2^{+/+}$).

Figure 4.2 Treatment with scAAV9-SMN resulted in increased muscle fiber size. **(A)** Average tibialis anterior muscle fiber size (in μm^2) at P5 following treatment with 2×10^{11} viral particles. An unpaired two-tailed Student's *t* test showed a significant difference between untreated SMA animals and unaffected animals ($p=0.020$) and unaffected animals and IV treated animals ($p=0.031$). Representative muscle cross sections (10×objective) of **(B)** ICV treated ($n=2$), **(C)** IV treated ($n=4$), **(D)** untreated SMA ($Smn^{-/-};SMN2^{+/+}$) ($n=5$), and **(E)** unaffected animals ($Smn^{+/-};SMN2^{+/+}$) ($n=5$) are shown.

to express high levels of SMN in disease-relevant tissues, even in the context of an advanced symptomatic stage of disease.

Muscle Fiber Size is Moderately Increased in Treated Animals

To determine if the increase in SMN resulted in improved muscle morphology in the treated animals, we harvested animals at P5 to examine the average size of their tibialis anterior muscle fibers. Although there is minimal improvement in IV treated animals, ICV treated animals display a slight improvement in muscle fiber area (**Figure 4.2A**). From the muscle sections, we were able to see that the tibialis anterior muscle was generally smaller in size in the untreated animals as compared with that in the treated and unaffected animals (**Figure 4.2B–E**). Despite the improvement in muscle fiber size in the treated mice, gross motor function was not dramatically improved as animals living past 10 days were able to walk only short distances before fatiguing (data not shown).

scAAV9-SMN Treated Animals Gain Significantly More Weight

Untreated SMA animals gain minimal body weight during their life span. They are typically born at ≈ 1.5 g and reach a maximum weight of ≈ 2.2 g, averaging a weight gain of $\approx 19\%$ (**Figure 4.3**). In contrast, ICV and IV treated animals were able to achieve 3-4 g on average, and in some instances exceeded 4 g. From P3 to their peak weight, ICV treated animals gained an average of 85% of their body weight and IV treated animals gained 27%, compared with the $\approx 19\%$ increase of the untreated SMA controls (**Figure 4.3B**).

scAAV9-SMN Treated Animals Live Significantly Longer

In addition to being weighed daily, the animals were monitored for survival. The animals that received the scAAV9-SMN via ICV and IV injections lived significantly longer than the untreated controls (**Figure 4.4A** and **Figure 4.5**). Consistent with the weight analysis, animals that received the scAAV9-SMN vector via an ICV administration exhibited a significant extension in survival compared with the animals that received the same total number of scAAV9-SMN viral particles via an IV injection (**Figure 4.4A**). ICV treated animals lived an average of 17 days, with the longest lived animals living beyond weaning age. The IV treated animals lived an average of 10 days with a maximum life span of $\approx$ 17 days. Consistent with previous reports, the untreated SMA animals had an extremely short life span of $\approx$ 7 days (**Fig. 4.4B**). Although the treated animals lived significantly longer than the untreated animals, we did not see a complete rescue of the SMA phenotype as was previously reported in the SMN Δ 7 model. These results demonstrate that scAAV9-SMN vector can profoundly impact the progression of disease in this extremely severe model that is symptomatic at birth; however, a complete rescue does not occur using scAAV9-SMN viral titers that effectively rescued the SMN Δ 7 mouse model.

Discussion

Increasing SMN protein levels in the CNS is the most straightforward approach when designing SMA therapeutics. Based on the unique opportunities inherent to the existence of *SMN2*, many therapeutic strategies have focused on modulating *SMN2* gene expression, including modulating exon 7 splicing, increasing transcription, stabilizing *SMN2* mRNA, and/or increasing the stability of the *SMN2*-derived protein

Figure 4.3 scAAV9-SMN treated animals gained more weight than untreated SMA animals. **(A)** Average weight (g) of animals at each day of life. Each treatment group is significantly different from the others by one-way ANOVA ($p < 0.0001$). Tukey's multiple comparison test: untreated vs. ICV, $p < 0.01$; IV vs. ICV, $p < 0.001$. **(B)** Percent body weight gained from P3 to peak weight of untreated, IV treated, and ICV treated SMA animals ($Smn^{-/-};SMN2^{+/+}$). Unpaired two-tailed Student's t test: untreated vs. ICV, $p = 0.007$; IV vs. ICV, $p = 0.011$.

Figure 4.4 IV and ICV scAAV9-SMN–treated animals lived significantly longer than untreated SMA animals. **(A)** Kaplan-Meier survival curve of untreated, IV treated, and ICV treated SMA animals (*Smn*^{-/-}; *SMN2*^{+/+}). Log-rank (Mantel-Cox) test: untreated vs. IV, $p=0.027$; untreated vs. ICV, $p=0.002$; IV vs. ICV, $p=0.006$. **(B)** Average survival. Untreated, 7.25 days; IV, 9.3 days; and ICV, 17 days. Unpaired two-tailed Student's t test: untreated vs. ICV, $p=0.005$; IV vs. ICV, $p=0.016$.

[166]. Recently, therapeutics designed to increase SMN protein through gene therapy have resulted in dramatic extensions in survival. These studies have demonstrated that supplying exogenous SMN via a viral vector is a highly effective tool for treating an animal model of SMA [102,109-111]. To date, all of the published gene therapy studies have been conducted in the SMN Δ 7 mouse model, demonstrating that neonatal delivery of scAAV-SMN is able to rescue the SMN Δ 7 model to a near-normal weight, phenotype, and a range in survival from $\approx$ 65 to 200+ days. In each of these studies, the vector was administered pre-symptomatically, generally within 48 hrs of birth. Injecting at this early time point allows the viral genome to be expressed before the animals develop overt disease symptoms around P7 [65]. Remarkably, a delay in administering the vector resulted in a profound decrease in efficacy, such that administration of the vector at P5 resulted in a maximum survival of $\approx$ 30 days, and delivery after onset of symptoms had no effect [102].

Recent genetic experiments examined the requirement of SMN postsymptomatically. Using a Cre-lox system, SMN was induced at various time points pre- and postsymptomatically [168]. These results demonstrate that pre-symptomatic expression of SMN dramatically rescues the phenotype, whereas a significant benefit can still be achieved if induction initiates at P8. This is in contrast to the work with the gene therapy vectors that showed a narrower therapeutic window [102]. This distinction could be due to several variables between the experimental approaches, including the overall level of SMN induction in disease-relevant tissues, the inability of the vector to efficiently cross the blood–brain barrier at later time points, and the temporal

requirements of each organ system to achieve therapeutically relevant levels of SMN expression. As anticipated, in the *SMN2* mouse model, our results strongly suggest that the therapeutic window that would lead to a complete rescue of the SMA phenotype is likely inaccessible after birth.

This is the first time ICV and IV administrations have been examined using the same viral vector, scAAV9-SMN. Our results demonstrate that the ICV mediated delivery is more efficient in partially rescuing the SMA phenotype. As SMA is primarily a disease of the CNS, this is not altogether surprising. The ICV injection physically bypasses the blood–brain barrier; therefore, a high dose of vector is present immediately within the CNS. Additionally, scAAV9 has a broad cellular tropism, and a significant fraction of the vector is likely absorbed in peripheral tissues following an IV injection. Therefore, IV injection likely results in less CNS-penetrating vector and a subsequent lesser rescue of the primary motor neuron defect. The *Smn*^{-/-};*SMN2*^{+/+} model is extremely severe, and it is known that SMN expression is also necessary in the periphery. One possibility to explain the effectiveness of the ICV administration is that a fraction of the virus crosses the blood–brain barrier at P1, providing a low level of transduction in peripheral organs. Previous work with scAAV8 suggests that peripheral organs are important for a complete rescue. For example, when scAAV8-SMN was delivered via ICV injection, the extension in survival was not as long as scAAV9-SMN delivered via IV injection. Although the motor neuron uptake was high for scAAV8, it has been previously documented that cardiac tissue is poorly transduced by AAV8 vectors [169,170]. This likely contributes to the shortened life span of the ICV scAAV8-treated animals relative to IV treated animals

and highlights the importance of examining multiple delivery methods for a single vector. Our results demonstrate that, in a direct comparison of ICV and IV delivery of scAAV9-SMN, ICV delivery results in a more profound rescue of the SMA phenotype.

Here we demonstrate that injection of scAAV9-SMN provides significant therapeutic benefit in an extremely severe SMA model, indicating that postsymptomatic delivery of scAAV9-SMN can be highly beneficial. Despite this unprecedented extension of survival, we do not observe a full rescue to normal life span like that seen in the SMN Δ 7 model. It is possible that *in utero* delivery would be the only means to achieve a full rescue of the severe model. The extremely low levels of SMN present in this model also appear to impact development in a global manner; therefore, a complete rescue, other than at the genetic level, may not be achievable. From this and the previous studies noted, we conclude that pre-symptomatic treatment is clearly superior to postsymptomatic treatment. However, it is encouraging to see that postsymptomatic treatment still provides a dramatic reduction in disease severity.

CHAPTER FIVE: DEFINING THE THERAPEUTIC WINDOW IN A SEVERE MODEL OF SMA USING SCAAV9-SMN

The work presented in this chapter was conducted equally between J. Glascock and K. Robbins.

Abstract

Spinal muscular atrophy (SMA) is caused by the loss of a single gene, *Survival Motor Neuron-1 (SMN1)*, which results in the rapid deterioration of motor neuron integrity and function, most often leading to infantile death. Administration of self-complementary adeno-associated virus expressing full-length SMN cDNA (scAAV-SMN) has proven an effective means to rescue the SMA phenotype in SMA mice, either by intravenous (IV) or intracerebroventricular (ICV) injection at very early postnatal time points. We have recently shown that ICV delivery of scAAV9-SMN is more effective than the same dose of vector administered via an IV injection, thereby providing an important framework to examine a timeline for rescuing the disease and determining the therapeutic window. SMN Δ 7 mice were injected with scAAV9-SMN via ICV injection on a single day from day 2 (P2) through day 8 (P8). At each delivery point, scAAV9-SMN decreased disease severity, ranging from a near complete rescue (P2) to a significant, albeit to a lesser degree, improvement in which animals lived ~150% longer (P8). While our study demonstrated that a maximal benefit is obtained when treatment is delivered during pre-symptomatic stages, significant therapeutic benefit was still achieved after the onset of symptoms.

Introduction

Spinal muscular atrophy (SMA) is caused by the functional, homozygous loss of *Survival Motor Neuron-1*, *SMN1* [9]. In humans there has been an inversion and duplication event such that a nearly identical copy gene, *SMN2*, exists [10]. However, *SMN2* cannot compensate for the loss of *SMN1* due to a silent C to T transition at the 5' end of exon 7 [11]. This change causes a mis-splicing event such that the vast majority of pre-mRNA transcripts from *SMN2* lack exon 7 [15-17]. Subsequently, *SMN2* produces low levels of full-length SMN protein and primarily generates the alternatively spliced isoform, SMN Δ 7 [11]. The SMN Δ 7 protein is very unstable and is rapidly degraded leaving it unable to protect from SMA. Low levels of the ubiquitously expressed full-length SMN protein result in the preferential loss of alpha motor neurons, a hallmark pathology of SMA .

Due to its monogenic nature, vector-based gene replacement is a viable therapeutic for the treatment of SMA. Of the many therapeutics tested in animal models of SMA to date, scAAV9-mediated *SMN1* replacement has resulted in the most profound improvements in lifespan and motor phenotype. When delivered within 48 hours of birth, scAAV9-SMN is able to completely rescue the lifespan of the SMN Δ 7 mouse model extending survival from ~14 to 200+ days [102,109-111]. Of the AAV serotypes investigated for the treatment of SMA, AAV9 has proven to be the most efficacious as it is able to cross the blood brain barrier when delivered systemically and transduce a variety of neuronal lineages, including motor neurons [89].

Unfortunately, because SMA is not on the newborn screening panel, human SMA patients often remain undiagnosed until the onset of symptoms. Therefore, the success

of any therapeutic which reaches the clinic is contingent upon the stage of disease at which increased SMN levels are still able to produce a functional benefit. Thus, understanding the temporal requirements of SMN during disease progression is of utmost importance for the design and development of effective therapeutics. It is important to determine if the therapeutic window is limited to pre-symptomatic stages of disease or if treatment after the onset of symptoms and extensive cellular pathology is still beneficial.

As scAAV9-SMN has been the most successful therapeutic tested in animal models and has been approved for clinical trials slated to begin in early 2014, we have chosen to use it as our therapeutic agent of choice for examining the therapeutic window. Using the SMN Δ 7 mouse model [65], we delivered scAAV9-SMN via ICV injection at sequential time points, from 48 hours post birth to after the onset of symptoms. We chose a dose of $1e^{11}$ viral genomes (vg)/animal as this dose has been shown to rescue lifespan when given at P2 [102]. Each animal was injected at a single time point, P2 through P8. Early SMN induction provided the most benefit whereas injection at later time points decreased the efficacy of this therapy. Importantly, all treated mice lived significantly longer than non-injected controls demonstrating that increasing SMN does confer a functional benefit after the onset of symptoms. However, SMN replacement at the earliest time point (P2) provided the most robust phenotypic rescue with the fewest early deaths.

Our study provides an exhaustive investigation into the therapeutic window of opportunity in the SMN Δ 7 mouse model. On one hand, it highlights the importance of

early, pre-symptomatic intervention for maximal therapeutic benefit. On the other hand, it provides encouraging, clinically relevant evidence that treatment after the onset of symptoms can still significantly mitigate disease progression, albeit to a lesser degree.

Material and Methods

Animal Handling and Genotyping

All animals were housed and treated according to the guidelines of the Animal Care and Use Committee at the University of Missouri. Pups were genotyped on their day of birth as previously described [155].

Virus Production

scAAV9-SMN viral vector was produced as described previously [73]. HEK293T cells were triple transfected in the presence of polyethyleneimine (PEI) (1mg/ml), pH 5.0. The scAAV plasmid was constructed to express the open reading frame of human *SMN1* cDNA (NCBI accession number NM_000344) under the control of the CAG promoter. scAAV9-SMN viral vector was purified using three cesium chloride density-gradient centrifugation steps. Quantification of viral genomes was performed using SYBR[®] Green and primers to amplify the promoter region, forward (5'-CCGGTGGTGGTGCAAATCAAAGAA-3') and reverse (5'-AGCAGAAGTAACACTTCCGTACAGGC-3').

Intracerebroventricular Injection of scAAV9-SMN or scAAV9-GFP

Intracerebroventricular (ICV) injection was performed on neonates to administer scAAV9-SMN therapy directly into the CNS as previously described [149]. Mice were

administered a single injection of $1e^{11}$ vg on one of the following days, P2 through P8. Briefly, a glass-pulled needle was inserted through the frontal plate (lateral to the metopic suture and rostral to the coronal suture) into the left or right ventricle of the neonatal mouse to deliver a 5 μ l bolus of viral vector. Unaffected animals were injected with scAAV9-GFP on P2 or P7.

Phenotypic Assessment

All treated and untreated mice were monitored daily to evaluate survival, weight gain and motor function as assessed by the time to right test. The time to right test consists of placing a mouse on its back and determining the time it takes for them to right themselves onto all four paws with a maximum allotted time of 30 seconds.

