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Development of a Novel Strategy to Treat Spinal Muscular Atrophy

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Development of a Novel Strategy to Treat Spinal Muscular Atrophy

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Abstract

Spinal muscular atrophy (SMA) is an autosomal recessive disorder caused by reduced levels of the survival motor neuron (SMN) protein, which results in degeneration of motor neurons, leading to muscle weakness, and, ultimately, death. To provide an effective therapy for SMA, SMN expression must be restored in motor neurons. The goal of our research is to develop protein-based therapeutics for SMA. Protein transduction domain (PTD), from the human immunodeficiency virus-transactivator of transcription (HIV-TAT), mediates the transduction of any polypeptide to which they are fused. We produced bacterial expression cassettes of PTD-SMN. The PTD-SMN is able to transduce cells *in vitro*, reaching the nucleus and forming punctate structures similar to that of endogenous SMN. Internalization of PTD-SMN results in an increased nuclear-staining foci (gems) number. Importantly, this PTD-SMN co-localizes with coiled bodies, indicating PTD-SMN is able to localize to the correct region of the nucleus. However, we were unable to detect interaction of PTD-SMN with SMN known binding partners, suggesting that majority of PTD-SMN has low activity. Our results suggest that PTD-SMN produced from a prokaryotic source may be useful for SMA therapy, but optimization of protein production is required before this therapy can reach its full potential.

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List of Abbreviations

A549 cells - human lung epithelial carcinoma cells
AAV - adeno-associated virus
Ad - Adenovirus
AIDS - acquired immunodeficiency syndrome
BBB - brain blood barrier
BHK cells - baby hamster kidney cells
CAR - coxsackie and adenovirus receptor
cDNA - complementary deoxyribonucleic acid
CHO cells - Chinese hamster ovary cells
CMV - cytomegalovirus
CNS - central nervous system
CPPs - cell penetrating peptides
CsCl - cesium chloride
DNA - deoxyribonucleic acid
DTT - dithiothereitol
EDTA - ethylenediaminetetraacetic acid
EIAV - equine infectious anemia virus
EMG - electromyography
FACS - fluorescence-activated cell sorting
FBS - fetal bovine serum
FITC - fluorescein isothiocyanate
FLAG - epitope tag DYKDDDDK
G-CSF - granulocyte colony-stimulating factor
GM-CSF - granulocyte-macrophage colony-stimulating factor
GPIIb/IIIa – platelet glycoprotein IIb/IIIa
hAd5 - human adenovirus type 5
HDAC - Histone deacetylases
HEK-293 cells - human embryo kidney cells
HeLa cells - human epithelium-derived adenocarcinoma cells
HEPES - 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HIV-1 - human immunodeficiency virus type 1
hnRNP – hetero nuclear ribonucleoprotein
hSMN - human survival motor neuron gene
IPTG - Isopropyl β-D-1-thiogalactopyranoside
ITR - inverted terminal repeats
MEM - Minimum essential medium
MOI - multiplicity of infection
mRNAs – messenger ribonucleic acid
MYC - myelocytomatosis epitope tag
NAIP - neuronal apoptosis inhibitor protein gene
NLS - nuclear localization signal
NMJ – neuromuscular junction
NS0 cells - mouse myeloma cells
PBS - phosphate buffer saline

PC12 cells – cell line derived from pheochromocytoma of the rat adrenal medulla
PCR - polymerase chain reaction
PRT - Protein replacement therapy
PTDs - protein transduction domains
PVDF – polyvinylidene difluoride membrane
RIPA – radioimmunoprecipitation buffer
RNA – ribonucleic acid
RNAi – ribonucleic acids interference
SDS-PAGE - sodium dodecyl sulfate polyacrylamide gel electrophoresis
SMA - Spinal muscular atrophy
SMN – survival motor neuron
snRNAs – small nuclear ribonucleic acids
snRNPs – small nuclear ribonucleic proteins
SR – serine/ arginine- rich splicing
Tat - transactivator of transcription
TBST – Tris-buffered saline tween-20
TFNR – transcription factor-like nuclear regulator
US FDA - United States Food and Drug Administration
UTR – untranslated region
VPA- valproic acid

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Chapter 1 – Introduction

1.1 General Introduction

Since the disclosure of the first draft of the human genome sequence in 2001, it has been estimated that our genome contains 25,000- 45,000 genes (Lander, et al, 2001; Pennisi, 2003), of which 2,500 have been identified as disease- causing genes (McKusick, 2009). As pointed out by Leader et al, every gene coded by the 3 billion base pairs contained in the human genome can potentially mutate, resulting in several thousand disorders (Leader, et al, 2008). Availability of the complete human genome sequence opens new possibilities for the development of novel therapeutics to target disease-causing genes. Unfortunately, there are still 1,500 known monogenic phenotypes for which the defective gene remains unknown (McKusick, 2009).

Among the immensurable list of genetic disorders, inherited neuromuscular diseases, such as muscular dystrophies, amyothrophic lateral sclerosis, congenital myopathies, spinal muscular atrophies and others, comprise a group of diseases affecting skeletal muscle and motor neurons, which present unique challenges in the search for effective therapies (Arnett, et al, 2009a). From childhood to adulthood onset of the disease, patients are restricted in their motions, condemning them to a life of continuous limitations and, in the worst cases, to early death. Fortunately, with the advent of new technologies, there are new and promising treatment strategies for these disorders, such as stem cell and gene therapy, and RNA-modification therapy (Arnett, et al, 2009b; O'Connor, et al, 2006). Furthermore, a novel strategy that is gaining power in the pharmaceutical industry is protein therapy, which has proven effective in some diseases (Ashton, 2001; Ho, et al, 2003).

In the following thesis, I present a complete description of my project, which was focused on developing a new treatment for SMA, the number one killer of infants under the age of two; hoping to lessen the devastating symptoms of this neuromuscular disorder.

1.2 Spinal muscular atrophy

1.2.1 Introduction

Spinal muscular atrophies comprise a group of genetically heterogeneous disorders, characterized by degeneration of lower motor neurons of the spinal cord and brainstem motor nuclei, resulting in symmetrical weakness and wasting of skeletal muscle (Dubowitz, 1991). The majority of patients present with an autosomal disorder with proximal manifestation in muscle weakness, leading to paralysis and ultimately death. With an incidence of 1 in 6,000 to 10,000 live births (McAndrew, et al, 1997; Pearn, 1978; Roberts, et al, 1970), and a carrier frequency of 1:35 (Cusin, et al, 2003; Feldkotter, et al, 2002), SMA is considered one of the most common genetic disorders that leads to death in early childhood.

1.2.2 Clinical features of SMA

Based on the variable clinical manifestations, achieved motor abilities, and age of onset of the disease, the International SMA Consortium classified SMA into four categories, from type I to type IV (Table 1.1). Briefly, type I SMA is the most severe form, which manifests within the first 6 months of life; patients suffer from severe muscle weakness and hypotonia that result in death at a very early age. Type II SMA is a milder form, although it is considered an intermediate form, since patients lack the ability to walk, and life

expectancy largely depends on the degree of respiratory distress. Type III and IV SMA present symptoms during childhood and adulthood, respectively, do not usually present a limited life span, and patients are capable of performing tasks such as walking or standing (Sumner, 2006). In addition, reports of patients with very severe muscle weakness and respiratory distress at birth are considered type 0, associated with death at birth (Russman, 2007).

Table 1.1. Clinical classification of SMA.

SMA Type	Age of Onset	Development Characteristics	Approximate Age of Death
0	At birth	Very severe muscle weakness	At birth
I (Werdnig-Hoffmann)	< 3-6 months	Never able to sit or maintain posture	< 2 years
II (Intermediate form)	> 6 months	Able to sit, unable to walk or stand unaided	Adolescence or adulthood
III (Kugelberg-Welander)	< 3 years >	Able to sit and walk	Lifespan is not reduced
IV (Adult form)	> 30 years	Mildly affected	Lifespan is not reduced

1.2.3 Molecular genetic basis of SMA

By linkage analysis of several families suffering from type I, II and III SMA, the mutant gene responsible for the disorder was mapped to the 5q12-q13 region (Melki, et al, 1990). Not only does this region harbor the gene for SMA, *SMN*, but also the neuronal apoptosis inhibitor protein gene (*NAIP*), the basal transcritption factor subunit *p44* gene, and the *H4F5t* gene, which are all present in telomeric and centromeric copies in a large inverted duplication (Wirth, 2000) (Fig.1.1). Due to repeated sequences, pseudogenes, and a retro-transposable element located within this region, unequal rearrangements between highly homologous elements occur, generating deletions, duplications or gene conversion events (Tizzano, 2001). Nevertheless, it has been clearly demonstrated that the only causative gene for SMA is *SMN*.

In humans there are two copies of the *SMN* gene, of which, the telomeric copy or *SMN1* undergoes conversion events, homozygous loss, or mutations, resulting in SMA; whereas the centromeric copy of *SMN* or *SMN2* is unaffected (Burghes, 1997; Lefebvre, et al, 1995). Deletions of exon 7 and 8 in *SMN1* occurs in ~95% of patients; whereas nonsense, frameshift, or missense mutations have also been reported (Lefebvre, et al, 1995; Parsons, et al, 1998), demonstrating that the *SMN1* gene is crucial for pathogenesis. *SMN2*, an almost identical gene to *SMN1*, fails to fully compensate for the lack of *SMN1*, due to a translationally silent mutation which changes a cytosine to a thymidine at position +6 of exon7, disrupting a putative exonic splicing enhancer, and resulting in the synthesis of an unstable protein not able to self-oligomerize or interact with other proteins (Lorson, et al, 1999; Monani, et al, 1999). In support to the role of *SMN* as a disease determining gene,

deletion of *SMN* in mice, which only carry a single copy, leads to early embryonic lethality (Schrank, et al, 1997).

Whereas the *SMN1* gene produces 100% of the full length protein, *SMN2* produces only 10% full-length with the remaining 90% lacking exon 7. Although 5-10% of the normal population lacks the centromeric copy of *SMN*, all patients with SMA retain the *SMN2* gene (Sumner, 2006; Wirth, 2000) (Fig. 1.2). In fact, most patients with SMA type I carry one or two copies of *SMN2*, while more than 80% of type II SMA patients carry three *SMN2* copies, and almost 100% of patients with type III SMA carry three or four *SMN2* copies (Feldkotter, et al, 2002), demonstrating that *SMN2* copy number has a very important modifying effect on disease severity.

Furthermore, there exist asymptomatic individuals who lack *SMN1* gene, but retain an increased *SMN2* dosage (Mailman, et al, 2002; Prior, et al, 2004). Other genes such as *NAIP* modify the severity of SMA; mutations in *NAIP* lead to a more severe SMA phenotype, regardless of the *SMN2* copy number (Jedrzejowska, et al, 2009; Watibayati, et al, 2009).

Figure 1.1: Schematic representation of the inverted duplication in the 5q region, containing 4 duplicated genes, flanked by HRAD17 and TFNR. Adapted from Wirth, B., Human Mutation (2000), 15, 228-237.

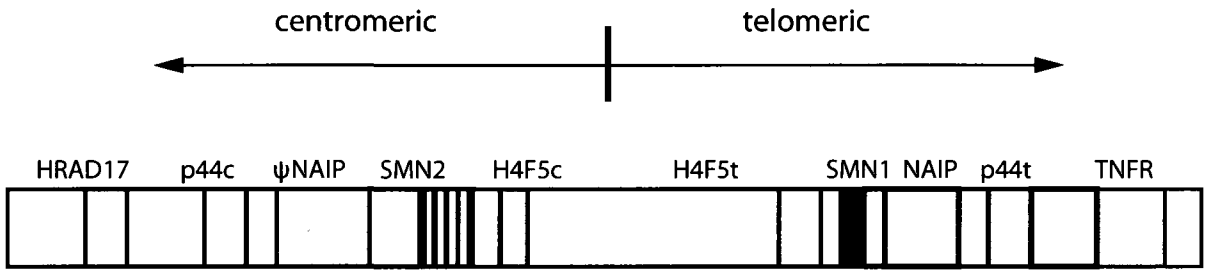
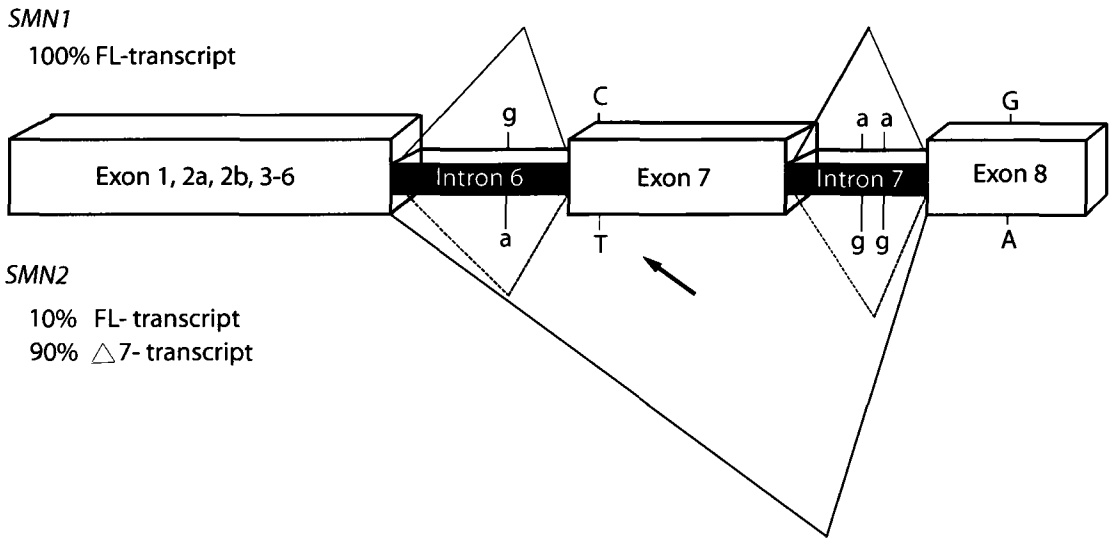

centromeric
telomeric
HRAD17
p44c
ψNAIP
SMN2
H4F5c
H4F5t
SMN1
NAIP
p44t
TNFR

Figure 1.2: Schematic representation of *SMN1* and *SMN2* genes. The two genes differ in five nucleotides, without translational consequences, except for the C to T transition in exon 7. *SMN1* produces 100% of full length transcript, and *SMN2* only produces 10%. Adapted from Wirth, et al, Seminars in Pediatric Neurology (2006), 13, 121-131.


SMN1
100% FL-transcript
Exon 1, 2a, 2b, 3-6
Intron 6
Exon 7
Intron 7
Exon 8
g
a
C
T
a a
g g
G
A
SMN2
10% FL- transcript
90% △7- transcript

1.2.4 The survival motor neuron protein

The *SMN* gene codes for a 38 kDa protein that is ubiquitously expressed. The protein is diffusely localized in the cytoplasm; whereas, in the nucleus, SMN protein is concentrated in punctate structures, called gems, which are in close proximity to Cajal or coiled bodies (Liu, et al, 1996). Cajal bodies participate in the biogenesis of small nuclear ribonucleoproteins (snRNPs), including their maturation, storage, and transport (Matera, et al, 1998). In all fetal tissues, gems and coiled bodies are observed as separate structures (Young, et al, 2001), which reach complete co-localization by adulthood (Young, et al, 2000). Interestingly, the number of gems is dramatically reduced in type I SMA patients, revealing an inverse correlation between the number of gems and severity of the condition (Coovert, et al, 1997).

1.2.4.1 SMN in RNA metabolism. Initial immunocytochemistry complemented with co-immunoprecipitation analysis of SMN, led to the discovery that SMN protein is part of a highly stable multiprotein complex that contains proteins involved in RNA metabolism (Liu, et al, 1997), Gemins2-8 (Baccon, et al, 2002; Carissimi, et al, 2006; Charroux, et al, 1999; Charroux, et al, 2000; Gubitz, et al, 2002; Pellizzoni, et al, 2002), and unrip proteins (Carissimi, et al, 2005). Currently, the best characterized function of SMN is related to the biogenesis of snRNPs. snRNPs are the major components of the spliceosome, the machinery that carries out pre-messenger RNA (pre-mRNA) splicing. Newly exported small nuclear ribonucleic acids (snRNAs) (U1, U2, U4, U5, U11, U12, and U4atac) in the cytosol, interact with 7 common Sm proteins, core components of snRNPS, and other proteins specific to each snRNAs to form snRNPs; which, after processing of the 5' and 3' end of the snRNAs,

are imported into the nucleus (Yong, et al, 2004). This complex is assembled through direct interaction of Sm proteins with snRNAs mediated by the SMN protein, providing specificity to the process (Fischer, et al, 1997; Meister, et al, 2001; Pellizzoni, et al, 2002; Yong, et al, 2004). Taken together, these findings suggest that when SMN expression is decreased, snRNPs assembly is affected.

Moreover, lower assembly and activity of snRNPs is directly proportional to the levels of *SMN* protein in *SMN*-deficient models (*in vitro* and *in vivo*) and SMA patients, demonstrating that there exists a strong correlation between levels of *SMN* protein and snRNP biogenesis (Zhang, et al, 2008; Gabanella, et al, 2007; Pellizzoni, et al, 1998; Wan, et al, 2005; Zhang, et al, 2008). Accordingly, *SMN* deficiency results in a different repertoire of snRNAs and messenger RNAs (mRNAs), leading to defects in pre-mRNA and a subsequent decrease in several transcripts, strongly suggesting that SMA might be a splicing disease, which not only affects motor neurons, but also other tissues (Anderson, et al, 2004; Zhang, et al, 2008). However, if clinical manifestations of SMA patients are a direct consequence of the detrimental splicing defects caused by the reduced levels of *SMN*, motor neurons should not be the only tissue affected. It is evident that further studies are required to elucidate the overall involvement of snRNPs in motor axon defects.

1.2.4.2 SMN in motor neurons. Several studies have tried to understand why motor neurons are more susceptible than any other tissue to the loss of *SMN*. There is evidence of the presence of the SMN protein in axons and growth cones of motor neurons, which peaks during embryonic development, but decreases post-natal and remains unchanged during adulthood (Fan, et al, 2002; Jablonka, et al, 2001; Pagliardini, et al, 2000; Rossoll, et al,

2002). In line with the role of SMN in RNA processing, SMN has been found to interact with two heterogenous nuclear ribonucleoproteins (hnRNP), hnRNP-R and hnRNP-Q, which are proteins involved in mRNA editing, transport and splicing in motor neurons (Rossoll, et al, 2002). Specifically, it has been reported that hnRNP-Q is a crucial component of the splicing machinery for pre-mRNA splicing, interacting only with wild type SMN but not the SMN mutant forms (Mourelatos, et al, 2001; Rossoll, et al, 2002). These studies strongly support an important role for SMN in RNA metabolism in this cell type.

Studies of cultured forebrain neurons from chick embryos and rat spinal cord showed that SMN protein form granules which are actively transported in neuronal processes and growth cones, dependent on both microtubules and microfilaments, and that the SMN lacking exon 7 mutant leads to impaired granule formation and generation of shorter neurites (Zhang, et al, 2003). Growth cone motility, axon outgrowth, and guidance are partly governed by actin filaments, microtubules, and their associated proteins (Dent, et al, 2003). As a consequence, reduced axonal growth observed in SMA models *in vitro* and *in vivo* could be caused by the malformed distribution of actin proteins. For example, β-actin protein is concentrated in growth cones of PC12 cells and primary motoneurons; whereas in SMN-deficient motor neurons, β-actin is deficient in growth cones and distal parts of the axon (Rossoll, et al, 2003). β-actin protein localization to growth cones depends largely on the axonal transport of the β-actin mRNA (Kislauskis, et al, 1997). The hnRNP-R protein interacts with the 3' untranslated region (UTR) of the β-actin mRNA, which is important for β-actin transport; this interaction is mediated by SMN (Rossoll, et al, 2003).

In addition to interacting with hnRNPs and actin, SMN also associates with profilin I and II isoforms, which are small actin-binding proteins and regulators of actin dynamics

(Bowerman, et al, 2007; Giesemann, et al, 1999; Sharma, et al, 2005). The proline-rich motifs at the carboxy terminus of SMN interact with both isoforms of profilin, but with higher affinity with profilin II, which is predominantly expressed in motor neurons (Giesemann, et al, 1999). Knockdown of profilin I and II inhibites neurite outgrowth of PC12 cells (Sharma, et al, 2005). Furthermore, it has been shown that deficiency in SMN leads to altered expression of profilin II, manifesting in upregulation of the RhoA/ROCK actin remodeling pathway and defects in neuronal integrity, such as shorter neurites and increase swelling along neurites (Bowerman, et al, 2007). These reports suggest that SMN deficiency compromises the cytoskeleton integrity of neurons.

Altogether, not only is SMN important for the assembly of snRNPs, but also for motor neuron outgrowth and maintenance, as evidenced by several studies which convincingly showed the role of SMN in mRNA transport along the axons, as well as its interaction with actin-binding proteins (Sumner, 2006).

Furthermore, based on the main features of the disease, which is weakness of the skeletal muscle, one can predict that skeletal muscle is also a target of the low levels of SMN. In order to gain more understanding of which organ is more affected, motor neurons or skeletal muscle, animal models represent an excellent tool to understand the phenomenon better.

1.2.5 Animal models of SMA

The development of animal models of SMA not only has yielded important insights into the disease pathogenesis, but also it has provided essential *in vivo* systems for identifying and validating the efficacy of potential therapeutics. The *SMN2* gene is unique to humans; most other organisms possess a single copy of the *SMN* gene (Monani, et al, 2000; Schmid, et al, 2007). Nematode, fly and mouse models with no functional SMN protein have a uniformly early embryonic lethal phenotype. To gain more insight into the characteristics of SMA and how defects in *SMN* lead to disease, several animal models have been designed, which have provided a better understanding of SMN biology.

Strong evidence of the vital importance of *SMN* is the embryonic lethality of mice caused by depletion of the *SMN* gene (Schrank, et al, 1997). In these mice, the expression of the *SMN* gene is inactivated by placing a *lacZ* gene fused in-frame to the first 40 nucleotides of exon 2. The resulting embryos do not implant; however, they do reach the early morula stage due to the maternal *SMN* contribution, whose depletion coincides with the death of mutant embryos. This report confirms the requirement of *SMN* for survival as it has been suggested by previous studies.

To better understand the SMA pathology, generation of mouse models that mimic SMA was performed by several scientists. Two research groups demonstrated independently that expression of human *SMN2* in a null *SMN* background mice can correct the SMA-like phenotypes (Hsieh-Li, et al, 2000; Monani, et al, 2000). Specifically, $SMN^{-/-}$;*SMN2* mice carrying one copy of *SMN2* die within the first 6h of birth; whereas, eight copies of *SMN2* allows survival longer than 10 months of age (Monani, et al, 2000). Moreover, expression of SMN2 protein is dramatically reduced in spinal cord compared to other tissues, suggesting

that there could be tissue-specific factors that modulate expression of the *SMN2* gene, resulting in a pathological condition (Hsieh-Li, et al, 2000). At the same time, generation of milder forms of SMA corresponding to type II and III has been reported (Le, et al, 2005; Monani, et al, 2003). To illustrate, introduction of a transgene carrying a missense mutation in exon 1 of the *SMN* gene into the severe SMA genetic background, generates type III SMA mice which suffer from motor neuron degeneration, muscle atrophy and abnormal electromyography (EMG) patterns (Monani, et al, 2003). Additionally, transgenic expression of *SMNΔ7*, the major product of *SMN2*, on a severe SMA background, $SMN^{-/-}$;*SMN2* (1 copy), increases lifespan from 5 to 13 days on average, agreeing with previous observations that expression levels of SMN is determinant for the severity of the condition (Le, et al, 2005).

