Spinal Muscular Atrophy: From Animal Model to Potential Treatments

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Abstract- Spinal muscular atrophy (SMA) is an autosomal recessive disease characterized by degeneration of the anterior horn cells of the spinal cord leading to muscular paralysis and atrophy. SMA is the second commonest genetic cause of death in childhood affecting approximately 1 in 10,000 live births, but no effective treatment is currently available. Due to gene deletions, mutations, or conversions, the telomeric copy of the *survival of motor neuron (SMN)* gene is abnormal in more than 96% of patients with clinically typical SMA. The identification of SMN-interacting protein strongly suggests that it is involved in the assembly of the spliceosome, processing of pre-mRNA splicing, transcription, and metabolism of ribosomal RNA. Mouse models of human SMA have been established through a combination of knockout and transgenic techniques. These SMA-like mice genotypically and phenotypically mimic SMA patients. They should be useful in elucidating the physiological functions of SMN protein, understanding the pathophysiology of the disease, and also providing a biological system for use in drug testing or stem cell and gene therapies. Recent advances concerning SMN functions and the potentialities of different SMA therapies are discussed.

Key Words: SMA, SMN protein, SMN functions, Drug screening, Gene therapy, Cell replacement therapy

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INTRODUCTION OF SPINAL MUSCULAR ATROPHY

Spinal muscular atrophy (SMA) is characterized by the degeneration of motor neurons of the spinal cord associated with muscle paralysis and atrophy. Clinical diagnosis of SMA is based on progressive symmetric weakness and atrophy of the proximal muscles. Childhood SMA is an autosomal recessive disorder that represents one of the most common genetic causes of death in childhood with a carrier rate of 1-3% in the general population and an incidence of 1 in 6,000-10,000 newborns[1-3]. Based on the age of onset of symptoms, achievement of motor milestones, and age at death, SMA has been subdivided into three clinical types[4]. The acute form, Werdnig-Hoffmann disease (type I), is characterized by the onset of severe muscular weakness and hypotonia in the first few months of life and the inability to sit or walk. Fatal respiratory failure usually occurs within the first 2 years. Type II SMA is characterized by the onset of proximal muscular weakness before 18 months of age. Children are able to sit

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but not walk unaided, and they survive beyond 4 years of age. In type III SMA (Kugelberg-Welander disease), patients have predominantly proximal muscular weakness, resulting from skeletal muscle denervation, which starts after the age of 18 months. They can walk independently until the disease progresses, and survive into adulthood.

SURVIVAL OF MOTOR NEURON GENES

The characterization of the SMA determining locus on chromosome 5q13 revealed a chromosomal region with an inverted duplication[5]. More than 96% of SMA patients have deletions or intragenic mutations in the telomeric copy of the *survival of motor neuron (SMN1)* gene, whereas mutations in the centromeric copy of this gene *(SMN2)* show no clinical consequence, indicating that *SMN1* is associated with SMA[6-8]. *SMN2* is still present in all of those SMA patients but is not able to compensate for the *SMN1* gene defect. Only five nucleotides distinguish *SMN1* from *SMN2* genes without any effect on their amino acid sequences. The sequences and functions of the *SMN1* and *SMN2* gene promoters are almost identical[9]. However, full-length transcripts are almost exclusively produced by *SMN1*, whereas the predominant forms encoded by *SMN2* loss exon 3, 5, or 7, especially exon 7. One nucleotide located in the exon 7 of *SMN2* is responsible for the alternative splicing of this exon[10]. The truncated transcript lacking exon 7, produced by the *SMN2* gene, should encode a protein lacking the last C-terminal 16 residues (SMNΔ7). However, SMNΔ7 is unstable both *in vitro* and *in vivo,* which explains why SMNΔ7 protein does not compensate for the *SMN1* defect[11]. Dosage analysis has also shown that the amount of SMN protein is significantly decreased in patients with severe SMA[12].