Tissue Collection

To harvest tissues, mice were euthanized using isofluorine followed by cervical dislocation. Brain, spinal cord, skeletal muscle, heart, kidney, liver, and spleen were collected and immediately flash-frozen in liquid nitrogen for those tissues used for protein analysis or submerged in 4% paraformaldehyde if used for histological examination of GFP distribution.

Western Blotting

Tissues were collected at specified time points and immediately frozen in liquid nitrogen. SMN immunoblots were performed using a mouse monoclonal anti-SMN antibody (BD Biosciences; 1:2000) and anti- β -actin rabbit antibody (Sigma; 1:2000) [138,157]. Western blot analysis was performed in triplicate and representative blots are shown. Westerns were quantitated using Fujifilm MultiGauge software.

Peripheral Viral Expression Analysis

Unaffected animals were injected with scAAV9-GFP on P2 or P7. Unaffected animals not injected with the GFP virus were used as a negative control. All tissues harvested for peripheral transduction analysis were harvested on P6 or P11.

Results

ICV Injection of scAAV9-SMN Increased Survival of SMN Δ 7 Mice

A single, ICV injection of 1×10^{11} vg was able to significantly extend the lifespan of all treatment groups (**Figure 5.1A**). As expected, earlier treatment resulted in a more robust lifespan rescue. Pups injected on P2 experienced no early deaths and all mice lived past 130 days. As treatment was delayed, the average lifespan steadily declined (**Figure 5.1B**). Overall, mice injected with scAAV9-SMN at earlier time points had a greater extension in survival compared to mice injected at later time points. Mice from all groups lived significantly longer than non-injected SMA controls which lived on average, 13 days with a median survival of 14 days (**Figure 5.1C**).

ICV Injection of scAAV9-SMN Increased Weight Gain of SMN Δ 7 Mice

Weight gain was analyzed as the average weight for a given group across days (**Figure 5.2A**) and also as the average percent weight gained from birth to peak (**Figure 5.2B**). A statistical comparison of weight gain between groups is described in the *p*-table in **Figure 5.2C**. At birth, all SMA pups and unaffected pups were similar in weight and there were no obvious phenotypic differences until around day 7 (**Figure 5.3**). However, by P11 all treated mice weighed significantly more than non-injected SMA animals. Even the late injected mice (P7 and P8) weighed significantly more, although this difference

C

	P2	P3	P4	P5	P6	P7	P8	NI
P2		0.0000	0.1090	0.0136	0.0402	0.0000	0.0000	0.0000
P3	***		0.0432	0.0000	0.7188	0.0000	0.0000	0.0000
P4	n.s.	*		0.0755	0.1348	0.0000	0.0000	0.0000
P5	*	n.s.	n.s.		0.9950	0.0181	0.0000	0.0000
P6	*	n.s.	n.s.	n.s.		0.0811	0.0004	0.0000
P7	****	****	****	*	n.s.		0.0002	0.0001
P8	****	****	****	****	***	***		0.0001
NI	****	****	****	****	****	****	****	

Figure 5.1 ICV injection of scAAV9-SMN significantly increases the survival of SMN Δ 7 mice when administered at early time points. **(A)** Kaplan-Meier survival curve of untreated SMN Δ 7 mice and those injected with $1e^{11}$ vg of scAAV9-SMN on a single day P2-P8. **(B)** Symbols represent minimum, maximum, and median survival of mice in each group. Line represents the average survival. **(C)** *P*-table comparing all treated and untreated groups. *P*-values were calculated using the logrank Mantel-Cox test. NI = non-injected. (*n.s.* no significance, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$)

C

	P2	P3	P4	P5	P6	P7	P8	NI
P2		0.2203	0.1395	0.0000	0.0000	0.0000	0.0000	0.0000
P3	n.s.		0.6311	0.0001	0.0001	0.0000	0.0000	0.0000
P4	n.s.	n.s.		0.0010	0.0009	0.0000	0.0000	0.0000
P5	****	****	***		0.8927	0.1701	0.0118	0.0056
P6	****	****	***	n.s.		0.2081	0.0095	0.0042
P7	****	****	****	n.s.	n.s.		0.1236	0.0474
P8	****	****	****	*	**	n.s.		0.0027
NI	****	****	****	**	**	*	**	

Figure 5.2 SMN Δ 7 mice treated with scAAV9-SMN gain weight throughout their lifespan.

(A) The average weight per group on each day. n_i = initial number of animals in each group. (B) Percent weight gained from birth to peak. SMN Δ 7 mice treated with scAAV9-SMN gain more weight than non-injected controls. (C) *P*-table demonstrating statistically significant differences in average weight gained from birth to peak of all mice in the treated and non-treated groups. Student's t-tests were performed to compare the average percent weight gained from birth to peak between groups. (n.s. no significance, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$, **** $p \leq 0.0001$).

Figure 5.3 Representative images of SMNΔ7 mice. **(A)** Phenotypic differences are not apparent at birth and the pups of the various genotypes cannot be distinguished by observation. Unaffected ($Smn^{+/+};SMN2^{+/+};SMN\Delta7^{+/+}$), Heterozygous ($Smn^{+/-};SMN2^{+/+};SMN\Delta7^{+/+}$), and SMA ($Smn^{-/-};SMN2^{+/+};SMN\Delta7^{+/+}$). **(B)** At P9, it can be readily observed that the pup injected at the earliest time point (P2) is comparable to the unaffected heterozygous littermate and the pups injected at the latest time points (P7 and P8) are smaller.

was not as great as the difference observed between early injected mice and the non-injected SMA controls. Around P10, the weight of P8 and non-injected SMA mice peaked and declined from thereon. By P14, P8 injected animals weighed significantly less than mice from all other treatment groups.

All treated animals exhibited a significantly higher percent weight gain from birth to their peak weight compared to non-injected SMA animals. (**Figure 5.2B**). Treated animals tended to fall into two groups. Those injected on P2, P3 or P4 displayed similar weight gain from birth to peak, and there was no significance when compared with each other. Similarly, animals injected on P5, P6 or P7 had comparable percent weight gain and also did not show significance when compared to one another.

Early Treatment Improved Motor Function in Mice Injected with scAAV9-SMN

Mice have a natural instinct to right themselves onto all four paws immediately after being placed on their back. Young, healthy neonates achieve this ability within the first week of life [159]. Thus, observing this ability in mice serves as an early test of motor function, known as the time-to-right test. Mice were given 30 seconds to perform this test. Over time, a greater percentage of animals from groups P2 through P7 gained the ability to right themselves. However, this was not the case for the untreated animals and those injected at the latest time point, P8 (**Figure 5.4A**).

Of the mice that are able to right themselves, the early-injected animals tended to right faster than those injected at later time points and the non-injected SMA controls. On P14, righting time increased with delayed treated and untreated animals were no longer able to right themselves within the 30 seconds allotted (**Figure 5.4B**).

Overall, earlier treatment produced more robust motor function improvements as measured by time-to-right.

Increased SMN Protein Was Observed in Animals Treated with scAAV9-SMN

SMN Δ 7 mice were injected on P2 or P7 and their tissues harvested on P11. As expected, P2 injected mice had equal or higher levels of SMN protein as compared to unaffected mice due to the large dose of viral particles injected (**Figure 5.5A-D**). Mice injected on P2 exhibited significantly higher SMN levels in the brain and spinal cord compared to P7 injected mice (**Figure 5.5A,B**). Both P2 and P7 injected animals had significantly more SMN protein than age-matched, non-injected SMA control mice. Robust SMN protein induction was also observed in peripheral organs such as the heart (**Figure 5.5C**). In the heart tissue, both P2 and P7 injected animals express significantly more SMN than non-injected controls ($p = 0.0006$ and $p = 0.0079$, respectively). Furthermore, scAAV9-SMN administration on P2 resulted in significantly higher SMN levels in the heart compared with the P7 group ($p = 0.0023$). The same pattern of induction was observed in muscle, with P2 injected animals displaying more robust SMN protein levels than P7 treated mice (**Figure 5.5D**).

It is possible that the P2 treatment group had more robust protein, as compared to the P7 group, because the virus was allowed to express for 5 days longer. To address this, P2 treated animals were harvested on P6 in order to compare protein levels after equal times of viral expression in both the P2 and P7 groups. The robust protein levels observed in P2 injected animals harvested at P11 were still observed in P2 injected mice harvested at P6 (**Figure 5.5E,F**).

Figure 5.4 Animals treated at early time points gain the ability to right themselves prior to animals injected at later time points. **(A)** Percentage of animals able to right themselves. **(B)** Individual time to right on P14. At two weeks, earlier treated animals perform better on the time to right test compared to those injected at later time points. There is a high degree of variability observed within groups on day 14; where some mice are able to right themselves more quickly than others, and some animals never gain the ability to turn.

A

B

C

D

Figure 5.5 SMN protein induction in SMN Δ 7 mice injected at early and late time points. SMN protein levels in (A) brain, (B) spinal cord, (C) heart, and (D) muscle of mice injected with scAAV9-SMN at P2 and P7 and harvested at P11. To account for potential differences in time of viral expression, additional animals were injected on P2 and harvested on P6 and protein levels were examined in (E) brain and (F) spinal cord. Quantification of the Western blots ($n = 3$ for each group) is shown as the average SMN/actin ratio.

Peripheral Distribution of scAAV9-SMN and Transduction Analysis

To determine the extent to which scAAV9-SMN traverses the blood-brain-barrier (BBB) and is distributed throughout the periphery, we utilized the scAAV9-GFP vector. Unaffected mice were injected with scAAV9-GFP on P2 or P7 and the heart, liver, spleen, and kidney tissues were harvested on P6 or P11 (**Figure 5.6**). Results confirm that scAAV9 is well distributed throughout the periphery with especially strong GFP expression in the liver and heart tissues after both P2 and P7 injections. This indicates that scAAV9-SMN is able to escape the BBB, even when injected at later timepoints.

Discussion

SMA is caused by low levels of SMN protein [11]. In humans, the *SMN2* gene, despite its inability to compensate for the loss of *SMN1*, is a critical disease modifying gene as it is able to produce low levels of full-length SMN protein [2]. Therapeutic approaches for the treatment of SMN can be broadly categorized into two groups: those that modulate *SMN2* splicing to generate more full-length transcripts and direct SMN replacement. Designing an effective therapeutic using either strategy requires knowledge of the tissue-specific and temporal requirements for SMN.

In an effort to elucidate the latter, we performed a thorough analysis of SMN replacement via scAAV9 at sequential postnatal timepoints in the *SMN Δ 7* mouse model of SMA. First, we confirmed that SMN replacement is able to fully rescue this model when given on P2 as has been previously reported [102]. Second, we observed that as treatment is delayed, the resulting phenotypic benefit achieved is diminished. However,

Figure 5.6 Unaffected mice injected with scAAV9-GFP exhibit substantial GFP expression in peripheral organs when injected on either P2 or P7.

even when treatment was administered when overt SMA-like symptoms were present (P8) a significant, albeit lesser, phenotypic improvement was seen.

It is encouraging to see that our investigation into the therapeutic window produced similar results to transgenic approaches previously described. In an inducible transgenic model, whereby SMN is induced by administration of doxycycline, embryonic or early postnatal induction of SMN resulted in a dramatic rescue of SMA mice. Conversely, later induction (P6) resulted in a significantly reduced rescue suggesting that there is a critical window in which SMN must be expressed in order to effectively rescue [171]. Similarly, in a mouse model harboring a cre-inducible SMN rescue allele, early induction (P4) of SMN resulted in the most profound rescue. Induction at later time points (P6,P8,P10) resulted in a decreasingly substantial phenotypic rescue, with mice receiving SMN induction at P10 not being significantly different than SMA mice in terms of lifespan [168].

Other therapeutic approaches to investigate temporal requirements of SMN have shown similar results as well. However, those conducted have yielded similar results to our study and the aforementioned transgenic approaches. A study investigating scAAV9-SMN IV delivery at four postnatal timepoints (P1, P2, P5, and P10) in the SMN Δ 7 model showed that injection at P1 and P2 produced a full lifespan rescue. At P5 the rescue is much less pronounced. By P10 delivery of scAAV9-SMN no longer produces any phenotypic benefit [102]. The key difference between this study and ours lies in the injection technique as the same viral dose was used (1e11 vg). ICV injection at

P5 resulted in ~30% of mice surviving past 100 days, while IV injection produced a maximum lifespan of 30 days highlighting the importance of route of delivery [112,113].

In the severe mouse model, in which symptoms are evident at birth, delivery of 2e11 vg at P1 produces a significant extension of lifespan (7.5 to 17 days) [113]. Thus, delivery of scAAV9-SMN after the onset of symptoms in both the SMN Δ 7 and severe models is able to modestly improve survival; however, not to the same extent as delivery prior to symptom onset.

Another therapeutic approach, antisense technology, is often utilized in SMA research to increase SMN protein levels by modulating *SMN2* splicing. Antisense oligomers (ASOs) to block an intronic splice silencer, ISS-N1, have been demonstrated to significantly increase full-length *SMN2* transcript levels and SMN protein in the brain and spinal cord of the SMN Δ 7 model [125,172]. When the ISS-N1 ASO was administered by ICV injection on P1, survival was extended from 15 to 100 days. However, delaying delivery until P4 decreased the efficacy of the antisense therapy. Furthermore, IV administration on P1 resulted in survival comparable to P1 ICV treatment. However, when delivery was delayed until P4, the IV injected group exhibited decreased survival compared to the ICV treated group.

Taken together, these studies highlight the need for early, pre-symptomatic, therapeutic intervention in order to achieve maximum therapeutic benefit. However, in the extent that these results can be extrapolated to clinic, our study and the others mentioned offer hope for therapeutic benefit even in symptomatic patients.

Understanding why scAAV9-SMN efficiency diminishes when treatment is

delayed would shed light on disease pathogenesis and aid in the development of AAV vectors as therapeutics. One possibility is the inability of the virus to escape from the CNS following an ICV injection at later timepoints. As many peripheral organs and tissues have been reported to be affected in both SMA patients and animal models (for review see[30]), the ability scAAV9 to cross the BBB is likely necessary to achieve a full lifespan rescue. It is known that scAAV9 crosses the blood-brain-barrier (BBB) in P2 neonatal mice [90]. However, the ability of scAAV9 to permeate the blood-brain-barrier at later timepoints is not known. To investigate this, we injected scAAV9-GFP into unaffected animals and observed GFP expression in peripheral organs (**Figure 6**). Our findings demonstrate the even when scAAV9-GFP is delivered on P7, robust GFP expression in all peripheral organs examined is observed. Thus, inability of the virus to cross the BBB is not the reason for the lack of rescue observed in mice treated at later timepoints.

As low levels of SMN result in the specific loss of lower motor neurons, SMN replacement in those cells is critical for AAV-mediated gene therapy. Previous studies have shown that following a P2 IV injection of scAAV9-GFP, approximately 42% of lumbar spinal motor neurons were transduced [90]. The number of motor neurons transduced after ICV injection at P2 is unknown. However, it is expected that transduction levels would be equal to or more than that observed following an IV injection. It is not known if or how transduction levels change as treatment is delayed. Reduced motor neuron transduction could explain a lack of phenotypic improvement when treatment is delayed.

Additionally, the extent of motor neuron loss in the SMN Δ 7 model reported in the literature varies greatly. Some publications report no motor neuron loss at P4 [65] while others observe up to a 50% reduction in motor neurons at the same timepoint [27]. Even if motor neurons are present, they may be functionally compromised. Therefore, it remains a possibility that an insufficient population of functional motor neurons are available at later timepoints, rendering SMN replacement less beneficial.