SMA mouse models also provided useful information as to which tissue is more affected by the reduction of SMN protein. Using a Cre-LoxP system to delete exon 7 from specific cell types (Sauer, et al, 1988), it was demonstrated that the full-length *SMN* is essential for the survival of neurons (Frugier, et al, 2000) and muscle (Cifuentes-Diaz, et al, 2001b). Conditional deletion of the murine *SMN* exon 7 targeted to neurons results in a SMA phenotype; severe motor defects associated with tremors are observed, and denervation of skeletal muscle (Frugier, et al, 2000). Similarly, deletion of *SMN* exon 7 targeted to skeletal muscle leads to muscle necrosis with a dystrophic phenotype causing muscle paralysis and death, suggesting a primary role of *SMN* in muscle (Cifuentes-Diaz, et al, 2001a). Despite the evidence that reduction of SMN expression in skeletal muscle generates severe muscular dystrophy, Gavrilina et al showed that rescue of severe SMA mice can be achieved through

up-regulation of *SMN* in neurons, but not in muscle, indicating that motor neurons are the primary target of deficient SMN expression (Gavrilina, et al, 2008).

Drosophila has emerged as a useful model system to elucidate fundamental cellular mechanisms of neurodegenerataive diseases, due to similarities shared with humans (Marsh, et al, 2006). Missense mutations in the *SMN* gene of *Drosophila melanogaster* cause inability of the protein to self-associate (Chan, et al, 2003). Larvae mutants develop a progressive loss of motility and increased uncoordinated movement before death. In addition, rescue of phenotypes is only possible if SMN is overexpressed in muscles and neurons, suggesting that SMN is required at both sides of the neuromuscular junction (NMJ). Likewise, directed-*SMN* ribonucleic acid interference (RNAi) in *Drosophila* leads to abnormal NMJ structure (Chang, et al, 2008). Specifically, reduction of SMN in muscle and neuron reveals a reduction in the number of synaptic boutons and a decrease of neurotransmitter receptors. Furthermore, screening of transposon insertions in the *Drosophila* genome identified a novel mutation which impairs jumping and flying, due to a specific *SMN* knockdown in the thorax muscle (Rajendra, et al, 2007). More importantly, it was observed that *SMN* mutant myofibers do not form thin filaments, and that SMN colocalizes with sarcomeric actin in myofibers. Based on these observations, this report provides evidence of the involvement of SMN in muscle and a likely muscle specific function.

McWhorter et al developed a SMA zebrafish model system using antisense morpholino technology (McWhorter, et al, 2003). Zebrafish have a well-characterized nervous system and relatively simple neuromuscular organization. *SMN* mutant embryos developed motor-axon specific pathfinding defects, without altering other neuronal cell types, or affecting muscle development. Ubiquitous knockdown of *SMN* specifically targets

motor axon outgrowth causing a dramatic increase in motor axon branching, strongly suggesting that SMN reduction primarily affects motor neurons. However, a study done by Winkler et al suggests that motor axon defects are not a direct consequence of SMN reduction; for instead, depletion of Gemin2, a component of the SMN complex, mimics motor axon defects as described above, and that coinjection of snRNAs into SMN or Gemin2 mutants equally ameliorate SMA-like phenotypes (Winkler, et al, 2005). Moreover, a more recent study has shown that transgenic zebrafish with human *SMN* driven by a motoneuron specific promoter ameliorates the phenotypes observed in NMJ formation at the pre-synaptic side, although survival is not improved (Boon, et al, 2009). SV2, a pre-synaptic protein at the NMJ, is dramatically reduced in *SMN* zebrafish mutants, suggesting SMN plays a role in NMJ formation, although it is unclear whether this is a direct or indirect consequence of SMN reduction.

Despite the contributions that animal models have made in understanding SMA pathology, it is quite obvious that results obtained from these models are not consistent; in fact, some contradict. Therefore, as Burghes warns, it is crucial to critically analyze the results, and pay particular attention to the techniques of development of these animal models (Burghes, 2009).

Table 1.2. Characteristic of SMA animal models.

Species	Mutation	Phenotype
Drosophila Melanogaster	Missense mutation in *SMN* gene	Inability of *SMN* to self-associate
	RNA interference *SMN* knockdown	Abnormal neuromuscular junction
	SMN mutation directed to thorax muscles	Lack of the ability to jump or fly
Zebrafish	Ubiquitous *SMN* knockdown using antisense morpholines	Motor axon defects
Mouse	$SMN^{-/-}$	Embryonic lethality
	$SMN^{-/-}$;*SMN2*	Number of copies of *SMN2* determines severity of the condition
	Conditional deletion of *SMN* exon7 to neurons	Denervation
	Conditional deletion of *SMN* exon7 to skeletal muscle	Muscle necrosis

1.2.6 Current treatment or disease management

Even though intense research has led to insights of SMA pathology and biology, it has not found a cure for SMA. In order to ameliorate the conditions and prevent rapid progression of the disease, supportive care is crucial. Respiratory, nutritional, and orthopedic management are required to prevent or diminish insufficient respiratory muscle strength, undernutrition and obesity, and progression of scoliosis respectively (Burnett, et al, 2009). The most prevalent feature of a SMA patient is respiratory insufficiency, due to weakness of muscles involved in breathing. The use of ventilators has really made a difference in smaller patients. In addition, progressive development of scoliosis can be retarded by the use of braces, especially if applied early. Further, spinal fusion surgery is often recommended in order to prevent major deformities. Altogether, SMA physicians agree on an early treatment of patients before more dramatic symptoms appear, and rapid progression of disease is inevitable.

1.2.7 Therapeutic prospects of SMA

In spite of its devastating outcome, SMA has an advantage over other genetic diseases in that there is a duplicate of the *SMN* gene, which can be targeted for therapeutic purposes. A variety of strategies to treat SMA have been proposed in order to ameliorate the symptoms, or even cure the disease. Among these strategies include the up-regulation of the *SMN* gene by activating *SMN2*, stem cell technology, use of neuroprotective agents, gene therapy and protein replacement therapy. Briefly, I will discuss the most relevant studies about the above mentioned strategies.

1.2.7.1 Restoration of the *SMN2* gene. Correction of the splicing mechanism of *SMN2* can increase the expression of the full length SMN2 protein. As shown by Brichta, et al, 0.5 to 500 μM of Valproic acid (VPA) increases the level of the FL-SMN2 2-4 fold. They report that this increase could be due to upregulation of the most important serine/arginine-rich splicing (SR)- like splicing factor, Htra2-β1; however, they also suggest that upregulation of the FL-SMN2 is due to higher transcriptional activators, such as SR proteins (Brichta, et al, 2003). VPA is known to act as a histone deacetylase inhibitor which has gained the approval of the United States Food and Drug Administration (US FDA). Histone deacetylases (HDAC) inhibitors regulate the level of histone acetylation, a post-translational modification of nucleosomal histones that in most case enhances gene transcription (Jenuwein, et al, 2001). Histone acetylation promotes gene transcription by relaxing chromatin structure and facilitating access to DNA by the transcriptional machinery, whereas histone deacetylation promotes a condensed chromatin state and transcriptional repression. HDAC inhibitors have the capacity to act on the promoter of *SMN2*, activating the gene, and increasing the levels of FL-SMN produced from *SMN2* (Kernochan, et al, 2005). More specifically, by using chromatin immunoprecipitation (ChIP), it was learned that HDAC inhibitors activate the *SMN2* promoter and modify histone acetylation of the *SMN2* gene. Treatment of SMA patient fibroblasts showed a ~2-fold increase in acetylated H3 histone levels upon treatment with 2nM of VPA, strongly suggesting the role of acetylation in *SMN2* expression regulation.

1.2.7.2 Neuroprotective agents. Since one of the hallmarks of SMA is degeneration of motor neurons, methods to protect them have also emerged. Riluzole is a candidate drug that exhibits neurotrophic activity, promotes neuronal survival, and enhances axonal pathfinding; however, when riluzole was administered in mutant mice carrying a deletion of *SMN* exon 7

directed to neurons, a very modest increase in survival was observed as compared to control animals, and no improvement in motor neuron performance was observed (Haddad, et al, 2003). In addition, positive effects of salbutamol have been reported in an open pilot study in patients with muscle disorders. Treatment of type II SMA patients with salbutamol, designed to establish tolerability and clinical response, showed an improvement in motor activity after 6 and 12 months, although the authors claim that these results might be due to other factors than direct administration of salbutamol, considering the small study size and placebo effects (Pane, et al, 2008).

1.2.7.3 Gene and Stem Cell Therapy. A strategy that could theoretically lead to the cure of SMA by replacing the defective gene is gene therapy. Delivery of the human *SMN* via infection with lentivirus equine infectious anemia virus (EIAV) pseudotyped with the rabies glycoprotein for retrograde axonal transport restored SMN expression in motor neurons of SMA mice (Azzouz, et al, 2004). Multiple injections of the pseudotyped EIAV in muscles involved in mobility, respiration, and feeding, reduced motor neuron death and resulted in a modest increase in survival by 3 days compared to the control-treated mice (Azzouz, et al, 2004). Expression of human *SMN* using adenovirus vectors in primary fibroblasts derived from SMA patients led to an increase in SMN protein, restoring dramatically the number of gems, as well as relocalization of Gemin2 (DiDonato, et al, 2003). A more recent study with adeno-associated virus (AAV) demonstrated that delivery of human *SMN1* utilizing AAV-serotype 2/8 directly to the central nervous system (CNS) increased SMN expression up to 4-fold (Passini, et al, 2009). Treated mice had a higher number of motor neurons compared to controls, although this increase was moderate. The authors showed that even with a low number of motor neurons, SMA-treated mice had dramatically improved motor activity,

increasing the lifespan to 50 days as compared to 15 days in untreated mutant controls, a 233% increase.

More invasive therapies include administration of stem cells with the hope of replacing degenerated motor neurons. Neural stem cell transplation in a SMA mouse model resulted in a small increase of motoneurons, improved neuromuscular function, increased life span, and improved motor unit pathology (Corti, et al, 2008). Currently, Keirstead, a member of a stem cell therapy research team funded by the Families of SMA Foundation, derived motor neuron progenitors from human embryonic stem cells with 95% purity for the treatment of spinal cord injury (Keirstead, 2009). This early study holds great promise for the replacement of motor neuron loss in SMA patients in the future.

1.3 Protein Replacement Therapy

1.3.1 Introduction

Proteins are the most dynamic and diverse molecules in a cell, constituting more than 50% of the dry mass of the cell. The highly complex activities that take place in the cell are accomplished by a plethora of proteins, which function in a specific and coordinated manner (Garrett, et al, 2005). However, once this balance is altered by a missing or deficient protein, clinical symptoms may be produced (Jimenez-Sanchez, et al, 2001). Hence, in order to counteract this event or ameliorate progression of a disease, various therapeutic approaches have become available.

1.3.2 Proteins as therapeutic agents

Protein replacement therapy (PRT) is a strategy that consists of augmenting or replacing a defective or a missing protein. However, the benefits of PRT are not limited to restoring a deficient protein, since it can also be applied as medical tools for diagnostics of diseases (Leader, et al, 2008). As of mid-2002, the US FDA approved some 120 protein-based therapeutic agents (Walsh, 2003b), placing protein-based therapeutics as the fastest growing compounds within the pharmaceutical industry (Walsh, 2003a).

There are several reasons why proteins, which can be obtained from native sources and recombinant deoxyribonucleic acid (DNA) technology, are more advantageous than chemical compounds as therapeutics agents. To illustrate, proteins have an innate specific function which prevents them from interfering with an unrelated pathway. This feature prevents the occurrence of rapid and strong adverse-effects in a cell (Leader, et al, 2008; Russell, et al, 1999). At the same time, sequence homology is a key factor to determine immunogenicity, and through recombinant technology, proteins can be designed to be less immunogenic (Schellekens, 2002). Furthermore, recombinant proteins can be manipulated such that they become more effective and efficient than their native counterparts. For instance, Cerezyme, an analogue of the human enzyme β-glucocerebrosidase, differs from placental glucocerebrosidase, imiglucerase, by additional mannose sugars at the glycosylation sites, making this product specifically recognized by endocytic carbohydrate receptors on macrophages, the cells that accumulate lipids in Gaucher disease (Friedman, et al, 1999). Moreover, based on the number of protein-based drugs approved during the last decade, it seems that the field of biopharmaceuticals is advancing successfully over other areas, such as gene therapy, antisense-based technology, and tissue engineering products

(Walsh, 2003a). For example, the clinical development and approval time was more than 1 year faster for 33 protein therapeutics, approved between 1980 and 2002 than for 294 small-molecule drugs approved during the same time period (Leader, et al, 2008). Finally, one of the greatest contributions of PRT has been to the treatment of rare "orphan" diseases (defined in the United States, as conditions that affect less than 200,000 individuals), such as cystic fibrosis, Crohn's disease, non-Hodgkin's lymphoma, and hemophilia (Ashton, 2001).

1.3.3 Potential uses or PRT

Application of proteins as effective medical treatments has been available for centuries. In fact, one of the greatest achievements in human history was the eradication of smallpox infection. In the late 18th century, Dr. Edward Jenner inoculated people with scabs that contained the vaccinia virus in order to prevent a deadly infection. Today, with the introduction of biotechnology, many new proteins for therapeutic purposes have been introduced. These agents include (i) factors that influence hematopoietic cells and blood coagulation, namely erythropoietin, granulocyte colony-stimulating factor (G-CSF), granulocyte-macrophage colony-stimulating factor (GM-CSF), clotting factors VIIa, VIII, and IX, glycoprotein (GP) IIb/IIIa inhibitors that affect platelet function, direct thrombin inhibitors, and antihemophilic factor; (ii) interferons and cytokines for anti-infective and cancer therapy; (iii) hormones and derivatives, in particular glucagon, somatotropin, calcitonin, somatostatin, thyroid-stimulating hormone, thyrotropin-releasing hormone, gonadotropin releasing hormone, leuteinizing hormone-releasing hormone, and oxytocin; (iv) enzymes and derivatives; and (v) recombinant proteins for vaccines (Ho, et al, 2003). Generation of these compounds confirms their validation as effective therapeutic agents.

Insulin, the most commonly used recombinant protein, became the first commercially available recombinant protein therapeutic approved by the US FDA in 1982. In 1922, insulin was first purified from bovine and porcine pancreas and used as a life-saving daily injection for patients with diabetes mellitus I (Banting, et al, 1991). Unfortunately, the challenges that the new therapy would encounter were soon realized. Among these include: first, the availability of animal pancreas for purification of insulin; second, the cost of insulin purification from animal sources, and third, the immune response of some patients to animal insulin. Nevertheless, these problems were soon overcome by producing human insulin by means of recombinant DNA technology, showing safety and hypoglycemic potency in healthy men (Keen, et al, 1980).

Another important example of protein therapeutics is the treatment of Gaucher's disease. Gaucher disease is one of the more than 40 lysosomal storage disorders, caused by the defective activity of acid β-glucosidase, resulting in accumulation of glucosylceramide in macrophages of liver, spleen and bone marrow, resulting in hepatosplenomegaly, cytopenias and bony disease (Beutler, 2006). Over the past 15 years macrophage-targeted enzyme reconstitution therapy has dramatically altered the disease by reversing disease manifestations.

Despite the efforts to generate safe and effective therapeutic proteins, enzyme reconstitution therapy has shown some adverse-effects. However, many of these have been controlled by decreasing the rate of infusions, along with administration of antihistamines or corticosteroids (Burrow, et al, 2007). In fact, the efficacy of the long-term effects of alpha-galactosidase A in Anderson-Fabry patients has been proven (Imbriaco, et al, 2009), as well as the prevention of disease progression in Gaucher patients after sustained intake of imiglucerase (Weinreb, et al, 2002).

1.3.4 Recombinant protein production

Another advantage of recombinant proteins is the capacity to choose a wide range of expression systems, from bacteria to transgenic plants (Table 1.3). Yet, when choosing an expression system, the desired features in the recombinant protein, mainly glycosylation of the final product, must be taken into consideration.

1.3.4.1 Prokaryotic expression. The first biopharmaceutical that gained marketing approval in 1982 was the genetically engineered insulin, produced in *E. coli*. Among the advantages of using bacteria to manufacture proteins, include its well known molecular biology, high levels of expression, ease of culture, rapid cell growth, and purification is also possible using the plethora of commercial kits available (Walsh, 2003b). However, if a functional protein is required, prokaryotic systems become a problem, since most proteins become insoluble in inclusion bodies and are very difficult to recover as functional proteins (Villaverde, et al, 2003). In addition, most, if not all, post-translational modifications are not available in bacteria, such as glycosylation, and thus the protein of interest may not be functional. Nevertheless, in several cases the lack of the carbohydrate component of glycoproteins does not limit their biological activity, confirmed by a series of recombinant proteins available in the market that have been produced by this means (Walsh, 2003b) (Table 1.4).

1.3.4.2 Eukaryotic expression. On the other hand, mammalian cells, including the immortalized Chinese hamster ovary (CHO) cells, cells derived from mouse myeloma (NS0), baby hamster kidney (BHK), human embryonic kidney (HEK-293) and human retinal cells, have become the system of choice for the production of recombinant proteins, due to their

capacity for proper folding, assembly, post-translational modifications, and genetic stability (Wurm, et al, 1999; Wurm, 2004). As of 2004, approximately 60-70% of all recombinant proteins that reached the market were produced in mammalian systems (Wurm, 2004), using non-viral, as well as, viral expression vectors (Baldi, et al, 2007) (Table 1.4). In recent years the productivity of recombinant proteins has reached levels of up to 5 grams per liter (Butler, 2005), indicating the effort that is being made to optimize production (Beutler, 2006). In spite of the advances in the production of recombinant protein from mammalian sources, there are still potential drawbacks of this approach. Specifically, slow growth, technical complexity, and more importantly, expense (Beutler, 2006).

Table 1.3. Expression systems employed for production of recombinant proteins.

Species	Most common cell lines used
E. coli	Strain K12
Yeast	*Saccharomyces cerevisiae*
Fungi	*Aspergillus*
Animal cell culture	CHO and BHK
Transgenic animals	Sheep and goat
Plant-based expression systems	Various
Insect cell culture systems	*Bombyx mori* and *Spodoptera frugiperda* cells

Adapted from Walsh, G. 2003, Biopharmaceuticals, Biochemistry and Biotechnology.

Other expression systems have gained acceptance, including transgenic animals (Han, et al, 2009; Pollock, et al, 1999) and plants (Aviezer D., et al, 2009; Fischer, et al, 2004), which provide the advantage of high- yield production, proper folding, low production costs, and elimination of contamination from bacterial or human pathogens. Yet, these systems still remain in development and are largely scrutinized due to the immune responses that a recombinant protein can produce due to the different host sources (Schellekens, 2002).

Yeast and baculovirus systems have been long investigated for the production of protein-based drugs. Specifically, *Pichia pastoris* expression systems confer several benefits including, economy, ease of manipulation, the ability to perform complex post-translational modifications, and high expression levels (Cereghino, et al, 2002); equally important, is the recent ability of glycoengineered yeast that allow for the generation of yeast strains capable of replicating the most essential glycosylation pathways found in mammals (Hamilton, et al, 2007). Furthermore, baculovirus expression systems have emerged as a new alternative for the high level production of recombinant proteins since humanized glycosylation of proteins have become available (Jarvis, 2003).

Nevertheless, although a number of attractive alternatives to generate recombinant proteins have been identified in the last years, bacteria *E. coli* and mammalian expression systems still remain the systems of choice; indeed, nine out of the 31 therapeutic drugs approved from 2003 to 2006 were produced in *E. coli*; whereas, 17 were obtained from mammalian cell lines (Walsh, 2006).

1.3.5 Current challenges of protein therapy

Despite the great advances in the production of protein-based drugs in recent years, including high yield, improved glycosylation, better delivery of proteins, substantial disadvantages prevent protein replacement therapy to expand to other genetic diseases. In fact, delivery of proteins as therapeutics is cumbersome due to poor oral bioavailability, protein denaturation in the digestive system, acid hydrolysis in the stomach, enzymatic degradation, poor adsorption due to size and polar/charge distribution (Leader, et al, 2008). However, by far the factor that most limits the use of recombinant proteins for clinical use is the inability of the proteins to enter cells and be targeted to the appropriate intracellular site (Russell, 1999).

Table 1.4. Some commercially available recombinant proteins and their production source.

Biopharmaceutical Product	Source
Tissue plasminogen activator (tPA)	*E. coli*, CHO
Insulin	*E. coli*
Interferon-α	*E. coli*
Interferon-γ	*E. coli*
Interleukin-2 (IL-2)	*E. coli*
Granulocyte colony-stimulating factor (G-CSF)	*E. coli*
Human growth hormone (hGH)	*E. coli*
Follicle-stimulating hormone (FSH)	CHO
Interferon-β	CHO
Erythropoietin	CHO
Glucocerebrosidase	CHO
Factor VIIa	BHK

Adapted from Walsh, G. 2003, Biopharmaceuticals, Biochemistry and Biotechnology.

1.4 Cell Penetrating Peptides (CPPs)

1.4.1 Introduction

There are certain diseases, such as cancer or neurodegenerative disorders, that require active compounds to be delivered intracellularly in order to exert their therapeutic effects (Sugita, et al, 2008). However, one of the restrictions for the efficient delivery of functional molecules is the innate hydrophobicity of the cell membrane, including the brain blood barrier (BBB), which prevents passage of large compounds (Egleton, et al, 1997; Lebleu, 1996; Schwarze, et al, 1999). Furthermore, a number of techniques currently employed for gene transfer such as electroporation, microinjection, cationic lipids, and viral vectors, fail to achieve a high transfection level. This is mainly due to the restrictions imposed by the cell membrane; in addition to the complicated manipulations, prominent cellular toxicity or immunogenicity that these methods promote (Vives, et al, 1997).

To circumvent membrane- related limitations for gene transduction, a series of small cationic peptides, called cell penetrating peptides (CPPs) or PTDs, have been shown to have the property to cross biologically active cellular membranes, a process commonly known as protein transduction (Schwarze, et al, 1999). This novel approach consists of tethering recombinant protein to PTDs covalently, to allow enhanced delivery of a protein to the cytoplasm and nucleus (Schwarze, et al, 2000). Among the most studied small peptides with transduction capacities are the third α-helix from Antennapedia homeodomain from *Drosophila melanogaster* (Derossi, et al, 1994), VP22 protein from herpes simplex virus (Elliott, et al, 1997), and the transactivator of transcription (Tat) protein of the human immunodeficiency virus type 1 (HIV-1).

1.4.2 PTD from HIV-1 TAT

The human immunodeficiency retrovirus type 1, responsible for the acquired immunodeficiency syndrome (AIDS), encodes for Tat protein, a small nuclear transcriptional activator of viral gene expression, which is essential for the transcription of genes and viral replication (Jeang, et al, 1999). Upon infection, HIV-1 integrates into the host genome in a random fashion, followed by transcriptional activation of the provirus by the Tat protein through the interaction with a *cis*-acting RNA element at the 5'end of viral messenger RNAs (Fittipaldi, et al, 2005). Additionally, Tat protein performs other activities, such as immunosuppression through the induction of cytokines (Viscidi, et al, 1989), modulation of survival, proliferation, and migration of different cell types, and induction of neurotoxicity in the central nervous system (Tyagi, et al, 2001). Most importantly, in 1988, two independent studies led to the identification of Tat protein as a molecule able to translocate across the cell membrane of any cell type when added exogenously to culture medium (Frankel, et al, 1988; Green, et al, 1988). These initial reports led to a series of studies to determine the protein sequence responsible for transduction, as well as the mechanism behind the translocatory properties of Tat protein.

Interestingly, the transductional property of the Tat protein does not depend entirely on the full sequence of the protein. In fact, previous studies have demonstrated that only a small sequence is required, which corresponds to a very basic region of the Tat amino acid sequence: Tyr-Gly-Arg-Lys-Lys-Arg-Arg-Gln-Arg-Arg-Arg (Vives, et al, 1997). Targeting of PTD fusion protein to the nucleus is achieved through the nuclear localization signal (NLS) (Gly-Arg-Lys-Lys-Arg) present within this domain (Nagahara, et al, 1998; Vives, et al, 1997).

1.4.3 Mechanism of PTD Internalization

Initial reports of PTD transduction were characterized by a general optimism with respect to the capacity of PTD fusion protein to cross the cell membrane of any tissue. High transduction efficiency into the cytosol and nucleus was claimed to occur in a temperature- and energy- independent manner as early as 5min post-incubation, suggesting direct penetration of the cell membrane (Herce, et al, 2007; Vives, et al, 1997). However, subsequent studies reported that the observed localization of PTD fusion protein to the cytoplasm and nucleus was a consequence of fixation artifacts: the positive protein binds the cell surface and gains entry when the cells are permeabilized during fixation, as opposed to the intrinsic transduction properties of PTD (Leifert, et al, 2002; Lundberg, et al, 2003). Accordingly, in order to avoid misinterpretation, biological assays as well as pre-treatment of cells with trypsin before fluorescence-activated cell sorting (FACS) could provide more accurate information regarding the potential of PTD as a delivery vehicle (Richard, et al, 2003).