PUTATIVE FUNCTIONS OF SMN PROTEIN

Although the expression levels are different, SMN protein is ubiquitously expressed in all tissues without any significant homology to other proteins, so it was difficult to speculate on its function when it was originally identified[5]. We now know that SMN protein associates with several molecules (Table) to form different complexes[13-34]. The SMN complexes are found both in the

Table. *List of SMN protein associating molecules and their putative functions*

SMN associated molecules	Putative biological functions
Gemin2/SIP1	Biogenesis of snRNPs[13]
Gemin3/DEAD box RNA helicase	Transcriptional regulation[14]
Gemin4	A cofactor of Gemin3[15]
Gemin5	Biogenesis of snRNPs or Sm protein rearrangements[16]
Gemin6	Biogenesis of snRNPs[17]
Gemin7	Biogenesis of snRNPs[18]
snRNAs U1 and U5	Biogenesis of snRNPs[13]
snRNPs Sm B/B', D1 and D3	Biogenesis of snRNPs[19,20]
Lsm4 and 6	Biogenesis of the snRNPs and mRNA decay[19,20]
Coilin	Recuritment of SMN complex to Cajal bodies[21]
ZPR1	Localization in nuclear bodies[22]
SMN	RNA splicing[23]
ZNF265	Pre-mRNA splicing[24]
hnRNP-R and gry-rbp/hnRNP-Q	mRNA editing, transport and splicing[25]
Fibrillarin and GAR1	Biogenesis of snoRNPs[26]
RNA helicase A	RNA polymerase II machinery[27]
RNA polymerase II	Transcription[27]
E2 of Epstein-Barr virus	Transcription activation[28]
E2 of papillomavirus	Regulation of gene expression[29]
P53	Transcription activation and apoptosis[30]
Bcl-2	Anti-apoptosis[31]
Osteoclast-stimulating factor	Osteoclast formation and bone resorption[32]
Fibroblast growth factor 2	Cell growth and differentiation[33]
Profilins	Control of actin dynamics[34]

snRNAs: small nuclear ribonuclear acids; snRNPs: small nuclear ribonuclear proteins; snoRNPs: small nucleolar ribonucleoproteins; hnRNP: heterogenous nuclear ribonucleoprotein.

cytoplasm and the nucleus, where they are concentrated in a structure known as gems, most often associated with or identical to Cajal bodies, depending on the cell type or tissue analyzed[12,35]. In cells isolated from SMA patients, the number of gems are significantly decreased compared to cells from healthy individuals, indicating that loss of gem formation may lead to SMA[13]. The identification of SMN-interacting proteins with known or putative functions strongly supports the theory that SMN is involved in the cytoplasmic assembly of snRNP into the spliceosome (a large RNA-protein complex that catalyzes pre-mRNA splicing), pre-mRNA splicing, transcription, and metabolism of ribosomal RNA[13-29]. However, SMN protein has also been shown to directly interact with other proteins, including Bcl-2 and P53, two proteins involved in apoptotic processes, suggesting that SMN may also play an important role in apoptosis[30,31].

There are still many questions related to the functions of SMN protein and the progression of SMA disease that need to be resolved. For example, it is not known how a partial deficiency of SMN protein, but not complete absence, leads to the SMA phenotype. Neither is it known which deficiency in the various putative functions of SMN is responsible for the SMA phenotype nor why spinal motor neurons are the most sensitive cells for reduced levels of SMN. In fact, researchers do not yet know the specific function of the SMN protein in spinal motor neurons. In addition, no specific and effective treatment is currently available for SMA patients. Thus, the development of a suitable animal model for SMA is not only important for understanding the pathophysiology of the disease but also for providing a biological system for use in drug testing or stem cell and gene therapies.