Another possibility for the lack of rescue may be the accumulation of irreversible neuromuscular junction (NMJ) defects. At P2, NMJs in SMA and unaffected littermates are indistinguishable. However, by P5 ~25% of all nerve terminals in SMA mice appear thick and swollen with abnormal NF aggregates. As the animals age, there is a progressive increase in the number of such defective terminal axons, reaching ~90% by P14. Additionally, SMA axons fail to form the fine terminal arbors characteristically found as early as P8 at normal NMJs. There have been varying reports as to the appearance of unoccupied endplates in SMA animals, with some groups reporting significant denervation and other reporting a lack thereof [67,173]. However, the absence of anatomically denervated endplates in SMA mice does not suggest that these endplates have maintained their functionality. Further investigation will be needed to determine if these NMJ defects contribute to the inability of sAAV9-SMN to rescue when administration is delayed.

Understanding the underlying reason as to the insufficiency of later therapeutic treatment to fully rescue may aid in the development of new therapeutics and the

betterment of existing ones. Nonetheless, early therapeutic intervention may be critical for therapeutic success.

CHAPTER SIX: RESTORATION OF SMN TO EMX-1 EXPRESSING CORTICAL NEURONS IS NOT SUFFICIENT TO PROVIDE BENEFIT TO A SEVERE MOUSE MODEL OF SPINAL MUSCULAR ATROPHY

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Abstract

Detailed analysis of neuromuscular junctions in SMA mice has revealed a selective vulnerability in a subset of muscle targets, suggesting that while SMN is reduced uniformly, the functional deficits manifest sporadically. Additionally, in severe SMA models, it is becoming increasingly apparent that SMA is not restricted solely to motor neurons. Rather, additional tissues including the heart, vasculature, and the pancreas contribute to the complete SMA-associated pathology. Recently, transgenic models have been utilized to examine the tissue-specific requirements of SMN, including selective depletion and restoration of SMN in motor neurons. To determine whether the cortical neuronal populations expressing the Emx-1 promoter are involved in SMA pathology, we generated a novel SMA mouse model in which SMN expression was specifically induced in Emx-1 expressing cortical neurons utilizing an Emx-1-Cre transgene. While SMN expression was robust in the central nervous system as expected, SMA mice did not live longer. Weight and time-to-right motor function were not significantly improved.

Introduction

Recently, SMA animal models have been developed to examine the temporal and spatial role of SMN in SMA development. The human skeletal actin (HSA) and prion promoters

(PrP) were used to drive SMN expression in severe SMA mice that typically die within the first week of life [65]. High levels of muscle expression from the HSA promoter failed to alter the life span of the animals; however, SMN expression driven by the pan-neuronal PrP cassette significantly extended survival beyond 150 days. The PrP cassette is widely expressed in the central nervous system including a broad range of neurons as well as astrocytes. Olig2-Cre mediated depletion of SMN within motor neurons and oligodendrocytes in an otherwise wildtype background led to an SMA phenotype that significantly impacted skeletal muscle and motor neuron pathology as well as motor neuron function [175]. Interestingly, however, Olig2-Cre mediated depletion of SMN did not lead to an early death similar to several SMA mouse models that express uniformly low SMN levels systemically. Hb9-driven restoration of SMN in motor neurons significantly improved the neuromuscular junction phenotypes in severe SMA mice; however, survival in Hb9-rescued mice was only extended ~5 days, to a total lifespan of ~2 weeks [26]. Similarly, Nestin-mediated restoration of SMN in neuronal populations (including motor neurons) does mitigate central and peripheral synaptic defects, however, the average life span is only extended from ~2.5 days to ~10 days [176]. These results suggest that while motor neurons are clearly important, additional cellular populations could contribute to the complex SMA pathology. Consistent with this, in severe SMA mice, a severe and progressive defect is present in proprioceptive reflexes that correlate with decreased function and number of synapses on motor neuron somata and proximal dendrites [27]. These results and the identification of peripheral defects in SMA mice [162,163,177-179] demonstrate that the comprehensive

pathological picture within severe SMA contexts is remarkably complex and will likely encompass not only motor neurons, but additional cells within the central nervous system as well as the periphery.

To further examine the role of SMN within the central nervous system, we generated a novel SMA mouse model in which SMN expression was specifically restored in cortical neurons utilizing an Emx-1-Cre transgene [180,181]. While SMN was expressed highly as anticipated, the overall pathology and survival of the SMA-Emx1-Cre mice was not significantly altered. While Emx-1 mediated induction of SMN does not alter the SMA phenotype, this work further delineates tissues which are and are not responsible for the complex SMA pathology.

Materials and Methods

Genotyping and Mouse Handling

Animals were handled according to the University of Missouri Animal Care and Use Committee approved protocols. Mice were bred according to the schematic presented in (**Figure 6.1B**). The day of birth was counted as P1 with neonates being genotyped within 24 h. Animals were genotyped using PCR conditions as previously described [155]. SMA mice were raised with 2 heterozygous siblings. Additional heterozygous and wild-type animals were culled at the time of injection in experimental cages to control for litter size.

Tissue collection for PCR

In order to harvest tissue for DNA purification, the vertebral column was separated from the torso and the spinal cord was removed. The brain was dissected

from the skull and divided into four equal sections. Total DNA was purified from the tissues using the Qiagen DNeasy Blood and Tissue Kit.

Western Blot Analysis

Tissues were harvested at indicated times and analysis was performed as previously described [138,157]. Mouse monoclonal anti-SMN (BDBiolabs), 1:2,000, was used for SMN detection. Equal protein amounts were loaded as analyzed by Bradford protein assay [182].

Motor Function Analysis

Time to right was measured from P5 to death. Mice were placed on their backs and given a maximum of 30 s to right themselves on to their paws. Inability to right within 30 s was considered failure.

Results

To determine if restoration of SMN levels in a subset of neurons, specifically Emx-1 expressing cortical neurons, impacted disease development in a relatively severe model of SMA we utilized a previously developed mouse strain that expresses a targeted mutation within the *Smn* gene (JaxStock#7951). The *Smn* gene has been modified such that it consists of *Smn* genomic sequence fused to the 3' end of the human *SMN2* gene, allowing for a Cre-mediated recombination event that replaces the human *SMN2* exon 7 sequence with the murine *Smn* exon 7 sequence [168]. The recombined allele effectively restored full-length SMN expression in a tissue-specific manner and does not generate the typical *SMN2* alternative splicing event. In the current model, the Emx-1 promoter was used to drive expression of Cre recombinase (JaxStock#5628) [180,183,184],

resulting in a restoration of full-length SMN protein in cortical neurons. In all other tissues, in the absence of Cre recombinase, the hybrid allele results in an *mSmn* gene analogous to human *SMN2* and produces low levels of full length SMN protein. This promoter drives the expression of Cre within the cortical neurons of the motor cortex, but not in the lower motor neurons of the spinal cord (**Figure 6.1A**). Mice homozygous for the non-recombined targeted mutation display a phenotype similar to that of the well-characterized *SMN Δ 7* mouse model (JaxStock#5025) [65]. Thus, the experimental mice express wild type levels of SMN only in the cortical neurons of the brain in the presence of Cre recombinase (**Figure 6.1B**).

To ensure that the Cre-mediated recombination event has in fact occurred in the experimental mice, PCR was used to identify the unique recombined genomic element (**Figure 6.2A,B**). As anticipated, the recombination event was easily detected in brain tissue in mice expressing Cre recombinase. Conversely, recombination was not detected in spinal cord tissue even in the presences of Cre recombinase, indicating that *Emx-1* Cre promoter was not expressing in lower motor neurons. To examine SMN levels following the recombination event, Western blot analysis was performed on brain extract from animals using an anti-SMN monoclonal antibody (**Figure 6.2C**). SMN levels were expectedly low in the SMA animals (*mSMN^{Tm3/Tm3}*, Cre⁻) and high in the unaffected control extracts (*mSMN^{Tm3/WT}*, Cre⁻), while SMN was increased in the experimental animals expressing Cre (*mSMN^{Tm3/Tm3}*, Cre⁺), demonstrating that the recombination event was efficient and resulted in increased SMN protein levels.

To determine whether an increase in SMN in *Emx-1* expressing cortical neurons

Figure 6.1 Emx1-mediated SMN expression in cortical neurons. **(A)** Schematic showing the Tm3 allele in the presence and absence of Cre recombinase. In the presence of Cre recombinase, WT mSMN exon 7 is expressed, leading to full-length SMN production. **(B)** Breeding schematic demonstrating the generation of the experimental animals used in this study.

Figure 6.2 (A) PCR, using wild type and mutant specific primers, confirms that the Cre-mediated recombination event occurs in the brain of Cre⁺ animals. Conversely, no Cre-mediated recombination is seen in the spinal cord of these animals. (B) In Cre⁺ animals, there is a detectable inversion event in the brain resulting in a 170 bp product, distinguishable from the 143 bp wild type product. (C) Western blot showing protein expression is increased to near normal levels in the brain of SMA-Emx1-Cre; Tm3^{+/+} mice following Cre recombinase mediated SMN induction.

Figure 6.3 (A) SMA-Emx1-Cre; Tm3^{+/+} animals do not display any significant increase in total body weight at any time point when compared to SMA control animals. (B) There is no significant difference in peak weight between Cre⁺ and control SMA animals. Both have a peak weight of ~4g. (C) Onset of weight loss for both SMA-Emx1-Cre; Tm3^{+/+} and control SMA animals occurs at ~P10.

decreased disease severity in the SMA mice, well characterized components of the disease phenotype were examined. In the SMA mice, disease progression is very severe and animals rarely gain much weight, typically no more than 4 g total body weight. To determine whether SMA-Emx1-Cre; Tm3^{+/+} animals exhibited increased weight gain, animals were weighed daily from birth until death. At no time point was there a statistically significant difference in the average weight between the SMA control and Emx1-SMN transgenic animals (**Figure 6.3A**). To examine these data more closely, two additional factors were analyzed: the peak weight (**Figure 6.3B**) and the onset of weight loss (**Figure 6.3C**). In each instance, SMA control and experimental SMA-Emx1-Cre; Tm3^{+/+} animals initiated weight loss at approximately the same time point, P10, and both groups achieved the same peak weight of approximately 4 g. Collectively, these results demonstrate that while weight is an important component of the SMA phenotype, there was no statistically significant difference between SMA and SMA-Emx1-Cre; Tm3^{+/+} animals, even though high levels of SMN were present in cortical neurons.

In addition to poor weight gain, the control SMA mice have a severely shortened life span, averaging approximately 14 days (**Figure 6.4**). To determine whether increasing SMN in cortical neurons driven by Emx1 extended survival, the lifespans for SMA control and SMA-Emx1-Cre; Tm3^{+/+} animals were determined. While there were modest differences in the Kaplan-Meier curves, the average lifespans for the two groups were nearly identical and were statistically insignificant ($P = 0.66$) (**Figure 6.4**). Furthermore, the SMA-Emx1-Cre; Tm3^{+/+} animals did not exhibit increased motor

function as measured by time-to-right assays compared to their untreated SMA littermates (data not shown). These results demonstrate that while a growing list of tissues and cell types have been implicated as contributing factors to the complex SMA phenotype, repletion of SMN specifically within cortical neurons mediated by the Emx-1 promoter, is not sufficient to modify the SMA phenotype.

Discussion

Only in this type of controlled genetic experiment was it possible to ask whether SMN restoration in Emx-1 expressing cortical neurons is sufficient to decrease disease severity. While our results indicate that SMN restoration in Emx1-positive neurons does not reduce disease severity, they are not wholly unexpected as the phenotype of this novel animal model is consistent with the clinical descriptions of SMA regarding the role of cortical neurons [185,186]. In this study, we restored SMN expression in Emx-1 expressing cortical neurons on a constitutively low full-length SMN background, closely resembling the well-characterized severe model of SMA referred to as SMN Δ 7. It is possible, however, that by restoring SMN expression to Emx-1 expressing cortical neurons in a less severe model, this could result in phenotypic improvement as the SMN Δ 7 model likely represents a relatively severe subpopulation of SMA patients analogous to severe Type I individuals. Less severe models have recently been described that appear to be more amenable to SMN-independent therapeutic strategies, such as the ROCK inhibitor Y-27632 [144,187]. Alternatively, it is possible that other subpopulations of cortical neurons and/or glia that do not express Emx-1 could contribute to disease pathology.

Figure 6.4 Mantel-Cox survival curve demonstrating that there are no significant differences between the lifespan of Cre⁺ and control SMA animals (P = 0.66).

Recent publications confirm the need to continue to investigate the role of other neuronal and glial cell types, especially those present in brain regions that are particularly vulnerable to the low SMN levels present in SMA such as the motor cortex, hippocampus, and dentate gyrus [167]. Somewhat surprisingly, the specific restoration of SMN in motor neurons in SMA mice, or the specific depletion of SMN in motor neurons in wildtype mice, results in relatively mild changes in pathology [26,175]. These results suggest that either that the drivers are insufficiently restoring/depleting SMN, or, that other cells contribute to SMA pathology. It is also necessary to look for overall transmission defects in the motor circuitry composed of these cells. Perhaps, the cells populations of the corticospinal tract between the brain and spinal cord, such as cortical neurons, are more resistant to low SMN levels than those cells involved in the reflex arc between the muscle and spinal cord where massive and progressive transmission failure has been reported [27]. Additionally, recent reports have shown a loss of the central synapses of the spinal cord, highlighting the need for the examination of the glial cell populations, such as astrocytes and microglia, that have key supporting roles at these and other synapses [29]. Continuing to look at the role of different neuronal and glial cell populations will help to further unravel the mechanism behind SMA development in addition to delineating tissue targets for potential therapeutics.

CHAPTER SEVEN: IDENTIFICATION OF SMA DISEASE-MODIFYING GENES AND MUTANT FORMS OF SMN

Abstract

We and others have shown that the *SMN Δ 7* mouse model of SMA can be rescued by delivering exogenous SMN via self complementary Adeno Associated Virus (scAAV). These studies demonstrate that the scAAV9 vector is a valuable tool for delivery of genes to the central nervous system and, more specifically, to motor neurons. With this foundation, the scAAV vector provides a means to investigate the role of other genes and genetic modifiers thought to play a role in the pathogenesis of SMA. In this report, we utilize scAAV to deliver genes and mutant forms of SMN reported to potentially modify the SMA disease phenotype. By doing so, we identify potential novel therapeutic targets.

Introduction

Several studies in recent years have demonstrated the efficacy of *SMN1* gene replacement via AAV vectors [102,109-112]. These studies provide validation that gene delivery using scAAV9 is an effective way to target CNS cells, especially motor neurons [102]. Furthermore, they demonstrate that systemic scAAV9-SMN delivery results in robust SMN protein expression, capable of rescuing a severe SMA mouse model. Collectively, these works provide a foundation upon which further development of scAAV, both as a therapeutic and as a tool, can be built.

