

Two-way Approach to Spinal Muscular Atrophy Therapy Development

Benoit B. Goulet

Department of Biochemistry, Microbiology and Immunology

Submitted in partial fulfillment of the requirements for the degree of Masters of Science

Faculty of Graduate Studies

University of Ottawa

Ottawa, Ontario, Canada

2013

Abstract

Spinal muscular atrophy (SMA) is the most commonly inherited neurodegenerative disease that leads to infant mortality worldwide. There are no known cures for SMA, but small increase in protein levels of SMN can be beneficial. We have developed adenoviral (Ad) vectors that express a human transgene of SMN and have tested their safety *in vitro*. We have demonstrated that these viruses can effectively express the transgene following cell entry and that the levels are relative to the virus dose. The viruses do not appear to impact the health and function of the cells, and are capable of increasing the number of Gems. We also attempted to change the tropism of the viruses through fiber protein modifications in order to target muscles and motor neurons. Our results suggest that a therapy based on an Ad-SMN fiber-modified vector may ultimately be successful in treating patients of SMA.

Acknowledgements

First, I would like to thank my supervisor, Dr. Robin Parks, whose guidance, support and patience have been paramount in this endeavor. I would also like to thank my thesis advisory committee members, Dr. Rashmi Kothary and Dr. Dennis Bulman, who helped me critically evaluate the results of my project. I would also like to thank current and past members of the Parks lab for their friendship, as well as all the assistance they have provided: Kathy Poulin, Adam Davidson, Andrea Giberson, Joe Burns, Carmen Wong, Natacha Provost, Emily McFall, Mélissa Geoffroy, Kalisa Campbell, Olga Vorobyova, Carin Christou, and Grace Tong.

On a personal level I would like to thank my parents, Michel Goulet and Marie Boucher, as well as my sister, Mélissa Goulet. Your constant and unwavering support was crucial in the last three years. Finally, I would like to express my deepest gratitude to Alexandra Waite. Without your love and support, I surely would have failed to see this project to fruition.

Table of Contents

Abstract	ii
Acknowledgements	iii
Table of Contents	iv
Lits of Abbreviations	vi
List of Figures	viii
List of Tables	ix
Chapter 1 – Introduction	
1.1 General Introduction	
1.1.1 What is SMA?	1
1.1.2 Disease Pathogenesis	4
1.1.3 SMN: Protein and Function	6
1.1.4 Animal models	7
1.1.5 Disease manifestations	10
1.2 Development of novel therapies for treatment and disease management	15
1.2.1 Choosing an appropriate vector for the development of a therapeutic strategy against SMA	18
1.2.2 Drawbacks and potential pitfalls of gene therapy for the treatment of SMA	18
1.3 Re-targeting of Adenovirus serotype 5 viral vectors	
1.3.1 Ad capsid structure	21
1.3.2 Ad binding and internalization	22
1.3.3 Re-targeting strategies for adenovirus	22
1.4 Rationale	27
1.5 Hypothesis	27
1.6 Objectives	28
Chapter 2 – Materials and methods	
2.1 Cell culture	29
2.2 Suspension cells and virus culture	29
2.3 Viral constructs	31
2.4 Immunoblot and antibodies	36
2.5 Cellular fractionation	37
2.6 Co-immunoprecipitation	38
2.7 Immunofluorescence and imaging	39
2.8 Protein stability	40
2.9 Cell growth assay	40
2.10 Promoter activity quantification by reporter gene expression	41
2.11 mRNA quantification and splicing analysis	42
2.12 Injection of chimeric adenovirus in Wild-Type mice	44
2.13 Tissue staining	45
2.14 β -Galactosidase detection in tissue	45

Chapter 3 – Supraphysiological expression of recombinant SMN from an adenovirus vector	
3.1 Introduction	47
3.2 Results	
3.2.1 Infection with Ad-SMN leads to supraphysiological levels of expression of SMN in several cell lines	49
3.2.2 Increases in Ad-SMN transcript level is proportional to the amount of virus	52
3.2.3 The stability of Ad-SMN is similar to the endogenous protein	53
3.2.4 The 50 kDa Ad-SMN is differentially spliced at the C-terminal	56
3.2.5 SMN protein produced from adenovirus vectors localizes correctly within the cell and interacts with its normal cellular protein partners	58
3.2.6 Over-expression of Ad-SMN does not have a negative impact on the function of target gene promoters or splicing	62
3.2.7 Over-expression of Ad-SMN does not negatively impact cell growth	67
3.2.8 SMN from an adenovirus vector can increase intracellular gem counts	69
3.3 Discussion	71
3.4 Future direction	79
3.5 Conclusion	79
Chapter 4 - Re-targeting of Adenovirus vectors by fiber modifications	
4.1 Introduction	81
4.2 Results	
4.2.1 Modified fiber viruses can infect A549 cells	83
4.2.2 Wild-type (Ad5-LacZ) and polylysine modified wild-type knob (Ad5spk-LacZ) viruses can be detected in the spinal cords and sciatic nerves of mice given intravenous injections	85
4.2.3 Viral constructs with the wild-type or modified (Ad5spk-LacZ) knobs, can readily infect muscle when directly injected into the tissue itself	88
4.2.4 Addition of the polylysine tag to the knob protein improves infectivity when the virus is delivered by intra-peritoneal injection.	91
4.3 Discussion	94
4.4 Future Direction	100
4.5 Conclusion	101
Reference list	102
Curriculum vitae	118

List of Abbreviations

A549 – Human lung epithelial carcinoma
AAV – Adeno-associated virus
AChR – Acetyl choline receptor
Ad - Adenovirus
AO – Antisense oligonucleotide
ATCC – American Type Culture Collection
BpA – Bovine growth hormone polyadenylation sequence
BSA – Bovine serum albumin
CAR - Coxsackie and adenovirus receptor
CMV - Cytomegalovirus
CNS – Central nervous system
CPE – Cytopathic effect
DMD – Duchenne’s muscular dystrophy
DMEM – Dulbecco’ modified Eagle’s medium
FBS – Fetal bovine serum
FITC – Fluorescein isothiocyanate
hdAd – Helper-dependant adenovirus
HeLa – Human epithelium-derived adenocarcinoma
His - Histidine
HIV – Human immunodeficiency virus
hnRNP-R - heterogenous nuclear ribonucleoprotein
hpi – Hours post-infection
HS – Horse serum
HSC – Hematopoietic stem cell
IGF-1 - Insulin-like growth factor 1
IGFALS - IGF-binding-protein acid labile subunit
IM - Intramuscular
IP – Intra-peritoneal
ITR – Inverted terminal repeats
IV - Intravascular
LacZ - β -galactosidase
MCMV – Murine cytomegalovirus
MEM – Minimum essential media
miRNA - MicroRNA
MLP – Major late promoter
MOI – Multiplicity of infection
NMJ – Neuromuscular junction
NP40 – Nonidet P-40
No-RevT – No-reverse transcriptase
pA - Polyadenylation
PBS – Phosphate buffered saline
PFA - Paraformaldehyde
PFU – Plaque forming units
pFU – Particle forming units

p(K) - Polylysine
PTD – Protein transduction domain
PVDF – Polyvinylidene difluoride membrane
qPCR – (Real-time quantitative PCR)
RFP – Red fluorescent protein
RGD - Arginine-Glycine-Aspartic acid
RhoA/ROCK - Ras homolog gene family member A/RhoA kinase
RT – Room temperature
RT-PCR – reverse-transcriptase PCR
sc-AAV – Self-complimentary adeno-associate virus
scFV – Single-chain antibody
sdAb – Single-domain antibody
shRNA – Short-harpin RNA
SMA – Spinal muscular atrophy
SMN – Survival of motor neuron
TA – Tibialis anterior
Tat – Transactivation of transcription
TIS – Transcription initial site
TRITC – Tetrahyrhodamine isothiocyanate
UbC – Human ubiquitin C
Usp11 – Ubiquitin specific peptidase like 1
UTR – Untranslated region
Vp/g – Viral particles/gram

List of Figures

Figure 1.1 - The genetic basis of SMA	3
Figure 2.1. Adenoviruses expressing recombinant SMN constructs	33
Figure 3.1. Ad-SMN infection can mediate high level protein expression in a variety of cell lines	51
Figure 3.2. mRNA levels correlate with high levels of protein expression mediated by Ad-SMN	53
Figure 3.3. The stability of virally derived SMN is similar to endogenous SMN	55
Figure 3.4. The 50 kDa Ad-SMN variant is differently spliced at the C-terminal end	57
Figure 3.5. SMN protein produced from adenovirus vectors localizes in the vicinity of Cajal bodies	60
Figure 3.6. Viral SMN has a similar sub-cellular distribution pattern as endogenous SMN and it interacts with SMN and Gemin2	61
Figure 3.7. Over-expression of Ad-SMN does not have a negative impact on the function of target gene promoters	65
Figure 3.8 Over-expression of Ad-SMN does not have a negative impact on splicing	66
Figure 3.9. Over-expression of Ad-SMN does not negatively impact cell growth	68
Figure 3.10. SMN from an adenovirus vector can increase intracellular gem counts	70
Figure 4.1. Modified fiber viruses can infect A549 cells	84
Figure 4.2. Wild-type (Ad5-LacZ) and polylysine modified wild-type knob (Ad5spk-LacZ) viruses can be detected in the spinal cords and sciatic nerves of mice given intravenous injections	87
Figure 4.3. Viral constructs with the wild-type or modified (Ad5spk-LacZ) knobs, can readily infect muscle when directly injected into the tissue itself	90
Figure 4.4. Addition of the polylysine tag to the knob protein improves infectivity when the virus is delivered by intraperitoneal injection	93

List of Tables

Table 1.1 Classification of the different types of Spinal Muscular Atrophies, age of onset, copies of <i>SMN2</i> , and the diagnostic features linked to each	5
Table 2.1 Characterization of Adenovirus vectors for targeting	35

Chapter 1 - Introduction

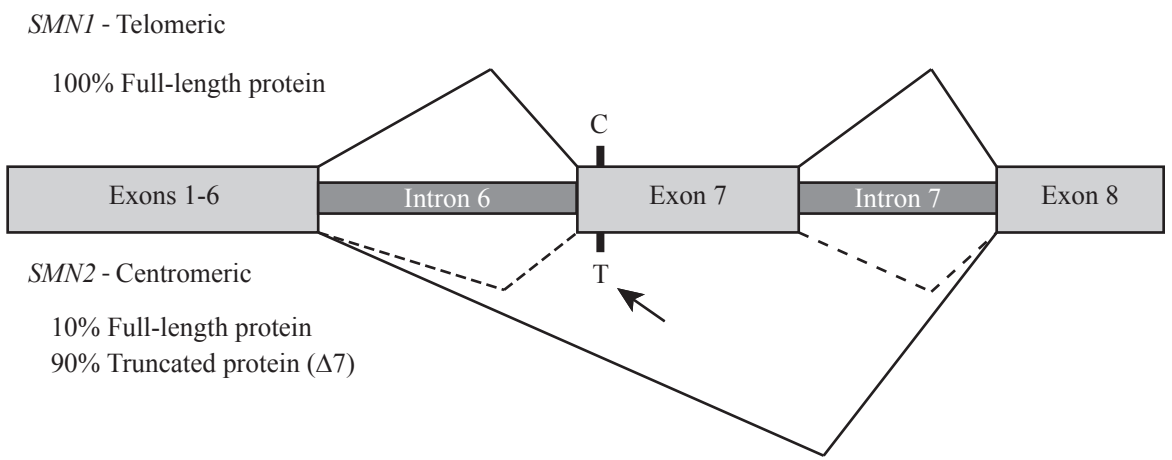
1.1 General Introduction

1.1.1 What is SMA?

Spinal Muscular Atrophy (SMA) is the most commonly inherited neurodegenerative disease that leads to infant mortality worldwide (Markowitz, Singh, & Darras, 2012; Roberts, Chavez, & Court, 1970). Approximately 1 in every 6000-10000 live births are affected by a form of the disease and the general population has a carrier frequency near 1 in 50 (McAndrew et al., 1997; Melki, 1997; Pearn, 1978). SMA is caused by a reduction in levels of full-length survival motor neuron (SMN) protein. The reduction in full-length SMN levels is caused by a homozygous deletion or mutation within the *SMN* locus (Lefebvre et al., 1995). Due to a duplication event during human evolutionary history, humans possess two copies of the *SMN* gene on the long arm of chromosome 5. The telomeric copy, called *SMN1*, is the main source of full-length SMN protein in healthy individuals and it is the mutated locus in diseased individuals. In 95% of cases, *SMN1* loss is caused by a homozygous deletion (Lefebvre et al., 1995; Rodrigues et al., 1995); but nonsense, frameshift, or missense mutations have also been reported (Burghes, 1997; DiDonato et al., 1997; Lefebvre et al., 1995; Parsons et al., 1998). These mutations are mostly found in the Tudor-domain (Kotani et al., 2007; Sun et al., 2005; Zapletalova et al., 2007) or YG-box sequences (Lorson et al., 1998; Pellizzoni, Charroux, & Dreyfuss, 1999; Shpargel & Matera, 2005; Wan et al., 2005). Additionally, in patients who possess a copy of *SMN1*, small deletions and/or point mutations have been identified (Martin, Valero, del Castillo, Pascual, & Hernandez-Chico, 2002). The centromeric copy, *SMN2*, differs from the telomeric copy by only five nucleotides. A Cytosine to Thymidine transition at the sixth position of exon seven changes the nature of the

mRNA splice site and causes the total loss of exon seven (Figure 1.1). The skipping of exon seven is the result of either a loss of an exon-splicing enhancer and/or the gain of an exon-splicing silencer (Cartegni & Krainer, 2002; Kashima & Manley, 2003). The truncated protein, called SMN Δ 7, cannot compensate for all functions of the full-length protein, is relatively unstable and is rapidly degraded (B. G. Burnett et al., 2009; Lorson, Hahnen, Androphy, & Wirth, 1999; Monani, Lorson, et al., 1999). The instability of the truncated *SMN2* protein is caused by the creation of a degradation signal when exon 7 is excluded during transcription (Cho & Dreyfuss, 2010). The modified exon splicing site, however, does not affect all transcripts from *SMN2*. Whereas the *SMN1* gene produces 100% of the full length protein, 5-10% of the mRNA transcripts from *SMN2* retain exon 7, giving rise to 5-10% full length SMN protein (Sumner et al., 2006; Wirth et al., 2006). To further illustrate the important role of *SMN2*, there exists a population of individual who are asymptomatic but who lack *SMN1* (Mailman et al., 2002; Prior, Swoboda, Scott, & Hejmanowski, 2004). This population remains disease free due to increase dosage from *SMN2*.

Figure 1.1 - The genetic basis of SMA. Due to a duplication event during human evolution, there are two copies of the SMN gene on the long arm of chromosome 5. The telomeric copy, termed *SMN1*, is the main source of full-length SMN protein in healthy individuals. The centromeric copy, *SMN2*, differs from *SMN1* by only five nucleotides. A transition (C to T) at the sixth position of exon seven changes the nature of the mRNA splice site and leads to preferential exclusion of exon seven from the mature mRNA. The resulting protein, SMN Δ 7, cannot compensate for all functions of the full-length protein, and is also rapidly degraded. Only 5-10% of the mRNA transcripts from *SMN2* retain exon 7, thus giving rise to 5-10% full-length SMN protein from this gene. Patients affected by SMA have mutations that eliminate *SMN1*, and the amount of full-length protein produced from *SMN2* is insufficient to prevent development of SMA. Based on figure by Wirth *et al.* (Wirth *et al.*, 2006).



1.1.2 Disease pathogenesis

The different types of SMA, their age of onset as well as certain diagnostic features are summarized in Table 1.1. Since the levels of full length SMN can be varied, the absolute copy number of SMN2 correlates the severity of SMA. In short, more copies of *SMN2* mean more full-length protein, which in turns leads to a decrease in disease severity (Lefebvre et al., 1997; Zapletalova et al., 2007). The most severely affected infants are affected by Type 0 SMA. This type of SMA is rarely seen as the pregnancies are not usually carried to term (Dubowitz, 1999; MacLeod, Taylor, Lunt, Mathew, & Robb, 1999). Type 1 SMA, or Werdnig-Hoffman disease, is the most severe type of SMA affecting children. Most of these patients are never able to sit and generally succumb to complications of their disease within the first three years of life. Studies have demonstrated that approximately 70% of all children affected by Type 1 SMA have two copies of the *SMN2* locus (Wirth et al., 2006). As demonstrated in Table 1.1, patients categorized as Type II or Type III (Kugelberg-Welander disease), while still being described as childhood SMAs, have less severe manifestations of the disease and later ages of onset, ranging from under 18 months to over 3 years of age (Munsat & Davies, 1992; Pearn, 1980). Approximately 82% of Type II patients have three copies of *SMN2* (Wirth et al., 2006). On the other hand, Type III patients have between three and five copies of *SMN2*; the higher copy number is correlated with a reduced disease severity and a later age of onset (Mailman et al., 2002; Ogino, Gao, Leonard, Paessler, & Wilson, 2003; Uchida et al., 2012). Finally, the adult onset SMA, Type IV, doesn't generally develop before 30 years of age. Adult patients normally present with very mild forms of distal limb muscle atrophies (Pearn, 1978), and genetic studies demonstrated that they have between 4 and 6 copies of *SMN2* (Wirth et al., 2006).

Table 1.1 Classification of the different types of Spinal Muscular Atrophies, age of onset, copies of *SMN2*, and the diagnostic features linked to each

SMA Type	Age of onset	Copies of SMN2	Diagnostic features
Type 0	Prenatal	N/A	Reduced movement <i>in utero</i> . Early neonatal death.
Type I (Werdnig-Hoffman disease)	Less than 6 months	70% of patients have 2 copies	Never able to sit. Death within three years.
Type II	Less than 18 months	82% of patients have 3 copies	Cannot sit or walk unaided
Type III (Kugelberg-Welander disease)	Less than 36 months	62% of patients have 2-3 copies	Can walk unassisted
	Over 36 months	65% of patients have 4-5 copies	
Type IV	Adulthood	4-6 copies	Mild proximal muscle weakness

1.1.3 SMN: Protein and Function

The SMN protein is 38 kDa and is found within the cytoplasm and nucleus of all cells (Burllet et al., 1998; Coover et al., 1997). In the nucleus, SMN oligomerizes, then interacts with the gemin group of proteins, as well as other protein, to form a multiprotein structure called gemini or coiled bodies or “gems” (Q. Liu & Dreyfuss, 1996). Furthermore, these structures are involved in the cytoplasmic assembly of small nuclear ribonucleoprotein complexes (Paushkin, Gubitz, Massenet, & Dreyfuss, 2002) and thus with the processing of pre-mRNA (Pellizzoni, Kataoka, Charroux, & Dreyfuss, 1998; Z. Zhang et al., 2008). The YG box, found on the C-terminus, mediates SMN oligomerization (Q. Liu, Fischer, Wang, & Dreyfuss, 1997; Lorson et al., 1998). Mutations in the domain affecting this characteristic have been described in SMA patients (Lorson et al., 1998; Pellizzoni et al., 1999). The Tudor domain mediates the interactions with SmB and fibrillarin (the other factors in the formation of snRNPs) and mutations in the *SMN1* gene have been detected in patients abrogating this ability (Kotani et al., 2007). Gems are found in close proximity to Cajal bodies, which are identified by positive staining with p80-coilin (Young et al., 2001; Young, Le, thi Man, Burghes, & Morris, 2000). For patients affected by SMA, tissue staining showed that a decrease in the number of gems in the nucleus of cells is proportional to the severity of the disease symptoms. In fact, studies have demonstrated that fibroblasts from Type I SMA patients have few or no gems (Coover et al., 1997).