Several groups embarked on a race to unveil the mechanism of PTD internalization; yet, efforts to elucidate this process have failed to come to a general consensus. To illustrate, it has been reported that translocation depends on the presence of heparan sulfate proteoglycans at the cell surface (Tyagi, et al, 2001), whereas, others have reported that transduction is a energy-dependent process, which occurs through clathrin- or caveolin-dependent endocytosis (Fittipaldi, et al, 2005; Mann, et al, 1991; Richard, et al, 2005), or that it involves a macropinocytotic pathway, which occurs on the cell surface of all tissues (Wadia, et al, 2004). The inconsistency in determining a unique mechanism for PTD internalization might suggest that there is not a unique mechanism that applies to all PTD

fusion proteins and, instead, internalization might depend on cell type as well as type of cargo (Sugita, et al, 2008).

1.4.4 Biological activity of PTD fusion proteins

Despite the skepticism of various groups regarding the intrinsic property of PTD to transduce cells, many other labs have provided compelling evidence of the therapeutic effects of PTD fusion proteins. Intraperitoneal administration of PTD fused to β-gal in a C57BL/6 mice showed presence of the protein as early as 20min (post-injection), and enzymatic activity at 4hr in all tissues, including brain and skeletal muscle (Schwarze, et al, 1999). In addition, TAT-utrophin delivered to dystrophin-deficient mice improved muscle function (Sonnemann, et al, 2009). Systemic administration of TAT-mini utrophin (TAT-μUtr), the truncated version of utrophin, resulted in transduction of most tissues examined, although the authors claim that there seems to be a predilection for certain tissues, since fluorescence-tagged TAT-μUtr was more prominent in a subset of organs. TAT-μUtr restored the dystrophin-glycoprotein complex, improved membrane stability and motor activity. TAT-caspase3 protein induced cell death specifically in HIV-infected cells, leaving uninfected cells unharmed (Vocero-Akbani, et al, 1999). Transduction of TAT-caspase3 occurred in 100% of cells *in vitro*, reaching a maximum concentration in less than 20min, demonstrating that protein transduction is concentration dependent. Furthermore, delivery of a TAT fusion protein to hypoxic regions of tumours resulted in a decrease in tumour size *in vivo* (Harada, et al, 2002). Studies in culture showed that TAT-oxygen-dependent degradation-caspase-3 fusion protein transduced only to hypoxic regions of cells, as it was expected; whereas, intraperitoneal injections (i.p.) reduced the mass of tumours in tumour-

bearing mice approximately in a 38% by day 3, with no apparent side effects. Ngb, a neuroglobin, which is upregulated following oxygen deprivation and protects neurons from hypoxia, fused to the domain responsible for the translocatory activity of the HIV-TAT protein, increases cell viability under hypoxic conditions in primary cultured cortical neurons (Zhou, et al, 2008). Furthermore, apoptosis was decreased; suggesting TAT-PTD may be a viable therapy for CNS diseases.

Gene therapy has also benefited by the properties of PTD. To enhance adenovirus tropism, several research groups generated modified-adenovirus with the TAT peptide. For wild type virus, the interaction between the coxsackie and adenovirus receptor (CAR) and fiber protein of the unmodified human adenovirus type 5 (hAd5) mediates entry into cells. Genetic manipulation of the fiber protein of the hAd5 with the highly basic region of the TAT protein resulted in a higher transduction in cells lacking the CAR, such as blood cells, vascular smooth muscle cells, and subcutaneous tumours, which are refractory to conventional hAd5 infection (Han, et al, 2007; Kurachi, et al, 2007). Moreover, incorporation of the TAT motif into the HI loop or the 3' end of the fiber protein does not affect binding of TAT-modified hAd5 to CAR-positive cells, indicating that TAT binding is independent of CAR interaction. A more recent study has suggested that transduction of TAT-hAd occurs through cellular heparan sulfate proteoglycans and macropinocytosis, confirming that entry into cells is CAR-independent (Yoshioka, et al, 2008).

1.4.5 Cytotoxicity of PTD

The most common toxic effects observed upon treatment with CPPs are (i) disruption of the cell membrane, and (ii) side-effect as a result of a direct interaction between the CPP fused protein with an unrelated molecule. CPPs from D*rosophila* and herpes simplex virus cause a greater degree of membrane disruption, as well as reduced cell viability as compared to PTD from HIV-1 Tat (Sugita, et al, 2008; Zorko, et al, 2005). *In vitro* studies suggest that a concentration of 100μM of PTD should not be exceeded in order to prevent unwanted effects, such as cell death (Jones, et al, 2005). Full length TAT protein induces apoptosis, along with immunological responses, such as production of cytokines and cytokine receptors, and it would not be unexpected that the TAT PTD- domain could elicit the same type of responses (Westendorp, et al, 1994). Fortunately, it has been shown that PTD from the HIV-Tat protein does not promote activation of the immune system (Leifert, et al, 2002), suggesting that PTD may not be suitable for vaccination purposes (Leifert, et al, 2002).

1.4.6 Future prospects of PTD transduction

Efficient delivery of recombinant proteins inside cells is hampered by the hydrophobic nature of the cell membrane. CPPs confer the possibility that a therapeutic protein could be targeted to the proper compartment. Despite several studies performed to date to prove the biological effect of PTD fusion proteins, it is quite evident that this technology is still in its infancy, and functional studies showing the therapeutic effects of PTD fusion proteins are limited.

1.5 Rationale

Limited levels of the survival motor neuron protein below the threshold lead to development of spinal muscular atrophy. Studies in mouse models strongly suggest that reconstitution of higher levels of SMN leads to an increased survival and alleviation of symptoms. Protein replacement therapy represents a novel strategy to correct SMA phenotypes. Restoration of SMN levels requires the efficient delivery of SMN to the inside of cells which is hampered by the cell membrane. However, small protein transduction domains have the capacity to cross biologically active membrane, allowing a feasible protein therapy strategy. In the present study, I evaluated the capacity of a SMN recombinant protein fused to the PTD to transduce cells *in vitro*.

1.6 Hypothesis

Recombinant SMN protein fused to a PTD domain will be able to translocate across the cell membrane in culture. Furthermore, delivery of the fused PTD-SMN will correct the phenotypic deficiencies of SMA models *in vitro*.

1.7 Objectives

The immediate aims to prove my hypothesis are: (i) to design and construct PTD-SMN protein, (ii) to express and purify PTD-SMN protein, and (iii) to prove efficient transduction of PTD-SMN into cells.

Chapter 2- Materials and Methods

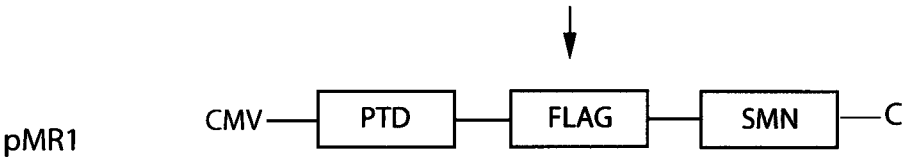
2.1 Construction of PTD-SMN and FLAG-SMN

Using polymerase chain reaction (PCR) and other standard recombinant DNA techniques, we generated bacterial expression cassettes which code for PTD-SMN or FLAG-SMN recombinant proteins fused to FLAG and Myc tags. Briefly, PTD oligo (5' GGCCGCAATGTACGGAAGAAAAAAGAGAAGGCAAAGAAGGAGA 3', 5' GGCCCCTCTCCTTCTTTGCCTTCTCTTTTTTCTTCCGTACATTG 3') was ligated to the 5' end of FLAG-SMN in pCD2-SMN vector (DiDonato, et al, 2003), which had been previously digested with NotI, generating pMR1. To incorporate Myc and 6xHistidine (His) tags onto the recombinant protein in a mammalian vector, pCD2-SMN was used as a template to amplify and clone the fragment of the FLAG-SMN (5' ATAGCGGCCGCCACCATGGATTAC 3', 5' GCGAAGCTTATTTAAGGAATGTGAGCACCTTC 3') into a pcDNA3 vector (Invitrogen, Carlsbad, CA)), creating pMR3. In order to create a bacterial expression cassette that expresses the cDNA of *SMN*, the Myc and 6xHis tags from pMR3 were incorporated into the pET-21d vector (EMD Biosciences, San Diego, CA), creating pMR7. Next, the NcoI-BclI 971-base pair fragment of pMR3 was cloned into pMR7 to make the FLAG-SMN-Myc-His construct, pMR9. The PTD-FLAG-SMN fragment obtained from pMR1, upon digestion with NotI and BglII, was cloned into the pET-21d vector to create pMR12. To add the PTD domain to pMR9 construct, the BamHI-NotI fragment of pMR9, FLAG-SMN-Myc-His, was cloned into pMR12, generating pMR15. Representative structures of the constructs are shown in Fig. 2.1.

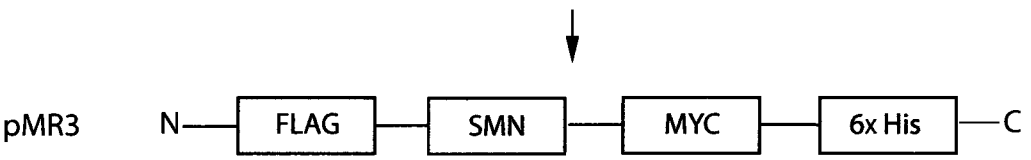
Both PTD-SMN and FLAG-SMN, cloned into the prokaryotic expression vector, pET 21d, are fused to a polyhistidine tag for purification purposes. Plasmids were transformed in *E. coli* DH5α competent cells by heat shock method, and large scale preparation of DNA was performed by alkaline lysis with purification by cesium chloride (CsCl) buoyant density centrifugation (Sambrook, et al, 1989). Constructs were sequenced by StemCore Labs (Ottawa Hospital Research Institute, Ottawa, ON) to confirm their identity.

Figure 2.1: Schematic structures of plasmids to generate PTD-SMN bacterial expression cassette. The final product, PTD-SMN, encodes for the PTD domain, followed by a FLAG epitope tag, the sequence of the cDNA of the human *SMN1*, the Myc epitope tag, and lastly, six histidine residues. pMR1 and pMR3 are mammalian vectors, which express the *SMN* gene under the regulation of the CMV promoter. pMR9, pMR12, and pMR15 express the SMN gene incorporated into the pET21-d bacterial expression vector.

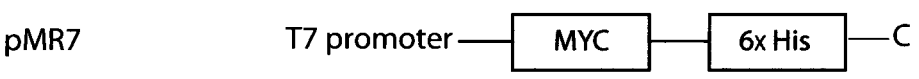
(1) Incorporation of PTD oligo into pCD2-SMN



(2) Incorporation of FLAG-SMN sequence from pCD2-SMN into pcDNA3

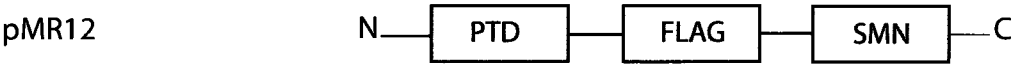


(3) Incorporation of Myc-6x His from pMR3 into pET-21d vector

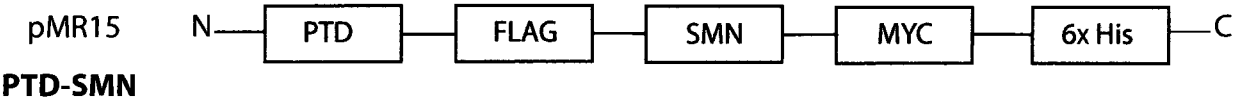


(4) Incorporation of FLAG-SMN from pMR3 into pMR7

(5) PTD-FLAG-SMN fragment from pMR1 cloned into pET-21d



(6) FLAG-SMN-Myc-His fragment from pMR9 cloned into pMR12



2.2 Bacterial growth

Transformed *E. coli* BL21 strain were allowed to grow in 25ml of Luria Broth (Thermo Fisher Scientific, Waltham, MA) media containing 50μg/ml of carbenicillin (Novagen, Madison, WI), overnight at 37°C under constant agitation. Next day, a 500ml of Luria Broth media containing 50mg/ml of carbamicillin was inoculated with the overnight culture at a 1:60 dilution until the bacteria reached an optical density (OD_{600}) of 0.6, after which induction of recombinant protein took place.

2.3 Fusion proteins purification

The PTD-SMN and FLAG-SMN fusion proteins were purified from soluble bacterial protein under native or denaturing conditions using Nickel (Ni^{2+}) columns, according to Qiagen protocols (Germantown, MD). Briefly, once bacteria reached an OD_{600} of ~ 0.6, recombinant proteins were overexpressed in *E. coli* strain BL21 upon induction with 1mM of isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4hr at 37°C. Bacterial pellets were lysed with lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM Imidazole, 1X protease inhibitor cocktail [Roche, Basel, Switzerland]), 1mg/ml lysozyme pH 8.0) for 30min at 4°C, followed by sonication 6 times for 10s each time, with 5s pauses between, in a Sonics & Materials sonicator (Newtown, CT). Next, bacteria lysates obtained from both purification procedures were harvested by centrifugation using a JA-25.50 rotor at 10,000g for 30min at 4°C in a Beckman J-25I centrifuge (GMI, Inc., Ramsey, MN). The purification procedure was performed using a vacuum system. Cleared lysates were applied to 1.5ml pre-washed columns. Under native condition, upon addition of lysate, columns were washed 2 times with

binding buffer (50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole). Eluate of recombinant proteins was obtained by gravity in elution buffer (50mM NaH_2PO_4, 300mM NaCl, 250mM imidazole, 1X protease inhibitor cocktail pH 8.0). Following purification, recombinant proteins were dialyzed overnight against phosphate buffer saline (PBS) (Sigma-Aldrich, St. Louis, MO) at 4°C. Recombinant protein expression and purification were evaluated by Coomassie blue staining of 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and by Western blot analysis. For immunoblotting, proteins were separated on a 12% SDS-PAGE gel followed by electrophoretic transfer onto an Immobilon polyvinylidene difluoride membrane (PVDF, Millipore, Billerica, MA). The primary antibody used was mouse-anti-FLAG (1:15,000) or mouse-anti-Myc (1:10). In addition to native purification, bacteria cells were subjected to denaturing purification. Briefly, lysis of bacteria required the use of strong denaturant agents: upon induction, bacteria pellets were resuspended in buffer B (8M urea; 0.1M NaH_2PO_4; 0.01M Tris-Cl; pH 6.3) for 1hr at r.t. The protein was then purified using a similar protocol as described above for native purification, except buffer B was used as the washing buffer, and buffer E (8M urea; 0.1M NaH_2PO_4; 0.01M Tris-Cl; pH 4.5), as the eluate buffer.

2.4 Cell culture

HeLa cells (human epithelium-derived adenocarcinoma; American Type Culture Collection [ATCC] CCL-2) were maintained in Dulbecco's modified Eagle's medium (DMEM) (Sigma-Aldrich), A549 (human lung epithelial carcinoma: ATCC CCL-185), and 293 cells (Graham, et al, 1977) were maintained in Minimum essential medium (MEM) (Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), 1%

antibiotic/antimycotic (Invitrogen), and 1% Glutamax-1 (Invitrogen). Cells were grown in tissue culture dishes (Sarstedt, Postfach, Germany), incubated at 37°C in a humidified 5% CO_2 atmosphere in a SANYO incubator (SANYO, Wood Dale, IL).

2.5 Protein Incubation with cells *in vitro*

Semi-confluent (~80%) A549 or HeLa cells were incubated with various amounts of purified PTD-SMN or FLAG-SMN, ranging from 12 to 600nM, in PBS for 8hr at 37°C, after which cells were either lysed or fixed for immunoprecipitation or immunocytochemistry analysis respectively.

2.6 Western blot analysis

HeLa cells seeded at $0.1x10^6$ per well in a 12-well dish (Corning Incorporated, Corning, NY), allowed to grow to semi-confluency, and treated with protein as described above, were washed with PBS and lysed with ice-cold modified radioimmunoprecipitation (RIPA) buffer (50mM Tris-HCl [pH 7.4], 150mM NaCl, 1mM EDTA, 1% Nonidet P-40 [NP-40], 1X protease inhibitor cocktail) or SDS-PAGE buffer (2X Laemmli sample buffer [2% SDS, 20% glycerol, 5% 2-mercaptoethanol, 0.01% bromophenol blue, 62.5mM Tris HCl pH 6.8]). Lysates were separated by a 12% SDS-PAGE and transferred to an Immobilon membrane using a semidry blot apparatus. The membrane was incubated overnight or for 1hr at 4°C in blocking solution (5% nonfat milk in TBST: 20mM Tris [pH 7.6], 137mM NaCl, and 0.1% Tween 20), rinsed in TBST, and then incubated in blocking solution with the corresponding primary antibody for 1hr at r.t under constant agitation. The membranes were subsequently

washed 3X in TBST for 5min each. To detect the presence of recombinant proteins, membranes were probed with anti-FLAG antibody (1:15,000), and anti-tubulin antibody was used for loading control (1:15,000), which were detected using enhanced chemiluminescence (GE Healthcare, Uppsala, Sweeden) captured on film (Thermo Scientific, Waltham, MA).

2.7 Cellular fractionation

HeLa cells were seeded at $2.2x10^6$ in a 10-cm dish (Sarstedt) and allowed to grow to semi-confluency. The cells were then incubated with PTD-SMN and FLAG-SMN for 8hr at 37°C and subjected to cytoplasmic and nuclear fractionation according to a modified nuclear extract protocol (Friedmann, et al, 2007). Briefly, cells were washed with ice-cold PBS, after which, cells were incubated in buffer A (10mM HEPES pH 7.4, 1.5mM $MgCl_2$, 10mM KCl, 0.5mM DTT, 0.1% Triton X-100, 1X protease inhibitor cocktail), for 10min at 4°C. Next, cells were dounce homogenized using a type A pestle (Kontes Glass CO., Vineland, NJ), followed by centrifugation at 800g for 5min at 4°C in a eppendorf microcentrifuge 5417 R (Westbury, NJ). The supernatant was retained as the cytoplasmic fraction. In order to obtain a clean nuclei sample, the pellet obtained from the above procedure was resuspended in buffer A and overlayed on 4% sucrose for centrifugation at 1400g for 20min at 4°C. Subsequently, the pellet was resuspended in buffer C (20mM HEPES pH 7.9, 25% glycerol, 0.42M NaCl, 1.5mM $MgCl_2$, 0.2mM EDTA, 0.5mM DTT, 1X protease inhibitor cocktail), and subjected to constant rocking for 30min at 4°C. Next, samples were centrifuged at 14000 revolutions per minute (rpm) for 10min at 4°C, after which nuclei extracts (supernatant) were separated on a 12% SDS-PAGE gel and transferred onto a PVDF membrane. To detect the presence of recombinant proteins, blots were probed with an anti-FLAG antibody (1:15,000).

2.8 Immunocytochemistry and imaging

A549 or HeLa, were seeded at $0.1x10^6$ in a 12-well, and allowed to grow to semi-confluency (80%) on coverslips. The cells were treated with protein as described above. Subsequently, cells were fixed in ice-cold 4% paraformaldehyde in PBS for 20min at room temperature (RT), permeabilized with 0.25% Triton-X 100 in PBS for 5min at r.t, blocked with 10% of bovine serum albumin (BSA) in PBS for 30min at 37 °C, and stained with the appropriate antibodies in 3% BSA at 37°C. When a double-staining procedure was carried out, a complete staining-procedure was performed before continuing with a different primary antibody. After staining, the cells were incubated with Hoescht, 864105, in PBS for 5min at r.t. The primary antibodies used were as follows: rabbit-anti-Myc (1:200), mouse-anti-Flag (1:500), mouse-anti-coilin (1:50), and mouse-anti-SMN (1:100); whereas, the secondary antibodies used were: goat anti-mouse TRITC (1:100) or goat anti-rabbit FITC (1:200). Immunofluorescent detection was performed using a Zeiss LSM510 confocal microscope (Carl Zeiss Canada Ltd., Toronto, ON). Images were captured by LSM software, version 4.2, and processed with Adobe Photoshop 7.0 software (Adobe Systems Incorporated, San Jose, CA).

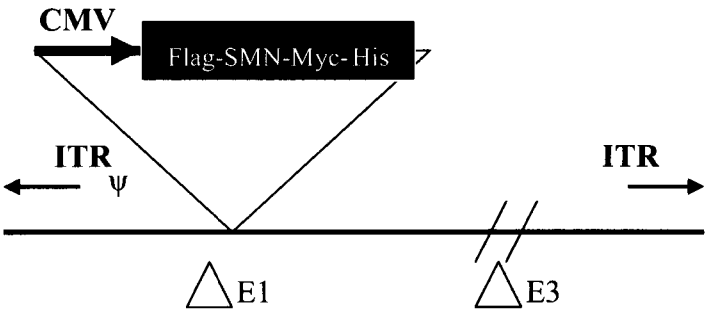
2.9 Construction of adenoviral vectors

We designed and engineered an E1/E3-deleted Ad vector, which expresses the complementary DNA (cDNA) of the human *SMN* linked to FLAG and Myc epitope tags under the regulation of the cytomegalovirus (CMV) promoter. To incorporate a six-histidine residue onto the carboxy terminus of the recombinant protein, the 980-sequence fragment obtained from the digestion of pMR3 with NotI and BclI, was ligated with the digested

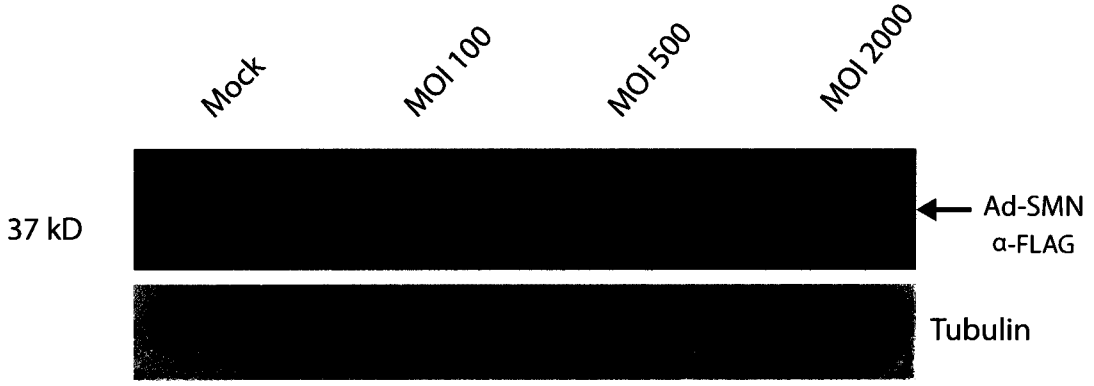
pCD2-SMN with NotI an BclI, an Ad shuttle vector plasmid (DiDonato, et al, 2003), generating pMR6. The expression cassette of *SMN* was cloned into an adenovirus genomic plasmid, by bacterial recombination, as described by Chartier et al (Chartier, et al, 1996)). The resulting plasmid, pMR8, was propagated using 293 cells and purified using cesium chloride (CsCl) gradient centrifugation, and titered by standard techniques (Friedmann, et al, 2007).

Figure 2.2: Schematic structure of E1/E3-deleted Ad-SMN encoding *SMN*. (A) Ad-SMN contains the *SMN* gene under the regulation by the murine cytomegalovirus immediate early promoter enhancer (CMV). We have included an N-terminal FLAG epitope and a carboxy-terminal Myc-His tag on *SMN* to aid in detection. Ad5 inverted terminal repeats (ITR) and packaging signal (ψ) are also shown. (B) Expression of Ad-SMN in 293 cells at different MOIs. Cells were lysed 24hr-post infection with SDS-PAGE buffer. SMN protein produced from the Ad vector was identified using mouse-FLAG antibody, and tubulin was identified using mouse-anti-tubulin antibody.

A.


CMV
Flag-SMN-Myc-His
ITR
ψ
ITR
E1
E3

B.