Interestingly, several genes and genetic modifiers have been suggested to play a role in the pathogenesis of SMA. Prior even to the identification of the *SMN* gene, there

were reports of SMA siblings with discordant phenotypes, all with some degree of clinical manifestations [188]. With the discovery of the *SMN1* gene, it was observed that unaffected siblings could have a homozygous deletion of *SMN1* just like their symptomatic siblings and that different types of patients were present in the same family [21,189-193]. We now know that *SMN2* is the most critical disease-modifying gene, as *SMN2* copy number strongly correlates with disease severity [2]. However, several papers have reported a lack of association between *SMN2* copy number and phenotype variability in siblings with homozygous deletion of or mutations in the *SMN1* gene [2,192,194,195]. In most of the families described, the less affected sibling was female. This lead may people to suggest that gender-related protective factors may influence the SMA phenotype. However, there have also been reports of discordant pairs of brothers and pairs of sisters. Furthermore, families where males were more severely affected than female siblings have also been documented [2,195]. The occurrence of discordant siblings argues heavily for the presence of modifying genes.

It has been hypothesized that Plastin 3, an F-actin bundling protein, is a gender-specific SMA modifying gene. In 2008, it was discovered that unaffected *SMN1*-deleted females have significantly higher Plastin 3 (PLS3) expression than their affected siblings [49]. PLS3 is important for axogenesis by increasing F-actin levels. Overexpression of PLS3 was shown to rescue the axon length and outgrowth defects associated with *SMN* down-regulation in primary SMA motor neurons and in zebrafish [49,196]. However, SMA mice, and likely human patients, do not have motor neuron axon outgrowth defects in vivo, but rather fail to develop and maintain mature NMJs (for review see

[197]). F-actin is involved in actin caging of synaptic vesicles of the reserve pool and their transport to active zones [198]. The generation of a conditional *PLS3*-overexpressing mouse and its breeding onto an SMA background aided in the elucidation of the biological mechanisms underlying *PLS3*-mediated SMA protection [199]. Overexpression of *PLS3* on a severe SMA background increased motor neuron soma size and proprioceptive inputs compared to SMA mice. Furthermore, *PLS3* increased presynaptic F-actin amounts, rescued synaptic vesicle and active zone content, restored the organization of readily releasable pools of vesicles, and increased the quantal content of neuromuscular junctions (NMJs). NMJs in *PLS3* overexpressing animals were improved as was their survival and motor function [199]. *PLS3* seems to act independently of *SMN*, making it a potential therapeutic target not only for SMA, but for other motor neuron diseases as well.

Another potential genetic modifier, a micro-RNA (miRNA), known as miR-9, has been shown to be down-regulated in the motor neurons of the severe mouse model of SMA. miR-9 is a highly conserved neuronal-specific miRNA shown to be involved in many aspects of neurobiology. In order for neurons to properly function, neurofilament gene expression must be tightly coordinated. Haramati et al. has shown that a neurofilament heavy subunit, NEFH, previously implicated in motor neuron degeneration, is specifically up-regulated in miR-9 deficient motor neurons. Furthermore, they demonstrate that NEFH is a target of miR-9 [200]. NEFH harbors nine miR-9 binding sites. In this way, miR-9 is involved in the tight regulation of proper neurofilament

stoichiometry. Therefore, low levels of miR-9 in SMA motor neurons may be contributing to their demise.

Candidate plasticity-related gene 15 (cpg15) is highly expressed in the developing ventral spinal cord and can promote motor axon branching and neuromuscular synapse formation [201,202]. Cpg15 colocalizes with SMN in axons and is locally translated in growth cones [44]. HuD is a neuron-specific binding protein and binding partner of SMN and cpg15 [44-46]. It has been shown that SMN deficiency reduces cpg15 mRNA levels in neurons. Recently, it was shown that cpg15 is an mRNA target for the SMN-HuD complex [44]. When cpg15 is overexpressed, axonal deficits seen in SMN deficient zebrafish can be partially rescued. Therefore, it seems that cpg15 may be a downstream effector of SMN in neurons and may serve as a critical disease modifier through its role in axon extension and terminal differentiation.

In 2007, an alternatively spliced *SMN* isoform, axonal-SMN, was identified [203]. Axonal-SMN (a-SMN) originates from the retention of *SMN* intron 3, resulting from the alternative splicing of the *SMN1* gene in humans. In the spinal cord, a-SMN is selectively expressed in developing motor neurons and mainly localized in axons and dendritic extensions. Overexpression of a-SMN in neuronal cells stimulates motor neuron axonogenesis in a time-dependent fashion and induces axonal-like growth even in non-neuronal cells, such as HeLa. Functional mapping of a-SMN has shown that exons 2b and 3 are essential for the axonogenic effects seen in vitro. Because a-SMN is a product of the *SMN1* gene, its loss may be contributing to the disease pathogenesis.

In addition to modifying genes, mutant forms of SMN may be able to modify the disease pathogenesis and/or phenotype. It has been proposed that when *SMN2* is spliced to create the SMN Δ 7 product, a degradation signal is created at SMN Δ 7's C-terminal 15 amino acids [204]. In order to determine which amino acids were critical for the activity of the degradation signal, degron, a series of mutants were generated. It was found that a S270A mutation (SMN Δ 7^{S270A}) limits the proteasome degradation of SMN Δ 7 and significantly increases its stability. SMN Δ 7^{S270A} was able to rescue SMN-deficient cells and was functional in snRNP assembly assays [204]. These findings indicate the SMN Δ 7 is a functional SMN protein and that its stabilization may be able to lessen disease severity.

In this report, we examined these modifiers and mutant forms of SMN using scAAV. By engineering the scAAV viral vector to express these genetic modifiers, we were able to quickly and efficiently determine their effects *in vivo*. Doing so allowed for the direct examination of the role of these modifiers on disease progression and the SMA mouse phenotype. Investigating these modifying genes and mutant forms of SMN furthers our understanding of disease pathogenesis opens the door to new therapeutic targets for the treatment of this devastating disease.

Material and Methods

Genotyping and Mouse Handling

Animals were handled according to the University of Missouri Animal Care and Use Committee approved protocols. To generate SMN^{2B/-} pups, SMN^{2B/2B} animals were bred with *mSMN*^{+/-} animals obtained from the Jackson Laboratory. Pups were tail

snipped within 24 hours of birth and genotyped with primers and PCR conditions previously described [129]. Litters of treated SMA mice and untreated controls were culled to four or five pups to control for litter size.

Production of scAAV9 Viruses

Viral particles were produced via triple transfection in HEK 293T cells using polyethyleneimine prepared to a working concentration of 1 mg/ml, pH 5.0 [73]. The scAAV plasmid was constructed to express the open reading frame of each modifying gene. Viral particles were purified by three cesium chloride density-gradient ultracentrifugation steps and dialyzed against HEPES buffer. Viral particles were titered by quantitative PCR using SYBR Green.

In Vivo Injections

On P2, SMA (SMN^{2B/-}) mice were injected with $1e^{11}$ viral particles. IV injections were performed as previously described [149].

Motor Function Assays

A modified tube test was performed to assess the gross motor function of the specified animals [205]. The test was modified to use a large, swallow beaker, instead of a conical tube due to the large size of the animals tested. All animals tested were greater than 100 days of age.

Results and Discussion

In order to investigate the effects of the modifying genes and mutant forms of SMN outlined in the introduction, we began by creating scAAV9 vectors expressing each modifier/mutant. In addition to the modifiers/mutants, viral particles expressing full-

length *SMN1* cDNA and SMN Δ 7, the major product of the *SMN2* gene, were also generated. From previous experiments, both transgenic and viral vector based, we were able to anticipate the efficacy of these controls.

In this study, the recently described SMN^{2B/-} mouse model was utilized [50]. These mice represent an intermediate model of SMA, exhibiting a median lifespan of approximately 30 days and a maximal weight gain of 7 grams. In this model the second exonic splice enhancer of *mSmn* exon 7 was mutated by homologous recombination causing the alternative splicing of exon 7. As a consequence, exon 7 is skipped in the majority of transcripts mimicking the splicing of human *SMN2*. The affected, experimental mice are denoted as SMN^{2B/-}.

A viral dose of 1e11 vector genomes (vg) was delivered via intravenous (IV) injection of the facial vein [149]. The scAAV9-SMN vector fully rescued the lifespan and weight gain of SMN^{2B/-} pups (**Figure 7.1**). scAAV9-SMN Δ 7 produced a more modest effect with median lifespan extending to ~45 days (**Figure 7.2**). Weight gain was only marginally affected. These results were not unexpected given previous results in the *SMN2*-based severe and SMN Δ 7 mouse models. scAAV9-SMN is able to fully rescue SMN Δ 7 mouse model [102,109-113]. Transgenic overexpression of SMN Δ 7 cDNA is able to extend the lifespan of the severe mouse model 2-3 fold [65]. These constructs provide a framework to gauge the efficacy of the modifier/mutant vectors tested.

With the exception of a-SMN and SMN Δ 7^{S270A}, all the tested modifiers, mir9, plastin3, and cpg15, conveyed modest ~50% extension in lifespan, while weight gain was relatively unaffected (**Figure 7.2**). These results were similar to those seen with

SMN Δ 7. Interestingly, these modifiers affect different pathways implicated in SMA pathogenesis. This may point towards the underlying complexity of SMA disease development and progression.

During the course of these experiments, we observed that a small number of mice significantly outperformed others in their respective treatment groups (**Figure 7.2**). These so called “escapees” were also observed in the untreated, SMN^{2B/-}, group. The phenomenon was also observed in the colony of Dr. Rashmi Kothary who generated this model [50]. We believe that the long-lived treated animals are intrinsic escapees, for whom treatment produces a superficially exaggerated extension of lifespan.

Notably, axonal SMN significantly worsened the SMA phenotype (**Figure 7.1,7.2**). To control for toxicity issues arising from the preparation of the virus, the virus was administered to unaffected, SMN^{2B/+} animals and no adverse effects were observed. a-SMN contains exon 2a and 2b which are part of the homodimerization domain [203]. Because of the inclusion of intron 3, a frameshift occurs resulting in a truncated protein lacking critical functional domains, such as the tudor and profillin interacting domain [203]. Therefore, it is possible that overexpression of a-SMN has a dominant negative effect. Future dose titration studies may help to elucidate the mechanism underlying this effect.

The most profound effect was seen following the delivery of the SMN mutant, SMN Δ 7^{S270A} which extended lifespan to 200+ days (**Figure 7.2**). Likewise, these animals displayed weight gains similar to unaffected mice (**Figure 7.1**). SMN Δ 7^{S270A} treated mice displayed gait abnormalities and progressive hind limb weakness. Gross motor function

was analyzed using a modified tube test [205]. In this test, SMN Δ 7^{S270A} treated animals were unable to perform as well as unaffected controls or FL-SMN treated animals (**Figure 7.3**). This suggests that while SMN Δ 7^{S270A} is able to rescue the lifespan of these mice it is unable to abrogate the motor defects characteristic of SMA, although it does delay their onset.

Previous work has shown the S270A mutation is able to significantly increase the stability of the SMN Δ 7 protein [204]. However, the molecular basis for this added stability is unknown. It is tempting to speculate that the serine residue present in wild type SMN Δ 7 is phosphorylated, leading to its degradation by the proteasome. When mutated to an alanine, this residue is no longer able to be phosphorylated and subsequently, SMN is more stable. In support of this hypothesis, it has been shown that inhibition or knockdown of the kinase, glycogen synthase kinase 3 (GSK-3), increased the stability of the SMN protein [206]. In this manner, this residue could be involved the regulation of SMN protein stability via phosphorylation. Therapeutics directed at the inhibition of the SMN specific kinases may be a valuable approach.

Understanding the involvement of modifier genes may shed light on the pathways involved in SMA pathogenesis. Furthermore, it open up new therapeutic avenues and targets for a disease which, as of yet, remains without an effective treatment or cure.

Acknowledgments

The SMN^{2B/2B} animals were a kind gift from the laboratory of Dr. Rashmi Kothary.

Figure 7.1 All modifier treated animals gained significantly more weight than untreated SMN^{2B/-} animals. There was no significant difference between unaffected SMN^{2B/+} mice and mice treated with scAAV9-S270A.

Figure 7.2 All modifier treated animals lived significantly longer than untreated $SMN^{2B/-}$ animals with the exception of scAAV9-aSMN treated mice, who had a significantly shorter lifespan*. Statistical comparison of lifespan by Mantel-Cox: untreated v. SMN $p=0.0001$, v. SMN Δ 7 $p=0.0002$, v. miR9 $p=0.0003$, v. Plastin3 $p=0.0021$, v. S270A $p<0.0001$, v. aSMN $p<0.0001^*$, and v. cpg15 $p<0.0001$.

Figure 7.3 scAAV9-S270A treated mice display motor function defects in the modified tube test. All animals tested were >100 days of age. Graph shows the percentage of animals who fell in the specified time (i.e 20% of scAAV9-S270A treated animals fell between 0-4 seconds).

CHAPTER EIGHT: CONCLUDING DISCUSSION

AAV Gene Therapy: Challenges When Entering the Clinic

AAV-mediated SMN gene replacement is a highly efficacious treatment for rescuing the phenotype of SMA mice. Historically, a median lifespan improvement of 25-50% was considered a successful therapeutic. Now, AAV-based approaches as described herein have shown the ability to completely rescue the lifespan of SMA mice. In that respect, there is tremendous potential for the use of AAV to restore *SMN1* to human SMA patients.

In this report, we have shown that viral dose, route, and timing of delivery dramatically influence AAV vector efficiency in animal models. These factors are just a portion of those that will need to be considered when moving AAV vectors into the clinic. Below some of these additional challenges, aside from the obvious issues of patient safety and cross-species efficacy, will be addressed.

First, can the large quantities of clinical-grade vector needed be routinely produced? Generation of quantities of viral particles needed to treat human patients would likely be on the order of 10^{14} viral genomes per patient, a quantity difficult to obtain with traditional production methods. Fortunately, with recent advancements in large-scale production generation of vector quantities of that degree may be possible [207,208]. However, the cost associated with these methods, tens of thousands of dollars per patient, could be prohibitive and further improvement of viral production methods to drive down costs would be of great benefit. Furthermore, standardization of

AAV production and compliance with good manufacturing practice must be implemented in order to compare clinical results and ensure patient safety [209].

Second, what patient population should be the recipient of AAV-based gene replacement and must treatment be given before the onset of symptoms? The work shown herein has demonstrated that early, pre-symptomatic viral delivery is able to produce a much more robust phenotypic improvement in SMA mice than when treatment is delayed until after the onset of symptoms [113]. Furthermore, scAAV9 transduction of motor neurons is most effective in neonates meaning that the primary target cell, motor neurons, may no longer be accessible if treatment is delayed [90]. Additionally, because motor neurons are lost as the disease progresses, treatment must be administered at a time when sufficient motor neurons remain functional in order to minimize muscle paralysis and atrophy. Currently, SMA is not screened for at birth and children are rarely diagnosed until symptoms are present. Thus, the question will SMN replacement via AAV be effective in children after symptoms are present? And if so, could it be even more effective if administered prior to symptom onset? If our results in mice are predictive, pre-symptomatic identification of SMA patients may become essential to the successful use of AAV-mediated gene therapy.

The first clinical trials, beginning in 2014, are enrolling only the most severely affected, Type I patients. The families of these patients are generally more willing to undertake substantial risks, such as enrollment in a clinical trial, than those of less severely affected patients. However, efficacy in these patients may be compromised due to their disease severity at the point of treatment. Type II and III patients have a

slower disease progression and may be more well-suited to this type of therapeutic. The outcome of these first clinical trials will give insight into the ability of AAV-mediated SMN replacement to correct disease after the onset of symptoms.