In the cytoplasm of cultured neurons, SMN is found in granules in the axons and growth cones (Fan & Simard, 2002). However, these granules have yet to be observed definitively *in vivo* in neurons (Briese, Esmaili, & Sattelle, 2005). Despite the absence of granules, there is significant evidence to establish that SMN is abundantly found at the

growth cone of motor neurons as well as in the postsynaptic portion of the neuromuscular junction (NMJ) in muscle (Fan & Simard, 2002; H. Zhang et al., 2006; H. L. Zhang et al., 2003). SMN is involved in the transport of β -actin mRNA to neuronal growth cones (Todd, Morse, et al., 2010; Todd, Shaw, Morse, Stebbings, & Young, 2010; H. Zhang et al., 2006) through its interaction with hnRNP-R (heterogenous nuclear ribonucleoprotein) (Rossoll et al., 2002). SMN can also modulate the RhoA/ROCK (Ras homolog gene family member A/RhoA kinase) pathway through its relationship with profilin II (Bowerman et al., 2009; Bowerman, Shafey, & Kothary, 2007). Taken together, this points to a role for SMN in motor axon outgrowth through its ability to regulate actin dynamics (Bowerman et al., 2009; Boyer, Bowerman, & Kothary, 2010). Recent evidence demonstrated that SMN could directly modulate the levels of certain mRNA transcripts, such as the arginine methyltransferase CARM1 (Sanchez et al., 2013). Furthermore, SMN protein levels can affect which species of a certain mRNA are dominant, such as the ubiquitin specific peptidase like 1 (Usp11) protein (H. Liu, Shafey, Moores, & Kothary, 2010). Based on the evidence, it is obvious that reduced concentrations of SMN protein can severely affect multiple functions that are intrinsic to the normal functioning of cells. However, it is not clear which of these functions is the primary deficit in SMA, or even if perhaps the disease is caused by some other as yet uncharacterized function of SMN protein.

1.1.4 Animal models

It is important to understand the differences between the animal models that are commonly used in the study of SMA, since each has its advantages and disadvantages. Each model can yield important insights into disease pathogenesis. They can also provide an *in vivo* testing ground for therapeutics or as drug screens for disease modifiers.

The most commonly used animals are rodent models. Mice genetically only have one copy of the SMN gene and knockout animals of the single gene are not viable (Schrank et al., 1997). When the *SMN* gene is disrupted in otherwise wild-type mice, the resulting embryos reach the morula stage but do not implant. The initial survival of the embryos is due to the presence of residual maternal SMN protein and their death coincides with the depletion of this stock (Schrank et al., 1997). To circumvent this lethality, models have been created that express human *SMN2* transgenes, in order to supply small amounts of SMN protein simultaneous to the knock out of the endogenous gene (Hsieh-Li et al., 2000; Monani, Coover, & Burghes, 2000). This model, named *SMN*^{-/-};*SMN2* allows mice pups to come to term, but they mimic very severe SMA. Disease onset normally occurs at post-natal day 2 (P2), with death from symptoms at P6/7 (Monani et al., 2000). Interestingly, the expression level of *SMN2* in the spinal cord is dramatically reduced when compared to other tissues, indicating the potential presence of tissue-specific gene modifiers (Hsieh-Li et al., 2000). A second slightly less severe model was created to extend the lifespan of the mice by approximately another week. By adding a human *SMN* cDNA transgene lacking the seventh exon, the onset of symptoms was delayed, allowing for a longer manipulation period. This delayed onset severe model was named *SMN*^{-/-};*SMN2*; Δ 7 (Le et al., 2005) or Delta7 (Δ 7). Even with the slight delay in onset, the window for assessing the effectiveness of therapeutics still remains rather small. Fortunately, a mutant allele of *SMN* termed *SMN*^{2B} was recently described (DiDonato et al., 2001; Hammond et al., 2010). In brief, the endogenous mouse *SMN* allele contains a three nucleotide substitution in the exon splicing enhancer of exon 7. This allele preferentially produces *SMN* mRNA lacking exon 7. When this allele is present in conjunction with a null allele, the resulting mice (*SMN*^{2B/-}) express an

intermediate phenotype (Bowerman et al., 2009; Bowerman, Beauvais, Anderson, & Kothary, 2010; Bowerman, Murray, Beauvais, Pinheiro, & Kothary, 2011; H. Liu et al., 2010), remaining phenotype-free until post-natal day 10 (P10) and have a median life expectancy of 28 days. This will become a very important model to test therapies as the delayed onset facilitates animal handling during procedures and improvements in movement and lifespan are easily notable.

On the other hand, invertebrate models are useful models to mimic human neurogenetic disorders (Muqit & Feany, 2002). Chan et al. (Chan et al., 2003) described a naturally occurring *Drosophila* model. This mutant expressed very low levels of SMN in its progeny due to residual zygotic expression of the maternal gene. Most importantly, this model closely mimics human degeneration. Finally, the same type of naturally occurring mutants with maternal zygotic expression was described in zebrafish (Boon et al., 2009). The resulting phenotype is similar to that found in the *Drosophila* model, as well as in human patients. Finally, an *SMN* orthologue was found in *Caenorhabditis elegans*. When this gene is knocked-out, subsequent progeny develop defect in germ cell maturation as well as locomotive defects (Miguel-Aliaga et al., 1999).

Despite the wealth of abundance in animal models to study SMA and to help develop therapeutic, there lacks an overall conformity in the methods used to develop these animals. This leads to a disparity in the results obtained between, for example, two different animal models. Therefore, it is crucial to critically analyze results and keep in mind the methods in which they were obtained (Burghes & Beattie, 2009).

1.1.5 Disease manifestations

The majority of research up to date has focused on the effects of reduced levels on the development function, and maintenance of the motor neuron. At the molecular level, several genes are aberrantly spliced in mouse models of SMA (H. Liu et al., 2010; Lotti et al., 2012; Pellizzoni et al., 1998; Z. Zhang et al., 2008). In the severe mouse models of SMA, motor axon development and axonal outgrowth is normal until embryonic day 10.5 demonstrating that lack of SMN does not affect the early stages of neuronal development. As the mice age, there is an increase in synapses lacking motor axon input. Eventually, NMJ denervation leads to motor neuron death (McGovern, Gavrilina, Beattie, & Burghes, 2008). Structurally, these mice presented with poor terminal arborization and aberrant aggregation on intermediate filaments in the neurons (Kariya et al., 2008). It has been suggested that these two defects could serve as early biomarkers of the disease. Functionally, the NMJs remained enervated, however, the motor neurons were victims of abnormal synaptic transmission (Kariya et al., 2008; Kong et al., 2009). A leading cause of this abnormality was the reduction in the probability of vesicle release (Kong et al., 2009). More recently, the lack of SMN protein led to a reduction in NMJ plasticity and remodelling following induced paralysis in the SMN^{2B/-} mouse model (Murray, Beauvais, Bhanot, & Kothary, 2012). Remodelling failed due to a lack of nerve-directed re-organization of the acetylcholine receptors (AChR) at the NMJ, which is most likely, a result of a decreased rate in axonal growth. While SMA remains primarily a disease of the motor neurons, continual research as well as breakthroughs in lifespan extension of patients and animal models has uncovered evidence for the involvement of other tissues.

Recent work has demonstrated that there is a disruption in the connection between proprioceptive neurons and motor neurons (Mentis et al., 2011). Proprioceptive neurons are important in the grand scheme of things, as they are the neurons that provide sensory signals to motor neurons. Furthermore, they appear to be affected earlier than motor neurons in the progression of the disease. These observations would suggest that the original defects in SMA might originate in this subset of neurons, contrary to popular opinion. On the other hand, restoring SMN expression in motor neurons specifically eliminated any signs of disease progression (Gogliotti et al., 2012). This includes the maintenance of the synaptic connection between proprioceptive and motor neurons. Thus, the deterioration of the connection between these two types of neurons is likely caused by the malfunction of motor neuron signalling. It is important to note however, that a reduction in SMN protein levels can affect other types of neurons during disease progression, either directly or indirectly.

A significant amount of data has been generated to make a case that reduced SMN protein levels also has an effect on normal muscle function and development (For review see (Boyer et al., 2010)). *In vitro* studies using C2C12 cells, highlighted several problems caused by artificial depletion of SMN protein (Shafey, Cote, & Kothary, 2005). In culture, the C2C12s proliferated at a slower rate without an increase in cell death. Furthermore, there were defects in myoblast fusion which caused myotube malformation. Most importantly, the severity of these abnormalities was directly correlated with the decrease in SMN dosage. These results were corroborated in SMA patient myoblasts (Martinez-Hernandez et al., 2009), further cementing the potential muscle role in SMA disease pathogenesis. A follow up *in vitro* C2C12 study demonstrated that SMN directly interacted with key muscle proteins: annexin II and myosin regulatory light chain (Shafey, Boyer, Bhanot, & Kothary, 2010).

Taken together, these results demonstrated that SMN probably has muscle-specific roles that contribute to disease pathogenesis. Additionally, in the previous study describing motor neuron defects at the NMJ, it was also observed that there was a significant lack of maturation in the AChR clusters on the muscle side of the NMJ (Kariya et al., 2008). During development of the motor endplate, the adult form slowly replaces the foetal form of the AChR. This process is normally completed by P14 (Missias, Chu, Klocke, Sanes, & Merlie, 1996). In the Kariya *et al.* (Kariya et al., 2008) study, this switch failed to occur. It was further characterized by the lack of the distinct “pretzel”-like shape of the mature NMJ. Unfortunately, muscle-specific expression of transgenic SMN protein in severe SMA mouse models only led to minor increases in lifespan (Gavrilina et al., 2008). In essence, normal muscle-specific levels of SMN protein are important for muscle function and maintenance, but they cannot circumvent the progression of the disease without sufficient expression in other key tissues.

Recently, several studies have implicated peripheral tissues in the pathogenesis of SMA: pancreatic islets, cardiac muscles, and hepatocytes. First, in the new SMN^{2B/-} mouse model, pancreatic biopsies demonstrated that there was an inherent imbalance in the types of cells that composed the pancreatic islets (Bowerman et al., 2012). The imbalance favored glucagon producing α -cells to the detriment of insulin producing β -cells. Thus, the affected mice tended to be hyperglycemic, hyperglucagonemic, as well as glucose resistant. Significantly, post-mortem examinations of diseased Type I SMA children brought to light similar pancreatic cell fate abnormalities, which were matched to clinical observations of glucose intolerance in those children. Second, observations of the muscle cells defects previously discussed led to the discovery of heart defects, in the SMN Δ 7 SMA mouse model

(Bevan et al., 2010; Gogliotti et al., 2012; Shababi et al., 2012). At P14, the mice had bradycardia, characterized by a decrease in heart contractility and dilated cardiomyopathy. As the pancreatic defects, this cardiac defect was also corroborated in severe SMA human patients (Rudnik-Schoneborn et al., 2008). It does remain unclear however what role, if any, a depletion of SMN protein plays in this heart phenotype. A likely explanation may be that as a result of the neuronal pathology, the cardiomyocytes are subject to reduced autonomic signalling, and this, more than the lack of SMN, results in the observed pathogenesis. Finally, the liver dysfunction was first noticed in Type I SMA patients (Millino et al., 2009). In this study, post-mortem gene expression analysis of Type I patients discovered that there was a major disruption of the insulin-like growth factor 1 (IGF-1) signalling pathway. Coincidentally, researchers who were attempting to develop an antisense oligonucleotide (AO) therapy for SMA found that the therapeutic was much more effective in severe SMA mice when administered systemically, compared to a direct central nervous system (CNS) injection (Hua et al., 2011). The AO had a propensity for accumulating in the liver and further testing led the researchers to find that there were reduced levels of circulating IGF-1, despite the fact that the liver gene expression was not affected. Eventually, it was established that a protein that stabilizes IGF-1, IGF-binding-protein acid labile subunit (IGFBALS), was depleted in the affected mice.

All in all, the bulk of the studies enumerated here demonstrate that, although SMA can be principally thought of as a disease that affects motor neurons, there are other pathologies that occur in other tissues and cells that all contribute to the severity of the disease. These “secondary” defects, as well as other undiscovered deficiencies, will become a more prominent issue as the research helps extend the lifespan of severely affected

individuals (Bowerman et al., 2012). It also further illustrates the need for a therapy that not only restores SMN levels in neurons, but is available to all other tissues that may require it.

1.2 Development of novel therapies for treatment and disease management

Despite the research and advancements in the field of SMA, there is still no cure for this devastating disease. In fact, the medical interventions are aimed at alleviating symptoms of respiratory distress, as well as provide nutritional and orthopedic support to the patients (B. G. Burnett et al., 2009). The most common physical symptom of SMA is respiratory insufficiency, due to the weakness of the muscles involved in breathing. For this reason, the use of ventilators has made a drastic difference in the lives of SMA patients (Iannaccone, 2007). Most of the recent research in the field of SMA concentrates on developing new therapeutic options to treat and/or cure the disease. There are multiple avenues being investigated and they can be broken down into two main categories. The first option aims to modify the expression of SMN protein in order to increase the availability of full-length protein in cells and tissues. SMN-dependant therapies can be further broken down into gene and protein therapies. The other category of treatments does not involve modulating the levels of SMN protein. They achieve their benefits through enhancing motor neuron survival and/or function. In the scope of this thesis however, we will only discuss those therapies that concentrate on viral vectors and/or administration of purified therapeutic proteins.

Over the years, several groups have successfully used gene therapy approaches to deliver a “good”, or replacement copy of SMN to tissue cultures or animal mouse models of SMA (Azzouz et al., 2004; DiDonato, Parks, & Kothary, 2003; Foust et al., 2010; Glascock, Osman, et al., 2012; Glascock, Shababi, Wetz, Krogman, & Lorson, 2012; Passini et al., 2010; Valori et al., 2010). In the beginning, lentivirus (Azzouz et al., 2004) and adenovirus (Ad) (DiDonato et al., 2003) vectors were used as vehicles to deliver a full-length copy of the SMN gene to patient derived fibroblasts. This treatment effectively restored Gem counts in

the cells while at the same time helped re-localize Gemin2 protein to Gems. As previously discussed, the Gem count in patient cells is inversely proportional to disease severity. In essence, a higher Gem count is generally correlated with a less severe form of SMA. The lentivirus vector was further utilized in the treatment of SMN Δ 7 mouse models. At P2, the mice received injections in several muscle groups and expression of SMN was restored in motor neurons through retrograde transport of the virus. Despite the subsequent prevention of motor neuron death, only a small increase of 38% in lifespan was observed in the treated mice (from 13 to 20 days for untreated and treated mice respectively) (Azzouz et al., 2004).

More recently, several groups have achieved significant lifespan extension in animal models of SMA by using adeno-associated virus-based (AAV) vehicles (Foust et al., 2010; Passini et al., 2010; Valori et al., 2010). Using a self-complimentary adeno-associated virus serotype 9 (sc-AAV9), Valori *et al.* (Valori et al., 2010) injected P1 SMN Δ 7 to deliver a codon-optimized copy of human SMN. As a result, approximately 80% of treated animals had a median lifespan extension to 69.1 days, when compared to 13.9 for untreated animals. As an added bonus, one mouse lived to more than 190 days. In this study, a one time facial vein injection restored SMN levels in muscle as well as the spinal cord of the animals and helped them outperform their untreated littermates in behavioural tests such as motor coordination, balance, gait analysis, and geotaxis. It is important to note however that there was significant accumulation of the virus in the animals' hepatocytes. Despite this observation, no tests to verify liver function or availability of IGF-1 were performed. Another group achieved similar improvements in lifespan (from 13 to 199 days) by injecting the sc-AAV9 vector in the temporal vein (Dominguez et al., 2011). Additionally, Foust *et al.* (Foust et al., 2010), injected an sc-AAV9 virus intravenously in P1 SMN $^{-/-}$;SMN2 animals.

These injections improved lifespan to above 250 days, when compared to 15.5 days for untreated controls, while restoring normal motor neuron function. In this study, the authors also aimed to determine whether there was a “window of opportunity” in which to provide treatment for the mice. The experiments were thus repeated with other litters at P5 and P10 respectively. The mice treated at P5 only had partial correction of motor neuron function, while P10 treatment had very little effect, demonstrating that there is an opportune window for gene therapy in mice. A fourth group injected the mice at P0 with an sc-AAV8 vector directly into the CNS. As with the three previous studies, the animals’ lifespan was extended to more than 157 days. Furthermore, skeletal muscle physiology was restored, as evidenced by an increase in myofiber size as well as the recovery of a traditional NMJ structure. A comparison of delivery methods of an sc-AAV9-SMN vector, between intracranial and intravenous injections, in SMN Δ 7 mice showed that intracranial injections yielded much larger lifespan extension. Animals given intracranial injections lived approximately 200 days, while those given intravenous injections lived only approximately 30 days (Glascock, Shababi, et al., 2012). When this study was repeated in the more severe SMN^{-/-};SMN2 animal model, the results were much less impressive. Untreated animals lived approximately 7 days, while the intravenous injections improved lifespan by 1 day and intracranial injections improved by 10 days (Gogliotti et al., 2012). In conclusion, gene replacement strategies are capable of restoring muscle and neuron functions while extending lifespan, even in the most severe mouse models. Furthermore, the therapies were more effective if they were delivered earlier and directly into the CNS. Going forward, these factors need to be taken into account when hatching a plan for the development of a successful gene therapy strategy for SMA.

Obviously, other methods have been used in order to develop treatment regimens against SMA. These range from drugs that enhance the expression of full-length SMN and SMN Δ 7 such as the synergistic combination of bortezomib (a ubiquitin proteasome system antagonist) and trichostatin A (a histone deacetylase inhibitor) (Kwon, Motley, Fischbeck, & Burnett, 2011), all the way to the use of embryonic stem cell-derived neural stem cells (Corti et al., 2008; Corti et al., 2010). However, as these were not viral vector based therapies, they fall beyond the scope of this thesis.