Mock
MOI 100
MOI 500
MOI 2000
37 kD
Ad-SMN
α-FLAG
Tubulin

2.10 Co-immunoprecipitation

HeLa cells were seeded at $2.2x10^6$ in a 10-cm dish (Sarstedt) and allowed to grow to semi-confluency. The cells were incubated with PTD-SMN for 8hr at 37°C or infected with Ad-SMN or Ad-control for 18hr at 37°C, and subsequently lysed with ice-cold modified RIPA buffer. The cell lysates were pre-cleared with 20µl of 50% protein G slurry (Millipore) for 30min at 4°C. Co-immunoprecipitations were performed by incubating 250µg of total cellular protein in modified RIPA buffer with 1µg of a rabbit polyclonal Myc antibody, under constant rocking. Next, complex was mixed with 20µl of 50% protein G slurry for 2h at 4°C. Samples were washed 5X in modified RIPA buffer and denatured in SDS-PAGE buffer. Samples were separated on a 12% SDS-PAGE and transferred to PVDF membranes. Membranes were probed with anti-FLAG (1:15,000), anti-gemin2 (1:1000), or anti-SMN (1:12,500) antibodies.

2.11 Antibodies

Murine monoclonal antibodies to human SMN and p80 coilin (BD Bioscience, Franklin Lakes, NJ) were kindly provided by Dr. Rashmi Kothary (Ottawa Hospital Research Institute [OHRI]). The mouse monoclonal (M2) antibody to the FLAG epitope was purchased from Sigma-Aldrich. The mouse monoclonal anti gemin2 and rabbit polyclonal to Myc were obtained from Abcam (Cambridge, MA). The mouse monoclonal Myc clone 9ε1θ was a kind gift of Dr. Michael Rudnicki (OHRI). The mouse monoclonal alpha-tubulin antibody was purchased from Calbiochem (Gibbstown, NJ). The secondary antibodies goat anti mouse and goat anti rabbit IgG HRP conjugate used for immunoblotting were from Bio Rad (Hercules, CA). The secondary antibodies goat anti mouse TRITC and goat anti rabbit FITC, used for

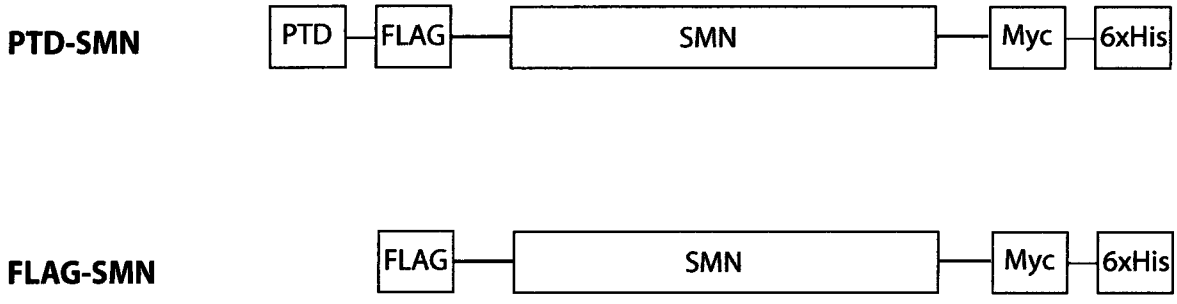
immunocytochemistry, were obtained from Jackson Immunoresearch Laboratories (West Grove, PA).

Chapter 3 - Results

3.1 Construction of PTD-SMN

To correct SMA phenotypes in SMA models *in vitro* and *in vivo*, the expression of SMN needs to be increased, which could be achieved by different strategies. In the present project, we constructed a *SMN* bacterial expression cassette that produces a SMN protein with the capacity to cross biologically active membranes. To allow efficient transduction of the SMN protein *in vitro*, we employed the basic eleven-amino acid PTD sequence, responsible for the translocatory properties of the HIV-TAT protein (Schwarze, et al, 2000; Vives, et al, 1997). The PTD domain was fused in -frame to cDNA of *SMN1*, producing the PTD-SMN fusion protein. The construct was cloned into the pET21d vector as described in Materials and Methods. To aid in assessing expression and incorporation of the PTD-SMN protein, the fusion protein was linked to a FLAG epitope tag at the N-terminus and a Myc epitope tag at the carboxy terminus. Additionally, the construct PTD-FLAG-SMN-Myc was incorporated upstream of a six-histidine domain. The presence of histidine residues allowed purification of recombinant proteins by Ni^{2+} affinity chromatography columns. This construct will be referred to simplify as PTD-SMN. To confirm the functionality of PTD-SMN protein, we engineered another SMN recombinant protein for use as a control. FLAG-SMN fusion protein lacks the PTD domain, but retains the FLAG, Myc epitope tag and the histidine residues. Figure 3.1 shows a schematic representation of the constructs.

Figure 3.1: Schematic representation of the PTD-SMN and FLAG-SMN fusion proteins. The construct PTD-SMN harbors the PTD motif cloned in-frame with the cDNA of hSMN; whereas FLAG-SMN lacks the PTD domain, and is used as a control. The proteins also contain an N-terminal FLAG epitope tag and a carboxy Myc epitope tag SMN to facilitate detection. Six histidine residues were linked to the carboxy terminus of the constructs for purification purposes using Ni^{2+} affinity chromatography columns.


PTD-SMN
PTD
FLAG
SMN
Myc
6xHis
FLAG-SMN
FLAG
SMN
Myc
6xHis

3.2 Expression of PTD-SMN

The expression of the PTD-SMN protein in *E.coli* BL21 strain was analyzed by examining induced bacteria lysates by means of coomassie blue staining of SDS-PAGE gels. Figure 3.2A shows the expression of PTD-SMN fusion protein obtained from total cell lysates upon addition of IPTG at 0hr; IPTG-induced cultures revealed a novel band with an apparent molecular mass of 42kD. Furthermore, the intensity of the band corresponding to PTD-SMN increases with longer induction time. This result is consistent with the presence of a protein with a similar size of the PTD-SMN protein, suggesting that the full length PTD-SMN fusion protein can be bacterially produced.

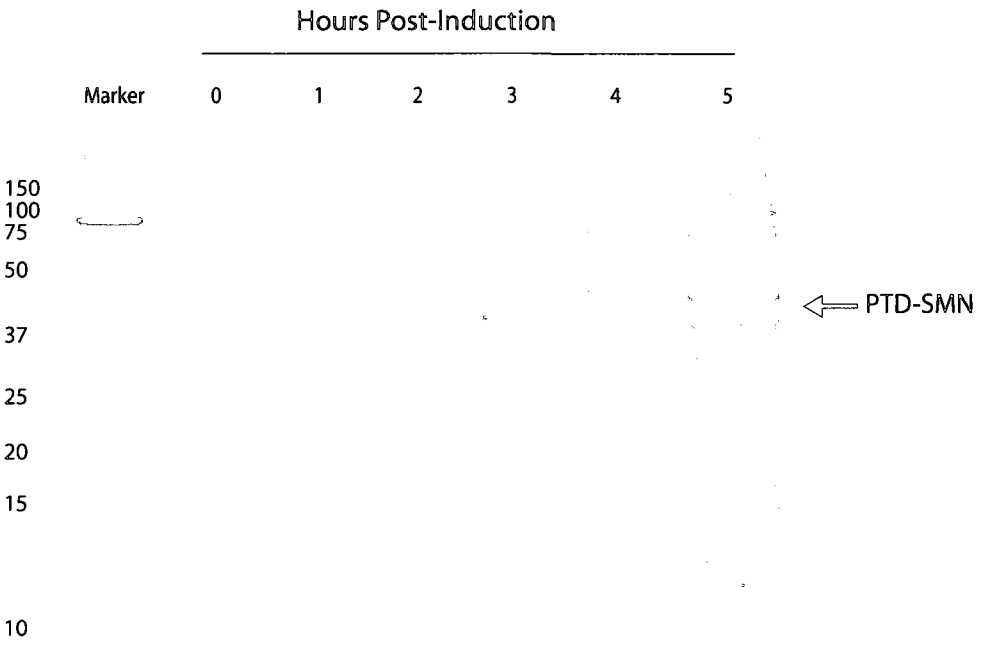
We also examined the effect of PTD-SMN fusion protein expression on bacterial cell growth in order to determine if PTD-SMN might have any toxic effects, which could limit recombinant protein yields. As shown in Figure 3.2B, induced-bacteria cultures grew with a shorter exponential phase than the non-induced samples, and unlike control bacteria, they reach the stationary phase as early as 1hr post-induction, remaining stable for up to 5hr; strongly suggesting that expression of the fusion protein is toxic to cells. However, it is important to mention that addition of IPTG, required for the induction of the recombinant protein, might contribute negatively on bacteria growth, independent of PTD-SMN expression.

Analysis of protein cell lysates and bacterial growth indicates that even though PTD-SMN expression is toxic to bacterial cells, production of protein is not halted. For example, although the bacteria stop growing after induction (Fig. 3.2B), there is a continual increase in protein isolated at latter time points (Fig. 3.2A). In fact, this observation agrees with previous studies on the influence of bacterial growth rate on recombinant gene expression, stating that

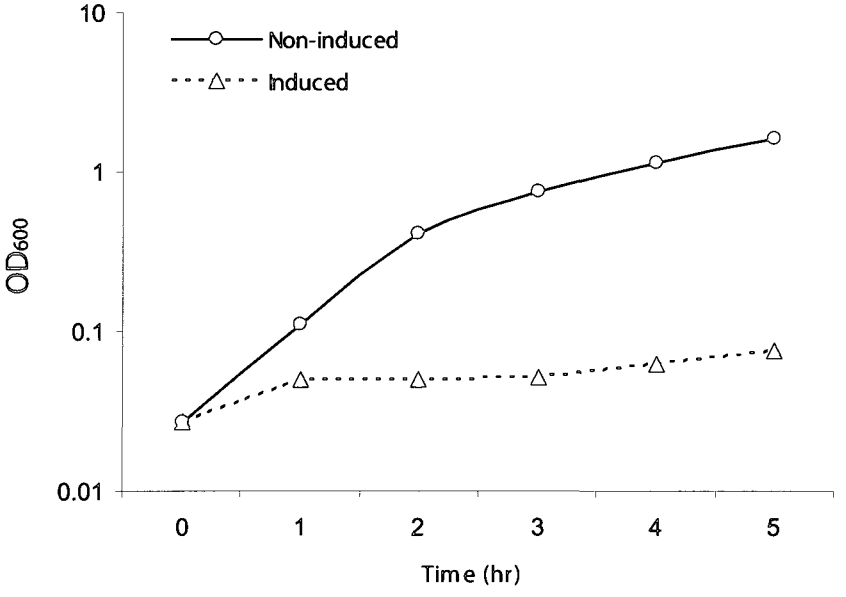
expression level of a recombinant protein does not necessarily correlate with cell growth (Woestenenk, et al, 2004).

Figure 3.2: Time-course expression of bacterially produced PTD-SMN recombinant protein. (A) Coomassie blue staining analysis of bacteria lysates expressing PTD-SMN upon induction with 1mM of IPTG. Loaded cell lysates correspond to non-induced bacteria at 0hr and induced bacteria up to 5hr. Amount loaded represent 1/20 part of total lysate. (B) Bacterial growth curve of non-induced versus induced bacteria. Bacteria were grown at 37°C, and OD_{600} was measured spectrophotometrically at 1-hour intervals. The graph is representative of 2 independent experiments.

A.


Hours Post-Induction
Marker
0
1
2
3
4
5
150
100
75
50
37
25
20
15
10
PTD-SMN

B.

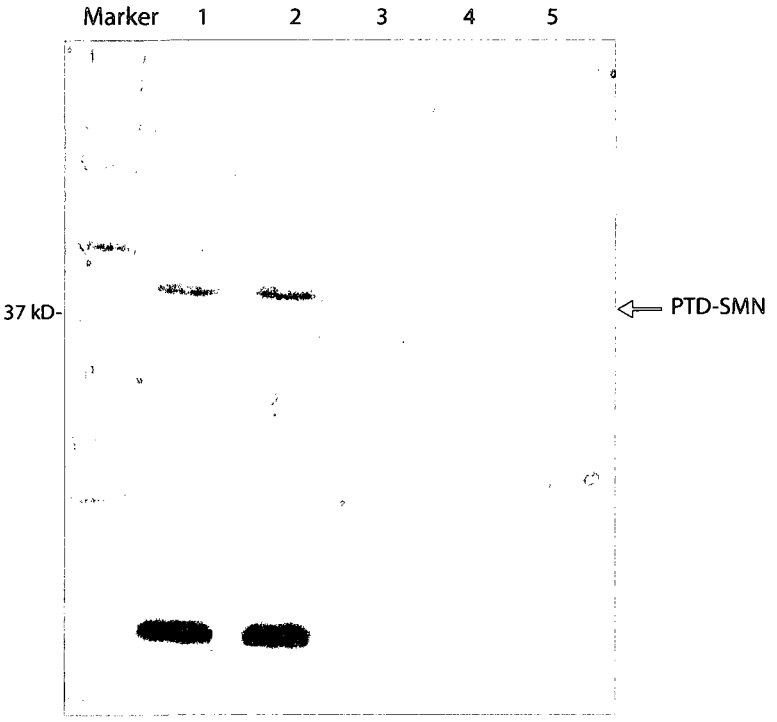

10
1
0.1
0.01
OD600
Non-induced
Induced
0
1
2
3
4
5
Time (hr)

3.3 Purification of PTD-SMN

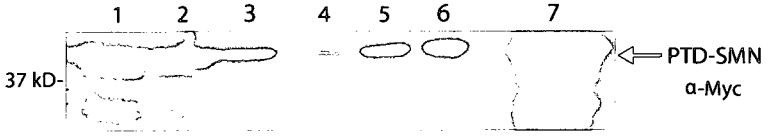
Following the induction of expression, PTD-SMN and FLAG-SMN were purified by Ni^{2+} affinity chromatography under native conditions. Bacteria were allowed to grow to an OD_{600} of 0.6 before induction of expression of recombinant *SMN* by addition of 1mM of IPTG. Bacteria were then incubated for an additional 4hr before harvesting the cells, reaching an OD_{600} of approximately 1.1. Bacterial samples obtained from the different fractions during the native purification process were examined by means of coomassie blue staining of SDS-PAGE gel and Western blot. Figure 3.3A indicates that the PTD-SMN fusion protein is present in the soluble fraction; however, Western blot analysis showed that most of the fusion proteins were localized to the insoluble fraction, possibly in inclusion bodies (Fig. 3.3B). Furthermore, it was observed that the fraction corresponding to the purified recombinant protein is highly contaminated by bacterial proteins, which could affect the therapeutic effects of PTD-SMN. Western blot analysis of the purification process indicates that a fraction of PTD-SMN is degraded during the purification process, as additional bands of low molecular weight are present; although, degradation was not a feature observed on a regular basis (Fig. 3.3B).

Figure 3.3: Purification of PTD-SMN. Four hours post-induction, bacteria were lysed for purification purposes at an OD_{600} of 1.1 ($1.62x10^{10}$ cells) (A) Coomassie-blue staining analysis of PTD-SMN purification procedure. Following expression in a 500ml culture volume of *E.coli*, PTD-SMN was purified using Ni^{2+} chromatography columns under native conditions (lysis and wash at 20mM imidazole; elution at 200mM imidazole). On this gel, cleared lysate represent 1/3000 part of total lysate; washes represent 1/1000 part of total volume, and eluate represent 1/600 part of the total volume of elution. 1& 2: cleared lysate; 3: flow through; 4: wash; 5: eluate. (B) Immunoblot analysis of PTD-SMN purification procedure. 1: cleared lysate; 2: flow through; 3& 4: first and second wash; 5 & 6: first and second eluate ; 7: bacterial pellet. PTD-SMN was detected with a mouse monoclonal Myc antibody.

A.



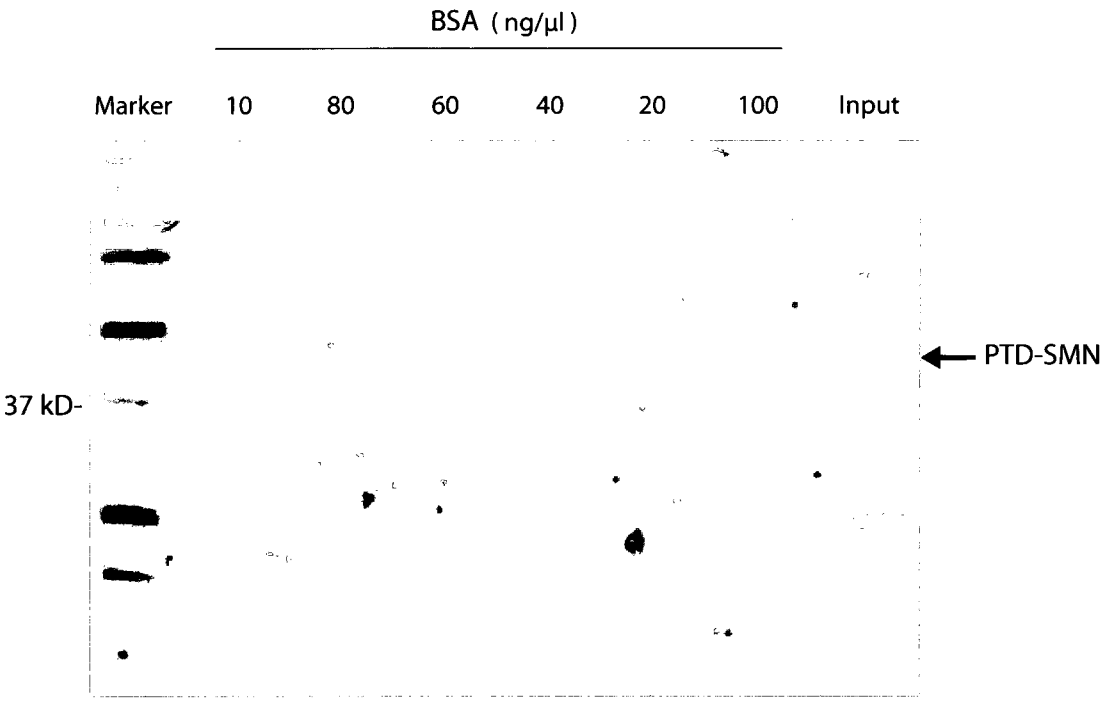
B.



3.4 Determination of PTD-SMN concentration

Protein concentrations were initially determined using Bradford assay and SDS-PAGE gel analysis. However, Bradford analysis overestimated protein concentrations due to the presence of bacterial proteins in our protein purified samples; therefore, a sample of the purified protein was compared to serial dilutions of BSA by gel electrophoresis followed by coomassie staining (Fig. 3.4). From a 500ml culture of bacteria, our approximate recovery of PTD-SMN and FLAG-SMN was 120μg and 450μg, at a concentration of 20μg/ml and 70μg/ml respectively. The amount of PTD-SMN recovered represents 8% from the total amount of protein in the preparation.

Figure 3.4: Determination of PTD-SMN concentration. Band intensities of a dilution series of BSA was compared to 10µl of the purified PTD-SMN protein. A 1/600 part of the total elution volume of PTD-SMN was loaded on a gel.

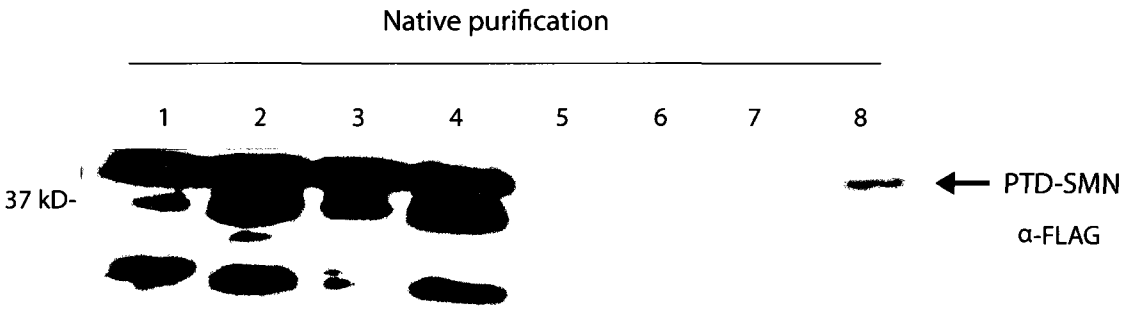

BSA (ng/μl)
Marker
10
80
60
40
20
100
Input
37 kD-
PTD-SMN

3.5 Attempts to improve yield and purity of PTD-SMN

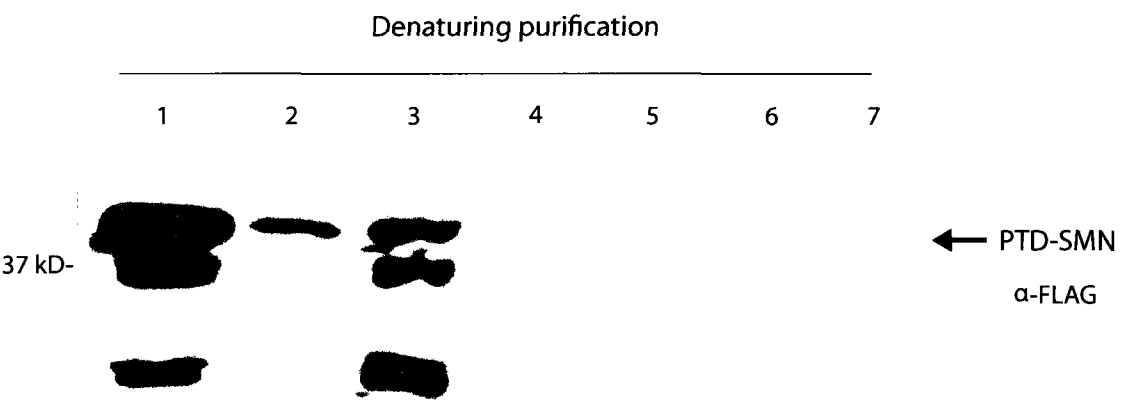
Low yields and bacterial proteins contaminants could compromise functional studies of PTD-SMN. Hence, we attempted to improve the purification conditions for the recombinant proteins in order to obtain higher yields and cleaner protein preparations. To circumvent these deficiencies, a series of steps were taken; which included denaturing purification procedure, reduction of the temperature during bacterial growth, and variation in imidazole concentrations during the nickel column wash steps. Previous studies reported that delivery of denatured TAT- recombinant protein to mice resulted in a broad expression of the β-galactosidase protein (Schwarze, et al, 1999). Therefore, we purified recombinant proteins under denaturing conditions, using strong denaturing agents such as urea, according to the manufacturers protocol (Qiagen). Western blot analysis of fractions from the denaturing procedure showed little to no recovery of purified protein as compared to native purification (Fig. 3.5A and 3.5B). Considering the complexity of the denaturing purification procedure, we chose to continue the purification procedure under the native methodology. Likewise, changes in temperature and imidazole did not improve the yield and purity of the protein.

Figure 3.5: Native vs denaturing PTD-SMN purification. (A) Immunoblot analysis of PTD-SMN purification using Ni^{2+} chromatography columns under native conditions (lysis and wash at 20mM imidazole; elution at 200mM imidazole) from a 25ml culture volume. 1: bacterial pellet; 2 & 3: cleared lysate; 4: flow through; 5, 6, & 7: sequential washes; 8: eluate Cleared lysate and washes represent 1/60 part of total volume of cleared lysate and washes respectively, and eluate represent 1/30 part of the total volume of elution. PTD-SMN was detected with a mouse FLAG antibody. (B) Immunoblot analysis of PTD-SMN purification using Ni^{2+} chromatography columns under denaturing conditions (8M urea; elution at pH 5.9) from a 25ml culture volume. 1: bacterial pellet; 2: cleared lysate; 3: flow through; 4, 5, & 6: sequential washes; 7: eluate. PTD-SMN was detected with a mouse FLAG antibody.

A.


Native purification
1
2
3
4
5
6
7
8
37 kD-
PTD-SMN
α-FLAG

B.