Third, will there be an immune response to the AAV capsid? Such a response could negate the effects of SMN replacement by effectively abrogating gene expression from the virus. Fortunately, because SMN is an endogenous protein and low levels are produced in SMA patients, there should not be an immune response to the SMN protein itself. Most adults are seropositive for AAV capsid antibodies as are infants and children [210,211]. There have even been reports of the infection of human embryos with AAV during pregnancy [212]. Young age is no guarantee of the absence of AAV antibodies. However, AAV antibody titers tend to be modest in infants and children, typically less than 1:50 [211]. Thus, young children may represent the most promising patient group for intravenous gene therapy [213]. It is predicted that antibody titers of greater than 1:200 would be prohibitive to vascular transduction. Approximately 30% of adults harbor AAV9 antibodies at titers at this level or greater [214].

Because immune responses are dose dependent, a vector with a highly efficient expression cassette will be needed to minimize the number of viral particles which must be administered. Codon optimized-sequences are one of the most common ways to maximize expression [215]. Further compounding the issue of immune response is the potential need for vector readministration. Ideally, a single injection would be sufficient for lifelong treatment. However, other AAV gene therapy studies conducted to date indicate that repeated administration may be necessary. In many preclinical and clinical

trials, neutralizing antibodies to AAV were produced after the first injection [216-218]. It is currently unknown if treatment in SMA patients would need to be repeated. Motor neurons are post-mitotic, but muscle has intrinsic potential for regeneration through a population of stem cells known as satellite cells. Therefore, transgene expression in muscle and other disease-relevant tissues containing proliferating cell populations may fade with time, especially in developing children, necessitating readministration for the maintenance of SMN levels.

If an immune response does occur, there are a number of potential strategies to address the problem. Induction of tolerance to AAV capsid, genetic modification of the AAV capsid antigen, temporary immunosuppression, and proteasome inhibition to block presentation of capsid antigen [219] are all methods which would be feasible approaches to combating the problem. An alternative approach, serotype switching, has been used in other disease contexts where repeated administration of AAV was needed with good results [220-223]. While serotype 9 is most commonly used for SMA applications, due to its ability to cross the blood-brain barrier, other serotypes could potentially be used if delivery strategies which allow for direct CNS administration are implemented.

Fourth, what cells need to be targeted for SMN replacement in order for gene therapy to be effective? Understanding exactly what cells and tissue types in which SMN must be replaced will be important for designing delivery strategies for AAV. Following intravenous injection in neonatal mice, AAV is able to easily cross the blood-brain barrier. AAV can cross in adult non-human primates as well, but less efficiently and with

changes in tropism from motor neurons to astrocytes. Therefore, more viral particles must be delivered to achieve the needed levels of transduction in motor neurons. Delivery of more virus in turn increases the likelihood of an immune response. For those reasons, direct CNS administration may be advantageous. In other clinical trials, including those for SMA, intrathecal injections have been well-tolerated in children [130]. However, intravenous administration has benefits as well. It is far less invasive and provides transduction of other peripheral organs which have been shown to be affected in SMA mouse models (for review see [158] and [30]). However, it remains to be seen if SMN replacement in those organs is necessary as there are certain peripheral defects seen in SMA mouse models that are not found in human patients, such as necrosis and cardiac defects [162,163,177]. Thus, identification of the cells and tissue types in which SMN must be replaced in human is critical as it will dictate the delivery strategy which will be most efficacious.

Fifth, what are the long-term effects of AAV expression? Once AAV is administered, its expression cannot be turned off should adverse events arise. For example, expression of endogenous SMN developmentally decreases while vector driven SMN is continuously expressed from a promoter not subject to intrinsic regulation. To that effect, it is possible that artificially high levels of SMN expression could become detrimental. Therefore, development of regulatable vectors might be beneficial.

Although AAV vectors are often assumed to persist as episomal concatemers, chromosomal integration does occur and can produce long-term expression of

transgenes [224,225]. Vector integration has been observed in various experimental settings, occurring at non-homologous sites of DNA damage or via homologous recombination [226]. There have been conflicting reports as to AAV induced tumorigenesis in mice [227,228]. While rarity of integration reduces the likelihood of insertional mutagenesis, the possibility remains. Approximately 0.1% of vector genomes have been reported to integrate [229,230]. While this number seems very small, an injection of $1e14$ viral particles would result in approximately $1e11$ integration events. Thus, the potential for AAV induced tumors raises concerns about its use in clinical applications. Two clinical trials utilizing an integrating retroviral vector for the treatment of severe combined immunodeficiency disease have reported the induction of leukemia in approximately 20% of treated patients [231]. It remains to be seen if AAV vectors are able to induce tumorigenesis in human patients, but there are adequate reasons to be concerned.

While there are certainly challenges which must be overcome and issues which will invariably have to be addressed, AAV-mediated SMN replacement has tremendous potential. Ways will be found around many of the technical obstacles outlined above and applicable knowledge will be gained from clinical trials not only in SMA, but in other diseases as well. It remains to be seen if the promise of this approach will be realized. However, should it be, a devastating disease for which there is currently no treatment may have an effective treatment and if we are lucky, a cure.

REFERENCES

- [1] T.O. Crawford, C.A. Pardo, The neurobiology of childhood spinal muscular atrophy, *Neurobiol Dis* 3 (1996) 97-110.
- [2] M. Feldkotter, V. Schwarzer, R. Wirth, T.F. Wienker, B. Wirth, Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy, *Am J Hum Genet* 70 (2002) 358-368.
- [3] J. Pearn, Classification of spinal muscular atrophies, *Lancet* 1 (1980) 919-922.
- [4] J.X. Chong, A.A. Oktay, Z. Dai, K.J. Swoboda, T.W. Prior, *et al.*, A common spinal muscular atrophy deletion mutation is present on a single founder haplotype in the US Hutterites, *Eur J Hum Genet* 19 (2011) 1045-1051.
- [5] A. Lewelt, T.M. Newcomb, K.J. Swoboda, New therapeutic approaches to spinal muscular atrophy, *Curr Neurol Neurosci Rep* 12 (2012) 42-53.
- [6] K. Zerres, S. Rudnik-Schoneborn, Natural history in proximal spinal muscular atrophy. Clinical analysis of 445 patients and suggestions for a modification of existing classifications, *Arch Neurol* 52 (1995) 518-523.
- [7] U.R. Monani, Spinal muscular atrophy: a deficiency in a ubiquitous protein; a motor neuron-specific disease, *Neuron* 48 (2005) 885-896.
- [8] C. Meldrum, C. Scott, K.J. Swoboda, Spinal muscular atrophy genetic counseling access and genetic knowledge: parents' perspectives, *J Child Neurol* 22 (2007) 1019-1026.
- [9] S. Lefebvre, L. Burglen, S. Reboullet, O. Clermont, P. Burlet, *et al.*, Identification and characterization of a spinal muscular atrophy-determining gene, *Cell* 80 (1995) 155-165.
- [10] C.F. Rochette, N. Gilbert, L.R. Simard, SMN gene duplication and the emergence of the SMN2 gene occurred in distinct hominids: SMN2 is unique to Homo sapiens, *Hum Genet* 108 (2001) 255-266.
- [11] C.L. Lorson, E. Hahnen, E.J. Androphy, B. Wirth, A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy, *Proc Natl Acad Sci U S A* 96 (1999) 6307-6311.
- [12] S. Lefebvre, P. Burlet, Q. Liu, S. Bertrand, O. Clermont, *et al.*, Correlation between severity and SMN protein level in spinal muscular atrophy, *Nat Genet* 16 (1997) 265-269.
- [13] U.R. Monani, C.L. Lorson, D.W. Parsons, T.W. Prior, E.J. Androphy, *et al.*, A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2, *Hum Mol Genet* 8 (1999) 1177-1183.
- [14] B. Lewin, Genes for SMA: multum in parvo, *Cell* 80 (1995) 1-5.
- [15] L. Cartegni, A.R. Krainer, Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1, *Nat Genet* 30 (2002) 377-384.
- [16] T. Kashima, J.L. Manley, A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy, *Nat Genet* 34 (2003) 460-463.

- [17] T. Kashima, N. Rao, J.L. Manley, An intronic element contributes to splicing repression in spinal muscular atrophy, *Proc Natl Acad Sci U S A* 104 (2007) 3426-3431.
- [18] C.L. Lorson, E.J. Androphy, An exonic enhancer is required for inclusion of an essential exon in the SMA-determining gene SMN, *Hum Mol Genet* 9 (2000) 259-265.
- [19] E. Parano, L. Pavone, R. Falsaperla, R. Trifiletti, C. Wang, Molecular basis of phenotypic heterogeneity in siblings with spinal muscular atrophy, *Ann Neurol* 40 (1996) 247-251.
- [20] S. Ogino, R.B. Wilson, B. Gold, New insights on the evolution of the SMN1 and SMN2 region: simulation and meta-analysis for allele and haplotype frequency calculations, *Eur J Hum Genet* 12 (2004) 1015-1023.
- [21] I. Cusco, M.J. Barcelo, R. Rojas-Garcia, I. Illa, J. Gamez, *et al.*, SMN2 copy number predicts acute or chronic spinal muscular atrophy but does not account for intrafamilial variability in siblings, *J Neurol* 253 (2006) 21-25.
- [22] T.W. Prior, K.J. Swoboda, H.D. Scott, A.Q. Hejmanowski, Homozygous SMN1 deletions in unaffected family members and modification of the phenotype by SMN2, *Am J Med Genet A* 130A (2004) 307-310.
- [23] K.K. Ling, R.M. Gibbs, Z. Feng, C.P. Ko, Severe neuromuscular denervation of clinically relevant muscles in a mouse model of spinal muscular atrophy, *Hum Mol Genet* 21 (2012) 185-195.
- [24] L. Kong, X. Wang, D.W. Choe, M. Polley, B.G. Burnett, *et al.*, Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice, *J Neurosci* 29 (2009) 842-851.
- [25] T.L. Martinez, L. Kong, X. Wang, M.A. Osborne, M.E. Crowder, *et al.*, Survival motor neuron protein in motor neurons determines synaptic integrity in spinal muscular atrophy, *J Neurosci* 32 (2012) 8703-8715.
- [26] R.G. Gogliotti, K.A. Quinlan, C.B. Barlow, C.R. Heier, C.J. Heckman, *et al.*, Motor neuron rescue in spinal muscular atrophy mice demonstrates that sensory-motor defects are a consequence, not a cause, of motor neuron dysfunction, *J Neurosci* 32 (2012) 3818-3829.
- [27] G.Z. Mentis, D. Blivis, W. Liu, E. Drobac, M.E. Crowder, *et al.*, Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy, *Neuron* 69 (2011) 453-467.
- [28] W.L. Imlach, E.S. Beck, B.J. Choi, F. Lotti, L. Pellizzoni, *et al.*, SMN is required for sensory-motor circuit function in *Drosophila*, *Cell* 151 (2012) 427-439.
- [29] K.K. Ling, M.Y. Lin, B. Zingg, Z. Feng, C.P. Ko, Synaptic defects in the spinal and neuromuscular circuitry in a mouse model of spinal muscular atrophy, *PLoS ONE* 5 (2010) e15457.
- [30] G. Hamilton, T.H. Gillingwater, Spinal muscular atrophy: going beyond the motor neuron, *Trends Mol Med* 19 (2013) 40-50.
- [31] F. Gabanella, C. Carissimi, A. Usiello, L. Pellizzoni, The activity of the spinal muscular atrophy protein is regulated during development and cellular differentiation, *Hum Mol Genet* 14 (2005) 3629-3642.

- [32] B. Schrank, R. Gotz, J.M. Gunnensen, J.M. Ure, K.V. Toyka, *et al.*, Inactivation of the survival motor neuron gene, a candidate gene for human spinal muscular atrophy, leads to massive cell death in early mouse embryos, *Proc Natl Acad Sci U S A* 94 (1997) 9920-9925.
- [33] Q. Liu, G. Dreyfuss, A novel nuclear structure containing the survival of motor neurons protein, *EMBO J* 15 (1996) 3555-3565.
- [34] D.D. Covert, T.T. Le, P.E. McAndrew, J. Strasswimmer, T.O. Crawford, *et al.*, The survival motor neuron protein in spinal muscular atrophy, *Hum Mol Genet* 6 (1997) 1205-1214.
- [35] L. Pellizzoni, Chaperoning ribonucleoprotein biogenesis in health and disease, *EMBO Rep* 8 (2007) 340-345.
- [36] S.J. Kolb, D.J. Battle, G. Dreyfuss, Molecular functions of the SMN complex, *J Child Neurol* 22 (2007) 990-994.
- [37] F. Gabanella, M.E. Butchbach, L. Saieva, C. Carissimi, A.H. Burghes, *et al.*, Ribonucleoprotein Assembly Defects Correlate with Spinal Muscular Atrophy Severity and Preferentially Affect a Subset of Spliceosomal snRNPs, *PLoS ONE* 2 (2007) e921.
- [38] N. Boulisfane, M. Choleza, F. Rage, H. Neel, J. Soret, *et al.*, Impaired minor tri-snRNP assembly generates differential splicing defects of U12-type introns in lymphoblasts derived from a type I SMA patient, *Hum Mol Genet* 20 (2011) 641-648.
- [39] Z. Zhang, F. Lotti, K. Dittmar, I. Younis, L. Wan, *et al.*, SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing, *Cell* 133 (2008) 585-600.
- [40] F. Lotti, W.L. Imlach, L. Saieva, E.S. Beck, T. Hao le, *et al.*, An SMN-dependent U12 splicing event essential for motor circuit function, *Cell* 151 (2012) 440-454.
- [41] W. Rossoll, G.J. Bassell, Spinal muscular atrophy and a model for survival of motor neuron protein function in axonal ribonucleoprotein complexes, *Results and Problems in Cell Differentiation* 48 (2009) 289-326.
- [42] H.L. Zhang, F. Pan, D. Hong, S.M. Shenoy, R.H. Singer, *et al.*, Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization, *J Neurosci* 23 (2003) 6627-6637.
- [43] M. Glinka, T. Herrmann, N. Funk, S. Havlicek, W. Rossoll, *et al.*, The heterogeneous nuclear ribonucleoprotein-R is necessary for axonal beta-actin mRNA translocation in spinal motor neurons, *Hum Mol Genet* 19 (2010) 1951-1966.
- [44] B. Akten, M.J. Kye, T. Hao le, M.H. Wertz, S. Singh, *et al.*, Interaction of survival of motor neuron (SMN) and HuD proteins with mRNA cpg15 rescues motor neuron axonal deficits, *Proc Natl Acad Sci U S A* 108 (2011) 10337-10342.
- [45] C. Fallini, H. Zhang, Y. Su, V. Silani, R.H. Singer, *et al.*, The survival of motor neuron (SMN) protein interacts with the mRNA-binding protein HuD and regulates localization of poly(A) mRNA in primary motor neuron axons, *J Neurosci* 31 (2011) 3914-3925.