1.2.1 Choosing an appropriate vector for the development of a therapeutic strategy against SMA

Ad-based vectors have been used extensively in strategies for molecular targeted therapeutics. In fact, Ad vectors possess many characteristics that make them an ideal choice compared to other vector types. In a biological sense, Ad is able to efficiently transduce a variety of dividing and quiescent cells. This phenomenon occurs both *in vivo* and *in vitro*. Other important characteristics of Ads are their amenability to genetic modification, large genetic payload capacity, and their ability to produce high titers of high quality virus (Beatty & Curiel, 2012). In terms of clinical side effects, these vectors have an impressive safety record, even if very high doses can lead to acute toxicity (Schnell et al., 2001). Finally, the most commonly used Ad vector in gene therapy is the human serotype 5 (Ad5) (Beatty & Curiel, 2012).

1.2.2 Drawbacks and potential pitfalls of gene therapy for the treatment of SMA

Ad5 vectors have several advantages that can be utilized in the development of a successful treatment strategy. To our knowledge however, no studies have been performed to demonstrate the safety of high expression of the SMN protein delivered by a viral vector in

non-target tissues. As was previously discussed, sc-AAV9 viruses administered by intravenous injection accumulated in the liver of the mice (Valori et al., 2010). The same holds true for Ad5 viruses. In fact, following an intravenous injection, liver cells sequestered more than 80% of the viral particles (Guo, Wang, Eisensmith, & Woo, 1996). While it is true that liver functions are diminished in SMA animal models and that they would benefit from the presence of exogenous SMN protein, a concentration of the therapeutic in this organ raises some red flags.

Evidence exists, in other disease models, demonstrating the first potential pitfall of a non-targeted gene therapy approach for the treatment of SMA. In a study evaluating the dangers of sustained high expression of short-harpin RNAs (shRNA) in hepatocytes, Grimm *et al.* (Grimm et al., 2006) found that several constructs caused dose-dependant liver injuries. Out of 49 tested constructs, 23 ultimately led to the demise of the test animals. The researchers determined that morbidity was associated with the downregulation of liver-derived microRNAs (miRNAs), indicating possible competition of the latter with shRNAs for limiting cellular factors required for the processing of various small RNAs. This finding is doubly troubling in regards to the treatment of SMA because SMN protein is known to regulate the assembly of RNA protein complexes thus indirectly impacting the pre-mRNA maturation process. It is possible that over-saturation of hepatocytes with Ad expressing an SMN transgene could affect these pathways in a manner similar to the effects observed in the Grimm *et al.* (Grimm et al., 2006) study. A second dangerous hurdle when using Ad5 is the acute toxicity it occasionally causes when it is administered systemically (Schnell et al., 2001). In non-human primates, it appears that the virus was sequestered in the Kupffer cells of the liver immediately following a systemic injection. Furthermore, the injection caused a

systemic release of interleukin-6 (IL-6) which led to an acute activation of macrophages and dendritic cells, followed by massive apoptosis. Finally, the clinical observations did not change whether the virus used was live or inactivated, which led the researchers to conclude that the acute toxicity reaction was caused by an interaction with one or several viral capsid proteins. It is thus important, when developing a potential gene therapy strategy against SMA, to verify the safety of high transgene expression both *in vitro* and *in vivo*, as well as mitigate some of the negative immunological effects. One strategy to achieve this last goal is the modification of viral tropism.

1.3 Re-targeting of Adenovirus serotype 5 viral vectors

1.3.1 Ad capsid structure

The human Ad virus has a 36-kb double stranded DNA genome, which is encapsulated by an icosahedral protein particle. Recent progress in the fields of X-ray crystallography and cryo-electron microscopy have allowed the scientific community to finely resolve the structural components of the Ad capsid (Fabry et al., 2005; Reddy, Natchiar, Stewart, & Nemerow, 2010; Saban, Silvestry, Nemerow, & Stewart, 2006). In total, the Ad capsid is composed of three major proteins: hexon, penton, and fiber; as well as several minor proteins: IIIa, VI, VIII, and IX. The hexon is the most abundant protein in the capsid. It is a homotrimeric protein and there are 720 copies in each virus. It's principal role is structural, but there are hypervariable regions which are exposed and confer some serotype variation (R. M. Burnett, 1985). The penton protein is found at all the vertices (12 in total) and it is composed of a homopentamer. Each penton has an RGD motif (Arg-Gly-Asp), which is involved in virion internalization. In addition to it's structural role, the penton also interacts with the last capsid component: the fiber protein (Beatty & Curiel, 2012). In the scope of this thesis, the most important capsid protein to consider is the fiber protein. Fiber is found as a homotrimer at each capsid vertex. It is noncovalently bound to the penton base by its N-terminus. The fiber protein is composed of two distinct subunits: a shaft domain and a globular knob domain. The knob domain is the primary cellular attachment site (Zubieta, Schoehn, Chroboczek, & Cusack, 2005) via its interaction with cell-surface receptors. Additionally, the length of the shaft domain can affect the virion's ability to interact with cell receptors and to enter the cell (Shayakhmetov & Lieber, 2000).

1.3.2 Ad binding and internalization

There are two main steps that occur in order to bring a virion from the outside to the inside of a cell: 1) attachment of the virus to its primary receptor and 2) internalization via molecular interaction. High-affinity interactions between the fiber knob domain and the appropriate cell surface receptor start off the whole process. For Ad5, this receptor is the coxsackie and Ad receptor (CAR). For other receptors relevant to this thesis, see Table 2.1. CAR, a member of the immunoglobulin superfamily, is involved in the formation of tight junctions (Coyne & Bergelson, 2006; Philipson & Pettersson, 2004). After the initial attachment, cellular αv integrin proteins interact with the penton's RGD motifs (Wickham, Mathias, Cheresch, & Nemerow, 1993), and induce cytoskeletal alterations that help bring the virion into the cell (Li, Stupack, Bokoch, & Nemerow, 1998; Li, Stupack, Klemke, Cheresch, & Nemerow, 1998). Eventually, the virus is internalized via a clathrin-coated vesicle, is fused to an endosome; the virus is released following endosomal acidification (Meier et al., 2002) and travels to the cell nucleus in order to undergo viral replication.

1.3.3 Re-targeting strategies for adenovirus

In SMA, the most affected tissues, motor neurons and muscles, are mostly refractory to Ad5 infection due to an apparent lack of CAR and/or αv integrin expression on the cell surface (Acsadi et al., 1994; Huard et al., 1995; Nalbantoglu, Pari, Karpati, & Holland, 1999). Thus, if the target cells exhibit low levels of CAR, a therapy utilizing Ad5 will lead to high transduction of non-target cells, and low transduction of target cells: a recipe for failure. The affinity of Ad5 for the liver following a systemic injection is also of importance for the success of a gene therapy. This interaction occurs via hepatic Kupffer cell uptake (Tao et al., 2001) and direct hepatocyte transduction, despite the observation that the liver does not have

a high distribution of CAR receptors (Wood et al., 1999). As discussed, this preference for liver targeting can lead to liver toxicity when using Ad-based vectors. In order to improve the efficacy of a gene therapy strategy for SMA, the tropism of Ad5 can be modified to simultaneously target diseased cells/tissues, while de-targeting non-pathogenic cells/tissues and limiting biodistribution of the virus.

A successful liver de-targeting was achieved after the discovery of the internalization method of Ad by the liver (Kalyuzhniy et al., 2008; Waddington et al., 2008). These studies demonstrated that the virus hexon capsid protein binds to the blood factor X. This factor then interacts with heparin sulfate, a cell surface receptor in hepatocytes and leads to virion internalization. The de-targeting was achieved by changing the hypervariable region of Ad5 with one native to Ad48. The newly acquired region does not interact with heparin sulfate/blood factor X and reduced virion uptake in the liver by a factor of 600. This is a good example of genetic modifications, which can be beneficial in the development of gene therapies. These genetic modifications can be of two main types: 1) the cloning of a targeting ligand into a protein of the Ad capsid, or 2) the swapping of capsid protein subunits, such as the fiber knob and/or shaft between Ad serotypes.

The first method has yielded several positive results and has been adapted to a multitude of approaches (Glasgow, Everts, & Curiel, 2006). In a study by Dmitriev *et al.* (Dmitriev et al., 1998), the authors added an RGD motif to the H-I loop of the Ad5 fiber knob protein. This modification caused the virions to be internalized in a CAR-independent pathway since this motif normally binds cellular αv integrins. The mutant vector was ultimately able to improve gene transfer to primary ovarian cancer cells, which normally have very low CAR distribution, by an order of 2-3 fold above control vectors. A second

study added a streak of 7 lysines (pK7) to the c-terminus of an Ad5 fiber (Wickham et al., 1997). This simple addition led to a 5-500 fold increase in the infectivity of macrophages, fibroblasts, T-cells, and endothelial cells. Most important for this thesis, it also drastically improved gene transfer to smooth muscle cells. Additionally, these results have been replicated with success in C2C12 cells as well as *in vivo* using a gutted helper-dependent Ad (hdAd) vector (Bramson et al., 2004). Although we only discuss recombinant vectors that have had their fiber proteins modified, it is important to note that other capsid proteins, such as pIX, can also be modified for re-targeting purpose (Poulin et al., 2010). In this study, the pIX protein was fused to either a single-chain antibody (scFV) or a single-domain antibody (sdAb). When directed to the endoplasmic reticulum, the scFV was poorly incorporated into the capsid. On the other hand, the sdAb was properly folded and highly incorporated. This led to the production of high titer virus preparations and enhanced viral infection of target cells. The only drawback of using the sdAb method is that it is limited by our knowledge of cell surface epitopes and our ability to produce antibodies against those epitopes. However, these vectors clearly demonstrate the feasibility of designing and producing Ads which can target tissue-restricted receptors to increase the efficiency and/or specificity of gene transfer.

The second vector modification method relies on the production of chimeric Ads. With chimeric vectors, the fiber knob domain (Shayakhmetov & Lieber, 2000), the shaft (Hidaka et al., 1999; Roelvink, Kovesdi, & Wickham, 1996; Roelvink et al., 1998; Shayakhmetov & Lieber, 2000), or the entire fiber (Bramson et al., 2004; V. N. Krasnykh, Mikheeva, Douglas, & Curiel, 1996; Shayakhmetov, Papayannopoulou, Stamatoyannopoulos, & Lieber, 2000) is genetically replaced with its structural counterpart from a different human serotype that recognizes an alternative cellular surface receptor

(Beatty & Curiel, 2012). These chimeric vectors have been successfully utilized in the development of cancer therapeutics, targeting cells that do not normally express CAR and/or αv integrins in ovarian carcinoma (Rein et al., 2011; Rocconi et al., 2007), prostate cancer (Murakami et al., 2010), and breast cancer (Stoff-Khalili et al., 2007). Guse *et al.* (Guse et al., 2012) recently developed Ad and hdAd vectors based on the Ad5 capsid, with an Ad3 fiber knob. These vectors had significantly increased transduction of skeletal muscle after intramuscular injections. This study is of significant importance because the vectors were developed in a strategy to treat Duchenne's Muscular Dystrophy (DMD), a disease phenotypically similar to SMA. In essence, a similar strategy could be adapted to treat SMA, especially with Ad's ability to go through retrograde transport to gain access to neurons.

The Ad fiber shaft is composed of repeats of up to 14 amino acids, forming β -sheet. Within the sheets, the number of repeats varies from 6 (Ad35) to 23 (Ad12) (Chroboczek, Ruigrok, & Cusack, 1995). It has been suggested that the shorter shafts allow the virions to enter the cells through direct interaction of the penton base with the cellular αv integrins (Roelvink et al., 1996; Roelvink et al., 1998). A chimeric Ad5 capsid combined with the shorter Ad9 fiber protein (which binds to CAR (Roelvink et al., 1998)), showed a dramatic decrease in infectivity of CAR/ αv integrin expressing cells (Hidaka et al., 1999). On the other hand, the infectivity of a second chimeric Ad5 vector with the short Ad7 fiber (non-CAR binding) was not affected regardless of the CAR expressing status of the cells. Similar results have been observed with a chimeric vector where the Ad35 fiber is incorporated into the Ad5 capsid (Shayakhmetov et al., 2000). In this case, the authors were able to abrogate the normal CAR-dependant entry of the virus by allowing it to interact with CD46 (Gaggar, Shayakhmetov, & Lieber, 2003). Shayakhmetov *et al.* attempted to further elucidate the role

of the fiber length in cell targeting. By combining CAR-interacting knobs (Ad5 and Ad9) and a CAR-independent knob (Ad35) with different length shafts, the authors were able to create chimeric vectors of every possible combination (Shayakhmetov & Lieber, 2000). For vectors that had Ad5/Ad9 knobs, long shafts were necessary for the transduction of cells expressing CAR/ α v integrins. In contrast, when these knobs were combined with short shafts, their absorption and infection capabilities were lowered. This reduction in infectivity was caused by a charge-dependent repulsion between the Ad5 capsid and the acidic cell surface proteins. Interestingly, the length of the shaft did not affect the infectivity of virions containing an Ad35 knob. This knob interacts with cells in a CAR-independent fashion. Overall, these chimeric studies all demonstrate that the fiber-CAR interaction is not the sole determinant of Ad tropism. Furthermore, CAR/ α v integrin-mediated infections are influenced by other factors, such as the length of the fiber shaft. These studies also confirmed the concept of targeting Ad vectors in the development of a gene therapy is a feasible goal; suffice to find the right combination of modifications for the desired outcomes.

1.4 Rationale

A reduction in levels of the survival motor neuron protein during development leads to the development of Spinal Muscular Atrophy. Patient observations as well as animal studies have demonstrated that even a small increase in available SMN protein can alleviate disease symptoms and improve quality of life. Gene therapy using adenovirus represents a novel strategy in the treatment of SMA. To date, no studies have been undertaken to demonstrate the safety of expressing exogenous SMN protein. Specifically, there are concerns with liver functions. Furthermore, when injected systemically, Ad vectors aggregate to the liver further compounding the safety concern. In SMA, the most important therapeutic targets are the motor neurons and skeletal muscle. These tissues are generally refractory to Ad serotype 5 infections, as they do not express the appropriate cell-surface receptors. Advances in the creation of chimeric Ad5 vectors, expressing modified fiber proteins from other Ad serotypes, have allowed researchers to target specific tissues that would normally be refractory to Ad injection. In the present study, I assessed the safety and efficacy of an Ad5 vector expressing recombinant full-length human SMN *in vitro*. I also experimented with chimeric Ads expressing different fibers to de-target the liver while targeting muscle and neuronal viral incorporation.

1.5 Hypothesis

All studies in this thesis are related to the development of a novel approach in the treatment of SMA. Thus, there are two distinct hypotheses:

- 1) High levels of recombinant SMN protein, delivered by an adenovirus vector, could negatively impact wild-type cells.

- 2) Shaft and knob modifications of the capsid fiber protein will improve targeting of the Ad vector to muscles and neurons.

1.6 Objectives

The immediate aims for each study are:

- 1) Safety and efficacy of Ad-SMN
 - a. Determine protein levels following infection. Establish the stability and interactions of Ad-SMN.
 - b. Determine the sub-cellular localization of Ad-SMN.
 - c. Examine effects on transcript availability and processing
- 2) Development of chimeric vectors
 - a. Determine whether the fiber modifications can target the vectors to specific cell types *in vivo*.
 - b. Determine an optimal delivery method

Chapter 2 – Materials and Methods

2.1 Cell Culture

The A549 cells (human lung epithelial carcinoma: American Type Culture Collection [ATCC] CCL-185) and 293 cells (Graham, Smiley, Russell, & Nairn, 1977) were grown in Minimum essential medium (MEM; Sigma-Aldrich), supplemented with 10% fetal bovine serum (FBS; Sigma-Aldrich), 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. HeLa cells (human epithelium-derived adenocarcinoma; ATCC CCL-2) and MN1 (Salazar-Gruesso, Kim, & Kim, 1991) were maintained in Dulbecco's modified Eagle's medium (DMEM; Sigme-Aldrich) supplemented as above. All cells were placed in tissue culture dishes (Sarstedt, Postfach, Germany), and incubated at 37°C in a humidified 5% CO₂ atmosphere within a SANYO incubator (SANYO, Wood Dale, IL).

2.2 Suspension cells and virus culture

Viruses used in Chapter 3 are shown in Figure 2.1, while viruses used in Chapter 4 are depicted in Table 2.1. These viruses were created using a combination of conventional cloning and bacterial RecA-mediated recombination (Chartier et al., 1996). All viruses were propagated using 293N3S cells (Graham, 1987) in a 3 L suspension culture of MEM, supplemented with 5% FBS, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin (Ross & Parks, 2009). Briefly, the 3 L suspension culture, at approximate concentration between 3×10^5 and 5×10^5 cells/ml, was concentrated by centrifugation (650g for 20 minutes at room temperature (RT)) to 80 ml and infected with approximately 20 ml of supernatant from a previously infected 15 cm of 293 cells. Following the two hour infection, the cells are returned to the 3 L spinner containing 1 L of spent media and 1 L of fresh 5% FBS MEM for 2-3 days, until complete cytopathic effect (CPE) is observed. After cell death

had occurred, cells were concentrated by centrifugation (650g for 20 minutes at 4°C) in 15 ml of 4% sucrose in 10 mM Tris, then treated with a series of incubation with 5% deoxycholate, 0.04 M MgCl₂, 0.02 mg/ml RNase A, and 0.02 mg/ml DNase I. The digestions were followed by a centrifugation (1000g for 10 minutes at 22°C) to pellet the cell debris. The supernatant was subjected to a series of cesium chloride gradient centrifugations. Briefly, a first gradient was prepared by carefully overlaying a 1.25 mg/ml cesium chloride solution on top of a 1.35 mg/ml cesium chloride solution. The viral preparation was added to the mixture and centrifuged for 1 hour at 35,000 RPM and 10°C in a swinging bucket rotor (SW41; Beckman Coulter). The viral band was extracted using a syringe and placed in another tube, which was then filled with the 1.35 mg/ml cesium chloride solution. This tube was heat sealed and centrifuged overnight at 35,000 RPM at 10°C (70.1Ti rotor; Beckman Coulter). The final viral band obtained from the gradient centrifugations was dialyzed for 24 hours against two 500 ml volumes of dialysis buffer of 10 mM Tris-HCl, pH 8.0. The virus was then diluted with 40% sucrose in 10 mM Tris-HCl, pH 8.0 to a final concentration of 4%, and stored at -80°C in small aliquots of 200 µl. Serial dilutions in phosphate buffered saline (PBS; Sigma-Aldrich), from 10⁻³ to 10⁻⁸, from the final virus preparations, were used to infect 6-well plates of confluent 293 cells (approximately 1x10⁶ cells/well) to determine viral titer. Cells were infected for 1 hour with frequent rocking, were over-layed with 4 ml of agarose solution containing 0.5% agarose, 1x MEM without phenol (Invitrogen), 5% FBS, 0.1 mg/ml streptomycin, and 100 U/ml penicillin, and incubated for 7-10 days. Plaques were counted visually to obtain a concentration of plaque forming units per milliliter (PFU/ml). Alternatively, particle counts were obtained instead of PFU counts due to the poor infectivity of the viruses in 293 cells, especially for viruses used in Chapter 4. Briefly, 20 µl of purified

vector was added to a final volume of 1 ml in 0.1% SDS-TE (10 mM Tris-HCl pH 7.5, 1 mM EDTA) and incubated for 10 minutes at 56°C. The absorbance at A_{260} was measured using a spectrophotometer. The viral concentration in particle forming units/milliliter (pFU/ml) is calculated based on the extinction coefficient of 1.1×10^{12} for wild-type Ad (Maizel, White, & Scharff, 1968). In order to infect cells, virus stocks were diluted to the appropriate multiplicity of infection (MOI) in PBS. Infected cells were incubated at 37°C for 1 hour. Plates were rocked at intervals of 15 minutes to redistribute the virus over the cells. Following incubation, cells were overlaid with fresh complete media and further incubated for 17 hours, unless stated otherwise.