Denaturing purification
1
2
3
4
5
6
7
37 kD-
PTD-SMN
α-FLAG

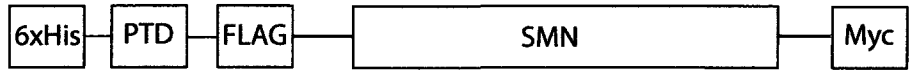
3.6 Optimization of PTD-SMN construct

Another factor that might contribute to the poor yield of PTD-SMN is the loss of protein during the first passage of the cleared lysate through the column (flow through). To increase the amount of protein bound to the chromatography columns, we investigated whether placing the His-tag at the amino-terminus could reduce protein elution during the first steps of purification, and thus increase protein concentration. The His-tag, originally placed at the carboxy-terminus of the construct, could be inaccessible due to the conformation of the recombinant protein. As shown in Figure 3.6B, expression of PTD-SMN with His-tag at the amino terminus did not seem to improve the yield of PTD-SMN, since much of the fusion protein is still lost during early steps of the procedure. Therefore, it was decided to continue using the initial construct, which had the six histine residues at the carboxy terminus. A schematic representation of the construct is shown in Figure 3.6A.

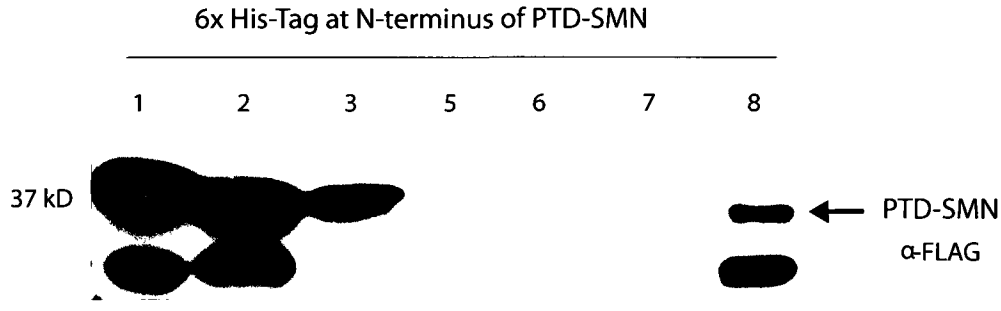
Despite the fact that PTD-SMN recombinant protein was contaminated with bacteria proteins, we began to characterize the function of the protein in tissue culture.

Figure 3.6: Purification of PTD-SMN containing an amino-terminal His tag. (A) Schematic representation of the PTD-SMN construct with the His tag at the amino terminus. (B) Immunoblot analysis of samples from 1: bacterial pellet; 2: cleared lysate; 3: flow through; 5, 6, & 7: sequential washes; 8: eluate. PTD-SMN was detected with a mouse monoclonal FLAG antibody.

A.


6xHis
PTD
FLAG
SMN
Myc

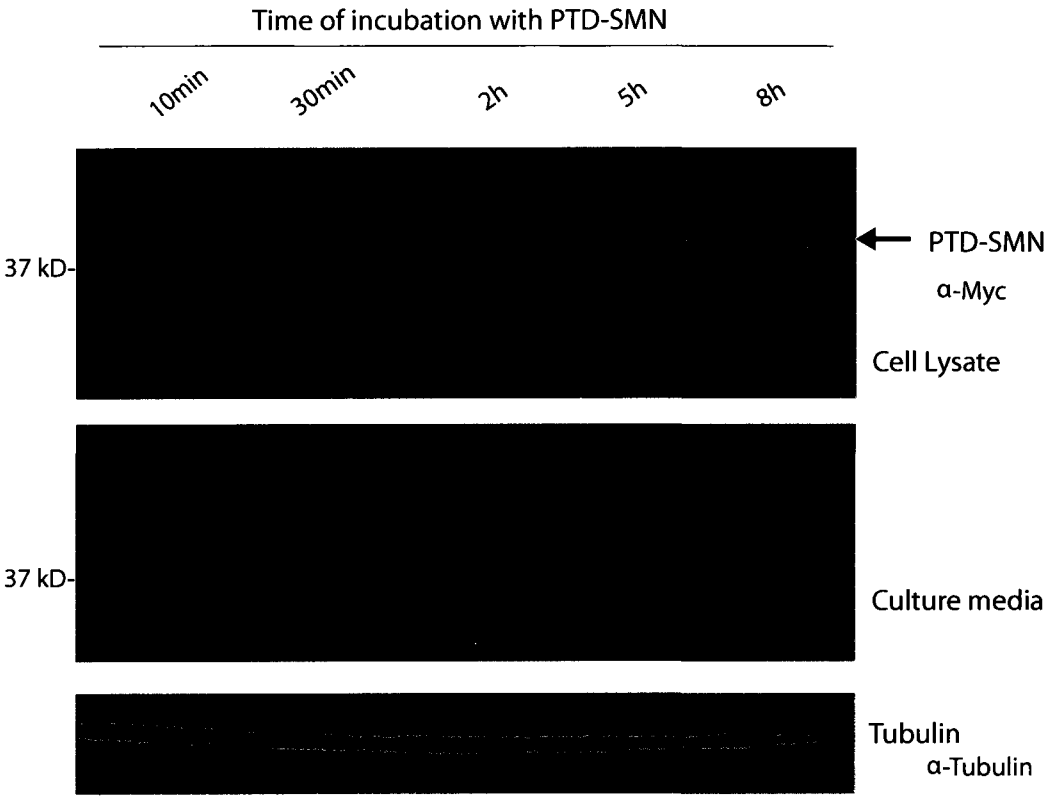
B.


6x His-Tag at N-terminus of PTD-SMN
1
2
3
5
6
7
8
37 kD
PTD-SMN
α-FLAG

3.7 Association-rate of PTD-SMN *in vitro*

To begin to examine the transduction capacity of the PTD-SMN fusion protein, we tested the binding capacity of the recombinant protein to cells in culture. PTD-SMN was added directly to cultured A549 cells at a final concentration of 82nM for 8hr at 37°C. Treated cells were harvested and lysed at the indicated time points. The amount of protein bound to cells was determined by immunoblot analysis, which showed that the recombinant protein was able to associate with the cells in a time dependent manner (Fig. 3.7). Specifically, as the incubation time increases, more protein is bound to cells; consequently, we observed that the amount of protein in the media is reduced as the amount of cell-associated protein increases. This data suggests that the binding capacity of PTD-SMN to cells *in vitro* is time-dependent.

Figure 3.7: Association-rate of PTD-SMN with cells in culture. Semi-confluent A549 cells were incubated for increasing time with 82nM of PTD-SMN. Immediately after the incubation period, the cells were washed extensively with PBS, lysed with Laemmli buffer, and an aliquot representing 1/10 of total lysate was separated by SDS-PAGE and immunoblotted for the Myc epitope tag on PTD, or tubulin (loading control).

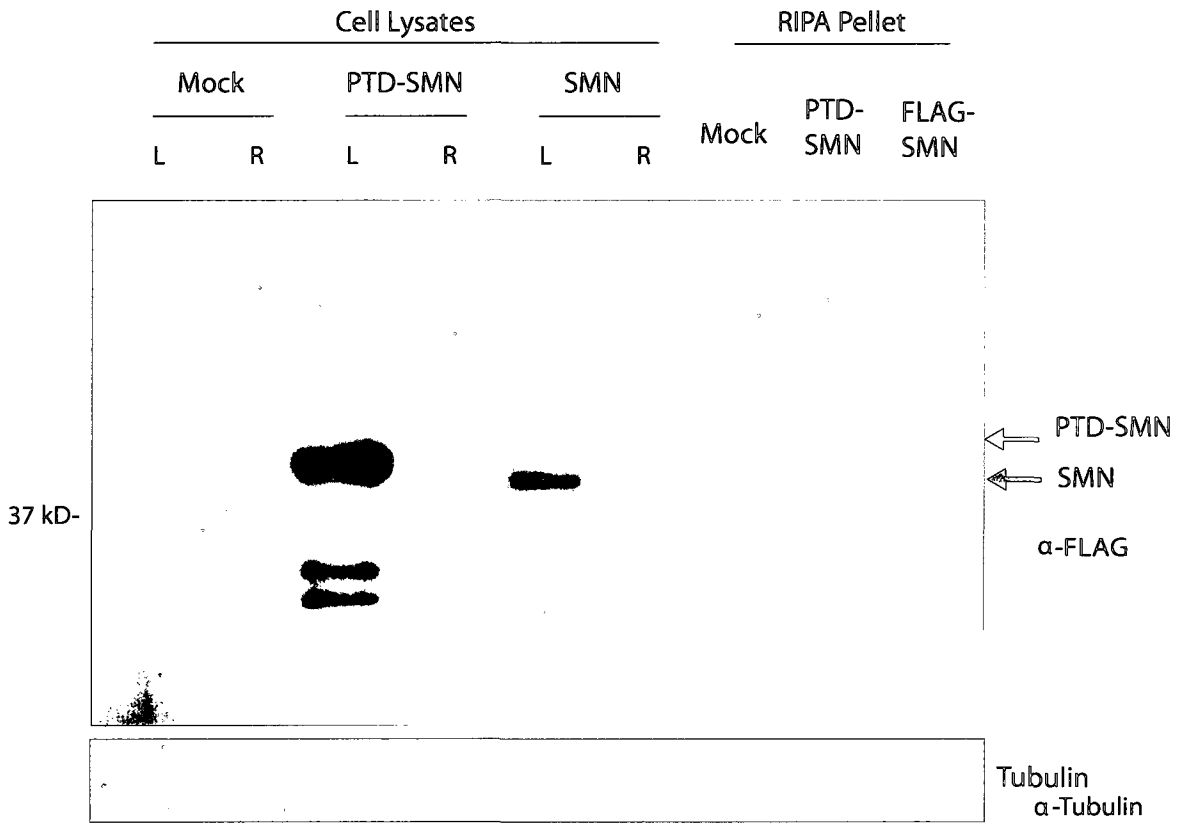

Time of incubation with PTD-SMN
10min
30min
2h
5h
8h
37 kD-
PTD-SMN
α-Myc
Cell Lysate
37 kD-
Culture media
Tubulin
α-Tubulin

3.8 Binding of PTD-SMN to cell surface

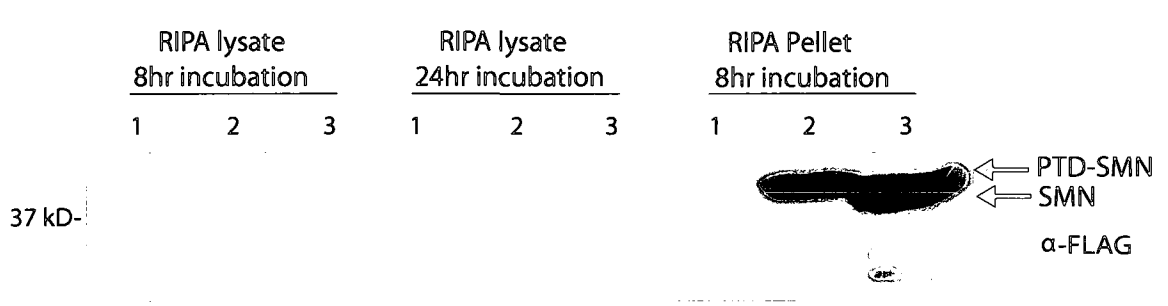
To further evaluate the cellular transduction of PTD-SMN *in vitro*, PTD-SMN and FLAG-SMN were added to the culture media of HeLa cells for 8hr at 37°C, at a final concentration of 40nM and 600nM respectively. After the incubation period, the cells were lysed with RIPA or SDS-PAGE buffer (2x Laemmli buffer), and the quantity of transduced protein associated with the cells determined by Western blot analysis. In this experiment, cells that were lysed with RIPA buffer had been previously incubated with 1X trypsin, in order to detach the cells from the culture dishes. Fig. 3.8A shows the bands corresponding to PTD-SMN and FLAG-SMN were found in the SDS-PAGE buffer lysates (L), whereas, RIPA lysates (R) from treated HeLa cells do not show any band corresponding to the recombinant proteins. This data is interesting; failure to observe PTD-SMN in RIPA lysates suggests that the fusion protein did not successfully cross the cell membrane, and may have been degraded by the treatment with trypsin. To further support this piece of information, we incubated HeLa cells with 12nM of PTD-SMN and 400nM of FLAG-SMN under conditions similar as the previous experiments. However, for this experiment, cells were not trysinized. As shown in Fig. 3.8B, recombinant protein is not found in the RIPA lysate but rather in the pellet. This pellet fraction contains the cell membrane and intact nucleus. Taken together, these data suggest that the majority of PTD-SMN is not taken up by the cells, but remains strongly attached to the cell surface.

Figure 3.8: PTD-SMN remains primarily bound to the cell surface. (A) Immunoblot analysis of HeLa cells incubated with PBS, PTD-SMN, or FLAG-SMN. HeLa cells were incubated with 40nM of PTD-SMN or 0.6μM of FLAG-SMN for 8hr, after which cells were washed extensively with PBS and lysed with RIPA or Laemmli buffer (SDS-PAGE buffer). Prior to RIPA lyses, cultured cells were incubated with 200μl of 1X trypsin for 2min at 37°C in order to detach cells from the tissue culture dish. Four μg of PTD-SMN and SMN, representing 11% and 34% of the total cellular protein obtained from RIPA lysates respectively, and 1/12.5 part of total lysate from Laemli lyses were applied to gel. PTD-SMN and FLAG-SMN were identified using mouse-FLAG antibody, and tubulin was identified using mouse-anti-tubulin antibody. (B) Immunoblot analysis of HeLa cells incubated with PBS, PTD-SMN, or FLAG-SMN. HeLa cells were incubated with 12nM of PTD-SMN or 0.4μM of FLAG-SMN for 8hr, after which cells were washed extensively with PBS, and lysed with RIPA buffer. Forty μg of total cellular protein obtained from RIPA lyses were applied to the gel. 1: PBS-treated cells; 2: PTD-SMN-treated cells; 3: FLAG-SMN-treated cells. R=RIPA; L= laemli.

A.


Cell Lysates
RIPA Pellet
Mock
PTD-SMN
SMN
L
R
L
R
L
R
Mock
PTD-SMN
FLAG-SMN
37 kD-
PTD-SMN
SMN
α-FLAG
Tubulin
α-Tubulin

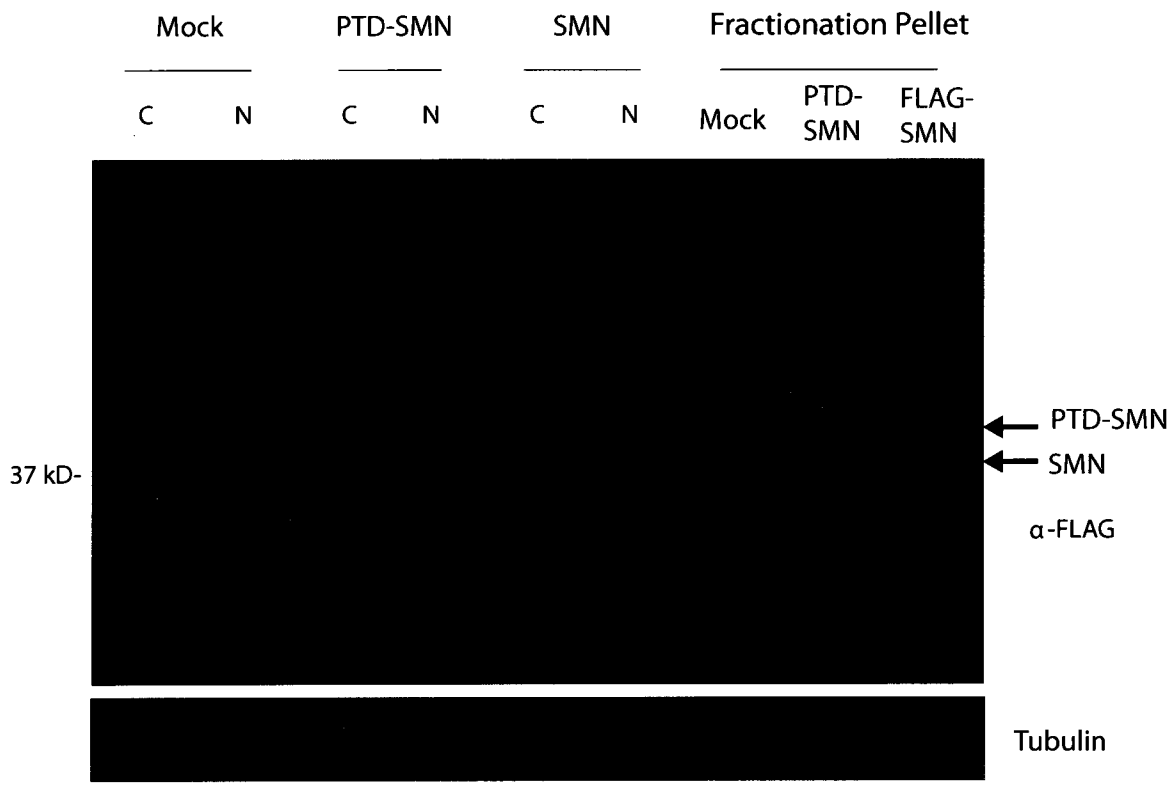
B.


RIPA lysate
8hr incubation
RIPA lysate
24hr incubation
RIPA Pellet
8hr incubation
1
2
3
1
2
3
1
2
3
37 kD-
PTD-SMN
SMN
α-FLAG

3.9 Cytoplasmic fractionation of PTD-SMN cultured cells

Although the majority of PTD-SMN appeared to remain bound to the cell membrane, and did not transduce to the interior of the cell, we undertook additional experiments to determine if a fraction of the protein was entering the cell. To this end, we performed cell fractionation experiment which would achieve two tasks. First, this procedure would concentrate the protein fractions, allowing us to more easily visualize small amounts of the protein by immunoblot. Secondly, we could determine the cellular location of the protein (nucleus versus cytoplasm). HeLa cells were incubated with PTD-SMN and FLAG-SMN for 8hr at 37°C, at a final concentration of 12nM, and subcellular fractions were prepared as described in the Materials and Methods section. Western blot analysis revealed a prominent PTD-SMN band in the cytoplasmic fraction of the PTD-SMN treated HeLa cells, which is absent from the nuclear fraction (Fig. 3.9). Importantly, recombinant FLAG-SMN protein fails to appear in the cytoplasmic and nuclear fraction. In addition, pellets obtained from the fractionation procedure were also analyzed. In agreement with previous experiments, we observed that majority of PTD-SMN and FLAG-SMN are largely present in the pellets, supporting previous observations that PTD-SMN is tightly bound to the cell membrane. Together, these data suggest that the vast majority of recombinant protein remains bound to the outside of the cell, and only a small fraction reaches the cytoplasm.

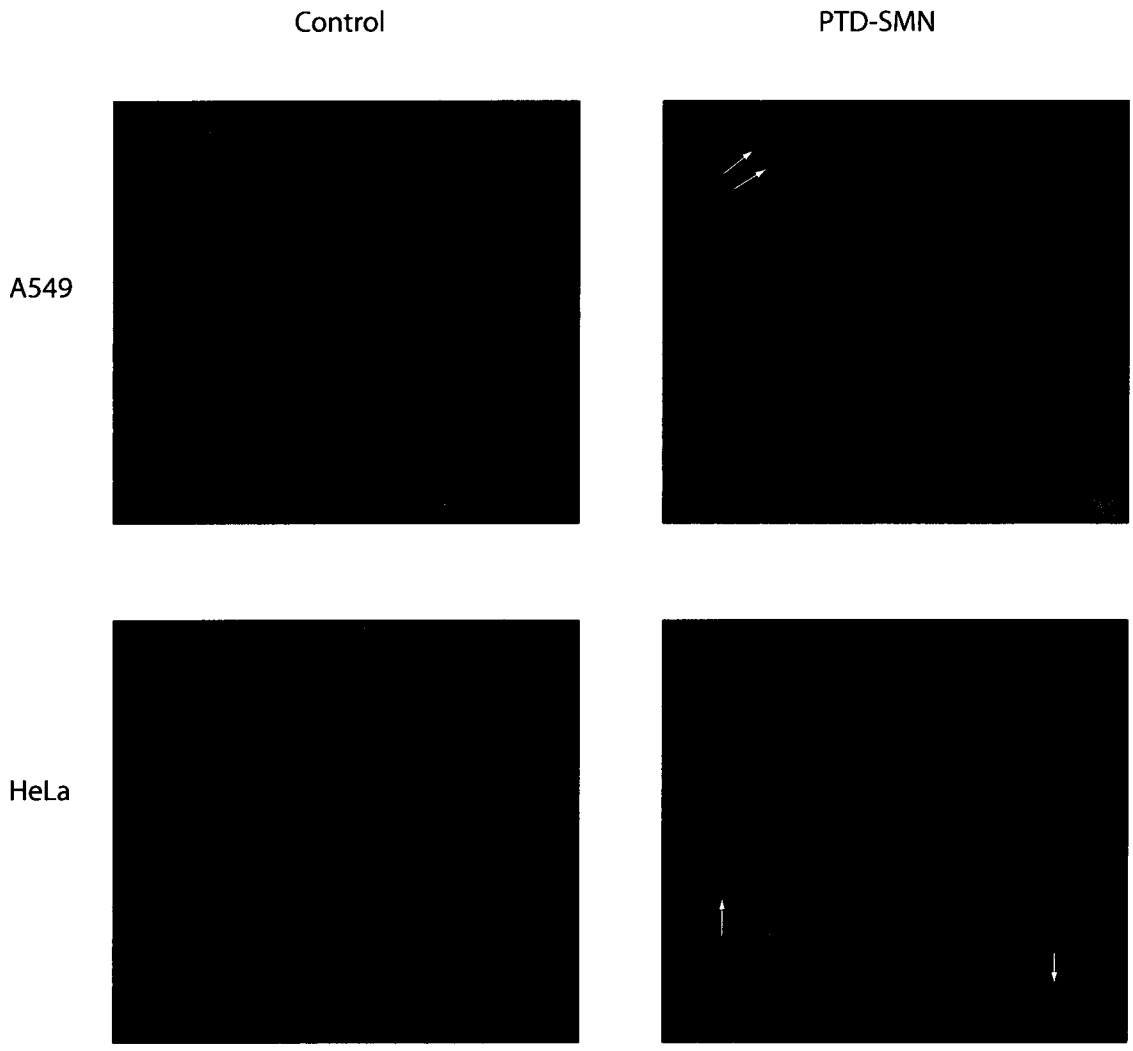
Figure 3.9: Cell fractionation of PTD-SMN-transduced cells. HeLa cells were incubated with 12nM of PTD-SMN or FLAG-SMN for 8hr, after which the cells were washed extensively with PBS. Aliquots of cytoplasmic and nuclear fractions were separated by SDS-PAGE. 1/12.5 parts of total cytoplasmic fraction, and 1/5 parts of the nuclear fraction were applied to the gel. PTD-SMN and FLAG-SMN were identified using mouse-FLAG antibody, and tubulin was identified using mouse-anti-tubulin antibody. C: cytoplasmic fraction; N: nuclear fraction


Mock
PTD-SMN
SMN
Fractionation Pellet
C
N
C
N
C
N
Mock
PTD-SMN
FLAG-SMN
37 kD-
PTD-SMN
SMN
α-FLAG
Tubulin

3.10 Transduction of PTD-SMN in culture

To visualize internalization of PTD-SMN in culture, immunocytochemistry analysis of cultured cells incubated with PTD-SMN was performed by confocal microscopy, which would allow us to distinguish between protein present on the surface or within the cell. A549 and HeLa cells were incubated with 160nM of purified protein for 8hr, and then processed for immunocytochemistry with a FLAG antibody. As observed in Fig. 3.10, incubation of cells with PTD-SMN results in significant fluorescent signal in both the cytoplasm and nucleus of the cells. Moreover, at least some of the protein in the nucleus was located in distinct punctate structures, reminiscent of gems; thus we will refer to them as "gem-like" structures. Consistent with this observation, fluorescence signal is not present surrounding the cell surface, as a halo, which might indicate that PTD-SMN was trapped at the cell membrane. Furthermore, transduction of PTD-SMN is observed in two different cell lines, suggesting that translocation of recombinant PTD-SMN fusion protein occurs independently of the cell line; although there are some reports that have claimed that TAT transduction might depend on the host cell type (Yang, et al, 2002). Overall, addition of a PTD to SMN protein allows for uptake of the protein by cells in tissue culture and entry into the nucleus.