- [46] L. Hubers, H. Valderrama-Carvajal, J. Laframboise, J. Timbers, G. Sanchez, *et al.*, HuD interacts with survival motor neuron protein and can rescue spinal muscular atrophy-like neuronal defects, *Hum Mol Genet* 20 (2011) 553-579.
- [47] W. Rossoll, S. Jablonka, C. Andreassi, A.K. Kroning, K. Karle, *et al.*, Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons, *J Cell Biol* 163 (2003) 801-812.
- [48] T.R. Cheever, E.A. Olson, J.M. Ervasti, Axonal regeneration and neuronal function are preserved in motor neurons lacking beta-actin in vivo, *PLoS ONE* 6 (2011) e17768.
- [49] G.E. Oprea, S. Krober, M.L. McWhorter, W. Rossoll, S. Muller, *et al.*, Plastin 3 is a protective modifier of autosomal recessive spinal muscular atrophy, *Science* 320 (2008) 524-527.
- [50] M. Bowerman, C.L. Anderson, A. Beauvais, P.P. Boyl, W. Witke, *et al.*, SMN, profilin IIa and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis, *Mol Cell Neurosci* 42 (2009) 66-74.
- [51] M. Bowerman, A. Beauvais, C.L. Anderson, R. Kothary, Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model, *Hum Mol Genet* 19 (2010) 1468-1478.
- [52] M.A. Lorson, L.D. Spate, M.S. Samuel, C.N. Murphy, C.L. Lorson, *et al.*, Disruption of the Survival Motor Neuron (SMN) gene in pigs using ssDNA, *Transgenic Research* 20 (2011) 1293-1304.
- [53] S. Duque, Porensky, P., Odermatt, P., Arnold, D., Nlend, R., Bevan, A., Foust, K., Schmelzer, L., Schumperli, D., Kaspar, B., Burghes, A., Delivery and Correction of SMA Using AAV Vectors in a Large Animal Model, Families of SMA International Meeting, Minneapolis, MN, 2012.
- [54] I. Miguel-Aliaga, E. Culetto, D.S. Walker, H.A. Baylis, D.B. Sattelle, *et al.*, The *Caenorhabditis elegans* orthologue of the human gene responsible for spinal muscular atrophy is a maternal product critical for germline maturation and embryonic viability, *Hum Mol Genet* 8 (1999) 2133-2143.
- [55] A.H. Burghes, C.E. Beattie, Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick?, *Nat Rev Neurosci* 10 (2009) 597-609.
- [56] S. Paushkin, B. Charroux, L. Abel, R.A. Parkinson, L. Pellizzoni, *et al.*, The survival motor neuron protein of *Schizosaccharomyces pombe*. Conservation of survival motor neuron interaction domains in divergent organisms, *J Biol Chem* 275 (2000) 23841-23846.
- [57] I. Miguel-Aliaga, Y.B. Chan, K.E. Davies, M. van den Heuvel, Disruption of SMN function by ectopic expression of the human SMN gene in *Drosophila*, *FEBS Lett* 486 (2000) 99-102.
- [58] Y.B. Chan, I. Miguel-Aliaga, C. Franks, N. Thomas, B. Trulzsch, *et al.*, Neuromuscular defects in a *Drosophila* survival motor neuron gene mutant, *Hum Mol Genet* 12 (2003) 1367-1376.

- [59] T.K. Rajendra, G.B. Gonsalvez, M.P. Walker, K.B. Shpargel, H.K. Salz, *et al.*, A *Drosophila melanogaster* model of spinal muscular atrophy reveals a function for SMN in striated muscle, *J Cell Biol* 176 (2007) 831-841.
- [60] M. Briesse, B. Esmaili, S. Fraboulet, E.C. Burt, S. Christodoulou, *et al.*, Deletion of *smn-1*, the *Caenorhabditis elegans* ortholog of the spinal muscular atrophy gene, results in locomotor dysfunction and reduced lifespan, *Hum Mol Genet* 18 (2009) 97-104.
- [61] T. Hao le, A.H. Burghes, C.E. Beattie, Generation and Characterization of a genetic zebrafish model of SMA carrying the human SMN2 gene, *Mol Neurodegener* 6 (2011) 24.
- [62] M.L. McWhorter, U.R. Monani, A.H. Burghes, C.E. Beattie, Knockdown of the survival motor neuron (*Smn*) protein in zebrafish causes defects in motor axon outgrowth and pathfinding, *J Cell Biol* 162 (2003) 919-931.
- [63] T.L. Carrel, M.L. McWhorter, E. Workman, H. Zhang, E.C. Wolstencroft, *et al.*, Survival motor neuron function in motor axons is independent of functions required for small nuclear ribonucleoprotein biogenesis, *J Neurosci* 26 (2006) 11014-11022.
- [64] U.R. Monani, M. Sendtner, D.D. Covert, D.W. Parsons, C. Andreassi, *et al.*, The human centromeric survival motor neuron gene (SMN2) rescues embryonic lethality in *Smn*(-/-) mice and results in a mouse with spinal muscular atrophy, *Hum Mol Genet* 9 (2000) 333-339.
- [65] T.T. Le, L.T. Pham, M.E. Butchbach, H.L. Zhang, U.R. Monani, *et al.*, SMN Δ 7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN, *Hum Mol Genet* 14 (2005) 845-857.
- [66] M. Osborne, Lutz, C., Reference Guide to Mouse Models of Spinal Muscular Atrophy, The Jackson Laboratory, 2013.
- [67] S. Kariya, G.H. Park, Y. Maeno-Hikichi, O. Leykekhman, C. Lutz, *et al.*, Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy, *Hum Mol Genet* 17 (2008) 2552-2569.
- [68] M. Osborne, D. Gomez, Z. Feng, C. McEwen, J. Beltran, *et al.*, Characterization of behavioral and neuromuscular junction phenotypes in a novel allelic series of SMA mouse models, *Hum Mol Genet* 21 (2012) 4431-4447.
- [69] U.R. Monani, M.T. Pastore, T.O. Gavrilina, S. Jablonka, T.T. Le, *et al.*, A transgene carrying an A2G missense mutation in the SMN gene modulates phenotypic severity in mice with severe (type I) spinal muscular atrophy, *J Cell Biol* 160 (2003) 41-52.
- [70] M. Michaud, T. Arnoux, S. Bielli, E. Durand, Y. Rotrou, *et al.*, Neuromuscular defects and breathing disorders in a new mouse model of spinal muscular atrophy, *Neurobiol Dis* 38 (2010) 125-135.
- [71] J.T. Gladman, T.W. Bebee, C. Edwards, X. Wang, Z. Sahenk, *et al.*, A humanized *Smn* gene containing the SMN2 nucleotide alteration in exon 7 mimics SMN2 splicing and the SMA disease phenotype, *Hum Mol Genet* 19 (2010) 4239-4252.

- [72] M.S. Cobb, F.F. Rose, H. Rindt, J.J. Glascock, M. Shababi, *et al.*, Development and characterization of an SMN2-based intermediate mouse model of Spinal Muscular Atrophy, *Hum Mol Genet* 22 (2013) 1843-1855.
- [73] J.C. Grieger, V.W. Choi, R.J. Samulski, Production and characterization of adeno-associated viral vectors, *Nat Protoc* 1 (2006) 1412-1428.
- [74] J.M. Timpe, K.C. Verrill, J.P. Trempe, Effects of adeno-associated virus on adenovirus replication and gene expression during coinfection, *J Virol* 80 (2006) 7807-7815.
- [75] T. Matsushita, S. Elliger, C. Elliger, G. Podsakoff, L. Villarreal, *et al.*, Adeno-associated virus vectors can be efficiently produced without helper virus, *Gene Ther* 5 (1998) 938-945.
- [76] M.W. Myers, C.A. Laughlin, F.T. Jay, B.J. Carter, Adenovirus helper function for growth of adeno-associated virus: effect of temperature-sensitive mutations in adenovirus early gene region 2, *J Virol* 35 (1980) 65-75.
- [77] R.M. Kotin, M. Siniscalco, R.J. Samulski, X.D. Zhu, L. Hunter, *et al.*, Site-specific integration by adeno-associated virus, *Proc Natl Acad Sci U S A* 87 (1990) 2211-2215.
- [78] R.T. Surosky, M. Urabe, S.G. Godwin, S.A. McQuiston, G.J. Kurtzman, *et al.*, Adeno-associated virus Rep proteins target DNA sequences to a unique locus in the human genome, *J Virol* 71 (1997) 7951-7959.
- [79] M. Penaud-Budloo, C. Le Guiner, A. Nowrouzi, A. Toromanoff, Y. Cherel, *et al.*, Adeno-associated virus vector genomes persist as episomal chromatin in primate muscle, *J Virol* 82 (2008) 7875-7885.
- [80] M. Stadtfeld, K. Hochedlinger, Without a trace? PiggyBac-ing toward pluripotency, *Nat Methods* 6 (2009) 329-330.
- [81] C. Zincarelli, S. Soltys, G. Rengo, J.E. Rabinowitz, Analysis of AAV serotypes 1-9 mediated gene expression and tropism in mice after systemic injection, *Mol Ther* 16 (2008) 1073-1080.
- [82] D. Duan, P. Sharma, J. Yang, Y. Yue, L. Dudus, *et al.*, Circular intermediates of recombinant adeno-associated virus have defined structural characteristics responsible for long-term episomal persistence in muscle tissue, *J Virol* 72 (1998) 8568-8577.
- [83] D.M. McCarty, Self-complementary AAV vectors; advances and applications, *Mol Ther* 16 (2008) 1648-1656.
- [84] D.M. McCarty, H. Fu, P.E. Monahan, C.E. Toulson, P. Naik, *et al.*, Adeno-associated virus terminal repeat (TR) mutant generates self-complementary vectors to overcome the rate-limiting step to transduction in vivo, *Gene Ther* 10 (2003) 2112-2118.
- [85] Z. Wang, H.I. Ma, J. Li, L. Sun, J. Zhang, *et al.*, Rapid and highly efficient transduction by double-stranded adeno-associated virus vectors in vitro and in vivo, *Gene Ther* 10 (2003) 2105-2111.
- [86] C. Ren, S. Kumar, D.R. Shaw, S. Ponnazhagan, Genomic stability of self-complementary adeno-associated virus 2 during early stages of transduction in mouse muscle in vivo, *Hum Gene Ther* 16 (2005) 1047-1057.

- [87] J.C. Grieger, R.J. Samulski, Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps, *J Virol* 79 (2005) 9933-9944.
- [88] M. Nonnenmacher, T. Weber, Intracellular transport of recombinant adeno-associated virus vectors, *Gene Ther* 19 (2012) 649-658.
- [89] S. Duque, B. Joussemet, C. Riviere, T. Marais, L. Dubreil, *et al.*, Intravenous administration of self-complementary AAV9 enables transgene delivery to adult motor neurons, *Mol Ther* 17 (2009) 1187-1196.
- [90] K.D. Foust, E. Nurre, C.L. Montgomery, A. Hernandez, C.M. Chan, *et al.*, Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes, *Nat Biotechnol* 27 (2009) 59-65.
- [91] P.L. Hermonat, N. Muzyczka, Use of adeno-associated virus as a mammalian DNA cloning vector: transduction of neomycin resistance into mammalian tissue culture cells, *Proc Natl Acad Sci U S A* 81 (1984) 6466-6470.
- [92] A. Srivastava, Replication of the adeno-associated virus DNA termini in vitro, *Intervirology* 27 (1987) 138-147.
- [93] X.S. Wang, S. Ponnazhagan, A. Srivastava, Rescue and replication signals of the adeno-associated virus 2 genome, *J Mol Biol* 250 (1995) 573-580.
- [94] S.K. McLaughlin, P. Collis, P.L. Hermonat, N. Muzyczka, Adeno-associated virus general transduction vectors: analysis of proviral structures, *J Virol* 62 (1988) 1963-1973.
- [95] R.J. Samulski, L.S. Chang, T. Shenk, Helper-free stocks of recombinant adeno-associated viruses: normal integration does not require viral gene expression, *J Virol* 63 (1989) 3822-3828.
- [96] X. Xiao, J. Li, R.J. Samulski, Production of high-titer recombinant adeno-associated virus vectors in the absence of helper adenovirus, *J Virol* 72 (1998) 2224-2232.
- [97] T. Matsushita, T. Okada, T. Inaba, H. Mizukami, K. Ozawa, *et al.*, The adenovirus E1A and E1B19K genes provide a helper function for transfection-based adeno-associated virus vector production, *J Gen Virol* 85 (2004) 2209-2214.
- [98] S.E. Reed, E.M. Staley, J.P. Mayginnes, D.J. Pintel, G.E. Tullis, Transfection of mammalian cells using linear polyethylenimine is a simple and effective means of producing recombinant adeno-associated virus vectors, *J Virol Methods* 138 (2006) 85-98.
- [99] J.C. Grieger, R.J. Samulski, Adeno-associated virus vectorology, manufacturing, and clinical applications, *Methods in Enzymology* 507 (2012) 229-254.
- [100] S.J. Gray, V.W. Choi, A. Asokan, R.A. Haberman, T.J. McCown, *et al.*, Production of recombinant adeno-associated viral vectors and use in in vitro and in vivo administration, *Curr Protoc Neurosci* Chapter 4 (2011) Unit 4 17.
- [101] N. Sanburn, K. Cornetta, Rapid titer determination using quantitative real-time PCR, *Gene Ther* 6 (1999) 1340-1345.
- [102] K.D. Foust, X. Wang, V.L. McGovern, L. Braun, A.K. Bevan, *et al.*, Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN, *Nat Biotechnol* 28 (2010) 271-274.

- [103] M. Okabe, M. Ikawa, K. Kominami, T. Nakanishi, Y. Nishimune, 'Green mice' as a source of ubiquitous green cells, *FEBS Lett* 407 (1997) 313-319.
- [104] A.N. Alexopoulou, J.R. Couchman, J.R. Whiteford, The CMV early enhancer/chicken beta actin (CAG) promoter can be used to drive transgene expression during the differentiation of murine embryonic stem cells into vascular progenitors, *BMC Cell Biol* 9 (2008) 2.
- [105] H. Niwa, K. Yamamura, J. Miyazaki, Efficient selection for high-expression transfectants with a novel eukaryotic vector, *Gene* 108 (1991) 193-199.
- [106] J. Miyazaki, S. Takaki, K. Araki, F. Tashiro, A. Tominaga, *et al.*, Expression vector system based on the chicken beta-actin promoter directs efficient production of interleukin-5, *Gene* 79 (1989) 269-277.
- [107] Y. Nitta, S. Kawamoto, C. Halbert, A. Iwata, A.D. Miller, *et al.*, A CMV-actin-globin hybrid promoter improves adeno-associated viral vector gene expression in the arterial wall in vivo, *J Gene Med* 7 (2005) 1348-1355.
- [108] S. Hermening, S. Kugler, M. Bahr, S. Isenmann, Increased protein expression from adenoviral shuttle plasmids and vectors by insertion of a small chimeric intron sequence, *J Virol Methods* 122 (2004) 73-77.
- [109] C.F. Valori, K. Ning, M. Wyles, R.J. Mead, A.J. Grierson, *et al.*, Systemic delivery of scAAV9 expressing SMN prolongs survival in a model of spinal muscular atrophy, *Sci Transl Med* 2 (2010) 35ra42.
- [110] E. Dominguez, T. Marais, N. Chatauret, S. Benkhelifa-Ziyyat, S. Duque, *et al.*, Intravenous scAAV9 delivery of a codon-optimized SMN1 sequence rescues SMA mice, *Hum Mol Genet* 20 (2011) 681-693.
- [111] M.A. Passini, J. Bu, E.M. Roskelley, A.M. Richards, S.P. Sardi, *et al.*, CNS-targeted gene therapy improves survival and motor function in a mouse model of spinal muscular atrophy, *J Clin Invest* 120 (2010) 1253-1264.
- [112] J.J. Glascock, M. Shababi, M.J. Wetz, M.M. Krogman, C.L. Lorson, Direct central nervous system delivery provides enhanced protection following vector mediated gene replacement in a severe model of spinal muscular atrophy, *Biochem Biophys Res Commun* 417 (2012) 376-381.
- [113] J.J. Glascock, E.Y. Osman, M.J. Wetz, M.M. Krogman, M. Shababi, *et al.*, Decreasing disease severity in symptomatic, *Smn*(-/-);*SMN2*(+/+), spinal muscular atrophy mice following scAAV9-SMN delivery, *Hum Gene Ther* 23 (2012) 330-335.
- [114] S. Benkhelifa-Ziyyat, A. Besse, M. Roda, S. Duque, S. Astord, *et al.*, Intramuscular scAAV9-SMN injection mediates widespread gene delivery to the spinal cord and decreases disease severity in SMA mice, *Mol Ther* 21 (2013) 282-290.
- [115] L. Samaranch, E.A. Salegio, W. San Sebastian, A.P. Kells, K.D. Foust, *et al.*, Adeno-associated virus serotype 9 transduction in the central nervous system of nonhuman primates, *Hum Gene Ther* 23 (2012) 382-389.
- [116] J.G. Chang, H.M. Hsieh-Li, Y.J. Jong, N.M. Wang, C.H. Tsai, *et al.*, Treatment of spinal muscular atrophy by sodium butyrate, *Proc Natl Acad Sci U S A* 98 (2001) 9808-9813.