2.3 Viral constructs

The genome structures of the viruses expressing SMN are summarized in Figure 2.1. The virus designated as Ad-C/SMN has been previously used (DiDonato et al., 2003). This study concluded that infection of fibroblasts with this vector led to very high levels of SMN expression. The virus contains the full-length, human SMN cDNA under regulation by the cytomegalovirus (CMV) immediate-early enhancer/promoter and the bovine growth hormone polyadenylation sequence, BpA. A second virus was created in which SMN is under the control of the human ubiquitin C (UbC) promoter (Ad-U/SMN). This promoter is normally used in lieu of viral promoters because it allows for more stable expression of the genes under its control (Christou & Parks, 2011; Wilber et al., 2005). A third SMN virus was created that contains a myc epitope tag on the C-terminus of the SMN protein. All of these viruses contain a Flag epitope tag on the N-terminus of the SMN. A virus (Ad-RFP), expressing a monomeric red fluorescent protein (RFP) under the control of the CMV

promoter and BpA (Poulin et al., 2010; Poulin et al., 2011), was used as a control to monitor the efficacy of all infections.

Figure 2.1. Adenoviruses expressing recombinant SMN constructs. All viruses are deleted of the Ad early region 1 (E1) and E3. All viruses contained the bovine growth hormone polyadenylation sequence (BpA) and were under the regulation of either the cytomegalovirus immediate-early enhancer/promoter (CMV) or the ubiquitin C (UbC) promoter. Ad-RFP contains the monomeric red fluorescent protein (RFP). Ad-C/SMN and Ad-U/SMN express the full-length human SMN cDNA tagged with a Flag epitope at the N-terminus under the control of the CMV and UbC promoters respectively. Ad-U/SMNmyc was based on the structure of Ad-U/SMN, with a myc epitope tag attached to the C-terminus. Also shown are the relative positions of the viral inverted terminal repeats (ITR) and DNA packaging elements (Ψ).



Ad-RFP



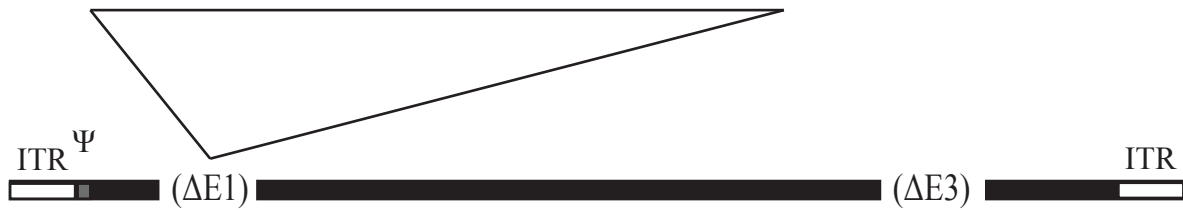
Ad-C/SMN



Ad-U/SMN



Ad-U/SMNmyc



A second set of viruses was also created to determine if targeting specific cell types through their cell surface receptors would be advantageous for a gene therapy strategy for SMA. These viruses are summarized in Table 2.1. All virus constructs were graciously provided by Dr. Dmitry M. Shayakhmetov (University of Washington) and have been previously described (Shayakhmetov et al., 2003; Shayakhmetov & Lieber, 2000; Shayakhmetov et al., 2000). The viruses were modified from the original forms to express *E. coli* β -galactosidase gene (*lacZ*) under the regulation of the murine cytomegalovirus immediate-early promoter/enhancer (MCMV) and the simian virus 40 polyadenylation sequence (pA) in place of the E1 region as described in (Willemsen, 2008). The fiber protein is expressed as the fifth protein controlled by the major late promoter (MLP). A total of four different forms of the adenovirus fiber protein were used in this study. A virus containing a fiber composed of the long Ad5 shaft (22 β -sheets, 37 nm) and the Ad5 knob was used as a control (Ad5-LacZ). Experimental viruses were composed of: the short Ad9 shaft (8 β -sheets, 11 nm) and the Ad5 knob (Ad5s-LacZ), as well as the shorter Ad35 shaft (6 β -sheets) and the Ad35 knob (Ad5/35-LacZ). A final virus was created by adding a polylysine tract [p(K)] to the H-I loop of the Ad5 knob protein as previously described (Bramson et al., 2004). Combined with the short Ad9 shaft, this virus formed the final experimental construct (Ad5spk-LacZ).

Table 2.1 Characterization of Adenovirus vectors for targeting

Viral Construct	Shaft	Length (nm)	Knob	Target
Ad5-LacZ	Ad5	22 B-sheets 37	Ad5	CAR
Ad5s-LacZ	Ad9	9 B-sheets 11	Ad5	CAR
Ad5/35-LacZ	Ad35	6 B-sheets	Ad35	CD46
Ad5spk-LacZ	Ad9	9 B-sheets 11	Ad5-p(K)	Heparan sulfate and CAR

2.4 Immunoblot and antibodies

Treated cells were washed once with PBS then lysed on ice for 5 minutes with a modified RIPA extraction buffer containing final concentrations of 50 mM Tris pH 8.0, 100 mM NaCl, 1 mM EDTA, 1% glycerol, 1% Nonidet P-40 (NP40), and protease inhibitors (Roche). The cell debris and crude extract were collected in a microcentrifuge tube, and cell debris was removed by centrifugation at 14 000 RPM for 10 minutes at 4°C. Lysate protein concentrations were determined by performing a Bradford assay (BioRad) according to the manufacturer's instructions using a serial dilution of bovine serum albumin (BSA; Sigma), as a standard curve. Protein extracts were diluted to a working concentration of 250 µg/µl in modified RIPA buffer. Samples were combined with SDS-PAGE loading buffer containing 62.5 mM Tris pH 6.8, 25% glycerol, 2% SDS, 0.1% bromophenol blue, 5% 2-mercaptoethanol, to a final protein concentration of 200 µg/µl. Prepared samples were boiled for 10 minutes then separated by electrophoresis through a 12% SDS-polyacrylamide gel. Following separation, the proteins were transferred to a polyvinylidene difluoride membrane (Immuno-PVDF, BioRad) using a semidry blot apparatus as recommended by the manufacturer (BioRad). The membranes were blocked with 5% milk powder in TBST (20 mM Tris pH 7.6, 137 mM NaCl, and 0.1% Tween 20) for 2 hours at RT or overnight at 4°C. After initial blocking, the membranes were incubated with primary antibody diluted to the appropriate concentration in blocking buffer (5% milk in TBST) for a minimum of 2 hours at RT under constant shaking, or overnight at 4°C on a rotator. The membranes were subsequently washed 3x for 5 minutes with TBST, probed with secondary antibodies at the appropriate dilutions in blocking buffer between 1-4 hours at RT with continuous shaking and washed thrice, again for 5 minutes with TBST. The secondary antibodies were

conjugated to horseradish peroxidase, which was visualized by chemiluminescent reaction (Pierce, Thermo Scientific) and captured on photographic film. The primary antibodies were specific to Flag epitope tag (1:5000; Rockland), SMN (1:10000; BD Transduction), Gemin2 (1:1000; Abcam 2E17), myc epitope tag (1:1000; Abcam), or tubulin (1:5000; Calbiochem). The conjugated secondary antibodies were either goat-anti-mouse (BioRad) or rabbit-anti-goat (BioRad).

2.5 Cellular fractionation

A549 cells were seeded at a density of 1×10^6 in a 10 cm dish (Sarstedt) and were grown overnight to semi-confluency. The cells were then infected at an MOI of 10 with Ad-RFP, Ad-C/SMN, and Ad-U/SMN for 1 hour at 37°C with frequent rocking. Following the hour, complete media was added to the cells and they were incubated for 17 hours. Cells were subjected to cytoplasmic and nuclear fractionation based on a modified protocol first published by Challberg and Kelly (1979) (Challberg & Kelly, 1979). Briefly, cells were washed with ice-cold PBS once after the removal of media, and scraped into 1 ml of PBS. Following collection, the cells were pelleted at 3000 x g for 5 minutes at 4°C, then gently resuspended in 1 ml of hypotonic solution (20 mM HEPES pH 7.4, 5 mM KCl, 0.5 mM MgCl₂, 0.5 mM dithiothreitol) containing a final concentration of 0.2 M sucrose. After resuspension, 1 ml of hypotonic solution and sucrose was layered onto the mix and gently inverted 5 times. This solution helped wash the cells to eliminate any contaminants from the media, while maintaining the integrity of the cell membrane. Once again, the cells were pelleted at 3000xg for 5 minutes at 4°C. The cells were gently resuspended in 500 µl of hypotonic solution that did not contain sucrose and incubated on ice for 10 minutes. This step lyses the cells, while maintaining the integrity of the nuclear membrane. To complete the

lyses process, the mixture was transferred to a pre-cooled Dounce homogenizer and the cells were mechanically disrupted with the plunger. The nuclei were separated from the cytoplasm by centrifugation at 2000xg for 5 minutes at 4°C. The supernatant obtained following this spin represents the cytoplasmic fraction. The nuclei were further processed and purified as follows. First, the nuclei were resuspended in 200 µl of nuclei resuspension buffer (50 mM HEPES pH7.5, 10% sucrose). Next, the nuclei were centrifuged through a sucrose cushion composed of 50mM HEPES pH7.5 and 0.88M sucrose at 2800xg for 10 minutes at 4°C. Finally, the nuclei were resuspended in 200 µl of nuclei resuspension buffer and were either frozen at -80°C for further use, or immediately lysed in 2x Laemmli buffer (20 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.1 mg/ml bromophenol blue, 5% β-mercaptoethanol) and analysed by SDS-PAGE.

2.6 Co-immunoprecipitation

A549 cells were seeded at a density of 1×10^6 in a 10 cm dish and were grown overnight to semi-confluency. The cells were then infected at an MOI of 10 with Ad-RFP, Ad-C/SMN, and Ad-U/SMN for 1 hour at 37°C with frequent rocking. Following the hour, cells were supplemented with complete media, incubated for 17 hours, and subsequently lysed with ice-cold NP40 buffer (50 mM Tris pH 7.5, 150 mM NaCl, 1% Nonidet P40, 0.5% sodium deoxycholate, protease tablet). NP40 buffer was preferred over RIPA buffer because it proved better at maintaining protein interactions. The cell lysates were pre-cleared with 5 µl of 50% Dynabead Protein G magnetic bead slurry (Invitrogen) for 30 minutes at 4°C with constant inversions. Co-immunoprecipitation was performed by incubating 250 µg of total cellular protein lysate in NP40 buffer with 1 µg of either rabbit polyclonal Flag or mouse polyclonal Gemin2 antibodies, with constant inversions at 4°C. Following the primary

incubation, 10 μ l of 50% Dynabead Protein G magnetic bead slurry was mixed with the protein-antibody complex and further incubated for 2 hours at 4°C. Beads were washed twice with NP40 buffer and the complex was denatured in 100 μ l 5x SDS-PAGE loading buffer and boiled for 5 minutes before SDS-PAGE and immunoblot analysis. Protein samples were separated using 12% polyacrylamide gels, transferred to PVDF membranes and probed with anti-Flag (1:5000), anti-Gemin2 (1:1000), or anti-SMN (1:10000) antibodies.

2.7 Immunofluorescence and imaging

A549 cells were seeded on 1 cm rounded glass cover slips (Fisher, Ottawa, Ontario) at a density of 1×10^5 cells per well in a 12-well dish. The next day, the cells were infected with Ad-RFP, Ad-U/SMN, and Ad-C/SMN at MOIs of 10, 100, and 1000. Eighteen hours later, the media was removed and the cells were gently washed once with ice-cold PBS, fixed with 4% fresh paraformaldehyde (PFA) in PBS for 10 minutes at RT, and then washed immediately with PBS on an orbital shaker. After two additional 5 minute washes with PBS, the cells were permeabilized with PBS containing 10% horse serum (HS) and 0.4% Triton X-100 for 30 minutes. The cover slips were then incubated with diluted primary antibodies; rabbit monoclonal, anti-Flag, M2 (Sigma Genosys, Oakville, ON; 1:500) and mouse-monoclonal, anti-coilin (BD Transduction Laboratories; 1:50) in PBS containing 0.5% BSA and 0.04% Triton X-100 overnight at 4°C. The next day, the coverslips were washed thrice with PBS for 5 minutes each, and incubated with secondary antibodies; tetrathylrhodamine isothiocyanate (TRITC), or fluorescein isothiocyanate (FITC) conjugated goat anti-mouse antibody (Jackson Immuno Research Laboratories, West Grove, PA; 1:100) and goat anti-rabbit antibody (Jackson Immuno Research Laboratories, West Grove, PA; 1:200) in PBS containing 0.5% BSA and 0.04% Triton X-100 for 1 hour at RT on an orbital shaker. Finally,

the cover slips were washed 3 times for 5 minutes with PBS containing 0.2 µg/ml Hoescht stain (Sigma Genosys, Oakville, Ontario), then applied to glass slides using DAKO fluorescent mounting medium (DAKO, Glostrup, Denmark). Immunofluorescent images were captured using a Zeiss Axioplan 2® fluorescence microscope and an Axiocam HR CCD camera. The images were ultimately processed using ZEN imaging software (Zeiss).

2.8 Protein stability

A549 cells grown in 35 mm dishes were infected at an MOI of 10 with Ad-U/SMN and Ad-C/SMN. A third set of cells treated with PBS served as uninfected controls. Cells were infected for 18 hours and overlaid with complete media supplemented with cycloheximide at a final concentration of 50 µg/ml. Samples were collected hourly using 5x SDS-PAGE loading buffer and analyzed by SDS-PAGE and immunoblot as previously described.

2.9 Cell growth assay

Confluent A549 cells grown in 10 cm dishes were infected at an MOI of 10 or 100 with Ad-RFP, Ad-U/SMN, or Ad-C/SMN. A dish treated with PBS was used as an uninfected control. Three hours after infection, the cells were trypsinized, counted, and re-seeded in 35 mm at a density of 1×10^5 cells/dish. Cell growth was monitored according to protocols that have been previously published (Hubberstey, Pavliy, & Parks, 2002; Ross et al., 2011). All treatments were performed in duplicate on a five day time course. All dishes were washed with 1 ml of PBS and fixed with 1 ml of β-Galactosidase fixative solution (0.2% glutaraldehyde, 2% paraformaldehyde, and 2 mM MgCl) for 10 minutes at RT. After fixing, the cells were washed thrice with PBS for 5 minutes and stained with a solution containing 0.1% crystal violet in water for 30 minutes on an orbital shaker. Excess stain was removed by thoroughly washing the plates under running water, and the plates were air-dried overnight. Following

the fifth day, the crystal violet was re-solubilized in a 20% acetic acid solution for 30 minutes. An aliquot of each sample was loaded on a 96-well plate and the absorbance at A_{595} was determined using a spectrophotometer. The original crystal violet solution was serially diluted to ensure that the assay was within the linear range of detection.

2.10 Promoter activity quantification by reporter gene expression

Using reporter gene constructs, we examined if SMN protein was able to affect the activity of different promoters in cells infected by the therapeutic viruses. Two plasmids covering the upstream regulator region of the human SMN gene (Monani, McPherson, & Burghes, 1999; Rouget et al., 2005) were obtained from Dr. Louis Simard (University of Manitoba). Briefly, the SMN promoter covered the sequence m46p125 (all distances in relation to the transcription initiation site (TIS), m (minus), and p (plus), and the 4kb-SMN (P4.0T) has a 3.7 kb fragment upstream of the TIS and 283 bp downstream. The m46p125, which was amplified by PCR, was introduced into a *EcoRI* restriction site that was added between the *BglIII* and *HindIII* in the multiple cloning cassette upstream of the luciferase reporter gene in the promoterless pGL3 vector (Rouget et al., 2005). Similarly, the P4.0T vector was constructed by isolating a PCR amplification of the human SMN promoter region 4.0kb upstream of the TSS and inserting it into the *KpnI* restriction site in the pGL3 vector (Monani, McPherson, et al., 1999). Two housekeeping promoters, elongation factor 1a (a gift from Dr. Shigekazu Nagata, Kyoto University (Mizushima & Nagata, 1990)), and mouse phosphoglycerate kinase (a gift from Dr Lucio Pastore, CEINGE-Biotecnologie Avanzate, Italy (Pastore et al., 1999)) were used as positive and a negative control respectively. These promoters controlled the expression of firefly luciferase and were transfected into the cells 24 hours before infection with Lipofectamine (Invitrogen) as per the

manufacturer's instructions. The next days, the cells were infected with either Ad-RFP or Ad-C/SMN at an MOI of 10. Eighteen hours after the start of the infection, the cells were collected in reporter lysis buffer (Promega) and the luciferase activity was measured with a luminometer (Turner BioSystems). The linear range of the assay was determined with a serial dilution of pure firefly luciferase.

A modified version of the CheckMate Mammalian Two-Hybrid System (Promega) was used to further evaluate the capabilities of SMN to modulate promoter activity. Dr. Yoichi Kawabe (Ottawa Hospital Research Institute, Canada) kindly provided the original plasmid constructs: pBIND, pACT, and pG5-luc. We generated modified versions of the assay's plasmids first by fusing the GAL4 DNA binding domain to SMN (pBIND-SMN). Briefly, the SMN gene was isolated from pCD2-SMN by enzyme digestion with *NotI* and *BglIII*. This fragment was then inserted into the *BamHI* restriction site located at the C-terminus of the GAL4 sequence of pBIND. We generated a positive control by combining the VP16 transcriptional activator from pACT and the GAL4 DNA binding domain (pBIND-VP16) into the *BamHI* restriction site of pBIND. Both of the plasmids, or an empty pBIND negative control, were co-transfected with the pG5-luc reporter plasmid (5 Gal4 binding sites driving the expression of firefly luciferase) into 35 mm dishes of A549 cells. Lysates were collected and analyzed for luciferase activity 24 hours after transfection, using the procedure previously described.