Figure 3.10: Internalization of PTD-SMN by tissue culture cells. A549 or HeLa cells were incubated with PBS (control) or 160nM of PTD-SMN for 8hr, washed extensively, fixed and prepared for immunofluorescence analysis using an anti-FLAG antibody (red) and DAPI (blue) to stain the nuclei. Confocal images clearly show that PTD-SMN is within the cell, both in the cytoplasm and in localized dots in the nucleus. PTD-SMN localizes to the nucleus and form "gem-like" structures. Gem-like structures are indicated with arrows.


Control
PTD-SMN
A549
HeLa

3.11 Distribution and effect of PTD-SMN *in vitro*

Full characterization of PTD-SMN includes determining whether PTD-SMN behaves as its endogenous counterpart protein. In order to evaluate this, we compared the distribution pattern of PTD-SMN with endogenous SMN. HeLa cells were incubated with PTD-SMN for 8hr at 37° at a final concentration of 12nM, and the localization of endogenous SMN and PTD-SMN was examined by immunofluorescence. Fig. 3.11 shows that the distribution pattern of PTD-SMN differs from endogenous SMN in that endogenous SMN is equally distributed along the cytoplasm, whereas PTD-SMN concentrates in aggregates throughout the cytoplasm. We also observed that PTD-SMN is localized to the nucleus, forming gem-like structures, as previously shown. Double-staining of PTD-SMN cultured cells for PTD-SMN and SMN fail to reveal any important information because we cannot distinguish between endogenous SMN and PTD-SMN.

To evaluate if PTD-SMN had any effect on the total number of gems, we quantified the number of gems in non-treated and treated cells. We observed that upon PTD-SMN treatment, there is a slight increase in the number of gems as compared to PBS treated cells (Table 3.11). Taken together, the majority of transduced PTD-SMN forms aggregates in the cytoplasm, whereas a very small portion of PTD-SMN reaches the nucleus, forming gem-like structures, as previously shown. It is believed that uptaken of PTD-SMN by the cells promotes formation of gems, as evidenced by gem counts.

Figure 3.11: Distribution and effect of PTD-SMN *in vitro*. HeLa cells incubated with PTD-SMN at a final concentration of 12nM were fixed and processed for immunocytochemistry analysis for PTD-SMN (green) and endogenous SMN (red). Confocal images show that PTD-SMN reaches the nucleus, forming gem-like structures, but fails to distribute in the cytoplasm as the endogenous SMN. Cells were double-stained for PTD-SMN and endogenous SMN using mouse-anti-SMN and rabbit-anti-Myc antibody.

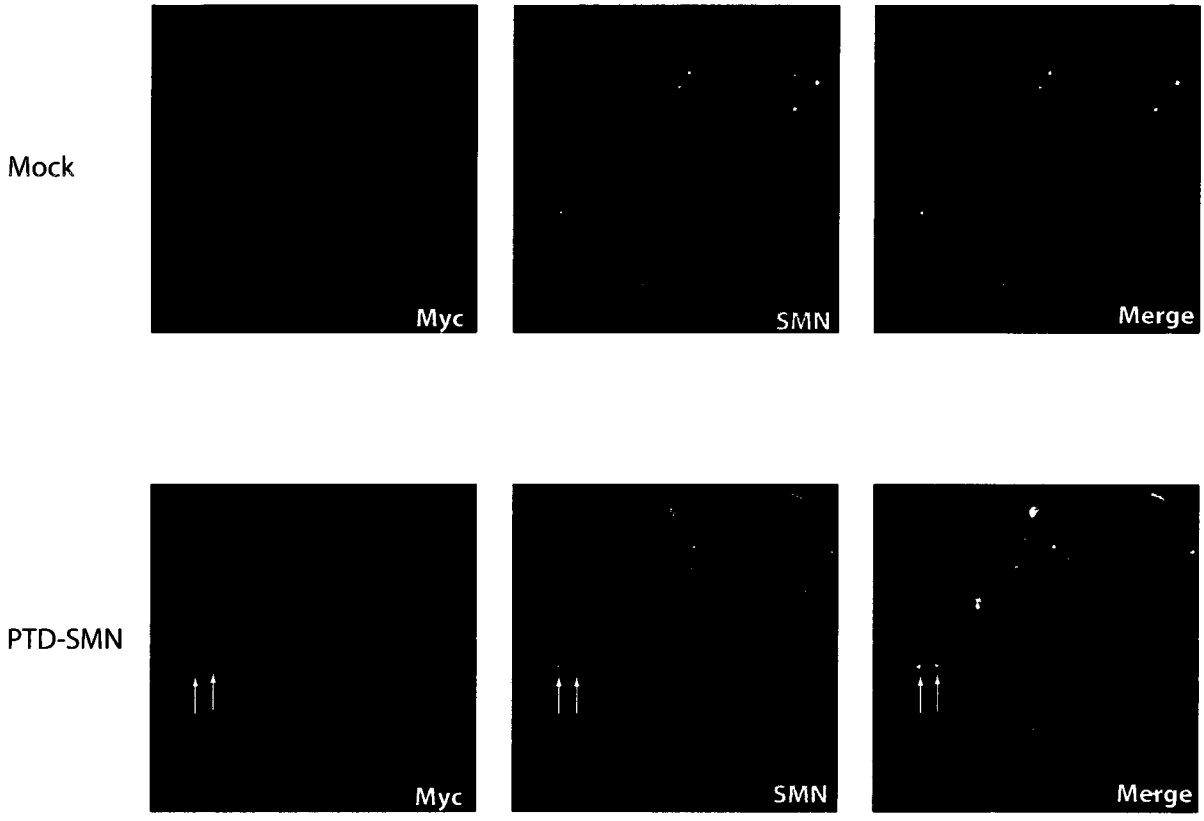

Mock
Myc
SMN
Merge
PTD-SMN
Myc
SMN
Merge

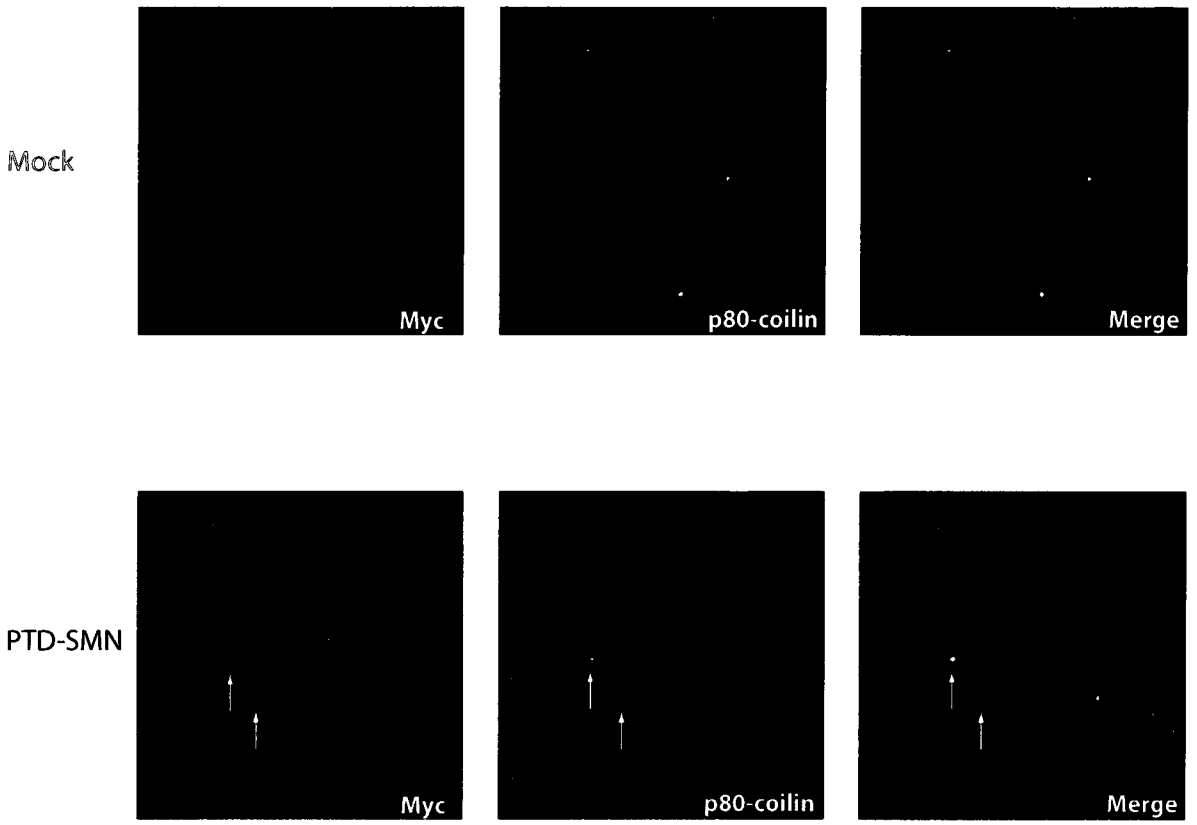
Table 3.11. Quantitation of the number of gems in cells treated with PTD-SMN.

Treatment	# cells	# cells with gems	# of cells with:					# gems	Gems/ 100 cells
			1 gem	2 gems	3 gems	4 gems	≥ 5 gems		
Mock	150	95	14	25	27	13	16	279	190
PTD-SMN	172	116	12	16	28	31	29	417	240

3.12 Localization of PTD-SMN to Cajal bodies in culture

Figure 3.10 suggests that a small fraction of PTD-SMN was able to cross the cell membrane. We wanted to investigate whether the internalized PTD-SMN was localized to the right structure within the cell. To determine if the punctate staining in the nucleus was due to correct localization of the exogenously added protein to gems, we treated Hela cells with PTD-SMN for 8hr at 37°C at a final concentration of 500nM and examined whether the protein co-localized with p80-coilin, a protein present in Cajal bodies which are quite often adjacent to gems (Young, et al, 2000). As shown in Fig. 3.12, we observed co-localization of PTD-SMN with p80-coilin, although we also observed additional discrete staining of both proteins. Therefore, a portion of the PTD-SMN that is internalized in cells correctly localizes within the nucleus. These data also suggest that the presence of the various tags on the amino and carboxy terminus of the SMN does not affect this localization.

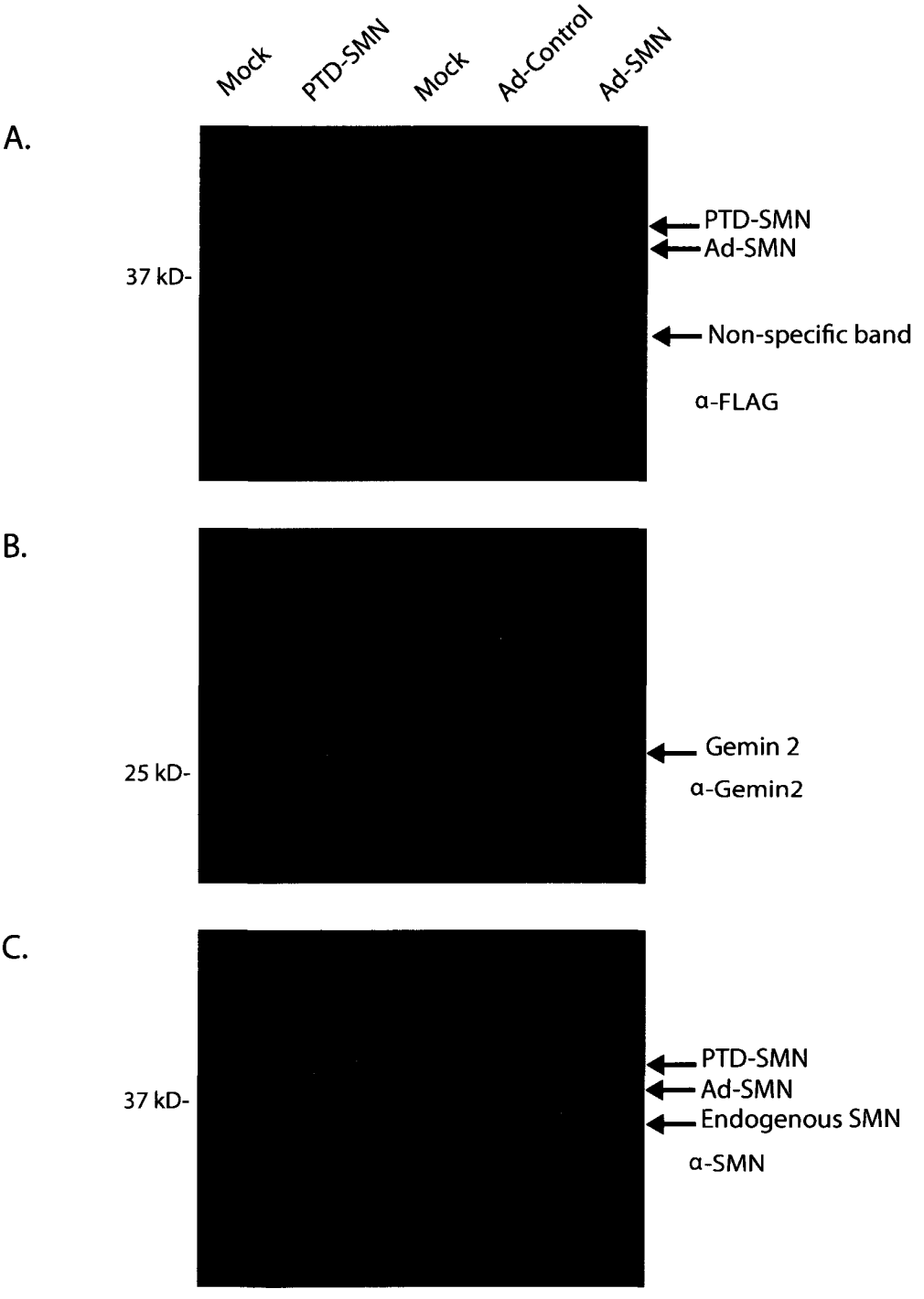
Figure 3.12: Cells treated with PTD-SMN show co-localization of the protein with p80-coilin. HeLa cells on cover slips in 35mm dishes were treated with 500nM of PTD-SMN for 8hr, washed extensively, fixed and subjected to immunofluorescence analysis for p80-coilin (red) and PTD-SMN (green) by confocal immunocytochemistry. In the cell, SMN-containing gems frequently overlap or are in close proximity to coiled bodies. Arrows indicate PTD-SMN that co-localizes with p80-coilin.


Mock
Myc
p80-coilin
Merge
PTD-SMN
Myc
p80-coilin
Merge

3.13 Interaction of PTD-SMN to SMN partners

The next objective of our project was to assess the ability of PTD-SMN to interact with SMN's normal cellular partners, such as Gemin2 or endogenous SMN. To this end, we performed co-immunoprecipitation studies of HeLa cells incubated with PTD-SMN at a final concentration of 25nM, or infected with an Ad vector which expresses the human *SMN* at a MOI of 3. As a control we used AdCA35, and E1/E3-deleted Ad encoding *lacZ*, as previously described (Addison, et al, 1997). The Ad vector was used as positive control in our experiment since protein produced from the virus has already been shown to interact with Gemin2 in the cell (DiDonato, et al, 2003). Immunoprecipitations were carried out with an anti-Myc antibody, and subsequently, immunoprecipitated proteins were resolved by SDS-PAGE, transferred to nylon membrane, and probed with antibodies that would recognize SMN protein partners. As shown in Fig. 3.13, the FLAG antibody specifically recognizes the PTD-SMN protein, as well as SMN produced from the Ad vector (Ad-SMN), indicating that pull down of the recombinant proteins was successful. However, Gemin2 and endogenous SMN do not precipitate along with PTD-SMN, as shown by the Western blot. In contrast, Gemin2 and endogenous SMN do interact with Ad-SMN, demonstrated by the positive signals on the corresponding membranes. Thus, at least at the detection level of this assay, PTD-SMN does not interact with SMN's normal cellular binding partners.

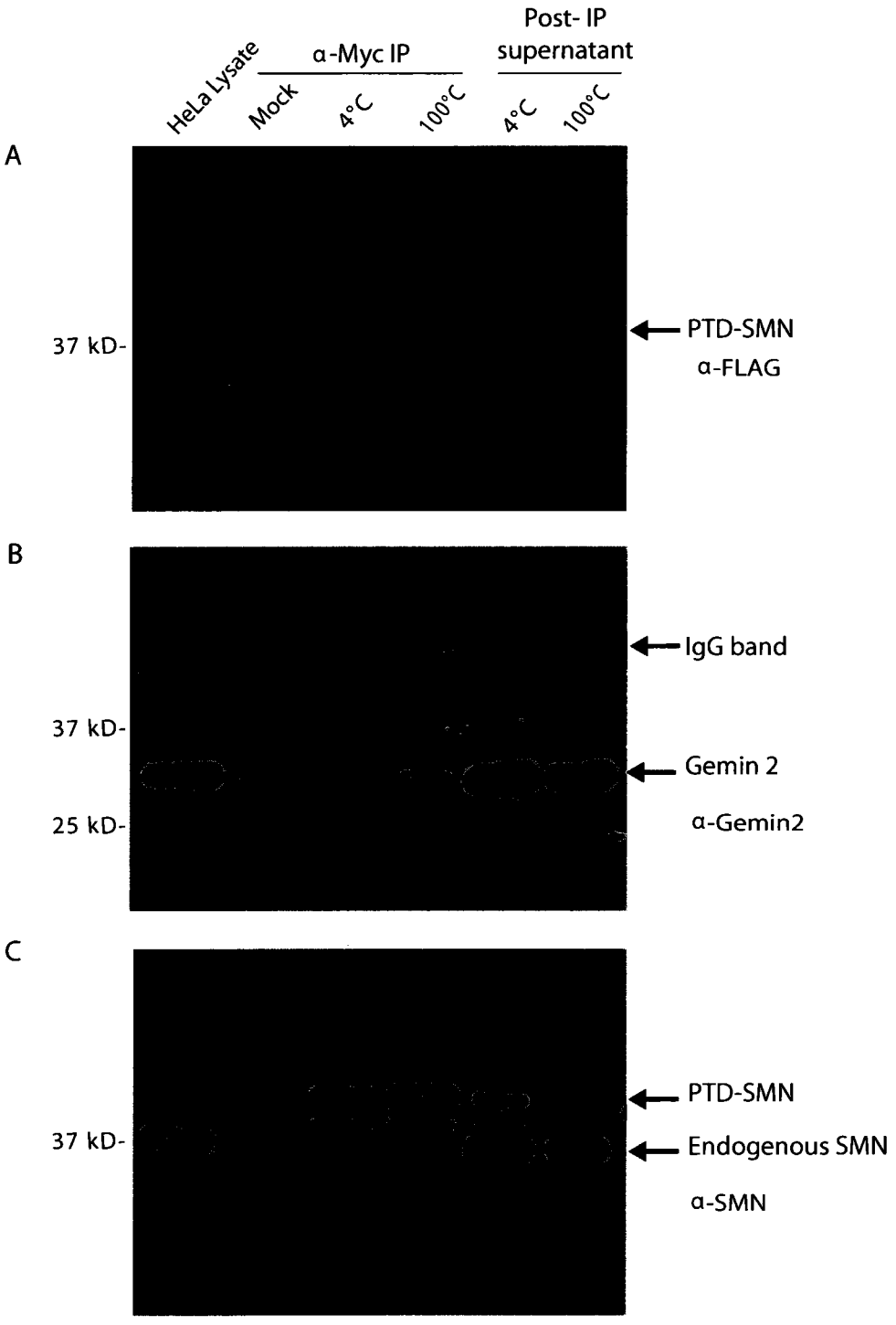
Figure 3.13: PTD-SMN does not interact with endogenous SMN or Gemin2 in cells. HeLa cells incubated with 25nM of PTD-SMN for 8hr at 37°C or infected with Ad-SMN at MOI of 3 for 18hr were lysed with RIPA buffer. Cellular extracts were immunoprecipitated with a rabbit-anti-Myc antibody. Bound proteins were analyzed by SDS-PAGE and by immunoblotting with antibodies that recognize (A) FLAG tag, (B) Gemin2 or (C) endogenous SMN. Western blot analysis of immunoprecipitates show that PTD-SMN does not interact with some of the SMN protein partners, Gemin2 and endogenous SMN.


Mock
PTD-SMN
Mock
Ad-Control
Ad-SMN
A.
37 kD-
PTD-SMN
Ad-SMN
Non-specific band
α-FLAG
B.
25 kD-
Gemin 2
α-Gemin2
C.
37 kD-
PTD-SMN
Ad-SMN
Endogenous SMN
α-SMN

3.14 Interaction of PTD-SMN to SMN partners in a free-membrane environment

Although we were unable to observe interaction of PTD-SMN with Gemin2 or endogenous SMN in our immunoprecipitation studies, we decided to take another approach to investigate whether our recombinant protein can associate with other proteins. We assumed that the cell membrane is a negative factor in PTD-SMN transduction; thus, we asked whether PTD-SMN could interact with its normal partners in a cell-free system. Non-treated Hela cells were lysed with modified RIPA buffer, and cleared lysates were incubated with 12nM of native or denatured PTD-SMN for 1hr or 16hr at 4°C, followed by co-immunoprecipitation procedure as described in Materials and Methods. The purpose of using denatured PTD-SMN, which was boiled for 5min prior to addition to the cell lysate, was to include a negative control, since it should be denatured and incapable of interacting. Fig. 3.14 indicates that we were able to immunoprecipitate PTD-SMN, but this protein did not interact with Gemin2 or endogenous SMN. Moreover, we observed that these two proteins are largely present in the unbound fraction, strongly suggesting that they do not interact with PTD-SMN.

Figure 3.14: PTD-SMN does not interact with endogenous SMN or Gemin2 in a cell-free system. Total cellular proteins (250μg) of HeLa cells incubated with 12nM of native or denatured PTD-SMN (boiled at 100°) for 1hr at 4°C were immunoprecipitated with a rabbit-anti-Myc antibody. Bound proteins were analyzed by SDS-PAGE and by immunoblotting with antibodies that recognize (A) FLAG tag, (B) Gemin2 or endogenous (C) SMN. Western blot analysis of immunoprecipitates show that PTD-SMN does not interact neither with Gemin2 nor endogenous SMN.


HeLa Lysate
Mock
α-Myc IP
4°C
100°C
Post- IP supernatant
4°C
100°C
A
37 kD-
PTD-SMN
α-FLAG
B
IgG band
37 kD-
Gemin 2
25 kD-
α-Gemin2
C
PTD-SMN
37 kD-
Endogenous SMN
α-SMN

3.15 Summary

In summary, we have expressed and purified bacterially produced PTD-SMN that corresponds to the expected molecular weight. Our protein is able to bind to cells *in vitro* in a time-dependent manner at 37°C. However, majority of PTD-SMN does not internalize into the cells, and only a small portion of the protein reaches the nucleus forming "gem-like" structures and localizes to coiled bodies. Furthermore, the PTD-SMN that reaches the nucleus promotes a slight increase in gem counts. Unfortunately, prokaryotic PTD-SMN protein does not efficiently interact with SMN's normal partners, revealing its limitation for therapeutic purposes.

Chapter 4 – Discussion & Future Directions

Spinal muscular atrophy, which is caused by homozygous deletion of the *SMN1* gene, is the number one killer of infants under the age of two. Although, much information has been accumulated since the discovery of the disease-causing gene, the question of why motor neurons specifically degenerate is still unanswered. However, motor neurons can be spared by increasing the cellular levels of the SMN protein. Thus, an effective treatment for SMA is to enhance or increase SMN expression.

Current management of SMA involves aggressive supportive care in order to prevent rapid disease-progression (Burnett, et al, 2009). In the search for a cure to SMA, several research labs have explored a variety of strategies to up-regulate SMN levels. Activation of *SMN2* gene transcription, application of embryonic stem cell and gene therapy, and use of neuroprotective agents are among some of the therapeutic prospects for SMA treatment. Although each approach is promising, there are important limitations with respect to their long term application.

In line with most prevailing strategies for the treatment of SMA, we aimed to develop protein therapeutics for SMA. Protein therapeutics, or protein replacement therapy, is designed to increase the levels of a defective or a missing protein. A well known therapy for lysosomal disorders, protein therapeutics have been demonstrated to be safe and effective, based on clinical, immunological, and therapeutic responses (Desnick, 2004). The administration to a patient of a purified protein to augment the levels of a missing or a deficient protein could correct disease phenotypes.