- [117] L. Brichta, Y. Hofmann, E. Hahnen, F.A. Siebzehrubl, H. Raschke, *et al.*, Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy, *Hum Mol Genet* 12 (2003) 2481-2489.
- [118] C.J. Sumner, T.N. Huynh, J.A. Markowitz, J.S. Perhac, B. Hill, *et al.*, Valproic acid increases SMN levels in spinal muscular atrophy patient cells, *Ann Neurol* 54 (2003) 647-654.
- [119] C. Andreassi, C. Angelozzi, F.D. Tiziano, T. Vitali, E. De Vincenzi, *et al.*, Phenylbutyrate increases SMN expression in vitro: relevance for treatment of spinal muscular atrophy, *Eur J Hum Genet* 12 (2004) 59-65.
- [120] E. Hahnen, I.Y. Eyupoglu, L. Brichta, K. Haastert, C. Trankle, *et al.*, In vitro and ex vivo evaluation of second-generation histone deacetylase inhibitors for the treatment of spinal muscular atrophy, *J Neurochem* 98 (2006) 193-202.
- [121] A.M. Avila, B.G. Burnett, A.A. Taye, F. Gabanella, M.A. Knight, *et al.*, Trichostatin A increases SMN expression and survival in a mouse model of spinal muscular atrophy, *J Clin Invest* 117 (2007) 659-671.
- [122] H.L. Narver, L. Kong, B.G. Burnett, D.W. Choe, M. Bosch-Marce, *et al.*, Sustained improvement of spinal muscular atrophy mice treated with trichostatin A plus nutrition, *Ann Neurol* 64 (2008) 465-470.
- [123] C.H. Ting, C.W. Lin, S.L. Wen, H.M. Hsieh-Li, H. Li, Stat5 constitutive activation rescues defects in spinal muscular atrophy, *Hum Mol Genet* 16 (2007) 499-514.
- [124] F. Farooq, F.A. Molina, J. Hadwen, D. MacKenzie, L. Witherspoon, *et al.*, Prolactin increases SMN expression and survival in a mouse model of severe spinal muscular atrophy via the STAT5 pathway, *J Clin Invest* 121 (2011) 3042-3050.
- [125] P.N. Porensky, C. Mitropant, V.L. McGovern, A.K. Bevan, K.D. Foust, *et al.*, A single administration of morpholino antisense oligomer rescues spinal muscular atrophy in mouse, *Hum Mol Genet* 21 (2012) 1625-1638.
- [126] J.H. Williams, R.C. Schray, C.A. Patterson, S.O. Ayitey, M.K. Tallent, *et al.*, Oligonucleotide-mediated survival of motor neuron protein expression in CNS improves phenotype in a mouse model of spinal muscular atrophy, *J Neurosci* 29 (2009) 7633-7638.
- [127] Y. Hua, K. Sahashi, G. Hung, F. Rigo, M.A. Passini, *et al.*, Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model, *Genes Dev* 24 (2010) 1634-1644.
- [128] M.A. Passini, J. Bu, A.M. Richards, C. Kinnecom, S.P. Sardi, *et al.*, Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy, *Sci Transl Med* 3 (2011) 72ra18.
- [129] T.H. Coady, C.L. Lorson, Trans-splicing-mediated improvement in a severe mouse model of spinal muscular atrophy, *J Neurosci* 30 (2010) 126-130.
- [130] K.M. Bishop, Chiriboga, C., Swoboda, K., Darras, B., Iannaccone, S., *et al.*, Development of ISIS-SMNRX for Spinal Muscular Atrophy, Families of SMA International Meeting, Anaheim, CA, 2013.
- [131] N. Naryshkin, Narasimham, J., Dakka, A., Gabbeta, V., Haley, M. *et al.*, Small Molecule Compounds Correct Alternative Splicing of the SMN2 Gene and

Restore SMN Protein Expression and Function Families of SMA International Meeting, Minneapolis, MN, 2012.

- [132] P.C. Chen, I.N. Gaisina, B.F. El-Khodori, S. Ramboz, N.R. Makhortova, *et al.*, Identification of a Maleimide-Based Glycogen Synthase Kinase-3 (GSK-3) Inhibitor, BIP-135, that Prolongs the Median Survival Time of Delta7 SMA KO Mouse Model of Spinal Muscular Atrophy, *ACS Chem Neurosci* 3 (2012) 5-11.
- [133] D.Y. Kwon, W.W. Motley, K.H. Fischbeck, B.G. Burnett, Increasing expression and decreasing degradation of SMN ameliorate the spinal muscular atrophy phenotype in mice, *Hum Mol Genet* 20 (2011) 3667-3677.
- [134] C.R. Heier, C.J. DiDonato, Translational readthrough by the aminoglycoside geneticin (G418) modulates SMN stability in vitro and improves motor function in SMA mice in vivo, *Hum Mol Genet* 18 (2009) 1310-1322.
- [135] Y. Hua, J. Zhou, Modulation of SMN nuclear foci and cytoplasmic localization by its C-terminus, *Cell Mol Life Sci* 61 (2004) 2658-2663.
- [136] V.B. Mattis, M. Bowerman, R. Kothary, C.L. Lorson, A SMNDelta7 read-through product confers functionality to the SMNDelta7 protein, *Neurosci Lett* 442 (2008) 54-58.
- [137] E.C. Wolstencroft, V. Mattis, A.A. Bajer, P.J. Young, C.L. Lorson, A non-sequence-specific requirement for SMN protein activity: the role of aminoglycosides in inducing elevated SMN protein levels, *Hum Mol Genet* 14 (2005) 1199-1210.
- [138] V.B. Mattis, R. Rai, J. Wang, C.W. Chang, T. Coady, *et al.*, Novel aminoglycosides increase SMN levels in spinal muscular atrophy fibroblasts, *Hum Genet* 120 (2006) 589-601.
- [139] V.B. Mattis, M.Y. Fosso, C.W. Chang, C.L. Lorson, Subcutaneous administration of TC007 reduces disease severity in an animal model of SMA, *BMC Neurosci* 10 (2009) 142.
- [140] V.B. Mattis, A.D. Ebert, M.Y. Fosso, C.W. Chang, C.L. Lorson, Delivery of a read-through inducing compound, TC007, lessens the severity of a spinal muscular atrophy animal model, *Hum Mol Genet* 18 (2009) 3906-3913.
- [141] M. Uehata, T. Ishizaki, H. Satoh, T. Ono, T. Kawahara, *et al.*, Calcium sensitization of smooth muscle mediated by a Rho-associated protein kinase in hypertension, *Nature* 389 (1997) 990-994.
- [142] M. Amano, M. Ito, K. Kimura, Y. Fukata, K. Chihara, *et al.*, Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase), *J Biol Chem* 271 (1996) 20246-20249.
- [143] M. Bowerman, D. Shafey, R. Kothary, Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity, *J Mol Neurosci* 32 (2007) 120-131.
- [144] M. Bowerman, L.M. Murray, J.G. Boyer, C.L. Anderson, R. Kothary, Fasudil improves survival and promotes skeletal muscle development in a mouse model of spinal muscular atrophy, *BMC Med* 10 (2012) 24.
- [145] T.O. Gavriliina, V.L. McGovern, E. Workman, T.O. Crawford, R.G. Gogliotti, *et al.*, Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice

- while muscle-specific SMN expression has no phenotypic effect, *Hum Mol Genet* 17 (2008) 1063-1075.
- [146] F.F. Rose, Jr., V.B. Mattis, H. Rindt, C.L. Lorson, Delivery of recombinant follistatin lessens disease severity in a mouse model of spinal muscular atrophy, *Hum Mol Genet* 18 (2009) 997-1005.
 - [147] C.J. Sumner, C.D. Wee, L.C. Warsing, D.W. Choe, A.S. Ng, *et al.*, Inhibition of myostatin does not ameliorate disease features of severe spinal muscular atrophy mice, *Hum Mol Genet* 18 (2009) 3145-3152.
 - [148] M. Bosch-Marce, C.D. Wee, T.L. Martinez, C.E. Lipkes, D.W. Choe, *et al.*, Increased IGF-1 in muscle modulates the phenotype of severe SMA mice, *Hum Mol Genet* 20 (2011) 1844-1853.
 - [149] J.J. Glascock, E.Y. Osman, T.H. Coady, F.F. Rose, M. Shababi, *et al.*, Delivery of Therapeutic Agents Through Intracerebroventricular (ICV) and Intravenous (IV) Injection in Mice, *J Vis Exp* (2011).
 - [150] K.A. Kienstra, D. Freysdottir, N.M. Gonzales, K.K. Hirschi, Murine neonatal intravascular injections: modeling newborn disease, *J Am Assoc Lab Anim Sci* 46 (2007) 50-54.
 - [151] M.S. Sands, J.E. Barker, Percutaneous intravenous injection in neonatal mice, *Laboratory Animal Science* 49 (1999) 328-330.
 - [152] M.A. Passini, J.H. Wolfe, Widespread gene delivery and structure-specific patterns of expression in the brain after intraventricular injections of neonatal mice with an adeno-associated virus vector, *J Virol* 75 (2001) 12382-12392.
 - [153] M.A. Passini, D.J. Watson, J.H. Wolfe, Gene delivery to the mouse brain with adeno-associated virus, *Methods Mol Biol* 246 (2004) 225-236.
 - [154] T.D. Baughan, A. Dickson, E.Y. Osman, C.L. Lorson, Delivery of bifunctional RNAs that target an intronic repressor and increase SMN levels in an animal model of spinal muscular atrophy, *Hum Mol Genet* 18 (2009) 1600-1611.
 - [155] T.H. Coady, T.D. Baughan, M. Shababi, M.A. Passini, C.L. Lorson, Development of a single vector system that enhances trans-splicing of SMN2 transcripts, *PLoS ONE* 3 (2008) e3468.
 - [156] A. Dickson, E. Osman, C.L. Lorson, A negatively acting bifunctional RNA increases survival motor neuron both in vitro and in vivo, *Hum Gene Ther* 19 (2008) 1307-1315.
 - [157] M. Shababi, J. Glascock, C.L. Lorson, Combination of SMN trans-splicing and a neurotrophic factor increases the life span and body mass in a severe model of spinal muscular atrophy, *Hum Gene Ther* 22 (2011) 135-144.
 - [158] Y. Hua, K. Sahashi, F. Rigo, G. Hung, G. Horev, *et al.*, Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model, *Nature* 478 (2011) 123-126.
 - [159] M.E. Butchbach, J.D. Edwards, A.H. Burghes, Abnormal motor phenotype in the SMN Δ 7 mouse model of spinal muscular atrophy, *Neurobiol Dis* 27 (2007) 207-219.

- [160] B.R. Snyder, S.J. Gray, E.T. Quach, J.W. Huang, C.H. Leung, *et al.*, Comparison of adeno-associated viral vector serotypes for spinal cord and motor neuron gene delivery, *Hum Gene Ther* 22 (2011) 1129-1135.
- [161] S.J. Gray, S.B. Foti, J.W. Schwartz, L. Bachaboina, B. Taylor-Blake, *et al.*, Optimizing promoters for recombinant adeno-associated virus-mediated gene expression in the peripheral and central nervous system using self-complementary vectors, *Hum Gene Ther* 22 (2011) 1143-1153.
- [162] A.K. Bevan, K.R. Hutchinson, K.D. Foust, L. Braun, V.L. McGovern, *et al.*, Early heart failure in the SMN Δ 7 model of spinal muscular atrophy and correction by postnatal scAAV9-SMN delivery, *Hum Mol Genet* 19 (2010) 3895-3905.
- [163] M. Shababi, J. Habibi, H.T. Yang, S.M. Vale, W.A. Sewell, *et al.*, Cardiac defects contribute to the pathology of spinal muscular atrophy models, *Hum Mol Genet* 19 (2010) 4059-4071.
- [164] S.E. Brogan, N.B. Winter, Patient-Controlled Intrathecal Analgesia for the Management of Breakthrough Cancer Pain: A Retrospective Review and Commentary, *Pain Med* (2011).
- [165] A.H. Burghes, V.L. McGovern, Antisense oligonucleotides and spinal muscular atrophy: skipping along, *Genes Dev* 24 (2010) 1574-1579.
- [166] M. Shababi, V.B. Mattis, C.L. Lorson, Therapeutics that directly increase SMN expression to treat spinal muscular atrophy, *Drug News Perspect* 23 (2010) 475-482.
- [167] T.M. Wishart, J.P. Huang, L.M. Murray, D.J. Lamont, C.A. Mutsaers, *et al.*, SMN deficiency disrupts brain development in a mouse model of severe spinal muscular atrophy, *Hum Mol Genet* 19 (2010) 4216-4228.
- [168] C.M. Lutz, S. Kariya, S. Patruni, M.A. Osborne, D. Liu, *et al.*, Postsymptomatic restoration of SMN rescues the disease phenotype in a mouse model of severe spinal muscular atrophy, *J Clin Invest* 121 (2011) 3029-3041.
- [169] K. Inagaki, S. Fuess, T.A. Storm, G.A. Gibson, C.F. McTiernan, *et al.*, Robust systemic transduction with AAV9 vectors in mice: efficient global cardiac gene transfer superior to that of AAV8, *Mol Ther* 14 (2006) 45-53.
- [170] L.T. Bish, K. Morine, M.M. Sleeper, J. Sanmiguel, D. Wu, *et al.*, Adeno-associated virus (AAV) serotype 9 provides global cardiac gene transfer superior to AAV1, AAV6, AAV7, and AAV8 in the mouse and rat, *Hum Gene Ther* 19 (2008) 1359-1368.
- [171] T.T. Le, V.L. McGovern, I.E. Alwine, X. Wang, A. Massoni-Laporte, *et al.*, Temporal requirement for high SMN expression in SMA mice, *Hum Mol Genet* 20 (2011) 3578-3591.
- [172] E.Y. Osman, P.F. Yen, C.L. Lorson, Bifunctional RNAs targeting the intronic splicing silencer N1 increase SMN levels and reduce disease severity in an animal model of spinal muscular atrophy, *Mol Ther* 20 (2012) 119-126.
- [173] L.M. Murray, L.H. Comley, D. Thomson, N. Parkinson, K. Talbot, *et al.*, Selective Vulnerability of Motor Neurons and Dissociation of Pre- and Post-Synaptic Pathology at the Neuromuscular Junction in Mouse Models of Spinal Muscular Atrophy, *Hum Mol Genet* (2007).