2.11 mRNA quantification and splicing analysis

A549 cells in 35 mm dishes were infected for 1 hour at an MOI of 10, 100, and 1000 with Ad-U/SMN or Ad-RFP. Seventeen hours later, total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and the concentration of

the resulting RNA was quantified using a NanoDrop 1000 spectrophotometer (Thermo Scientific). Synthesis of cDNA was performed using the M-MLV reverse transcriptase kit (Invitrogen) with RNase inhibitor (Applied Biosystems), random primers (Sigma) and 2 µg of RNA, according to manufacturer's instructions. Real-time quantitative PCR (qPCR) was performed on an Mx3000P QPCR System using the PerfeCTa SYBR Green FastMix Low ROX kit (Quanta). The PCR conditions were: 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds, 58°C for 1 minute, and 72°C for 1 minute. The primers were designed to either target full-length SMN (both the endogenous and exogenous copies) by amplifying exons 6-7 while endogenous SMN was specifically amplified with primers directed against the 3' untranslated region (UTR) and exon 9 (Total SMN, Forward Primer: 5'-CAC TTA CTA TCA TGC TGG CTG CCT; Reverse Primer: 5'-GAA TCT GGA CAT ATG GCA GGT GGT. Endogenous SMN, Forward Primer: 5'-ACG GTG GTG AGG CAG TTG A; Reverse Primer: 5'-AAA CTA CAA CAC CCT TCT ACA GC). All reactions were negatively controlled with a no-reverse transcriptase (no-RevT) and a no-template reaction. The specificity of the primers was confirmed by dissociation curve, and a standard curve was generated by pooling cDNA from uninfected samples and serially diluting by factors of 5 to 1/625.

Changes in splicing of the endogenous SMN transcript were investigated by reverse-transcriptase PCR (RT-PCR). cDNA was prepared as described above. The PCR conditions were: 95°C for 10 minutes, followed by 40 cycles at 95°C for 30 seconds, 59°C for 1 minute, 72°C for 1 minute, and a final extension of 10 minutes at 72°C. The primers that were used exon 6-8 (Forward: 5' – ACA TCA AGC CCA AAT CTG CTC CA; Reverse: 5'-AAA CTA CAA CAC CCT TCT ACA GC). The oligonucleotide binding site for the 3' UTR oligo is

not present in the viral constructs, thus limiting analysis exclusively to the endogenous gene. PCR products were separated by 1% agarose gel electrophoresis. In a similar manner, splicing of the *Usp11* transcript was examined using the synthetic oligonucleotides (Forward: 5'-AGT TCG GGT CCA CTG TAT GC; Reverse: 5'-GGC TTA CTT GGA GTG GGA CA) (H. Liu et al., 2010).

2.12 Injection of chimeric adenovirus in Wild-Type mice

Six week old C57Bl/6 mice were obtained from (Charles River, Sherbrooke, QC, Canada) and housed in the University of Ottawa vivarium. All animal experiments were approved by and performed according to the guidelines set by the Animal Research Ethics Board at the University of Ottawa (Ottawa, ON, Canada). Mice received an intra-vascular (tail vein, 300 μ l), an intra-peritoneal (200 μ l), or an intra-muscular (*Tibialis anterior* (TA), 25 μ l) injection of one of the viral constructs. Four different viruses were used (Table 2.1): Ad5-LacZ, Ad5s-LacZ, Ad5/35-LacZ, and Ad5spk-LacZ. Animals injected with equivalent volumes of PBS were used as negative controls. The intra-vascular injections were performed at a viral concentration of 5×10^8 viral particles/gram of body weight (Vp/g). The IP injections were also carried out at 5×10^8 Vp/g except for injections with Ad5-LacZ. This virus was used at 5×10^7 Vp/g because the concentration of the stock did not allow for the appropriate volumes at the higher working stock concentration. The viral load for the TA injections was lowered to 5×10^7 Vp/g, except for Ad5-LacZ, which was used at 5×10^6 Vp/g. Twenty-four hours after the injections, animals were sacrificed by intra-peritoneal injection with Euthanyl followed by cervical dislocation. The liver, diaphragm, and TA muscles were collected and flash frozen in liquid nitrogen before storage at -80°C for β -galactosidase assay. The spinal cord and the sciatic nerves were dissected and prepared for sectioning. Tissues were fixed in 2%

PFA fixative solution (2% paraformaldehyde, 0.08M Na₂HPO₄, 0.02M NaH₂PO₄, 0.002M MgCl₂, and 0.00125 M EGTA pH 7.4) for 1 hour at 4°C, washed three times in PBS for 5 minutes, then cryoprotected by incubation in a solution of 30% sucrose in PBS overnight at 4°C. Finally, tissues were embedded in OCT (Tissue-Tek) in plastic mounts, frozen on dry-ice and stored at -80°C until further processed.

2.13 Tissue staining

Tissues were sectioned to 14 µm in a cryostat, placed on glass slides and dried at RT for a minimum of 2 hours. The tissues were rehydrated by placing the slides in a coplin jar and submerging them in PBS twice for 10 minutes. To stain the tissue, the slides were submerged in X-gal stain solution (2 mM MgCl₂, 0.01% Sodium deoxycholate, 0.02% NP-40, 0.1M Phosphate buffer pH 7.3[0.43M NaH₂PO₄, 0.13M Na₂HPO₄], 0.1% X-gal, 5.4 mM Potassium ferrocyanide, 4.6 mM Potassium ferricyanide) overnight in a 37°C water bath. The next day, the slides were washed twice with PBS for 10 minutes and a glass coverslip was placed over the tissue with aqueous mounting medium (50% glycerol, 50% PBS). The slides were kept at RT until analyzed by light microscopy (ZEISS). Images were processed using ZEN software.

2.14 β-Galactosidase detection in tissue

The level of viral expression of the LacZ gene in the liver, TA, and diaphragm, was determined by a β-galactosidase assay. Whole livers were transferred to 15 ml bacterial culture tubes (Startsted) and 3 ml of PBS was added. Crude protein extracts were obtained by blending at ½ maximum intensity twice for 15 seconds with a 20 second break, sonicating at 1/3 maximum intensity, twice for 15 seconds with 10 second breaks, and centrifuged at 2000 RPM for 5 minutes at 4°C. TA muscle and diaphragm were crushed into a fine powder with a

pestle and mortar, in liquid nitrogen. The powder was resuspended in 500 μ l and 250 μ l lysis buffer respectively (Galacto-Star™ System, Applied Biosystems; 100 mM potassium phosphate pH 7.8, 0.2% Triton X-100) and tissue debris was pelleted by centrifugation at 16000g for 10 minutes at 4°C. The crude lysates were transferred to microcentrifuge tubes before storage at -80°C. Once all the samples were processed, the aliquots were thawed and heated at 50°C for 15 minutes in a waterbath to denature endogenous β -galactosidase. A 10 μ g aliquot of each lysate was mixed with 300 μ l of Reaction buffer (Applied Biosystems; 100 mM sodium phosphate pH 7.5, 1 mM magnesium chloride, 5% Sapphire-II enhancer, 1x Galacto-Star™ substrate), incubated at RT for 1 hour and read with a luminometer (Turner Biosystems). A serial dilution of lyophilized β -galactosidase reconstituted to 40 ng/ml to 1.28×10^{-2} ng/ml was used to verify the linear range of the assay.

Chapter 3 – Supraphysiological expression of recombinant SMN from an adenovirus vector

3.1 Introduction

As previously discussed, small increases in the amount of available full-length SMN protein can make a difference in the severity of SMA (Table 1.1). In mice models of SMA, gene therapy has made significant advances. Several groups have treated SMA through gene therapy with a “good” copy of the SMN gene with positive results (Azzouz et al., 2004; DiDonato et al., 2003; Foust et al., 2010; Glascock, Shababi, et al., 2012; Valori et al., 2010). Delivery of full-length SMN by a viral vector, self-complementary adeno-associated virus 9 (sc-AAV9), extended the lifespan of the severe mouse model (SMN Δ 7) from an average of 14 days to 69 days (Foust et al., 2010; Valori et al., 2010). Some mice lived as long as 150 days (Valori et al., 2010) and 250 days (Foust et al., 2010). In the Study by Valori *et al.* (2010) (Valori et al., 2010), the treated mice, while remaining smaller, continually gained weight well into adulthood at the same rate as wild-type litter controls. The treated mice also recovered musculature as well as motor-functions. Protein analysis demonstrated that in most tissues, the SMN protein levels were the same or higher than carrier controls.

Unfortunately, the nature of the vector used in this study led to high levels of off-target infection, specifically in the liver. To date, no studies have been performed to our knowledge that demonstrate the safety of such high expression of SMN in non-target tissues. Evidence exists, in other disease models however, demonstrating potential pitfalls for a non-targeted gene therapy approach to treating SMA. Grimm *et al.* (2006) (Grimm et al., 2006) attempted to deliver short hairpin RNA (shRNA) to hepatocytes by intravenous injection

with adeno-associated virus type 8 (AAV8). Out of 49 constructs tested, 36 caused dose-dependent liver injuries, while 23 ultimately caused death. These investigators determined that high concentrations of shRNA competed with endogenous microRNAs (miRNAs) in the liver and shut down a portion of the endogenous small RNA processing pathways.

There are two main factors that are worrisome for a potential gene therapy for SMA that utilizes an adenovirus vector. As previously mentioned, these types of vectors lead to high-level expression of the therapeutic in hepatocytes. Secondly, SMN is known to regulate the assembly of RNA protein complexes as well as the processing of pre-mRNA. Over-saturation of hepatocytes, and other non-target tissues, could affect these pathways in a manner similar to the effects observed in the Grimm *et al.* (2006) study.

Thus, we hypothesized that high levels of recombinant SMN protein, delivered by an adenovirus vector, could negatively affect wild-type cells. In order to elucidate the potential pitfalls, we infected different cells lines with different quantities of full-length SMN adenoviruses and analyzed (i) protein levels, stability and interactions (ii) localization, and (iii) the effects on transcript availability and processing.

3.2 Results

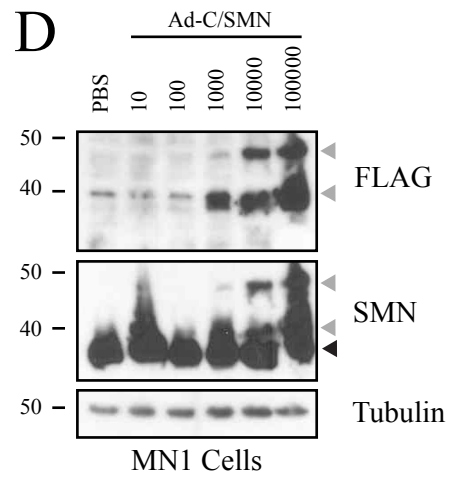
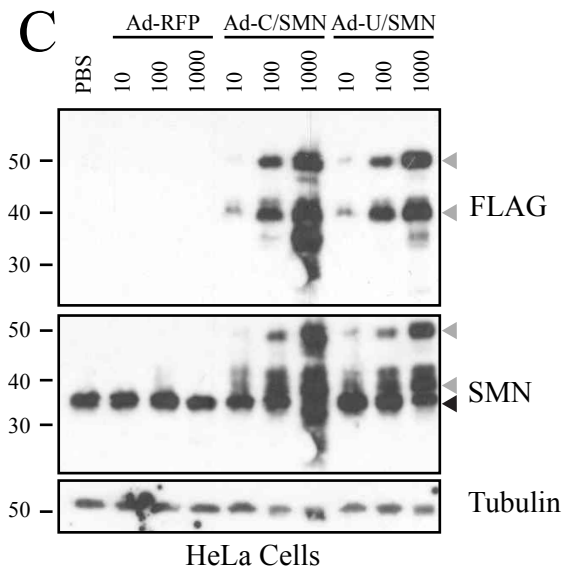
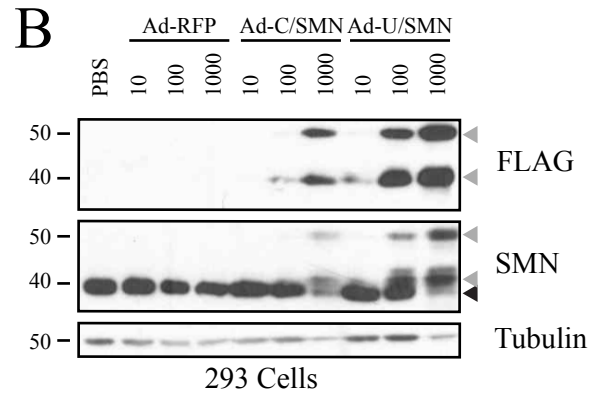
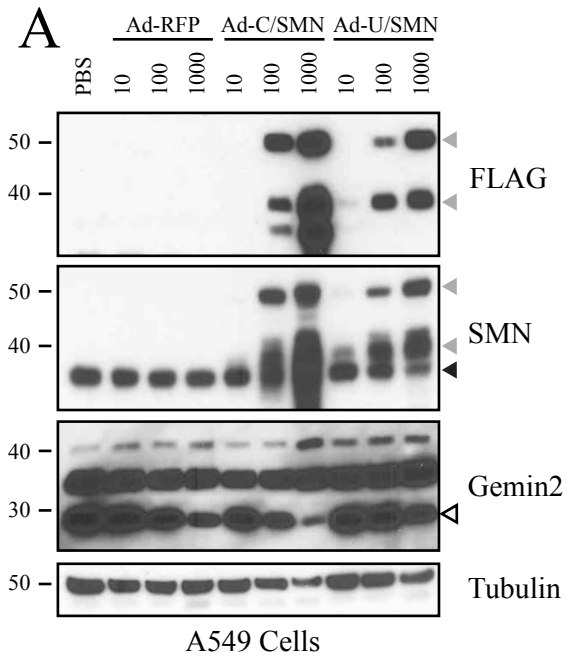
3.2.1 Infection with Ad-SMN leads to supraphysiological levels of expression of SMN in several cell lines

All cell lines (A549, HeLa, 293, and MN1) were seeded at a density of 1×10^5 cells/ml in 35 mm tissue culture dishes and allowed to grow to 90% confluence. The cells were then infected at varying MOI with Ad-RFP, -C/SMN, or -U/SMN and lysed 18 hours later. Whole protein extract were prepared from lysates, separated by SDS-PAGE and examined by immunoblot for exogenous SMN using the anti-FLAG antibody, total SMN, or Gemin2. Tubulin was used as a loading control. Cells infected with Ad-RFP were used as controls, and did not have a FLAG signal (Figure 3.1, Panels A, B, and C). In A549 cells, infection with both Ad-SMN viruses yielded FLAG signals at approximately 39 kDa (the expected size of FLAG-SMN; Figure 3.1A) that demonstrated a correlation in signal intensity with increasing MOI. Additionally, we observed a band at 50 kDa with both viruses and a band at 37 kDa with Ad-C/SMN. All cells including the Ad-RFP and untreated controls, when treated with the anti-SMN antibody, had a band at approximately 37 kDa representing endogenous SMN. Interestingly, cells treated with both Ad-SMN viruses had signals at 39 kDa and 50 kDa (Figure 4A), confirming that both the observed FLAG signals are in fact of viral origin and represent an SMN-derived protein species. The viral SMN signals also confirmed the relationship between increases in MOI and increases in Ad-SMN expression. By comparing the endogenous and exogenous SMN signal, we observed that the construct under the control of the CMV promoter produced very high levels of recombinant protein. On the other hand, the UbC promoter virus showed a 2-fold protein over-expression relative to endogenous protein at an MOI of 100. This was increased to approximately 10-fold over-

expression at an MOI of 1000. Signals for control antibodies, Gemin2 and Tubulin, decreased in protein extracts from cells infected at the highest MOI. These results were recapitulated in other cell lines, notably HeLa and 293 cells (Figure 3.1 B and C). Finally, viral SMN is detected in abundance in MN1 only in cells infected with a minimum of 100 viruses per cell (Figure 3.1D). The FLAG and SMN signals are both detectable, however, the expression pattern established in other cell lines only occurs with virus concentrations 100-fold higher.

Therefore, this data establishes that the viruses can effectively infect several different types of cells. The viruses are also able to express the transgene. Most importantly, we are able to control the relative amount of exogenous SMN in the treated cells by varying the amount of virus used at the time of infection. Unfortunately, MN1 cells would be more representative of the target cells for an SMA gene therapy. The inability of the viruses to infect these cells has rendered them useless in the scope of this study.

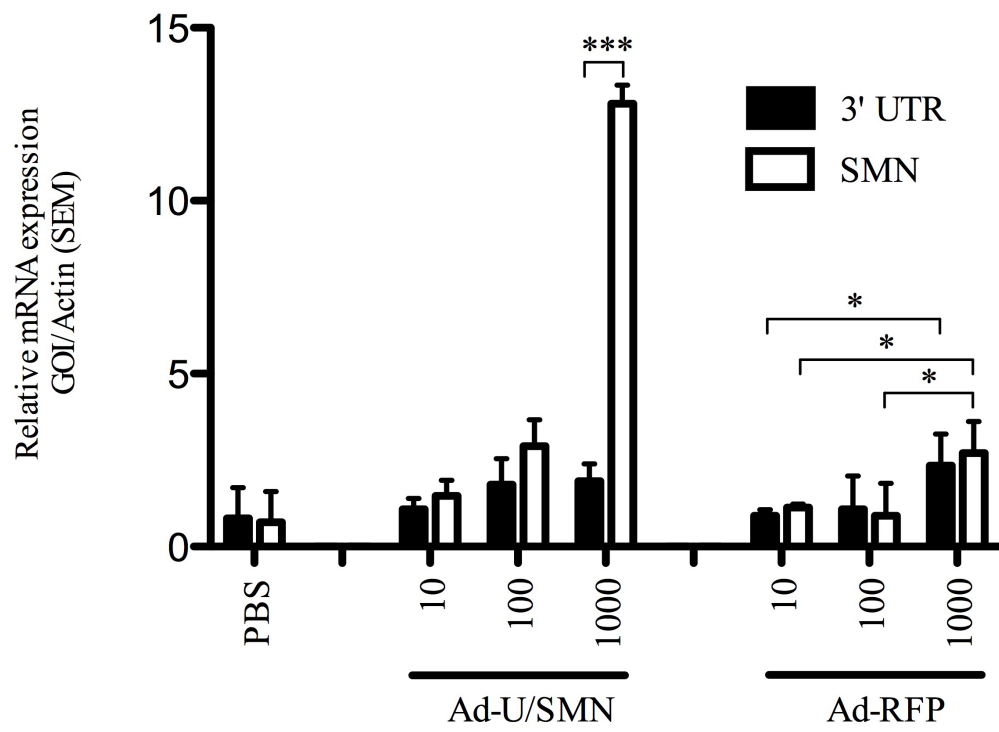
Figure 3.1. Ad-SMN infection can mediate high level protein expression in a variety of cell lines. A549 (Panel A), HeLa (Panel B), 293 (Panel C), and MN1 (Panel D) cells were infected with increasing MOI of Ad-RFP, Ad-C/SMN or Ad-U/SMN. Crude protein extract was prepared 18 hours after infection, separated by SDS-PAGE, and subjected to immunoblot analysis with antibodies to FLAG, SMN, Gemin2 or tubulin. The gray arrows (◄) denote the viral proteins while the black arrows (◄) represent the endogenous SMN protein. The white arrow (◄) identifies Gemin2. Representative results of multiple experiments.