The production of recombinant proteins can be achieved by a plethora of systems available. Among them, the two most widely used are the mammalian and prokaryote

systems. For the development of our project, we employed bacteria as the manufacturer of our recombinant protein, since it offers important advantages over other expression systems, such as low cost, high expression levels of recombinant protein, and ease of manipulation.

Once the purified protein is obtained, it needs to be delivered to its site of action; in the case of SMN, this is inside the cell. However, the intrinsic characteristic of a lipid membrane impedes the transport of most cargo, limiting the efficiency of protein delivery. A novel approach to deliver therapeutic compounds to the inside of the cell consists of linking the recombinant protein to short peptides that can translocate through the cell membrane. Amid the various protein domains with translocatory capacity so far identified, an eleven-amino acid sequence from the HIV-TAT protein, PTD, has been shown to be very effective. For the purpose of this project, we designed and engineered a recombinant SMN protein fused to the PTD domain to confer the capacity to traverse cell membranes.

The present study aimed at establishing whether protein therapy is suitable for treatment of SMA. Our efforts addressed whether the PTD-SMN fusion protein conserves the properties of the PTD domain and the SMN protein; that is, PTD-SMN has translocatory capacity and PTD-SMN behaves as endogenous SMN.

4.1 Bacteria can be used to generate PTD-SMN

Our study of the expression of the recombinant PTD-SMN fusion protein showed that PTD-SMN protein can be produced from bacteria. SDS-PAGE analyses of PTD-SMN revealed that this protein is mostly present in the insoluble fraction of the bacterial cell, most likely in inclusion bodies (Wadia, et al, 2004), restricting its availability for purification under native conditions. Furthermore, we observed that bacterial expression of the 42kD

protein could be toxic to bacterial cells (Fig. 3.2B), affecting their growth rate. Yet, it seems that reduced growth does not affect protein expression (Fig. 3.2A), suggesting that variation in cell growth does not correlate with the expression level of a recombinant protein (Woestenenk, et al, 2004). Despite obtaining a low yield of the recombinant protein, we were able to purify sufficient amounts of protein for our subsequent experiments. The low cost and ease of scalability still suggest that bacteria are a good choice for production of PTD-SMN.

4.2 Bacterially produced PTD-SMN binds to cells, but remains primarily bound to the cell membrane.

At first, we focused on evaluating whether PTD-SMN has retained the translocatory properties of the PTD domain. The first assay demonstrated that PTD-SMN exhibits in full the cell binding capacity of PTD. As observed in our Western blot analysis, PTD-SMN binding to cells is observed as early as 2hr and it maximizes at 8hr, indicating that PTD-SMN interaction is time-dependent (Fig. 3.7). Consistent with this observation, Tyagi et al showed internalization of glutathione *S*-transferase (GST) - TAT peaked at 10hr (Tyagi, et al, 2001). However, from our assay we were not able to determine whether transduction of the protein into the cell had occurred. Thus, a series of subsequent studies performed would disclose the functional capacity of PTD-SMN.

Western blot analysis of treated-cell lysates and cellular fractionation clearly demonstrated that the majority of PTD-SMN remains tightly bound to the cell surface (Fig.3.8 and 3.9). This is not unexpected, given the fact that internalization of PTD from HIV-TAT is mediated by heparin sulphate proteoglycans on the cell surface (Tyagi, et al, 2001), which are highly present in a wide variety of human, rodent and simian cell lines

(Fittipaldi, et al, 2005). Moreover, this interaction, which can be highly specific to relatively nonspecific, is not only determined by the ionic composition of the protein, but also requires conformational structure recognition (Fittipaldi, et al, 2005; Maccarana, et al, 1993); that is, PTD needs to be properly folded in order to interact with heparin sulphate proteoglycans for translocation to occur. TAT-fusion proteins are often localized to bacterial inclusion bodies (Wadia, et al, 2005). We have shown that bacterially produced PTD-SMN largely accumulates in inclusion bodies (Fig. 3.3B); which might be a consequence of its delay to reach a native conformation or to interact with folding modulators in the bacteria, resulting in the improper folding of the protein (Baneyx, et al, 2004). Therefore, if the vast majority of the produced PTD-SMN aggregates in inclusion bodies, only a small portion of the protein, present in the soluble fraction, would be properly folded. This would result in a limited transduction of the protein.

4.3 Some PTD-SMN reaches the nucleus and co-localized with Cajal bodies

Endogenous SMN is found diffusely throughout the cytoplasm, and in nuclear bodies called Cajal bodies, which overlap with gems in adults (Liu, et al, 1996; Young, et al, 2000). We observed that transduction of PTD-SMN in cultured cells resulted in localization of the fusion protein to the nucleus, forming small aggregates, which are reminiscent of gems (Fig. 3.10). Further assessment of internalization revealed that PTD-SMN co-localizes with p80-coilin, a marker of coiled bodies (Fig. 3.12). Targeting of SMN to Cajal bodies is mediated by the self-association of the SMN protein, which is dictated by the sequences encoded by exons 2b, 3 and 6 of *SMN* (Morse, et al, 2007). Access of PTD-SMN to the nucleus and localization to Cajal bodies suggests that PTD-SMN conserves the functional characteristics

of the endogenous SMN protein. These data indicate that the PTD domain and the SMN protein from PTD-SMN are fully functional: while PTD allows for uptake and internalization of the recombinant protein to the nucleus, the functionally active domains of the SMN protein enable targeting to Cajal bodies.

In addition, we evaluated the therapeutic effects of PTD-SMN. We observed that application of PTD-SMN caused a moderate increase in the number of gems (Table 3.11). These results indicate that ectopic expression of SMN and its localization to nuclear bodies promotes formation of gems. Likewise, DiDonato et al showed that overexpression of SMN levels in SMA patient fibroblast using Ad vectors leads to a corresponding increase in the number of gems (DiDonato, et al, 2003). Gem counts can be used as an indicator of disease severity, since the number of SMN-positive foci is correlated with SMA disease severity in fibroblasts from SMA patients (Coovert, et al, 1997). Therefore, an increase in gem formation could indicate that SMN protein levels are restored. The above result is indicative that PTD-SMN might be a viable strategy for the treatment of SMA.

In spite of the strong binding capacity of PTD-SMN to cell surface, we have shown that the small portion of PTD-SMN that is taken up by the cells has a positive impact in gem counts. This result suggests that in order to correct SMN deficiencies, only a very small amount of protein is required. However, we were aware that outcomes of the immunofluorescence assays could not be conclusive to evaluate the functionality of PTD-SMN; thus, we needed to further analyze the functional capacity of PTD-SMN.

4.4 Most bacterially-produced PTD-SMN cannot interact with normal cellular partners.

To evaluate whether PTD-SMN is able to interact with SMN cellular partners, immunoprecipitation studies were performed. The results revealed that the vast majority of PTD-SMN fails to interact with Gemin2 or endogenous SMN: we were not able to detect association between PTD-SMN with either of the two tested proteins (Fig. 3.13 and Fig. 3.14). However, to confirm whether bacterially produced PTD-SMN is unable to interact with SMN cellular partners, more sensitive assays would need to be conducted in order to detect the small amount of PTD-SMN that reaches the nucleus of the cell.

With this respect, the amount of protein used for each experiment is determinant for the outcome of the experiment. For instance, the concentration of PTD-SMN used for each assay varied considerably (i.e. 12nM- 500nM). Transduction of PTD-SMN and colocalization experiments needed 160nM and 500nM of PTD-SMN respectively. On the other hand, when investigating interaction of PTD-SMN with SMN cellular's partners, it was used a concentration of 25nM. There exists the possibility that a higher concentration of the recombinant protein is required in order to observe interaction and transduction of the protein *in vitro*.

Overall, PTD-SMN can be produced in bacteria, but only a low percentage retains biological activity. The small amount that does enter the cell is capable of reaching the nucleus, increasing the number of gems, and co-localizing with p80-coilin. These data suggest that PTD-SMN maybe viable therapeutic, but better production methods are required to obtain protein with higher biological activity.

4.5 Future Directions

Immediate future directions which could be addressed are to explore other strategies to improve PTD-SMN production in bacteria; for instance, genetic manipulation of the construct and host cell could improve the production of PTD-SMN (Ho, et al, 2003). Engineering of a SMN-Gemin2 fusion protein could promote the expression of a functional SMN protein: fusion to Gemin2 may allow for folding of SMN in the bacteria, since gemin2 is part of the SMN-complex, and it binds tightly to SMN (Lorson, 2009). Furthermore, expression of the PTD-SMN fused with thioredoxin, a heat-stable protein, would allow the release of the protein into the media by osmotic shock, facilitating its purification (Ho, et al, 2003). Also, it is possible that the presence of the FLAG tag (strong negative charge) immediately adjacent to the PTD (strong positive charge) affects the efficiency of uptake, as it has been previously reported (Jiang, et al, 2004). Thus, analysis of PTD-SMN in the absence of the FLAG tag should be pursued to determine if this molecule has enhanced translocatory capacity.

Even though nonviral gene transfer is the preferred approach to generate stable cell lines for manufacturing purposes, viral vectors for the expression of recombinant proteins in mammalian cells confers many advantages. Specifically, Ad vectors can be easily produced at high titers, and its biology is very well known (Altaras, et al, 2005). Previous studies on Ad vectors expressing SMN, demonstrated that the expressed SMN has functional activity (DiDonato, et al, 2003). In preliminary studies, we determined that purified SMN protein from E1/E3 deleted Ad remains tightly bound to endogenous SMN and Gemin2 (data not shown), strongly implying that even after the purification process, SMN remains active. This observation suggests that PTD-SMN produced in mammalian cells is an excellent alternative

that should be pursued in the future. In addition, insect cell-based system for the generation of PTD-SMN is an alternative, since it is commonly used to produce a wide range of recombinant proteins (Walsh, 2003).

Together, these future studies will aim to obtain a recombinant PTD-SMN that is fully functional in order to improve its therapeutic value.

4.6 Conclusion

In this study we developed a novel therapeutic strategy for the treatment of SMA. We designed and constructed a bacterial SMN protein with capacity to cross the cell membrane. Our results demonstrated that PTD-SMN is able to cross the cell membrane, increasing the number of gems, and localizing to Cajal bodies; although, most of the bacterially produced PTD-SMN remains bound to the cell surface. Future prospects are addressed to improve production methods and explore other recombinant protein expression systems. Validation of a proof-of-concept has shown that SMN protein replacement therapy could emerge as a novel therapy for SMA patients.

Reference List

Addison, C.L., Hitt, M., Kunsken, D., and Graham, F.L. (1997). Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. J. Gen. Virol. *78,* 1653-1661.

Altaras, N.E., Aunins, J.G., Evans, R.K., Kamen, A., Konz, J.O., and Wolf, J.J. (2005). Production and formulation of adenovirus vectors. Gene Therapy and Gene Delivery Systems *99,* 193-260.

Anderson, K.N., Baban, D., Oliver, P.L., Potter, A., and Davies, K.E. (2004). Expression profiling in spinal muscular atrophy reveals an RNA binding protein deficit. Neuromuscular Disorders *14,* 711-722.

Arnett, A.L.H., Chamberlain, J.R., and Chamberlain, J.S. (2009a). Therapy for neuromuscular disorders. Curr. Opin. Genet. Dev. *19,* 290-297.

Ashton, G. (2001). Growing pains for biopharmaceuticals. Nat. Biotechnol. *19,* 307-311.

Aviezer D., Brill-Almon E., Shaaltiel Y., Hashmueli S., Bartfeld D., Mizrachi S., Liberman Y., Freeman A., Zimran A., and Galun E. (2009). A plant-derived recombinant human glucocerebrosidase enzyme--a preclinical and phase I investigation. PLoS One *4,* e4792.

Azzouz, M., Le, T., Ralph, G.S., Walmsley, L., Monani, U.R., Lee, D.C.P., Wilkes, F., Mitrophanous, K.A., Kingsman, S.M., Burghes, A.H.M., and Mazarakis, N.D. (2004). Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy. J. Clin. Invest. *114,* 1726-1731.

Baccon, J., Pellizzoni, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). Identification and characterization of Gemin7, a novel component of the survival of motor neuron complex. J. Biol. Chem. *277,* 31957-31962.

Baldi, L., Hacker, D.L., Adam, M., and Wurm, F.M. (2007). Recombinant protein production by large-scale transient gene expression in mammalian cells: state of the art and future perspectives. Biotechnol. Lett. *29,* 677-684.

Baneyx, F., and Mujacic, M. (2004). Recombinant protein folding and misfolding in Escherichia coli. Nat. Biotechnol. *22,* 1399-1408.

Banting, F.G., Best, C.H., Collip, J.B., Campbell, W.R., and Fletcher, A.A. (1991). Pancreatic Extracts in the Treatment of Diabetes-Mellitus - Preliminary-Report. Can. Med. Assoc. J. *145,* 1281-1286.

Beutler, E. (2006). Lysosomal storage diseases: Natural history and ethical and economic aspects. Mol. Genet. Metab. *88,* 208-215.

Boon, K.L., Xiao, S., McWhorter, M.L., Donn, T., Wolf-Saxon, E., Bohnsack, M.T., Moens, C.B., and Beattie, C.E. (2009). Zebrafish survival motor neuron mutants exhibit presynaptic neuromuscular junction defects. Hum.Mol.Genet.

Bowerman, M., Shafey, D., and Kothary, R. (2007). Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity. Journal of Molecular Neuroscience *32,* 120-131.

Brichta, L., Hofmann, Y., Hahnen, E., Siebzehnrubl, F.A., Raschke, H., Blumcke, I., Eyupoglu, I.Y., and Wirth, B. (2003). Valproic acid increases the SMN2 protein level: a well-known drug as a potential therapy for spinal muscular atrophy. Hum. Mol. Genet. *12,* 2481-2489.

Burghes, A.H.a.B.,C.E. (2009). Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? Nat. Rev. Neurosci. *10,* 597-609.

Burghes, A.H.M. (1997). When is a deletion not a deletion? When it is converted. Am. J. Hum. Genet. *61,* 9-15.

Burnett, B.G., Crawford, T.O., and Sumner, C.J. (2009). Emerging treatment options for spinal muscular atrophy. Curr. Treat. Options Neurol. *11,* 90-101.

Burrow, T.A., Hopkin, R.J., Leslie, N.D., Tinkle, B.T., and Grabowski, G.A. (2007). Enzyme reconstitution/replacement therapy for lysosomal storage diseases. Curr. Opin. Pediatr. *19,* 628-635.

Butler, M. (2005). Animal cell cultures: recent achievements and perspectives in the production of biopharmaceuticals. Appl. Microbiol. Biotechnol. *68,* 283-291.

Carissimi, C., Baccon, J., Straccia, M., Chiarella, P., Maiolica, A., Sawyer, A., Rappsilber, J., and Pellizzoni, L. (2005). Unrip is a component of SMN complexes active in snRNP assembly. FEBS Lett. *579,* 2348-2354.

Carissimi, C., Saieva, L., Baccon, J., Chiarella, P., Maiolica, A., Sawyer, A., Rappsilber, J., and Pellizzoni, L. (2006). Gemin8 is a novel component of the survival motor neuron complex and functions in small nuclear ribonucleoprotein assembly. J. Biol. Chem. *281,* 8126-8134.

Cereghino, G.P.L., Cereghino, J.L., Ilgen, C., and Cregg, J.M. (2002). Production of recombinant proteins in fermenter cultures of the yeast Pichia pastoris. Curr. Opin. Biotechnol. *13,* 329-332.

Chan, Y.B., Miguel-Aliaga, I., Franks, C., Thomas, N., Trulzsch, B., Sattelle, D.B., Davies, K.E., and van den Heuvel, M. (2003). Neuromuscular defects in a Drosophila survival motor neuron gene mutant. Hum. Mol. Genet. *12,* 1367-1376.

Chang, H.C., Dimlich, D.N., Yokokura, T., Mukherjee, A., Kankel, M.W., Sen, A., Sridhar, V., Fulga, T.A., Hart, A.C., Van Vactor, D., and Artavanis-Tsakonas, S. (2008). Modeling Spinal Muscular Atrophy in Drosophila. PLoS One *3,* Article No.: e3209.

Charroux, B., Pellizzoni, L., Perkinson, R.A., Yong, J., Shevchenko, A., Mann, M., and Dreyfuss, G. (2000). Gemin4: A novel component of the SMN complex that is found in both gems and nucleoli. J. Cell Biol. *148,* 1177-1186.

Charroux, B., Pellizzoni, L., Perkinson, R.A., Shevchenko, A., Mann, M., and Dreyfuss, G. (1999). Gemin3: A novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems. J. Cell Biol. *147,* 1181-1193.

Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A., and Mehtali, M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. J. Virol. *70,* 4805-4810.

Cifuentes-Diaz, C., Frugier, T., Tiziano, F.D., Lacene, E., Roblot, N., Joshi, V., Moreau, M.H., and Melki, J. (2001). Deletion of murine SMN exon 7 directed to skeletal muscle leads to severe muscular dystrophy. J. Cell Biol. *152,* 1107-1114.

Coovert, D.D., Le, T., McAndrew, P.E., Strasswimmer, J., Crawford, T.O., Mendell, J.R., Androphy, E., Prior, T.W., and Burghes, A.H.M. (1997). The survival motor neuron protein (SMN) in spinal muscular atrophy (SMA). Am. J. Hum. Genet. *61,* 1926.

Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Ronchi, D., Saladino, F., Bordoni, A., Fortunato, F., Del Bo, R. *et al.* (2008). Neural stem cell transplantation can ameliorate the phenotype of a mouse model of spinal muscular atrophy. J. Clin. Invest. *118,* 3316-3330.

Cusin, V., Clermont, O., Gerard, B., Chantereau, D., and Elion, J. (2003). Prevalence of SMN1 deletion and duplication in carrier and normal populations: implication for genetic counselling. J. Med. Genet. *40,* e39.

Dent, E.W., and Gertler, F.B. (2003). Cytoskeletal Dynamics and Transport in Growth Cone Motility and Axon Guidance. Neuron *40,* 209-227.

Derossi, D., Joliot, A.H., Chassaing, G., and Prochiantz, A. (1994). The 3rd Helix of the Antennapedia Homeodomain Translocates through Biological-Membranes. J. Biol. Chem. *269,* 10444-10450.

Desnick, R.J. (2004). Enzyme replacement and enhancement therapies for lysosomal diseases. J. Inherit. Metab. Dis. *27,* 385-410.

DiDonato, C.J., Parks, R.J., and Kothary, R. (2003). Development of a gene therapy strategy for the restoration of survival motor neuron protein expression: implications for spinal muscular atrophy therapy. Hum. Gene Ther. *14,* 179-188.

Dubowitz, V. (1991). Chaos in classification of the spinal muscular atrophies of childhood. Neuromuscul. Disord. *1,* 77-80.

Egleton, R.D., and Davis, T.P. (1997). Bioavailability and transport of peptides and peptide drugs into the brain. Peptides *18,* 1431-1439.

Elliott, G., and OHare, P. (1997). Intercellular trafficking and protein delivery by a herpesvirus structural protein. Cell *88,* 223-233.

Fan, L., and Simard, L.R. (2002). Survival motor neuron (SMN) protein: role in neurite outgrowth and neuromuscular maturation during neuronal differentiation and development. Hum. Mol. Genet. *11,* 1605-1614.

Feldkotter, M., Schwarzer, V., Wirth, R., Wienker, T.F., and Wirth, B. (2002). 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,* 358-368.

Fischer, U., Liu, Q., and Dreyfuss, G. (1997). The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. Cell *90,* 1023-1029.

Fischer, R., Stoger, E., Schillberg, S., Christou, P., and Twyman, R.M. (2004). Plant-based production of biopharmaceuticals. Curr. Opin. Plant Biol. *7,* 152-158.

Fittipaldi, A., and Giacca, M. (2005). Transcellular protein transduction using the Tat protein of HIV-1. Adv. Drug Deliv. Rev. *57,* 597-608.

Frankel, A.D., and Pabo, C.O. (1988). Cellular Uptake of the Tat Protein from Human Immunodeficiency Virus. Cell *55,* 1189-1193.

Friedmann, T., and Rossi, J. (2007). Gene Transfer: Delivery and expression of DNA and RNA. A Laboratory Manual. Nucleic Acids Research *11,* 1475-1489.

Friedman, B.A., Vaddi, K., Preston, C., Mahon, E., Cataldo, J.R., and McPherson, J.M. (1999). A comparison of the pharmacological properties of carbohydrate remodeled recombinant and placental-derived beta-glucocerebrosidase: Implications for clinical efficacy in treatment of Gaucher disease. Blood *93,* 2807-2816.

Frugier, T., Tiziano, F.D., Cifuentes-Diaz, C., Miniou, P., Roblot, N., Dierich, A., Le Meur, M., and Melki, J. (2000). Nuclear targeting defect of SMN lacking the C-terminus in a mouse model of spinal muscular atrophy. Hum. Mol. Genet. *9,* 849-858.

Gabanella, F., Butchbach, M.E., Saieva, L., Carissimi, C., Burghes, A.H., and Pellizzoni, L. (2007). Ribonucleoprotein assembly defects correlate with spinal muscular atrophy severity and preferentially affect a subset of spliceosomal snRNPs. PLoS One *2,* e921.

Garrett, R.H., and Grisham, C.M. (2005). Biochemistry (USA: Thomson Learning, Inc.).

Gavrilina, T.O., McGovern, V.L., Workman, E., Crawford, T.O., Gogliotti, R.G., DiDonato, C.J., Monani, U.R., Morris, G.E., and Burghes, A.H.M. (2008). Neuronal SMN expression corrects spinal muscular atrophy in severe SMA mice while muscle-specific SMN expression has no phenotypic effect. Hum. Mol. Genet. *17,* 1063-1075.

Giesemann, T., Rathke-Hartlieb, S., Rothkegel, M., Bartsch, J.W., Buchmeier, S., Jockusch, B.M., and Jockusch, H. (1999). A role for polyproline motifs in the spinal muscular atrophy protein SMN - Profilins bind to and colocalize with SMN in nuclear gems. J. Biol. Chem. *274,* 37908-37914.

Graham, F.L., Smiley, J., Russell, W.C., and Nairn, R. (1977). Characteristics of a Human Cell Line Transformed by Dna from Human Adenovirus Type-5. J. Gen. Virol. *36,* 59-72.

Green, M., and Loewenstein, P.M. (1988). Autonomous Functional Domains of Chemically Synthesized Human Immunodeficiency Virus Tat Trans-Activator Protein. Cell *55,* 1179-1188.

Gubitz, A.K., Mourelatos, Z., Abel, L., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). Gemin5, a novel WD repeat protein component of the SMN complex that binds Sm proteins. J. Biol. Chem. *277,* 5631-5636.

Haddad, H., Cifuentes-Diaz, C., Miroglio, A., Roblot, N., Joshi, V., and Melki, J. (2003). Riluzole attenuates spinal muscular atrophy disease progression in a mouse model. Muscle Nerve *28,* 432-437.

Hamilton, S.R., and Gerngross, T.U. (2007). Glycosylation engineering in yeast: the advent of fully humanized yeast. Curr. Opin. Biotechnol. *18,* 387-392.

Han, T., Tang, Y., Ugai, H., Perry, L.E., Siegal, G.P., Contreras, J.L., and Wu, H. (2007). Genetic incorporation of the protein transduction domain of Tat into Ad5 fiber enhances gene transfer efficacy. Virology Journal *4,* 103.

Han, Z., Wu, S., Li, Q., Li, J., Gao, D., Li, K., Liu, Z.W., and Zhao, H. (2009). Efficient Human Growth Hormone Gene Expression in the Milk of Non-transgenic Goats. Folia Biol. *55,* 17-22.

Harada, H., Hiraoka, M., and Kizaka-Kondoh, S. (2002). Antitumor effect of TAT-oxygen-dependent degradation-caspase-3 fusion protein specifically stabilized and activated in hypoxic tumor cells. Cancer Res. *62,* 2013-2018.