- [174] A.S. Taylor, J.J. Glascock, F.F. Rose, Jr., C. Lutz, C.L. Lorson, Restoration of SMN to Emx-1 expressing cortical neurons is not sufficient to provide benefit to a severe mouse model of Spinal Muscular Atrophy, *Transgenic Research* 22 (2013) 1029-1036.
- [175] G.H. Park, Y. Maeno-Hikichi, T. Awano, L.T. Landmesser, U.R. Monani, Reduced survival of motor neuron (SMN) protein in motor neuronal progenitors functions cell autonomously to cause spinal muscular atrophy in model mice expressing the human centromeric (SMN2) gene, *J Neurosci* 30 (2010) 12005-12019.
- [176] A.J. Lee, T. Awano, G.H. Park, U.R. Monani, Limited Phenotypic Effects of Selectively Augmenting the SMN Protein in the Neurons of a Mouse Model of Severe Spinal Muscular Atrophy, *PLoS ONE* 7 (2012) e46353.
- [177] C.R. Heier, R. Satta, C. Lutz, C.J. DiDonato, Arrhythmia and cardiac defects are a feature of spinal muscular atrophy model mice, *Hum Mol Genet* 19 (2010) 3906-3918.
- [178] M. Bowerman, K.J. Swoboda, J.P. Michalski, G.S. Wang, C. Reeks, *et al.*, Glucose metabolism and pancreatic defects in spinal muscular atrophy, *Ann Neurol* 72 (2012) 256-268.
- [179] E. Somers, Z. Stencel, T.M. Wishart, T.H. Gillingwater, S.H. Parson, Density, calibre and ramification of muscle capillaries are altered in a mouse model of severe spinal muscular atrophy, *Neuromuscul Disord* 22 (2012) 435-442.
- [180] H. Guo, S. Hong, X.L. Jin, R.S. Chen, P.P. Avasthi, *et al.*, Specificity and efficiency of Cre-mediated recombination in Emx1-Cre knock-in mice, *Biochem Biophys Res Commun* 273 (2000) 661-665.
- [181] C.H. Chan, L.N. Godinho, D. Thomaidou, S.S. Tan, M. Gulisano, *et al.*, Emx1 is a marker for pyramidal neurons of the cerebral cortex, *Cerebral Cortex* 11 (2001) 1191-1198.
- [182] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding, *Anal Biochem* 72 (1976) 248-254.
- [183] J.A. Gorski, T. Talley, M. Qiu, L. Puellas, J.L. Rubenstein, *et al.*, Cortical excitatory neurons and glia, but not GABAergic neurons, are produced in the Emx1-expressing lineage, *J Neurosci* 22 (2002) 6309-6314.
- [184] M. Kummer, K. Kirmse, O.W. Witte, K. Holthoff, Reliable in vivo identification of both GABAergic and glutamatergic neurons using Emx1-Cre driven fluorescent reporter expression, *Cell Calcium* 52 (2012) 182-189.
- [185] E.P. Pioro, J.P. Antel, N.R. Cashman, D.L. Arnold, Detection of cortical neuron loss in motor neuron disease by proton magnetic resonance spectroscopic imaging in vivo, *Neurology* 44 (1994) 1933-1938.
- [186] M.A. Farrar, S. Vucic, H.M. Johnston, M.C. Kiernan, Corticomotoneuronal integrity and adaptation in spinal muscular atrophy, *Arch Neurol* 69 (2012) 467-473.
- [187] S.M. Hammond, R.G. Gogliotti, V. Rao, A. Beauvais, R. Kothary, *et al.*, Mouse survival motor neuron alleles that mimic SMN2 splicing and are inducible rescue embryonic lethality early in development but not late, *PLoS ONE* 5 (2010) e15887.

- [188] G. Bouwsma, N.J. Leschot, Unusual pedigree patterns in seven families with spinal muscular atrophy; further evidence for the allelic model hypothesis, *Clin Genet* 30 (1986) 145-149.
- [189] F. Capon, C. Levato, L. Merlini, C. Angelini, M.L. Mostacciuolo, *et al.*, Discordant clinical outcome in type III spinal muscular atrophy sibships showing the same deletion pattern, *Neuromuscul Disord* 6 (1996) 261-264.
- [190] J.M. Cobben, G. van der Steege, P. Grootsholten, M. de Visser, H. Scheffer, *et al.*, Deletions of the survival motor neuron gene in unaffected siblings of patients with spinal muscular atrophy, *Am J Hum Genet* 57 (1995) 805-808.
- [191] E. Hahnen, R. Forkert, C. Marke, S. Rudnik-Schoneborn, J. Schonling, *et al.*, Molecular analysis of candidate genes on chromosome 5q13 in autosomal recessive spinal muscular atrophy: evidence of homozygous deletions of the SMN gene in unaffected individuals, *Hum Mol Genet* 4 (1995) 1927-1933.
- [192] M. Jedrzejowska, J. Borkowska, J. Zimowski, A. Kostera-Pruszczyk, M. Milewski, *et al.*, Unaffected patients with a homozygous absence of the SMN1 gene, *Eur J Hum Genet* 16 (2008) 930-934.
- [193] P.E. McAndrew, D.W. Parsons, L.R. Simard, C. Rochette, P.N. Ray, *et al.*, Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number, *Am J Hum Genet* 60 (1997) 1411-1422.
- [194] Y. Harada, R. Sutomo, A.H. Sadewa, T. Akutsu, Y. Takeshima, *et al.*, Correlation between SMN2 copy number and clinical phenotype of spinal muscular atrophy: three SMN2 copies fail to rescue some patients from the disease severity, *J Neurol* 249 (2002) 1211-1219.
- [195] I. Cusco, E. Lopez, C. Soler-Botija, M. Jesus Barcelo, M. Baiget, *et al.*, A genetic and phenotypic analysis in Spanish spinal muscular atrophy patients with c.399_402del AGAG, the most frequently found subtle mutation in the SMN1 gene, *Hum Mutat* 22 (2003) 136-143.
- [196] T. Hao le, M. Wolman, M. Granato, C.E. Beattie, Survival motor neuron affects plastin 3 protein levels leading to motor defects, *J Neurosci* 32 (2012) 5074-5084.
- [197] J.N. Sleight, T.H. Gillingwater, K. Talbot, The contribution of mouse models to understanding the pathogenesis of spinal muscular atrophy, *Dis Model Mech* 4 (2011) 457-467.
- [198] A. Bleckert, H. Photowala, S. Alford, Dual pools of actin at presynaptic terminals, *J Neurophysiol* 107 (2012) 3479-3492.
- [199] B. Ackermann, S. Krober, L. Torres-Benito, A. Borgmann, M. Peters, *et al.*, Plastin 3 ameliorates spinal muscular atrophy via delayed axon pruning and improves neuromuscular junction functionality, *Hum Mol Genet* 22 (2013) 1328-1347.
- [200] S. Haramati, E. Chapnik, Y. Sztainberg, R. Eilam, R. Zwang, *et al.*, miRNA malfunction causes spinal motor neuron disease, *Proc Natl Acad Sci U S A* 107 (2010) 13111-13116.
- [201] A. Javaherian, H.T. Cline, Coordinated motor neuron axon growth and neuromuscular synaptogenesis are promoted by CPG15 in vivo, *Neuron* 45 (2005) 505-512.

- [202] E. Nedivi, G.Y. Wu, H.T. Cline, Promotion of dendritic growth by CPG15, an activity-induced signaling molecule, *Science* 281 (1998) 1863-1866.
- [203] V. Setola, M. Terao, D. Locatelli, S. Bassanini, E. Garattini, *et al.*, Axonal-SMN (a-SMN), a protein isoform of the survival motor neuron gene, is specifically involved in axonogenesis, *Proc Natl Acad Sci U S A* 104 (2007) 1959-1964.
- [204] S. Cho, G. Dreyfuss, A degtron created by SMN2 exon 7 skipping is a principal contributor to spinal muscular atrophy severity, *Genes Dev* 24 (2010) 438-442.
- [205] B.F. El-Khodori, N. Edgar, A. Chen, M.L. Winberg, C. Joyce, *et al.*, Identification of a battery of tests for drug candidate evaluation in the SMN Δ 7 neonate model of spinal muscular atrophy, *Exp Neurol* 212 (2008) 29-43.
- [206] N.R. Makhortova, M. Hayhurst, A. Cerqueira, A.D. Sinor-Anderson, W.N. Zhao, *et al.*, A screen for regulators of survival of motor neuron protein levels, *Nat Chem Biol* 7 (2011) 544-552.
- [207] N. Clement, D.R. Knop, B.J. Byrne, Large-scale adeno-associated viral vector production using a herpesvirus-based system enables manufacturing for clinical studies, *Hum Gene Ther* 20 (2009) 796-806.
- [208] R.H. Smith, J.R. Levy, R.M. Kotin, A simplified baculovirus-AAV expression vector system coupled with one-step affinity purification yields high-titer rAAV stocks from insect cells, *Mol Ther* 17 (2009) 1888-1896.
- [209] J.F. Wright, Manufacturing and characterizing AAV-based vectors for use in clinical studies, *Gene Ther* 15 (2008) 840-848.
- [210] N. Chirmule, K. Probert, S. Magosin, Y. Qian, R. Qian, *et al.*, Immune responses to adenovirus and adeno-associated virus in humans, *Gene Ther* 6 (1999) 1574-1583.
- [211] R. Calcedo, H. Morizono, L. Wang, R. McCarter, J. He, *et al.*, Adeno-associated virus antibody profiles in newborns, children, and adolescents, *Clin Vaccine Immunol* 18 (2011) 1586-1588.
- [212] T. Burguete, M. Rabreau, M. Fontanges-Darriet, E. Roset, H.D. Hager, *et al.*, Evidence for infection of the human embryo with adeno-associated virus in pregnancy, *Hum Reprod* 14 (1999) 2396-2401.
- [213] C.L. Halbert, A.D. Miller, S. McNamara, J. Emerson, R.L. Gibson, *et al.*, Prevalence of neutralizing antibodies against adeno-associated virus (AAV) types 2, 5, and 6 in cystic fibrosis and normal populations: Implications for gene therapy using AAV vectors, *Hum Gene Ther* 17 (2006) 440-447.
- [214] S. Boutin, V. Monteilhet, P. Veron, C. Leborgne, O. Benveniste, *et al.*, Prevalence of serum IgG and neutralizing factors against adeno-associated virus (AAV) types 1, 2, 5, 6, 8, and 9 in the healthy population: implications for gene therapy using AAV vectors, *Hum Gene Ther* 21 (2010) 704-712.
- [215] N. DiPrimio, S.W. McPhee, R.J. Samulski, Adeno-associated virus for the treatment of muscle diseases: toward clinical trials, *Curr Opin Mol Ther* 12 (2010) 553-560.
- [216] F. Mingozzi, J.J. Meulenberg, D.J. Hui, E. Basner-Tschakarjan, N.C. Hasbrouck, *et al.*, AAV-1-mediated gene transfer to skeletal muscle in humans results in dose-dependent activation of capsid-specific T cells, *Blood* 114 (2009) 2077-2086.

- [217] J.R. Mendell, L.R. Rodino-Klapac, X.Q. Rosales, B.D. Coley, G. Galloway, *et al.*, Sustained alpha-sarcoglycan gene expression after gene transfer in limb-girdle muscular dystrophy, type 2D, *Ann Neurol* 68 (2010) 629-638.
- [218] M.L. Brantly, J.D. Chulay, L. Wang, C. Mueller, M. Humphries, *et al.*, Sustained transgene expression despite T lymphocyte responses in a clinical trial of rAAV1-AAT gene therapy, *Proc Natl Acad Sci U S A* 106 (2009) 16363-16368.
- [219] J.D. Finn, D. Hui, H.D. Downey, D. Dunn, G.C. Pien, *et al.*, Proteasome inhibitors decrease AAV2 capsid derived peptide epitope presentation on MHC class I following transduction, *Mol Ther* 18 (2010) 135-142.
- [220] J. Adriaansen, Vervoordeldonk, M., Plence, P., Bakker, A., Gicquel, E., Jorgensen, C., Tak, P., Apparailly F., Switching of serotype can improve local AAV-mediated gene therapy in RA, 24th European Workshop for Rheumatology Research, Berlin, Germany, 2004.
- [221] F. Mingozzi, Y. Chen, S.L. Murphy, S.C. Edmonson, A. Tai, *et al.*, Pharmacological modulation of humoral immunity in a nonhuman primate model of AAV gene transfer for hemophilia B, *Mol Ther* 20 (2012) 1410-1416.
- [222] A.C. Nathwani, J.T. Gray, C.Y. Ng, J. Zhou, Y. Spence, *et al.*, Self-complementary adeno-associated virus vectors containing a novel liver-specific human factor IX expression cassette enable highly efficient transduction of murine and nonhuman primate liver, *Blood* 107 (2006) 2653-2661.
- [223] L. Wang, R. Calcedo, T.C. Nichols, D.A. Bellinger, A. Dillow, *et al.*, Sustained correction of disease in naive and AAV2-pretreated hemophilia B dogs: AAV2/8-mediated, liver-directed gene therapy, *Blood* 105 (2005) 3079-3086.
- [224] S.M. Young, Jr., R.J. Samulski, Adeno-associated virus (AAV) site-specific recombination does not require a Rep-dependent origin of replication within the AAV terminal repeat, *Proc Natl Acad Sci U S A* 98 (2001) 13525-13530.
- [225] C.C. Yang, X. Xiao, X. Zhu, D.C. Ansardi, N.D. Epstein, *et al.*, Cellular recombination pathways and viral terminal repeat hairpin structures are sufficient for adeno-associated virus integration in vivo and in vitro, *J Virol* 71 (1997) 9231-9247.
- [226] D.R. Deyle, D.W. Russell, Adeno-associated virus vector integration, *Curr Opin Mol Ther* 11 (2009) 442-447.
- [227] A. Donsante, D.G. Miller, Y. Li, C. Vogler, E.M. Brunt, *et al.*, AAV vector integration sites in mouse hepatocellular carcinoma, *Science* 317 (2007) 477.
- [228] P. Bell, L. Wang, C. Leberherz, D.B. Flieder, M.S. Bove, *et al.*, No evidence for tumorigenesis of AAV vectors in a large-scale study in mice, *Mol Ther* 12 (2005) 299-306.
- [229] D.M. McCarty, S.M. Young, Jr., R.J. Samulski, Integration of adeno-associated virus (AAV) and recombinant AAV vectors, *Annual Review of Genetics* 38 (2004) 819-845.
- [230] D.W. Russell, A.D. Miller, I.E. Alexander, Adeno-associated virus vectors preferentially transduce cells in S phase, *Proc Natl Acad Sci U S A* 91 (1994) 8915-8919.
- [231] A. Fischer, S. Hacein-Bey-Abina, C. Lagresle, A. Garrigue, M. Cavazana-Calvo, [Gene therapy of severe combined immunodeficiency disease: proof of principle of

efficiency and safety issues. Gene therapy, primary immunodeficiencies, retrovirus, lentivirus, genome], Bull Acad Natl Med 189 (2005) 779-785; discussion 786-778.

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