3.2.2 Increases in Ad-SMN transcript level is proportional to the amount of virus

We measured the relative levels of expression from Ad-U/SMN by qPCR using specifically designed primers that differentiated between total SMN and endogenous SMN (3' UTR, Figure 3.2). All values were normalized to β -actin and represent a sample size of 2 (n=2). The only statistically significant conclusion from this experiment is that there is an increase in total SMN transcript observed when the cells are infected at an MOI of 1000 with Ad-U/SMN. The endogenous SMN has a relative expression level of 1.90 (± 0.49) while total SMN has a relative expression level of 12.80 (± 0.53), representing a 6.7-fold increase ($p = 0.0045$). On the other hand, at an MOI of 100, the increase in total SMN (2.90 ± 0.77) is not significantly higher than the level of endogenous SMN (1.79 ± 0.75 ; $p = 0.41$). None of the other experimental conditions yielded a significant increase in transcript levels.

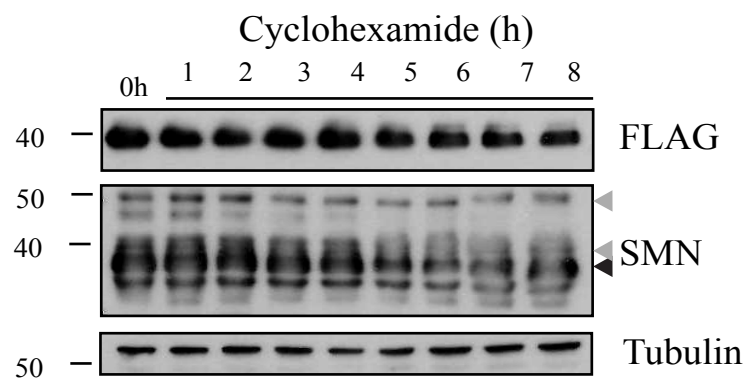
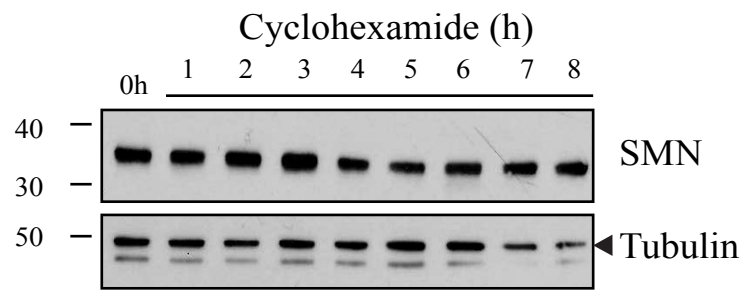
Figure 3.2. mRNA levels correlate with high levels of protein expression mediated by Ad-SMN. A549 cells were infected with an increasing MOI of Ad-RFP or Ad-U/SMN and total RNA was isolated 18 hours after infection using Trizol reagent. RNA was converted to cDNA, and the amount of total SMN transcript (viral + endogenous) or endogenous transcript determined by quantitative PCR using primers specific to each species. β -actin transcript levels were used to normalize expression data. Data represents n = 2.



3.2.3 The stability of Ad-SMN is similar to the endogenous protein

Cells that had previously been infected with Ad-C/SMN or PBS (18 hours earlier) were treated with cycloheximide (50 μ g/ml) in order to measure the stability of the two forms of SMN. Both endogenous and protein produced from Ad-SMN have a similar stability profile (Figure 3.3). Curiously, the amount of the 50 kDa variant previously described in Figure 3.1 only decreases slightly over 8 hours. Tubulin was used as a loading control as the half-life is approximately 15 hours (Ducommun & Wright, 1989). Therefore, virally produced SMN has a similar stability profile as endogenous protein.

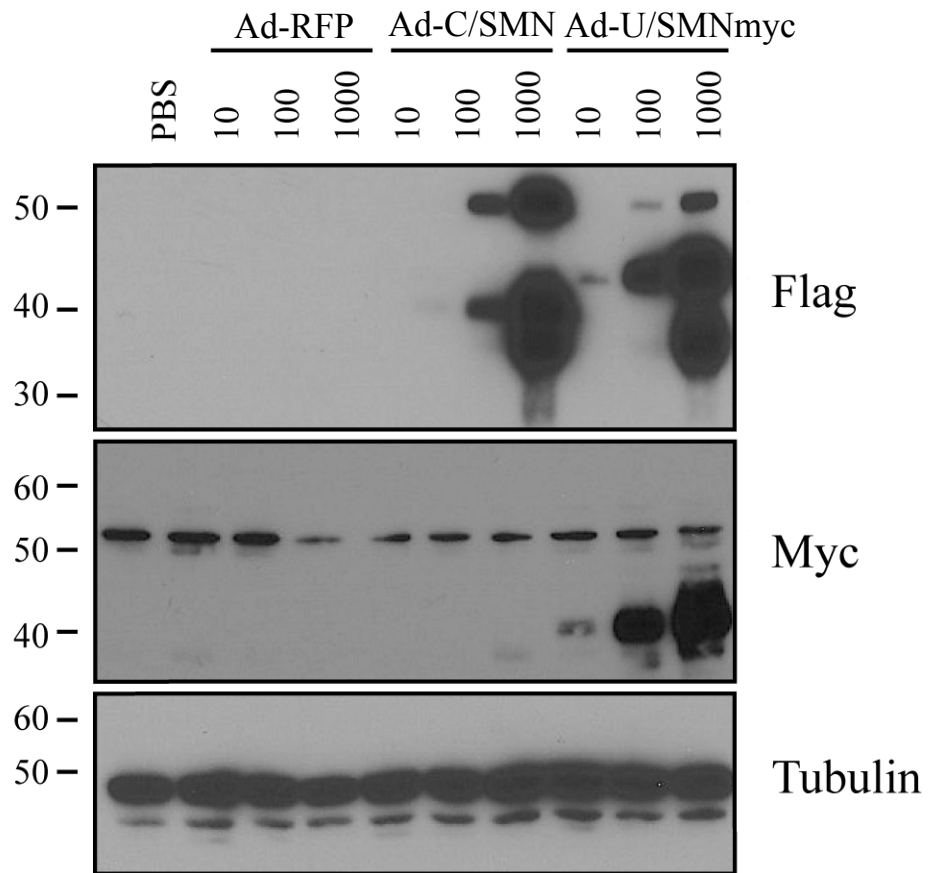
Figure 3.3. The stability of virally derived SMN is similar to endogenous SMN. A549 cells were infected with Ad-C/SMN at an MOI of 10 or cells treated with PBS were used as controls (Top panels). Eighteen hours later, the cells were treated with cycloheximide (50 $\mu\text{g/ml}$). Protein lysate was collected periodically over 8 hours and analyzed by immunoblot for FLAG, SMN, or Tubulin (bottom panels). Ad-SMN is denoted by the gray arrow (\blacktriangleleft), and endogenous SMN by the black arrow (\blacktriangleleft). Representative results of multiple experiments ($n > 3$)



3.2.4 The 50 kDa Ad-SMN is differentially spliced at the C-terminal

As seen in Figure 3.1, cells infected with Ad-C/SMN produced an SMN protein species that was detected using anti-FLAG at approximately 40 kDa and 50 kDa. We suspected that the 50 kDa SMN protein was differentially spliced. Thus, A549 cells were infected with Ad-C/SMN, Ad-U/SMNmyc, or Ad-RFP (and PBS) at varying MOIs. Protein lysates were collected 18 hpi and separated by SDS-PAGE. The samples were subjected to immunoblot analysis with antibodies against FLAG, Myc. Tubulin was used as a loading control. As expected, the Ad-C/SMN lysates were negative for the Myc epitope (Figure 3.4). As for the cells infected with Ad-U/SMNmyc, FLAG signal was detected at approximately 43 and 50 kDa. The smallest of these proteins also cross-reacted with the Myc epitope antibody while the 50 kDa did not. The signal observed at approximately 52 kDa is non-specific antibody binding as it is also observed in the uninfected as well as the Ad-RFP infected control cells. Tubulin was once again used as a control. Therefore, this data demonstrated that the C-terminus of the 50 kDa viral SMN variant no longer contained the Myc epitope tag. This protein is likely the result of an aberrant splicing event into the Ad backbone. Curiously, this phenomenon's occurrence increased as the concentration of virus used during infection increased, potentially demonstrating one of the pitfalls associated with over-expression of viral transgenes.

Figure 3.4. The 50 kDa Ad-SMN variant is differently spliced at the C-terminal end. A549 cells were infected with varying concentrations of Ad-C/SMN, Ad-U/SMNmyc, or Ad-RFP (or PBS). Protein lysates were collected 18 hours later, separated by SDS-PAGE and subjected to immunoblot analysis for FLAG, Myc, or Tubulin.



3.2.5 SMN protein produced from adenovirus vectors localizes correctly within the cell and interacts with its normal cellular protein partners.

We sought to determine whether SMN produced from an adenoviral vector could co-localize with p80-coilin in the nucleus of wild-type cells. A549 cells were infected with Ad-U/SMN at an MOI of 10 or 100 (or with PBS) and processed for immunofluorescence analysis 18 hours later. We observed distinct foci in the nucleus of cells analyzed with the anti-FLAG primary antibody, identifying exogenous SMN. Furthermore, the positive FLAG staining was found to co-localize with p80-coilin, a marker of Cajal bodies, in the nucleus of infected cells (Figure 3.5). Therefore, SMN protein from a viral source can be localized in proximity to its normal cellular partners. We also observed low-level FLAG staining in the cytoplasm of cells, which is also observed for endogenous SMN protein.

In addition, relative cellular distribution of SMN protein was determined by cell fractionation. A549 cells were infected with Ad-U/SMN, -C/SMN, -RFP or PBS and the nuclei were separated from the cytoplasmic fraction 18 hpi. In the control infections (PBS or Ad/RFP), endogenous SMN was detected at a roughly equal distribution between the two cellular compartments (Figure 3.6A). The presence of exogenous SMN protein did not affect the cellular distribution of SMN interacting protein such as Gemin2 or even endogenous SMN. This data demonstrates that as well as being found in proximity to Cajal bodies, a normal neighbor of endogenous SMN, virally produced SMN has the same sub-cellular distribution as endogenous SMN.

We investigated the ability of Ad-SMN to interact *in vitro* with endogenous Gemin2 by co-immunoprecipitation. A549 cells were infected with Ad-U/SMN, -C/SMN, -RFP, or

PBS and processed for co-immunoprecipitation 18 hours later. Immunoprecipitation with a FLAG antibody detected an interaction between exogenous SMN and endogenous SMN as well as an SMN-Gemin2 interaction only in cells treated with Ad-SMN (Figure 3.6B). The reciprocal assay, immunoprecipitating with a Gemin2 antibody, confirmed these results (data not shown). Taken together, these data show that viral SMN can interact with the same cellular partners as endogenous SMN.

Figure 3.5. SMN protein produced from adenovirus vectors localizes in the vicinity of Cajal bodies. A549 cells grown on glass cover slips were infected with Ad-U/SMN at an MOI of 10 or with PBS as an uninfected control. Eighteen hours later, the infected cells were fixed with 2% PFA and subjected to immunofluorescence treatment, identifying FLAG-tag (green) and p80-coilin (red). Heterochromatin was stained using Hoescht to identify the nuclei. Images were captured with confocal imaging.

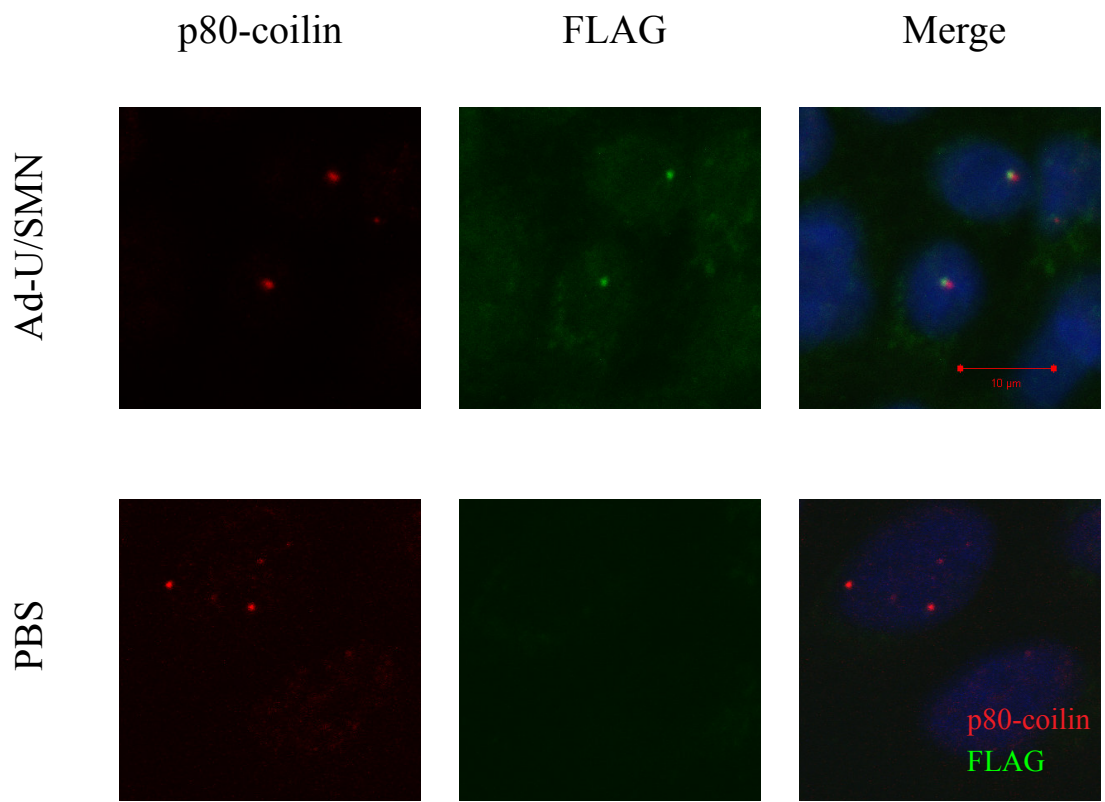
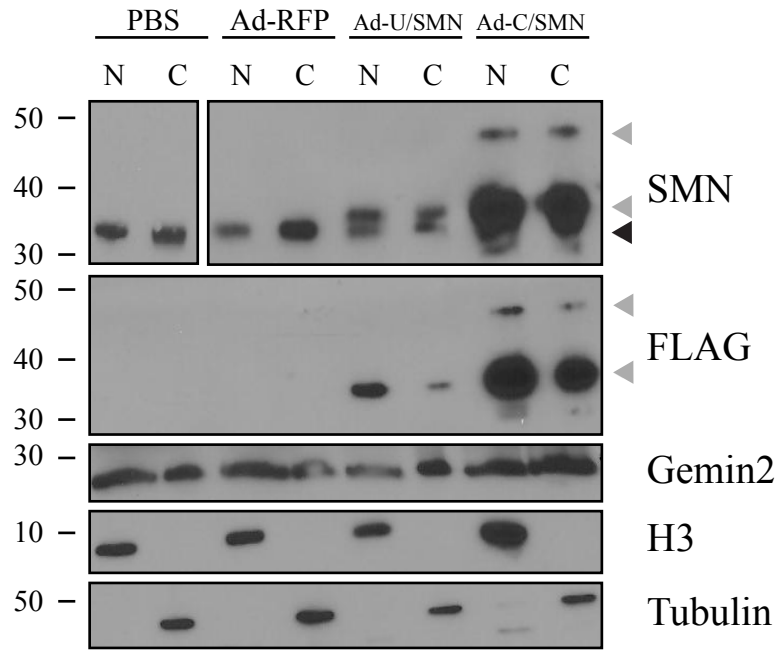
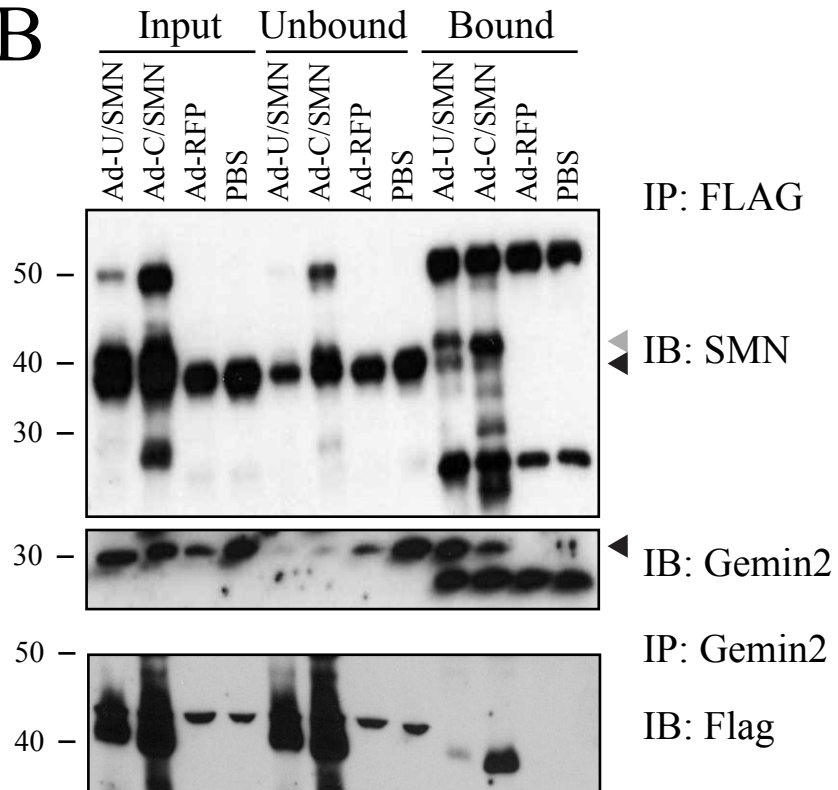


Figure 3.6. Viral SMN has a similar sub-cellular distribution pattern as endogenous SMN and it interacts with SMN and Gemin2. A549 cells were infected with Ad-U/SMN, -C/SMN, -RFP at an MOI 10 (or PBS) for 18 hours (Panel A). A549 cells were fractionated to obtain cytoplasmic and nuclear protein samples. The proteins were separated by SDS-PAGE and immunoblotted for FLAG, SMN, and Gemin2. Probing for histone 3 (H3, nuclear) and tubulin (cytoplasmic) helped assess the quality of the fractionation procedure. Following the same infection protocol, Protein lysates were collected and subjected to co-immunoprecipitation using the FLAG-tag (upper frames) or Gemin2 (bottom frames) antibodies (Panel B). The resulting protein extracts were analyzed by SDS-PAGE with antibodies against SMN, Gemin2 and FLAG. Gray arrows (◄) denotes exogenous protein while the (◄) marks endogenous protein.