Herce, H.D., and Garcia, A.E. (2007). Molecular dynamics simulations suggest a mechanism for translocation of the HIV-1 TAT peptide across lipid membranes. Proc. Natl. Acad. Sci. U. S. A. *104,* 20805-20810.

Ho, R.J.Y., and Gibaldi, M. (2003). Biotechnology and Biopharmaceuticals: Transforming Proteins and Genes into Drugs.

Hsieh-Li, H.M., Chang, J.G., Jong, Y.J., Wu, M.H., Wang, N.M., Tsai, C.H., and Li, H. (2000). A mouse model for spinal muscular atrophy. Nat. Genet. *24,* 66-70.

Imbriaco, M., Pisani, A., Spinelli, L., Cuocolo, A., Messalli, G., Capuano, E., Marmo, M., Liuzzi, R., Visciano, B., Cianciaruso, B., and Salvatore, M. (2009). Effects of enzyme-replacement therapy in patients with Anderson-Fabry disease: a prospective long-term cardiac magnetic resonance imaging study. Heart *95,* 1103-1107.Jiang, T., Olson, E.S., Nguyen, Q.T., Roy, M., Jennings, P.A., and Tsien, R.Y. (2004). Tumor imaging by means of proteolytic activation of cell-penetrating peptides. Proc. Natl. Acad. Sci. U. S. A. *101,* 17867-17872.

Jablonka, S., Bandilla, M., Wiese, S., Buhler, D., Wirth, B., Sendtner, M., and Fischer, U. (2001). Co-regulation of survival of motor neuron (SMN) protein and its interactor SIP1 during development and in spinal muscular atrophy. Hum. Mol. Genet. *10,* 497-505.

Jarvis, D.L. (2003). Developing baculovirus-insect cell expression systems for humanized recombinant glycoprotein production. Virology *310,* 1-7.

Jeang, K.T., Xiao, H., and Rich, E.K. (1999). Multifaceted activities of the HIV-1 transactivator of transcription, Tat. J. Biol. Chem. *274,* 28837-28840.

Jedrzejowska, M., Milewski, M., Zimowski, J., Borkowska, J., Kostera-Pruszczyk, A., Sielska, D., Jurek, M., and Hausmanowa-Petrusewicz, I. (2009). Phenotype modifiers of spinal muscular atrophy: the number of SMN2 gene copies, deletion in the NAIP gene and probably gender influence the course of the disease. Acta Biochim. Pol. *56,* 103-108.

Jenuwein, T., and Allis, C.D. (2001). Translating the histone code. Science *293,* 1074-1080.

Jimenez-Sanchez, G., Childs, B., and Valle, D. (2001). Human disease genes. Nature *409,* 853-855.

Jones, S.W., Christison, R., Bundell, K., Voyce, C.J., Brockbank, S.M.V., Newham, P., and Lindsay, M.A. (2005). Characterisation of cell-penetrating peptide-mediated peptide delivery. Br. J. Pharmacol. *145,* 1093-1102.

Keen, H., Glynne, A., Pickup, J.C., Viberti, G.C., Bilous, R.W., Jarrett, R.J., and Marsden, R. (1980). Human Insulin Produced by Recombinant Dna Technology - Safety and Hypoglycemic Potency in Healthy-Men. Lancet *2,* 398-401.

Keirstead, H.S., 2009. Motor neuron progenitors derived from human embryonic stem cells for clinical application. 13th Annual International Spinal Muscular Atrophy Research Group Meeting.

Kernochan, L.E., Russo, M.L., Woodling, N.S., Huynh, T.N., Avila, A.M., Fischbeck, K.H., and Sumner, C.J. (2005). The role of histone acetylation in SMN gene expression. Hum. Mol. Genet. *14,* 1171-1182.

Kislauskis, E.H., Zhu, X.C., and Singer, R.H. (1997). beta-actin messenger RNA localization and protein synthesis augment cell motility. J. Cell Biol. *136,* 1263-1270.

Kurachi, S., Tashiro, K., Sakurai, F., Sakurai, H., Kawabata, K., Yayama, K., Okamoto, H., Nakagawa, S., and Mizuguchi, H. (2007). Fiber-modified adenovirus vectors containing the TAT peptide derived from HIV-1 in the fiber knob have efficient gene transfer activity. Gene Ther. *14,* 1160-1165.

Lander, E.S., Linton, L.M., Birren, B., Nusbaum, C., Zody, M.C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W. *et al.* (2001). Initial sequencing and analysis of the human genome. Nature *409,* 860-921.

Le, T.T., Pham, L.T., Butchbach, M.E., Zhang, H.L., Monani, U.R., Coovert, D.D., Gavrilina, T.O., Xing, L., Bassell, G.J., and Burghes, A.H. (2005). SMNDelta7, 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,* 845-857.

Leader, B., Baca, Q.J., and Golan, D.E. (2008). Protein therapeutics: A summary and pharmacological classification. Nature Reviews Drug Discovery *7,* 21-39.

Lebleu, B. (1996). Delivering information-rich drugs - Prospects and challenges. Trends Biotechnol. *14,* 109-110.

Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., Benichou, B., Cruaud, C., Millasseau, P., Zeviani, M. *et al.* (1995). Identification and Characterization of a Spinal Muscular Atrophy-Determining Gene. Cell *80,* 155-165.

Leifert, J.A., Harkins, S., and Whitton, J.L. (2002). Full-length proteins attached to the HIV tat protein transduction domain are neither transduced between cells, nor exhibit enhanced immunogenicity. Gene Ther. *9,* 1422-1428.

Liu, Q., and Dreyfuss, G. (1996). A novel nuclear structure containing the survival of motor neurons protein. EMBO J. *15,* 3555-3565.

Liu, Q., Fischer, U., Wang, F., and Dreyfuss, G. (1997). The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. Cell *90,* 1013-1021.

Lorson, C.L. (2009). Spinal Muscular Atrophy Conference, 2009.

Lorson, C.L., Hahnen, E., Androphy, E.J., and Wirth, B. (1999). A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. Proc. Natl. Acad. Sci. U. S. A. *96,* 6307-6311.

Lundberg, M., Wikstrom, S., and Johansson, M. (2003). Cell surface adherence and endocytosis of protein transduction domains. Molecular Therapy *8,* 143-150.

Maccarana, M., Casu, B., and Lindahl, U. (1993). Minimal Sequence in Heparin Heparan-Sulfate Required for Binding of Basic Fibroblast Growth-Factor. J. Biol. Chem. *268,* 23898-23905.

Mailman, M.D., Heinz, J.W., Papp, A.C., Snyder, P.J., Sedra, M.S., Wirth, B., Burghes, A.H.M., and Prior, T.W. (2002). Molecular analysis of spinal muscular atrophy and modification of the phenotype by SMN2. Genetics in Medicine *4,* 20-26.

Mann, D.A., and Frankel, A.D. (1991). Endocytosis and Targeting of Exogenous Hiv-1 Tat Protein. EMBO J. *10,* 1733-1739.

Marsh, J.L., and Thompson, L.M. (2006). Drosophila in the Study of Neurodegenerative Disease. Neuron *52,* 169-178.

Matera, A.G., and Frey, M.R. (1998). Coiled bodies and gems: Janus or Gemini? Am. J. Hum. Genet. *63,* 317-321.

McAndrew, P.E., Parsons, D.W., Simard, L.R., Rochette, C., Ray, P.N., Mendell, J.R., Prior, T.W., and Burghes, A.H.M. (1997). Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number. Am. J. Hum. Genet. *60,* 1411-1422.

McWhorter, M.L., Monani, U.R., Burghes, A.H.M., and Beattie, C.E. (2003). Knockdown of the survival motor neuron (Smn) protein in zebrafish causes defects in motor axon outgrowth and pathfinding. J. Cell Biol. *162,* 919-931.

McKusick, V.A. (2009). Online Mendelian Inheritance in Man, OMIM (TM).*October 8th, 2009,*

Meister, G., Buhler, D., Pillai, R., Lottspeich, F., and Fischer, U. (2001). A multiprotein complex mediates the ATP-dependent assembly of spliceosomal U snRNPs. Nat. Cell Biol. *3,* 945-949.

Melki, J., Sheth, P., Abdelhak, S., Burlet, P., Bachelot, M.F., Lathrop, M.G., Frezal, J., and Munnich, A. (1990). Mapping of Acute (Type-I) Spinal Muscular-Atrophy to Chromosome 5q12-Q14. Lancet *336,* 271-273.

Monani, U.R., Coovert, D.D., and Burghes, A.H.M. (2000). Animal models of spinal muscular atrophy. Hum. Mol. Genet. *9,* 2451-2457.

Monani, U.R., Lorson, C.L., Parsons, D.W., Prior, T.W., Androphy, E.J., Burghes, A.H.M., and McPherson, J.D. (1999). A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. Hum. Mol. Genet. *8,* 1177-1183.

Monani, U.R., Pastore, M.T., Gavrilina, T.O., Jablonka, S., Le, T.T., Andreassi, C., DiCocco, J.M., Lorson, C., Androphy, E.J., Sendtner, M., Podell, M., and Burghes, A.H.M. (2003). 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,* 41-52.

Monani, U.R., Sendtner, M., Coovert, D.D., Parsons, D.W., Andreassi, C., Le, T.T., Jablonka, S., Schrank, B., Rossol, W., Prior, T.W., Morris, G.E., and Burghes, A.H.M. (2000). 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,* 333-339.

Morse, R., Shaw, D.J., Todd, A.G., and Young, P.J. (2007). Targeting of SMN to Cajal bodies is mediated by self-association. Hum. Mol. Genet. *16,* 2349-2358.

Mourelatos, Z., Abel, L., Yong, J.S., Kataoka, N., and Dreyfuss, G. (2001). SMN interacts with a novel family of hnRNP and spliceosomal proteins. EMBO J. *20,* 5443-5452.

Nagahara, H., Vocero-Akbani, A.M., Snyder, E.L., Ho, A., Latham, D.G., Lissy, N.A., Becker-Hapak, M., Ezhevsky, S.A., and Dowdy, S.F. (1998). Transduction of full-length TAT fusion proteins into mammalian cells: TAT-p27(Kip1) induces cell migration. Nat. Med. *4,* 1449-1452.

O'Connor, T.P., and Crystal, R.G. (2006). Genetic medicines: treatment strategies for hereditary disorders. Nature Reviews Genetics *7,* 261-276.

Pagliardini, S., Giavazzi, A., Setola, V., Lizier, C., Di Luca, M., DeBiasi, S., and Battaglia, G. (2000). Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. Hum. Mol. Genet. *9,* 47-56.

Pane, M., Staccioll, S., Messina, S., D'Arnico, A., Pelliccioni, M., Mazzone, E.S., Cuttini, M., Alfieri, P., Battinif, R., Main, M. *et al.* (2008). Daily salbutamol in young patients with SMA type II. Neuromusc. Disord. *18,* 536-540.

Parsons, D.W., McAndrew, P.E., Iannaccone, S.T., Mendell, J.R., Burghes, A.H.M., and Prior, T.W. (1998). Intragenic telSMN mutations: Frequency, distribution, evidence of a founder effect, and modification of the spinal muscular atrophy phenotype by cenSMN copy number. Am. J. Hum. Genet. *63,* 1712-1723.

Passini, M.A., Bu, J., Roskelley, E.M., Richards, A.M., Sardi, S.P., O'Riordan, C.R., Klinger, K.W., Shihabuddin, L.S., and Cheng, S.H. (2009). CNS delivery of human SMN1 by adeno-

associated virus is highly efficacious in a mouse model of spinal muscular atrophy type I. 13th Annual International Spinal Muscular Atrophy Research Group Meeting

Pearn, J. (1978). Incidence, Prevalence, and Gene Frequency Studies of Chronic Childhood Spinal Muscular-Atrophy. J. Med. Genet. *15,* 409-413.

Pellizzoni, L., Baccon, J., Rappsilber, J., Mann, M., and Dreyfuss, G. (2002). Purification of native survival of motor neurons complexes and identification of Gemin6 as a novel component. J. Biol. Chem. *277,* 7540-7545.

Pellizzoni, L., Kataoka, N., Charroux, B., and Dreyfuss, G. (1998). A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. Cell *95,* 615-624.

Pellizzoni, L., Yong, J., and Dreyfuss, G. (2002). Essential role for the SMN complex in the specificity of snRNP assembly. Science *298,* 1775-1779.

Pennisi, E. (2003). Bioinformatics - Gene counters struggle to get the right answer. Science *301,* 1040-1041.

Pollock, D.P., Kutzko, J.P., Birck-Wilson, E., Williams, J.L., Echelard, Y., and Meade, H.M. (1999). Transgenic milk as a method for the production of recombinant antibodies. J. Immunol. Methods *231,* 147-157.

Prior, T.W., Swoboda, K.J., Scott, H.D., and Hejmanowski, A.Q. (2004). Homozygous SMN1 deletions in unaffected family members and modification of the phenotype by SMN2. American Journal of Medical Genetics Part a *130A,* 307-310.

Rajendra, T.K., Gonsalvez, G.B., Walker, M.P., Shpargel, K.B., Salz, H.K., and Matera, A.G. (2007). A Drosophila melanogaster model of spinal muscular atrophy reveals a function for SMN in striated muscle. J. Cell Biol. *176,* 831-841.

Richard, J.P., Melikov, K., Brooks, H., Prevot, P., Lebleu, B., and Chernomordik, L.V. (2005). Cellular uptake of unconjugated TAT peptide involves clathrin-dependent endocytosis and heparan sulfate receptors. J. Biol. Chem. *280,* 15300-15306.

Richard, J.P., Melikov, K., Vives, E., Ramos, C., Verbeure, B., Gait, M.J., Chernomordik, L.V., and Lebleu, B. (2003). Cell-penetrating peptides - A reevaluation of the mechanism of cellular uptake. J. Biol. Chem. *278,* 585-590.

Roberts, D.F., and Court, S.D.M. (1970). Genetic Component in Child Mortality. Arch. Dis. Child. *45,* 33-&.

Rossoll, W., Jablonka, S., Andreassi, C., Kroning, A.K., Karle, K., Monani, U.R., and Sendtner, M. (2003). 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,* 801-812.

Rossoll, W., Kroning, A.K., Ohndorf, U.M., Steegborn, C., Jablonka, S., and Sendtner, M. (2002). Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? Hum. Mol. Genet. *11,* 93-105.

Russell, C.S., and Clarke, L.A. (1999). Recombinant proteins for genetic disease. Clin. Genet. *55,* 389-394.

Russman, B.S. (2007). Spinal muscular atrophy: Clinical classification and disease heterogeneity. J. Child Neurol. *22,* 946-951.

Sambrook, J., Fritsch, E.F., and and Maniatis, T. (1989). In Molecular Cloning: A Laboratory Manual (Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press).

Sauer, B., and Henderson, N. (1988). Site-Specific Dna Recombination in Mammalian-Cells by the Cre Recombinase of Bacteriophage-P1. Proc. Natl. Acad. Sci. U. S. A. *85,* 5166-5170.

Schellekens, H. (2002). Bioequivalence and the immunogenicity of biopharmaceuticals. Nature Reviews Drug Discovery *1,* 457-462.

Schmid, A., and DiDonato, C.J. (2007). Animal models of spinal muscular atrophy. J. Child Neurol. *22,* 1004-1012.

Schrank, B., Gotz, R., Gunnersen, J.M., Ure, J.M., Toyka, K.V., Smith, A.G., and Sendtner, M. (1997). 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,* 9920-9925.

Schwarze, S.R., and Dowdy, S.F. (2000). In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. Trends Pharmacol. Sci. *21,* 45-48.

Schwarze, S.R., Ho, A., Vocero-Akbani, A., and Dowdy, S.F. (1999). In vivo protein transduction: Delivery of a biologically active protein into the mouse. Science *285,* 1569-1572.

Sharma, A., Lambrechts, A., Hao, L.T., Le, T.T., Sewry, C.A., Ampe, C., Burghes, A.H.M., and Morris, G.E. (2005). A role for complexes of survival of motor neurons (SNM) protein with gemins and profilin in neurite-like cytoplasmic extensions of cultured nerve cells. Exp. Cell Res. *309,* 185-197.

Sonnemann, K.J., Heun-Johnson, H., Turner, A.J., Baltgalvis, K.A., Lowe, D.A., and Ervasti, J.M. (2009). Functional Substitution by TAT-Utrophin in Dystrophin-Deficient Mice. Plos Medicine *6,*

Sugita, T., Yoshikawa, T., Mukai, Y., Yamanada, N., Imai, S., Nagano, K., Yoshida, Y., Shibata, H., Yoshioka, Y., Nakagawa, S. *et al.* (2008). Comparative study on transduction and toxicity of protein transduction domains. Br. J. Pharmacol. *153,* 1143-1152.

Sumner, C.J. (2006). Therapeutics development for spinal muscular atrophy. NeuroRx. *3,* 235-245.

Yang, Y.H., Ma, J., Song, Z.Y., and Wu, M. (2002). HIV-1 TAT-mediated protein transduction and subcellular localization using novel expression vectors. FEBS Lett. *532,* 36-44.

Young, P.J., Le, T.T., Man, N.T., Burghes, A.H.M., and Morris, G.E. (2000). The relationship between SMN, the spinal muscular atrophy protein, and nuclear coiled bodies in differentiated tissues and cultured cells. Exp. Cell Res. *256,* 365-374.

Tizzano, E.a.B., M. (2001). Molecular bases of spinal muscular atrophy: the survival motor neuron gene. Contributions to Science *2,* 35-42.

Tyagi, M., Rusnati, M., Presta, M., and Giacca, M. (2001). Internalization of HIV-1 Tat requires cell surface heparan sulfate proteoglycans. J. Biol. Chem. *276,* 3254-3261.

Villaverde, A., and Carrio, M.M. (2003). Protein aggregation in recombinant bacteria: biological role of inclusion bodies. Biotechnol. Lett. *25,* 1385-1395.

Viscidi, R.P., Mayur, K., Lederman, H.M., and Frankel, A.D. (1989). Inhibition of Antigen-Induced Lymphocyte-Proliferation by Tat Protein from Hiv-1. Science *246,* 1606-1608.

Vives, E., Brodin, P., and Lebleu, B. (1997). A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. J. Biol. Chem. *272,* 16010-16017.

Vocero-Akbani, A.M., Vander Heyden, N., Lissy, N.A., Ratner, L., and Dowdy, S.F. (1999). Killing HIV-infected cells by transduction with an HIV protease-activated caspase-3 protein. Nat. Med. *5,* 29-33.

Wadia, J.S., and Dowdy, S.F. (2005). Transmembrane delivery of protein and peptide drugs by TAT-mediated transduction in the treatment of cancer. Adv. Drug Deliv. Rev. *57,* 579-596.

Wadia, J.S., Stan, R.V., and Dowdy, S.F. (2004). Transducible TAT-HA fusogenic peptide enhances escape of TAT-fusion proteins after lipid raft macropinocytosis. Nat. Med. *10,* 310-315.

Walsh, G. (2003a). Biopharmaceutical benchmarks - 2003. Nat. Biotechnol. *21,* 865-870.

Walsh, G. (2003b). Biopharmaceuticals: Biochemistry and Biotechnology (Chichester, England: John Wiley & Sons, Ltd.).

Walsh, G. (2006). Biopharmaceutical benchmarks 2006. Nat. Biotechnol. *24,* 769-U5.

Wan, L.L., Battle, D.J., Yong, J.S., Gubitz, A.K., Kolb, S.J., Wang, J., and Dreyfuss, G. (2005). The survival of motor neurons protein determines the capacity for snRNP assembly: Biochemical deficiency in spinal muscular atrophy. Mol. Cell. Biol. *25,* 5543-5551.

Watibayati, M.S., Fatemeh, H., Marini, M., Atif, A.B., Zahiruddin, W.M., Sasongko, T.H., Tang, T.H., Zabidi-Hussin, Z.A.M.H., Nishio, H., and Zilfali, B.A. (2009). Combination of SMN2 copy number and NAIP deletion predicts disease severity in spinal muscular atrophy. Brain Dev. *31,* 42-45.

Weinreb, N.J., Charrow, J., Andersson, H.C., Kaplan, P., Kolodny, E.H., Mistry, P., Pastores, G., Rosenbloom, B.E., Scott, C.R., Wappner, R.S., and Zimran, A. (2002). Effectiveness of enzyme replacement therapy in 1028 patients with type 1 Gaucher disease after 2 to 5 years of treatment: A report from the Gaucher Registry. Am. J. Med. *113,* 112-119.

Westendorp, M.O., Liweber, M., Frank, R.W., and Krammer, P.H. (1994). Human Immunodeficiency Virus Type-1 Tat Up-Regulates Interleukin-2 Secretion in Activated T-Cells. J. Virol. *68,* 4177-4185.

Winkler, C., Eggert, C., Gradl, D., Meister, G., Giegerich, M., Wedlich, D., Laggerbauer, B., and Fischer, U. (2005). Reduced U snRNP assembly causes motor axon degeneration in an animal model for spinal muscular atrophy. Genes Dev. *19,* 2320-2330.

Wirth, B. (2000). An update of the mutation spectrum of the survival motor neuron gene (SMN1) in autosomal recessive spinal muscular atrophy (SMA). Hum. Mutat. *15,* 228-237.

Wirth, B., Brichta, L., and Hahnen, E. (2006). Spinal Muscular Atrophy: From Gene to Therapy. Semin. Pediatr. Neurol. *13,* 121-131.

Woestenenk, E.A., Hammarstrom, M., van den Berg, S., Hard, T., and and Berglund, H. (2004). His tag effect on solubility of human proteins produced in *Escherichia coli*: a comparison between four expression vectors. Journal of Structural and Functional Genomics *5,* 217-229.

Wurm, F.M. (2004). Production of recombinant protein therapeutics in cultivated mammalian cells. Nat. Biotechnol. *22,* 1393-1398.

Wurm, F., and Bernard, A. (1999). Large-scale transient expression in mammalian cells for recombinant protein production. Curr. Opin. Biotechnol. *10,* 156-159.

Yong, J., Wan, L., and Dreyfuss, G. (2004). Why do cells need an assembly machine for RNA–protein complexes? Trends Cell Biol. *14,* 226-232.

Yoshioka, Y., Asavatanabodee, R., Eto, Y., Watanabe, H., Morishige, T., Yao, X., Kida, S., Maeda, M., Mukai, Y., Mizuguchi, H. *et al.* (2008). Tat conjugation of adenovirus vector broadens tropism and enhances transduction efficiency. Life Sci. *83,* 747-755.

Young, P.J., Le, T.T., Dunckley, M., thi Man, N., Burghes, A.H.M., and Morris, G.E. (2001). Nuclear Gems and Cajal (Coiled) Bodies in Fetal Tissues: Nucleolar Distribution of the Spinal Muscular Atrophy Protein, SMN. Exp. Cell Res. *265,* 252-261.

Young, P.J., Le, T.T., Man, N.T., Burghes, A.H.M., and Morris, G.E. (2000). The relationship between SMN, the spinal muscular atrophy protein, and nuclear coiled bodies in differentiated tissues and cultured cells. Exp. Cell Res. *256,* 365-374.

Zhang, H.L.L., Pan, F., Hong, D., Shenoy, S.M., Singer, R.H., and Bassell, G.J. (2003). Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. Journal of Neuroscience *23,* 6627-6637.

Zhang, Z., Lotti, F., Dittmar, K., Younis, I., Wan, L., Kasim, M., and Dreyfuss, G. (2008). SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. Cell *133,* 585-600.

Zhou, G.Y., Zhou, S.N., Lou, Z.Y., Zhu, C.S., Zheng, X.P., and Hu, X.Q. (2008). Translocation and neuroprotective properties of transactivator-of-transcription protein-transduction domain-neuroglobin fusion protein in primary cultured cortical neurons. Biotechnol. Appl. Biochem. *49,* 25-33.

Zorko, M., and Langel, U. (2005). Cell-penetrating peptides: mechanism and kinetics of cargo delivery. Adv. Drug Deliv. Rev. *57,* 529-545.

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2009 Travel award, University of Ottawa, Faculty of Graduate and Postdoctoral Studies ($250.00)

2008 Ontario Graduate Scholarship in Science and Technology ($15,000)

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PROFESSIONAL ACTIVITIES

Oral Presentations

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- Poster presentation at the 13th Annual International SMA Research Group Meeting. June 18-20, 2009, Cincinnati, Ohio.
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