GENERATING SMA MOUSE MODELS

In order to generate SMA-like mice, alternative methods other than traditional mouse *Smn* gene knockout had to be applied, because there is only a single copy of the *Smn* gene in the mouse genome and its targeted disruption resulted in early embryonic lethality[36-38]. An attempt to create a SMA mouse model by deleting exon 7 of the mouse *Smn* gene failed, because homozygous mice died during pre-implantation[37], even though a similar genetic state to that existing in human SMA patients was expected. This result indicated that exon 7-deficient mouse SMN protein is equally as unstable as human SMNΔ7. Heterozygous mice proved phenotypically normal as human carriers[37,38]. We thus developed transgenic mouse lines carrying the human *SMN2* gene and then crossed them with the heterozygous mice. The offsprings of these mice, with a homozygous mouse *Smn* gene knockout and human *SMN2* transgene, both genotypically and phenotypically mimicked human SMA patients[37]. However, increasing the copy number of *SMN2* gene induces a milder SMA phenotype in SMA-like mice similar to that displayed in human patients, indicating that *SMN2* modifies the severity of SMA symptoms[8,37-39]. From an analysis of SMN protein levels of SMA-like mice, we found that the full-length SMN protein was abundantly expressed in tissues other than the spinal cord[37]. These results indicate that there are tissue-specific factors that can modify the post-transcriptional processing of the *SMN2* transgenic transcripts; changing the site of production of full-length SMN protein to tissues other than the spinal cord, resulting in a decrease of full-length SMN protein in the spinal cord.

However, a tissue-specific mouse *Smn* knockout approach was also used to generate SMA mouse models. Using tissue-specific promoters, the mouse *Smn* gene was selectively deleted in neurons[40] and muscle fibers[41], respectively. The neuronal and muscular SMN deficient mice showed some clinical SMA phenotypes, suggesting that not only motor neurons but also skeletal muscles could be targets of SMA gene defects[40,41]. In such studies, the dysfunction of both motor neurons and skeletal muscles contributed to SMA pathogenesis.

POTENTIAL DRUG TREATMENTS FOR SMA

Since SMA patients possess an intact *SMN2* gene, it has been postulated that up-regulation of *SMN2* gene expression, preventing exon 7 splicing of *SMN2* transcripts, or stabilizing SMNΔ7 protein could result in attractive therapeutic strategies for SMA patients[42-45]. Recently, the serine/arginine-rich (SR) and SR-like pro-

teins, which can bind and stimulate an exonic splicing enhancer on *SMN2* transcripts, were found and used to restore full-length SMN protein expression[46,47]. In addition, analyses of promoter sequences of both *SMN1* and *SMN2* genes revealed several consensus-binding sites for transcription factors[9]. The identification of spliceosome-associated proteins that can prevent the exon 7 splicing of *SMN2* pre-mRNA and transcription factors, which are involved in the regulation of *SMN* gene expression, support the idea of screening for compounds that could increase full-length SMN protein expression via the *SMN2* gene. Several groups have generated biological systems, including cells derived from SMA patients or SMA-like mice and cells transfected with the *SMN2* gene, to identify compounds that are able to induce these modifications[42-45]. Molecules have been found, including interferons, sodium butyrate, sodium vanadate, and aclarubicin, that increase the amount of SMN protein produced by the *SMN2* gene, either by activating the *SMN2* gene promoter or preventing the alternative splicing of the *SMN2* exon 7 *in vitro*[42-45]. In addition, a large-scale high-throughput drug screening of chemicals, which could increase the amount of full-length *SMN2* mRNA and/or also prevent alternative splicing of *SMN2* exon 7, has begun[48]. However, the therapeutic effects of these compounds once identified, will have to be validated *in vivo*[43], before proceeding onto clinical trials for SMA patients.

POTENTIAL GENE THERAPIES FOR SMA

Over the past two decades, many gene transfer methods have been developed, including chemicals, viral vectors, liposomes, and electroporation. Historically, transfection of post-mitotic neurons has been labor-intensive, inefficient, unreliable, and cytotoxic[49]. An ideal method for the transfection of neurons should be highly efficient, allow for transfection constructs of various sizes, have limited cytotoxicity, and be easy and safe to perform[49]. The increasing use of recombinant viral vectors, including adenovirus (AdV) and adeno-associated virus (AAV), for transferring DNA into neurons is due to its extremely high infection efficiency as compared to non-viral methods. Since many recombinant viral vectors are replication-incompetent, most of them are relatively safe and easy to use[49]. In addition, intra-muscular injections of replication-defective recombinant AdV and AAV result in high-level recombinant gene expression, specifically in the motor and sensory neurons that innervate the inoculated muscles[50,51]. Neural expression of recombinant genes results from viral transport from injected muscles through retrograde exonal transport. Gene transfer can thus specifically target to particular regions of the brain or spinal cord by appropriate choice of the injected muscle.