A



B



3.2.6 Over-expression of Ad-SMN does not have a negative impact on the function of target gene promoters or splicing.

The data presented in Figure 3.1 initially led us to believe that the increases of exogenous SMN between MOI 10 and MOI 1000 infections in A549 cells were having a negative effect on the levels of endogenous SMN. We hypothesized that SMN protein was involved in a feed-back regulation loop in order to regulate its own protein levels. Using reporter plasmids containing the upstream regulator region of the SMN gene (Monani, McPherson, et al., 1999; Rouget et al., 2005), we attempted to understand whether high-level expression of the exogenous SMN could affect the endogenous promoter activity. As can be seen in Figure 3.7, the reporter plasmids behaved no differently whether they were exposed to high levels of control virus (Ad-RFP) or the therapeutic virus (Ad-C/SMN). No values reached a point of statistical significance (95% confidence) with a Student's T-test. This observation holds true for both control promoters (PGK and EF1 α). Thus, at this expression level and within these parameters, SMN is not involved in a regulation loop to control its own protein levels.

There is also evidence demonstrating that SMN can regulate gene expression and transcription of other genes (Strasswimmer et al., 1999; Zou et al., 2004). In order to verify whether SMN could act as a transcription factor, we modified a mammalian two-hybrid reporter assay. As can be seen in Figure 3.8A, co-transfecting with GAL4 linked to SMN (pBind-SMN) and the reporter construct pG5-luc, resulted in the same relative luminescence as pG5-luc alone or as co-transfecting pG5-luc with an empty GAL4 (pBind-empty). The only increase in activity observed was with pBind-VP16 (a positive control from herpes

virus), due to its inherently high transcriptional activity (315005 ± 76180.9 ; $p=0.003$). Therefore, it does not appear that SMN can act as a transcription factor on its own.

As previously discussed, SMN can modulate the pre-mRNA splicing of its own transcript (Jodelka, Ebert, Duelli, & Hastings, 2010). When levels of SMN decrease, exon 7 is skipped more often in the SMN2 gene, exacerbating the decrease in protein. We attempted to determine whether the opposite was also true: if SMN is over-expressed, will the proportions of SMN transcripts lacking exon 7 decrease. A549 cells were infected at varying MOI with Ad-RFP, -C/SMN, or -U/SMN and lysed 18 hours later. Total mRNA was extracted with TRIzol and cDNA was obtained by reverse transcription. A specific 3'UTR primer was used for the reverse transcription reaction in order to eliminate viral constructs from the analysis. The subsequent cDNA was then analyzed by PCR with primers flanking exon 7. This strategy allowed us to amplify both full-length endogenous SMN as well as endogenous SMN Δ 7. As can be observed in Figure 3.8B, infections with Ad-U/SMN, regardless of the viral concentration, did not affect the ratio between the full-length construct and SMN Δ 7. However, we can observe a change when cells were treated with increasing quantities of Ad-C/SMN. As the amount of virus is increased, the amount of SMN Δ 7 decreases. This is concomitant with an increase in full-length transcript. Unfortunately, the phenomenon is also observable when the cells are subjected to the same concentrations of control Ad-RFP virus. Thus, the change is likely caused by the presence of increasing quantities of virus as opposed to an increase in SMN protein.

Finally, the splicing pattern of ubiquitin specific peptidase like 1 (Usp11) protein was analyzed following infections with increased concentrations of Ad-SMN. When SMN protein levels are reduced, the medium form (M) of Usp11 is preferred over the small form (S)(H.

Liu et al., 2010). We chose to analyze this gene to represent general cellular splicing of other genes because the original change in splicing after SMN depletion was observed by our collaborators. We hypothesized that increasing the SMN protein levels would have the opposite effect, meaning that it would favor the S-form of Uspl1. A549 cells were infected at varying MOI with Ad-RFP, or -C/SMN. Whole protein extract were prepared from lysates, separated by SDS-PAGE and examined by immunoblot for Uspl1. No change from M to S-form is observed when the concentration of Ad-C/SMN is increased in A549 cells (Figure 3.8C). Thus, an increase in levels of exogenous SMN protein does not affect cellular splicing of a gene known to be affected in SMA.

Figure 3.7. Over-expression of Ad-SMN does not have a negative impact on the function of target gene promoters. A549 cells were transfected with luciferase reporter constructs controlled by two SMN promoter sequences (m46p125 or 4 kB), murine phosphoglycerate kinase (PGK) or elongation factor 1 α (EF1 α). The cells were infected with Ad-C/SMN or – RFP (MOI = 10) the next day and analyzed for luciferase activity 18 hpi. Data represents the average and standard error (n=2).

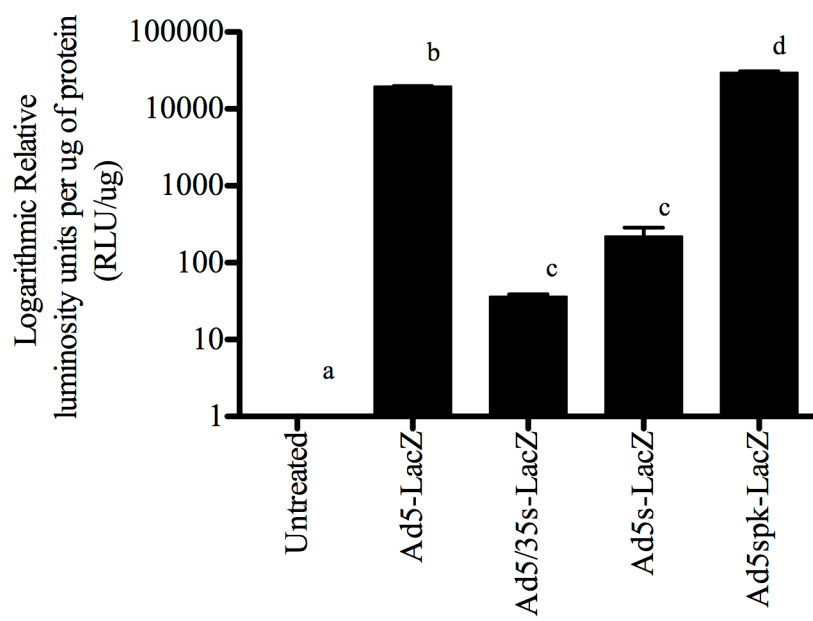
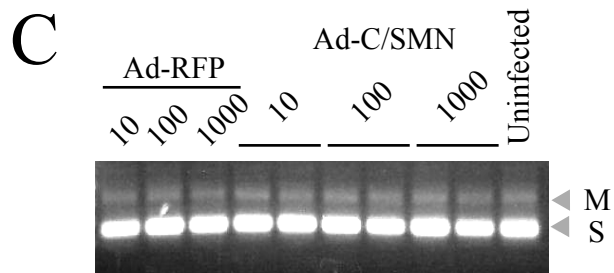
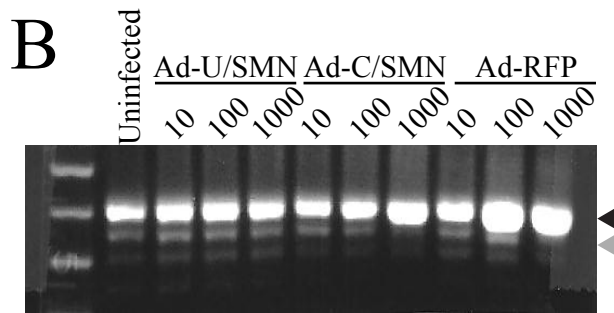
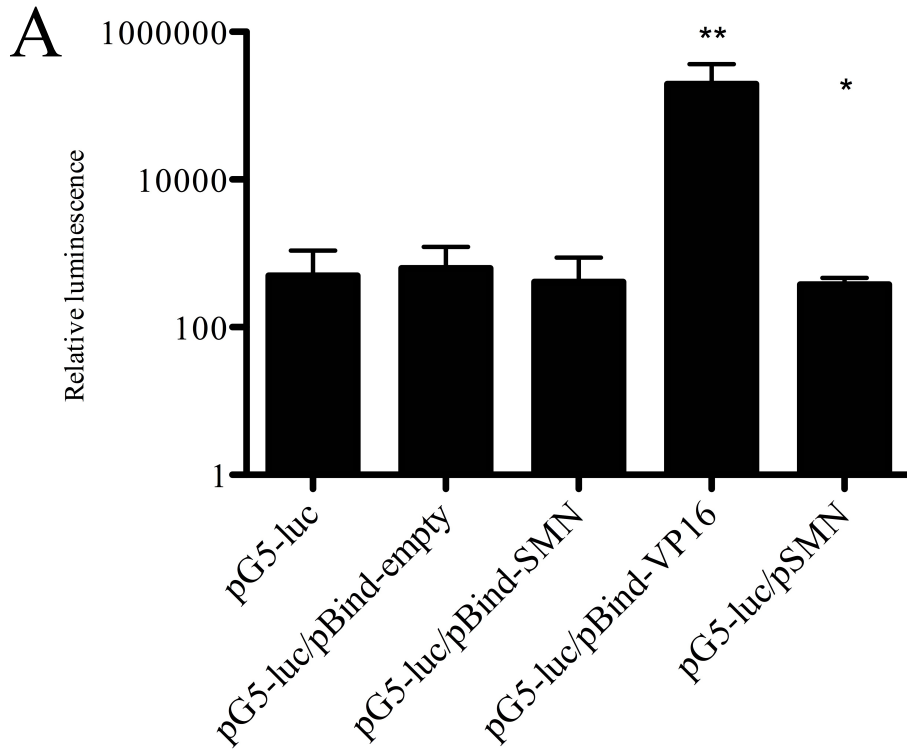


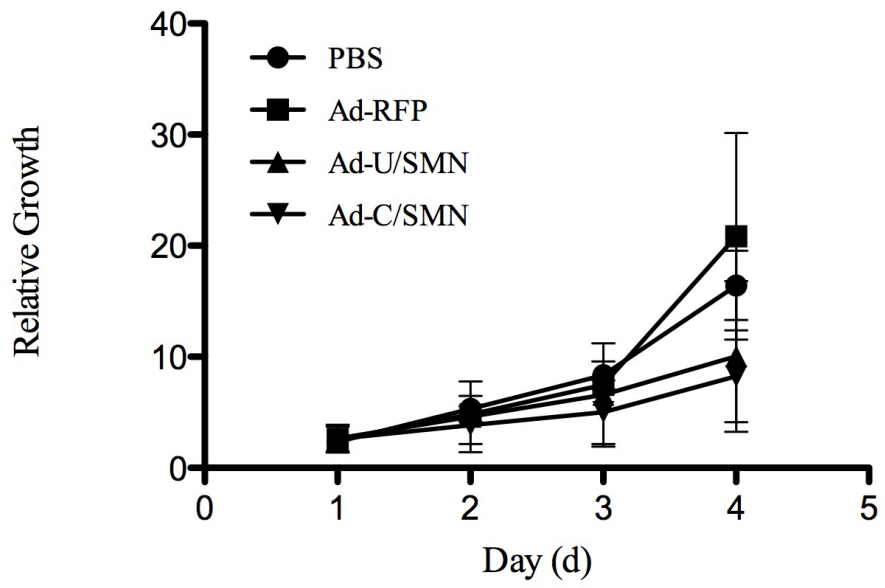
Figure 3.8 Over-expression of Ad-SMN does not have a negative impact on splicing. A549 cells were transfected with a luciferase reporter plasmid containing five GAL4 binding domains (pG5-luc) in conjunction with plasmids expressing GAL4 DNA binding domain alone (pBIND-empty), or fused to SMN (pBIND-SMN), VP16 (pBIND-VP16) or SMN without GAL4 (pSMN) (Panel A). Cells were lysed 24 hours after transfected and tested for luciferase activity. Data represents the average and standard error (n=2; Panel B). A549 cells were infected with varying concentrations of Ad-U/SMN, -C/SMN, or -RFP. Eighteen hours later, RNA was extracted using TRIzol and cDNA was generated using a primer specific to the 3' untranslated region (3' UTR) of endogenous SMN. The other primer bound in exon 6 of SMN. The PCR products were resolved using an agarose gel (1%) and visualized by ethidium bromide staining. The black arrow (◄) represents transcripts incorporating exon 7 while the gray arrow (◄) represents those lacking exon 7. A549 cells were infected with varying amounts of Ad-C/SMN or Ad-RFP and 18 hpi mRNA was extracted using TRIzol, and converted to cDNA (Panel C). *Usp11* splicing variants were analyzed by RT-PCR and the amplicons were resolved on a 1% agarose gel, stained with ethidium promide. M: medium. S: Small



3.2.7 Over-expression of Ad-SMN does not negatively impact cell growth

In order to establish whether high levels of virally produced SMN could negatively impact the overall health of cells, cell growth was assayed by infecting A549 cells with Ad-U/SMN, Ad-C/SMN or –RFP and monitoring the cells over a period of 4 days using crystal violet staining. No statistical differences were observed in the growth rates between cells that were left untreated or those treated with the viruses. However, we did observe a trend toward a slower growth rate when the cells were subjected to the therapeutic viruses (Figure 3.9). As none of the data points were statistically significant, we concluded that high levels of viral SMN protein did not affect the growth rate of cells.

Figure 3.9. Over-expression of Ad-SMN does not negatively impact cell growth. A549 cells grown in 10 cm were infected (MOI 10, 100) with Ad-U/SMN, -C/SMN, -RFP or PBS (uninfected) and allowed to recover for three hours. Following recovery, the cells were trypsinized and split into 35 mm dishes at a density of 1×10^5 cells/dish. Every day, for four days, cells were fixed and stained with crystal violet. At the end, the crystal violet was re-solubilized and the absorbance was read with a spectrophotometer. Each time-point was performed in duplicate. All data was normalized to day 1 (D1) and represents the average and standard error (n=3).



3.2.8 SMN from an adenovirus vector can increase intracellular gem counts

Several studies have shown that there is a positive correlation between the number of gems within the nucleus of a patient's cells and the severity of their disease (Coovert et al., 1997). In other words, more gems means a more positive outcome for the patients. We attempted to quantify whether infecting cells with increasing concentrations of Ad-SMN increased the number of gems. A549 cells grown on cover slips were infected with Ad-U/SMN, -C/SMN or PBS and 18 hpi were subjected to the same immunostaining protocol as previously described. As can be seen in Figure 3.10A, there is a proportional increase between positive FLAG staining and the concentration of virus used to infect. We counted the number of gems, represented by a co-localization of p80-Coilin and FLAG, per cell (3 representative field of views per slide) in order to quantify this increase (Figure 3.10B). The average uninfected A549 cell has $1.43(\pm 0.10)$ gems per cell. When either virus is used at an MOI of 10, the number of gems per cell is unchanged. There is, however, a significant increase when moving to the higher viral concentrations. For Ad-U/SMN, the gem counts increased to $2.49(\pm 0.46)$ at an MOI of 100 and $4.70(\pm 0.57)$ at an MOI of 1000, which represent p-values of 0.023 and 0.0007 respectively when compared to the same virus at an MOI of 10. The same trend holds true for Ad-C/SMN. The number of gems per cell increased to $2.00(\pm 0.20; p=0.02)$ at 100 MOI and $4.62(\pm 0.45; p=0.0003)$ at 1000 MOI. Overall, it appears that a ten-fold increase in viral concentration approximately doubles the number of visible gems per cell.

Figure 3.10. SMN from an adenovirus vector can increase intracellular gem counts. A549 cells grown on glass cover slips were infected with Ad-U/SMN, -C/SMN or PBS (uninfected) at an MOI of 10, 100, or 1000 (Panel A). Eighteen hours after infection, cells were fixed with 2% PFA and subjected to immunofluorescent treatment, identifying FLAG-tag (green) and p80-coilin (red). DNA was stained using Hoescht to identify the nuclei. Images were captured using confocal imaging. Total number of gems per cell were counted for each infection (Panel B). Data represents the average and standard error (n=3). Statistical significance represents Student's T-test: * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.001$.

3.3 Discussion

The infectivity of the viral constructs and their ability to mediate the expression of SMN was examined in common tissue culture cell lines. Several cell lines were used, including A549 cells and HeLa cells (non-permissive for Ad vector replication), 293 cells (permissive for Ad vector replication), and MN1 cells (primary motor neuron culture, non-permissive). A pattern of expression was established in A549 (Figure 3.1A) cells and confirmed with HeLa and 293s. The amount of protein detected increased proportionately when the amount of virus used at infection was increased. The ability to control the correlation between the expression level of exogenous SMN and the amount of virus used is extremely important. As was demonstrated in Table 1.1, even slight increases in SMN protein can lead to significant ameliorations in disease severity. This concept was further illustrated in the differences in the results between two SMA rescue experiments (Foust et al., 2010; Valori et al., 2010). In the study by Valori *et al.* (Valori et al., 2010), SMN^{-/-};SMN2 mice were given a systemic injection of 1.0×10^{11} genome copies of a viral vector at P2. This treatment regimen helped increase the animal's lifespan by 390%, from 13 days to more than 190 days. On the other hand, Foust *et al.* (Foust et al., 2010), administered five times more viral genomes (5×10^{11}), which led to an increased lifespan to 250 days. This suggests that there is a relationship between dose and efficacy, with higher doses providing a more robust therapeutic response. Our study has thus demonstrated that we can control exogenous SMN levels in cells by controlling the viral dose. As was previously discussed, a higher number of gems per cell correlate with better patient outcome (Coovert et al., 1997). Thus we established that an increase in Gems occurs proportionately to increases in viral dose (Figure

3.10). This result could explain in part the increases in lifespan seen in the previous rescue experiments (Foust et al., 2010; Valori et al., 2010).

Furthermore, we observed a decrease in the loading control genes at MOI 1000 for all viruses used in this experiment (Figure 4.1). This is indicative of vector toxicity at high concentrations, which can be caused by background level expression of viral genes and active virus replication due to the high concentration of Ad vector used for these infections (Nelson & Kay, 1997). Despite the vector toxicity, it does not appear that the SMN construct is toxic since the decrease observed in Gemin2 and tubulin is the same for the control virus expressing RFP (Ad-RFP) as for those expressing SMN(Ad-C/SMN, -U/SMN).