Reduced expression of a neurotrophic factor, ciliary neurotrophic factor (CNTF), in the nerves of patients with motor neuron diseases, such as amyotrophic lateral sclerosis (ALS) and SMA, in comparison to healthy individuals has been reported[52]. A lack of neuronal growth factors has been proposed as one possible etiology of motor neuron diseases, therefore, neurotrophic factors have long been considered as potential candidates for the treatment of human motor neuron diseases. Their clinical use, however, as injected recombinant protein, has been limited by toxicity and/or poor bioavailability[53]. In contrast, intra-muscular delivery of both neurotrophin-3 and CNTF genes by recombinant AdV and AAV-mediated gene transfer of glial cell line-derived neurotrophic factor, have been shown to exhibit therapeutic effects in progressive motor neuronopathy and ALS model mice, respectively[53,54]. Anti-apoptotic *bcl-2* gene expression via recombinant AdV, has also been shown to have a protective effect on the degeneration of motor neurons in an ALS mouse model[55]. Recently, it has been shown that recombinant AdV-delivered human *SMN1* gene expression to primary fibroblast cell lines, derived from SMA type I patients, can restore normal SMN expression patterns[56]. Therefore, recombinant viral vector-mediated gene-transfer of neurotrophic factors, anti-apoptosis gene products, and SMN protein offer new prospects for the treatment of SMA and other motor neuron diseases.

POTENTIAL CELL REPLACEMENT THERAPY FOR SMA

Stem cells have generated excitement for their applications in studying tissue and organ development, and

their potential to replace damaged cells[57]. Bone marrow stem cells (BMSC) are pluripotent cells able to differentiate into various cell types including skeletal muscles or neurons, *in vitro* and *in vivo*[57-59]. An expansion and mobilization in circulating BMSC from an *in vivo* treatment with bone marrow-stimulating factors have been shown to increase the amount of BM-derived neuronal cells in mouse brain[60]. From a study in model mice of a ischemic heart disease, BMSC, mobilized by bone marrow-stimulating factors, were found to target to the infarcted region of the heart, replicate, differentiate, and ultimately promote myocardial repair[61]. The same treatment also decreased the brain infarct volume and enhanced the survival rate in a rat model of cerebral ischemia[62]. Therefore, mobilization of primitive BMSC by cytokine treatment might offer a non-invasive therapeutic strategy for ischemic heart diseases and neurodegenerative diseases[60-62]. Alternatively, exogenous administration of bone marrow cells has been shown to experimentally repair brain tissue under some pathological circumstances, by differentiation of BMSC into both neurons and glial cells[59,63]. Since SMA is a motor neuron degenerative disease, endogenous and/or exogenous increases in BMSC may provide source of differentiated functional motor neurons to replace degenerated motor neurons. However, the endogenous BMSC from SMA patients still have *SMN1* gene defect, therefore, the BMSC should be isolated, transferred with potential therapeutic gene(s), and then transplanted back to SMA patients. SMA model mice could offer opportunities for testing transplanted and gene-transferred BMSC into functional spinal motor neurons, and then replacing damaged or lost cells of SMA-like mice.

PROSPECTS

Refined characterization of the degenerative process in SMA mice and the identification of the molecular pathway downstream of the defective SMN protein will provide further insights into SMA in the near future. Determining the pathophysiology of SMA and the functions of SMN protein should help in the design of potential targeted or non-targeted therapeutic molecules. Even now, genes of neurotrophic factors, anti-apoptosis proteins, and full-length SMN protein are potential candidates for the treatment of SMA. However, the recombinant viral vectors need to be further modified to provide a more convenient and safe gene transfer system. In combination with potential gene therapy, BMSC or neuron progenitor cells may be used as target cells for transplantations to cure neuron degenerative diseases, including SMA. The generated SMA-like mice should be a great platform to test candidate drugs and SMA therapies. The results of these studies will also be very useful for other degenerative disease therapies.

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