In order for a protein replacement therapy approach to be beneficial to the patients, it is important to demonstrate that the resulting protein is functional. Specific markers of functionality were chosen in order to elucidate the potential of virally produced SMN protein.

First, the stability of the exogenous SMN was compared to that of endogenous SMN protein. After the initial infections, cells were treated with cycloheximide. The cycloheximide treatment inhibits *de novo* protein synthesis, thus the stability of a given protein can be examined by harvesting protein lysate over a pre-determined period of time. In this case, an 8-hour time-course was chosen based on the half-life of endogenous SMN of approximately 4 hours (B. G. Burnett et al., 2009). There were no observable differences between endogenous and exogenous SMN protein. Without fully elucidating protein half-life, the two forms of the protein appear to behave in a similar fashion

In the nucleus, endogenous SMN is one of the most important members of a multiprotein structure called gemini of coiled bodies or “gems” (Cauchi, 2010; Q. Liu &

Dreyfuss, 1996). These structures play a role in the assembly of RNA protein complexes and processing of pre-mRNA (Pellizzoni et al., 1998). Positive staining for p80-coilin, a protein component of Cajal bodies, which are closely associated with gems in the nucleus (Young et al., 2001; Young et al., 2000), is a commonly used marker for an important cellular interaction partner of gems. Exogenous SMN, found by positively staining for FLAG, was routinely detected in proximity to p80-coilin in the nucleus of infected cells (Figure 3.5 and 3.10).

We attempted to establish the cellular distribution pattern for SMN in A549 cells, as this has not been previously published to the best of our knowledge. Cell fractionation was performed on infected and uninfected cells and we found that in untreated cells, SMN was evenly distributed between the cellular and nuclear compartments (Figure 3.6A). Interestingly, in the infected cells, we found that SMN protein levels were increased in both compartments equally. Additionally, overexpression of Ad-SMN does not affect the normal distribution of SMN-interacting proteins. These data, in conjunction with the immunofluorescence data, demonstrate that SMN produced from an Ad vector can localize in a manner similar to endogenous SMN.

Finally, the addition of tags to the SMN protein constructs could prevent normal protein-protein interactions, thus nullifying the positive aspects of treatment. In the formation of gems, SMN forms a homodimer before recruiting Gemins 2 through 8 as well as Unrip (Cauchi, 2010). SMN-Gemin2 is one of the most important protein interactions in the formation of gems. The ability of Ad-SMN to interact *in vitro* with endogenous Gemin2 was not affected by the addition of tags to the full-length SMN protein. Additionally, we also detected an interaction between exogenous SMN and endogenous SMN (Figure 3.6B),

demonstrating that the basic homodimer can in fact be a heterodimer formed by the interaction of a viral SMN protein and a cellular SMN protein.

When A549 cells were infected with increasing concentrations of Ad-SMN, we detected a FLAG signal at 50 kDa (Figure 3.1) that was not present in the cells infected with Ad-RFP. The therapeutic construct is expected to be approximately 40 kDa for Ad-U/SMN and –C/SMN. Small Ubiquitin-like Modifier (SUMO) proteins can be covalently bonded to proteins following transcription for many purposes, including transcriptional regulation and protein stability (Hay, 2005). Previous evidence demonstrated that SUMO-1 transiently colocalizes with SMN and Coilin in Cajal bodies (Navascues et al., 2008). Since SMN contains several putative SUMOylation sites, we investigated whether the 50 kDa species was covalently bound to SUMO-1 by co-immunoprecipitation. No evidence of SUMOylation of SMN was observed (Data not shown).

Following the confirmation that the therapeutic protein was not SUMOylated, we elucidated the composition of the C-terminus of the 50 kDa construct. We had already determined, with positive FLAG epitope tag detection, that the amino-terminus corresponded with the expected layout of the experimental construct (Figure 3.1). Ad-U/SMN and Ad-C/SMN do not however, have a tag at the C-terminus, thus, we created a third Ad vector that expressed a FLAG-SMN-Myc epitope tagged construct (Ad-U/SMNmyc; Figure 2.1). In essence, if the 50 kDa variant was a product of post-translational modification, both the N-terminus and C-terminus would be detectable in the final protein. On the other hand, if the exogenous SMN transcript was aberrantly spliced into the Ad vector, the C-terminus of the protein would be lost. The construct did lose the Myc epitope tag in the 50 kDa variant (Figure 3.4). In conclusion, these experiments demonstrate that the 50 kDa protein is a result

of mis-splicing of the exogenous SMN transcript. It is unclear what is replacing the myc epitope from the original construct. It is likely that the C-terminus of the 50 kDa species contains down-stream regions derived from the viral genome. In fact, this phenomenon has been previously described in Ad vectors (Nakai et al., 2007). The results of that study demonstrated that even in Ad vector lacking the E1A region of their genome (thus, replication incompetent), the viral pIX capsid protein is significantly co-expressed with the transgene. In some cases, when the transgene cassette is inserted in the right direction, the N-terminus of the transgene can be aberrantly spliced to form a fusion protein with pIX. Interestingly, pIX is approximately 14 kDa (Giberson, Davidson, & Parks, 2012). Thus, the 50 kDa protein variant may in fact be an SMN-pIX fusion protein.

A recent study has suggested that SMN can modulate its own promoter (Anderton et al., 2012). Whether this control was exercised directly or indirectly remains to be determined. The conclusion is, however, in keeping with the theory that SMN is regulated by feedback loops at several different checkpoints. In our experiments, we did not observe a change in the amount of endogenous SMN protein in the presence of the different Ad-SMN proteins (Figure 3.1). Furthermore, we did not observe a significant change at the transcript level (Figure 3.2). Despite these lack of changes, would high-level expression of the exogenous SMN affect the activity of the endogenous SMN promoter? Reporter constructs containing the upstream regulator region of the SMN gene (previously described (Monani, McPherson, et al., 1999; Rouget et al., 2005)) were obtained from collaborators and transfected in A549 cells that were subsequently infected (24 hours post-transfection) with Ad-RFP or Ad-C/SMN. As expected, the presence of exogenous SMN did not affect the luciferase expression controlled by the phosphoglycerate kinase (PGK) and elongation factor 1 alpha

(EF1 α) promoters (Figure 3.7). The luciferase activity was not different in the presence of Ad-SMN when its expression was under the control of the two SMN promoters. In the study by Anderton *et al.* (Anderton *et al.*, 2012), exogenous expression of SMN protein led to an increase in SMN transcript, possibly by increasing the inclusion of exon 7. We did not observe the same results as the increase in SMN transcripts was solely due to viral expression, instead of exon 7 exclusion.

In addition to regulating its own activity, SMN has been implicated in the direct regulation of gene expression and transcription. Firstly, SMN directly binds to the papillomavirus E2 transcription activator, increasing its activity in reporter assays (Strasswimmer *et al.*, 1999). Secondly, SMN can bind the mSin3A corepressor and once it is attached to DNA, can repress the expression of downstream reporter genes (Zou *et al.*, 2004). In a general transcription assay, SMN did not regulate gene expression and transcription (Figure 3.8A). In the study by Strasswimmer *et al.* (Strasswimmer *et al.*, 1999) it was established that SMN can act as a transcriptional activator by binding another protein, that is itself a transcription activator. Combining this observation with the results from our study demonstrates that although SMN can regulate gene expression, it cannot do so on its own. It needs to interact with another protein or protein complex to achieve this goal. Although this other factor was likely present in our experiments, it is possible that the addition of the BIND factor to the c-terminus of the SMN construct abrogated the oligomerization capabilities of SMN. This in turn would likely prevent the recruitment of any other necessary factor to influence transcription (Meister, Buhler, Pillai, Lottspeich, & Fischer, 2001; Paushkin *et al.*, 2002; Pellizzoni, Yong, & Dreyfuss, 2002).

On a different level, SMN can modulate pre-mRNA splicing of its own transcript (Jodelka et al., 2010) as well as the splicing pattern of several other genes (Baumer et al., 2009; H. Liu et al., 2010; Pellizzoni et al., 1998; Z. Zhang et al., 2008). Firstly, it was determined that reductions in the levels of SMN transcript actually increase the frequency of exon 7 skipping in the SMN2 gene (Jodelka et al., 2010; Ruggiu et al., 2012). As previously mentioned, the shorter version of SMN (lacking exon 7) is unstable and rapidly degraded. Thus, a reduction in SMN transcript levels led to a greater decrease of full-length protein. Secondly, it was determined that the reduced levels of SMN found in the SMN^{-/-};SMN2 mouse model resulted in a change in the splicing pattern of the Usp11 (ubiquitin specific peptidase like 1) pre-mRNA transcript (Baumer et al., 2009; H. Liu et al., 2010; Z. Zhang et al., 2008). A preference for the medium form of the Usp11 transcript occurred in the presence of reduced levels of SMN (H. Liu et al., 2010). In order to establish the safety of the different viruses, we analyzed both of these parameters for changes when Ad-SMN is over-expressed.

In our study, over-expression of SMN does not appear to change the ratio of full-length endogenous transcript and those missing exon 7. When cells are infected with Ad-U/SMN or -C/SMN, regardless of the concentration, the ratio was not affected (Figure 3.8B). It is always a possibility that the relationship that occurred between the reduction in SMN transcripts and an increase in exon 7 in the study by Jodelka *et al.* (Jodelka et al., 2010) does not occur in a reverse order. Meaning that an increase in SMN transcript, caused by the presence of the viruses, does not affect the inclusion of exon 7.

We used the transcript pattern of *Usp11* as an indicator of the health of general splicing in the cell when they are subjected to high levels of Ad-SMN. The splicing pattern of *Usp11* was analyzed by RT-PCR after reverse transcription of the mRNA. As can be seen

in Figure 3.8C, there was no change in the splicing pattern of *Usp11* in the presence of Ad-SMN. These data once again demonstrate that over-expression of exogenous SMN does not adversely affect splicing under our controlled conditions. Once again, our results do not correlate previously published data (H. Liu et al., 2010). As with the ratio of full-length SMN transcript to SMN Δ 7 transcript, the splicing of *Usp11* due to the lack of SMN may not occur in reverse in the presence of over-expressed SMN.

To sum up, the data presented in Figure 3.8 helped us demonstrate that increasing the level of SMN in a cell does not appear to impact any of the gene regulation or transcription aspects with which it is normally implicated. It is important to note that as far as general cellular splicing is concerned, *Usp11* was used as an indicator of the overall health of the process although it may not be the best or only gene that is mis-spliced in the presence of low levels of SMN. On the other hand, the data help us take another step forward in verifying the safety of the viral therapeutics in demonstrating that none of these parameters are obviously affected under the tested conditions.

The final step to show that Ad-U/SMN and Ad-C/SMN do not adversely affect the viability of cells, was to perform a cell growth assay and compare the growth rate between cells infected with the therapeutics and untreated cells. Several studies have shown that reducing the levels of SMN has a significant effect on cell function; whether these cells are motor neurons (Kariya et al., 2008; Kong et al., 2009; H. Liu et al., 2010; McGovern et al., 2008; Pellizzoni et al., 1998; Z. Zhang et al., 2008), skeletal and cardiac muscle cells (Bevan et al., 2010; Gogliotti et al., 2012; Hayhurst, Wagner, Cerletti, Wagers, & Rubin, 2012; Martinez-Hernandez et al., 2009; Rudnik-Schoneborn et al., 2008; Shababi et al., 2012; Shafey et al., 2005), or even liver and pancreas cells (Bowerman et al., 2012; Hua et al.,

2011). These effects were all characterized by disruption of cellular function and even death of the cells. We thus hypothesized that changes in levels of SMN, even increases, may disrupt cell viability and manifest itself as a decrease in growth rate. We did not observe any significant differences between the different cell treatments (Figure 3.9). Thus, we concluded that the therapeutic vectors were no more toxic than control Ad vectors.

3.4 Future direction

Now that Ad-U/SMN and Ad-C/SMN have shown that they can effectively and safely increase the amount of SMN protein *in vitro*, we will test them *in vivo*. Using the SMN^{2B/-} mouse model, we will investigate whether these vectors can help improve the lifespan and alleviate disease symptoms. Positive results will improve the animal's life expectancy past 28 days. Additionally, the treatments will positively influence physical traits of SMA, such as, the steadiness of gait, limb strength, and geotaxis.

3.5 Conclusion

In conclusion, we have shown that exogenous SMN delivered by adenovirus vectors is a safe and potentially useful therapeutic in the treatment of SMA. The levels of mRNA and protein of Ad-SMN correlate with the concentrations used at infection and the presence of over-expressed protein does not affect the gene regulation or transcription functions of endogenous SMN. Ad-SMN interacts with endogenous SMN as well as Gemin2, one of its normal cellular partners, despite the different tags attached to the constructs and it localizes to the same cellular areas as endogenous SMN. Furthermore, the high concentrations of Ad-SMN do not adversely affect cell growth. Finally, we demonstrated that an increase in Ad-

SMN correlates to an increase in Gem counts, opening the door for future therapeutic studies in phenotypic cells or animals.

Chapter 4 – Re-targeting of Adenovirus vectors by fiber modifications

4.1 Introduction

Virus based gene therapy often utilizes recombinant adenovirus as a vehicle for gene transfer, specifically: the human serotype 5 adenovirus (Ad5)(Hitt, Addison, & Graham, 1997). Binding of this serotype is mediated by the coxsackievirus and adenovirus receptor (CAR)(Bergelson et al., 1997; Bergelson et al., 1998; Tomko, Xu, & Philipson, 1997). Internalization of the viral capsid requires further interaction between the RGD motifs on the penton base and α_v integrins on the cell surface (Huang, Endo, & Nemerow, 1995; Mathias, Wickham, Moore, & Nemerow, 1994; Nemerow & Stewart, 1999; Wickham et al., 1993).

A major hurdle faced by gene therapies concerns the delivery of the gene product to the cells that need it most. In SMA, the major targets are motor neurons as they are traditionally seen as most affected during disease progression. One cannot ignore, however, the growing evidence for the important role SMN plays in other tissues such as skeletal muscle and the pancreas (Bowerman et al., 2012; Martinez-Hernandez et al., 2009). As such, a successful gene therapeutic for SMA needs to target these tissues while avoiding unnecessary off-target gene transfers (Hua et al., 2011; Valori et al., 2010). Unfortunately, the widespread distribution of CAR and α_v integrins precludes the use of wild-type Ad5 capsid as a valid tool. Additionally, Ads cannot efficiently transduce mature muscle due to an apparent lack of the appropriate receptors CAR and/or integrins (Acsadi et al., 1994; Huard et al., 1995; Nalbantoglu et al., 1999). Hence, the modification of Ad tropism remains an important goal in order to overcome the shortcomings of the vector.

There are currently three methods used in an attempt to circumvent CAR-dependant transduction of target cells. The first involves complexing the viral capsid with a bispecific antibody (Curiel, Agarwal, Wagner, & Cotten, 1991; Poulin et al., 2010; Wickham et al., 1996) or peptide ligands (Curiel, 1994; Romanczuk et al., 1999). A second method requires the modification of the knob portion of the Ad fiber. In this case, small targeting molecules are added to the H-I loop of the knob protein in order to change the natural Ad5 tropism (Bouri et al., 1999; Bramson et al., 2004; Jakubczak et al., 2001; Kirby et al., 2000; V. Krasnykh et al., 1998; Michael, Hong, Curiel, & Engler, 1995; Roelvink, Mi Lee, Einfeld, Kovesdi, & Wickham, 1999). Finally, a third strategy revolves around the different lengths of the shaft proteins between the Ad serotypes. It was postulated that the shorter shafts allow the capsid to directly interact with the appropriate cellular receptors, thus abrogating the need for fiber-dependant binding (Roelvink et al., 1996; Roelvink et al., 1998). Modification of the shaft length was demonstrated to be a successful strategy in several studies (Gall, Kass-Eisler, Leinwand, & Falck-Pedersen, 1996; V. N. Krasnykh et al., 1996; Miyazawa et al., 1999; Shayakhmetov & Lieber, 2000; Shayakhmetov et al., 2000).

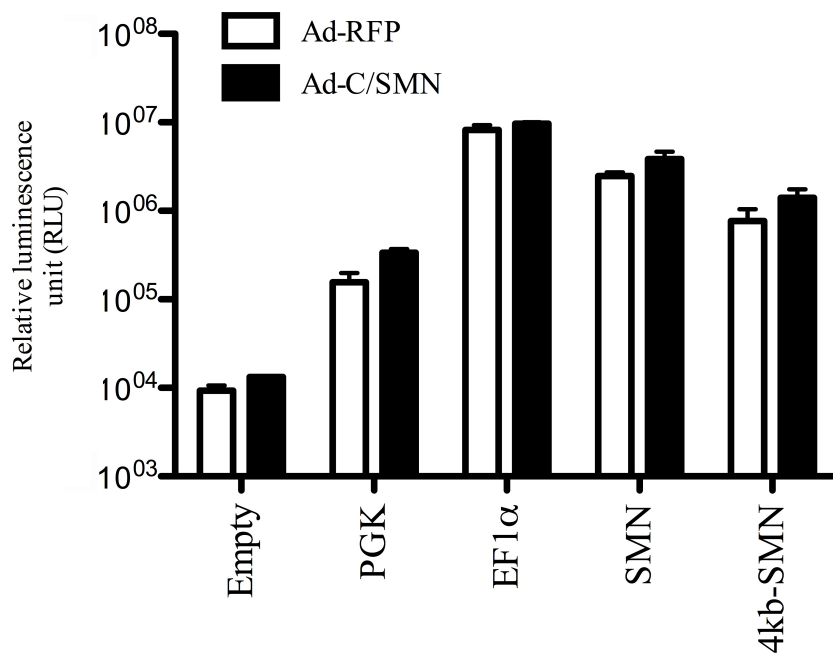
In this study, we aim to combine knob and shaft modifications in order to create a new adenoviral vector for the potential treatment of SMA. In order to be successful, this vector needs to target skeletal muscle and motor neurons and be administered in a way that makes it available to the cells that require supplemental SMN. Additionally, we will test three different delivery methods in order to determine which will be optimal in the administration of targeted gene therapies.

4.2 Results

4.2.1 Modified fiber viruses can infect A549 cells

The infective ability of the modified viruses was established *in vitro* before moving into animals. A549 cells were infected with the different viruses, 18 hours later were lysed and luminosity was assayed using a β -gal luminosity assay as previously described and values were normalized to the amount of protein found in the luminosity assay determined by Bradford assay. Normalized background luminosity was measured at $0.54(\pm 0.007)$; Figure 4.1). Ad5-LacZ and Ad5spk-LacZ increased relative luminosity 10000-fold to $19697.50(\pm 146.55)$ and $29853.53(\pm 998.60)$ respectively. Furthermore, these two values proved to be significantly different ($p=0.0006$) from each other. Less significantly, luminosity was increased approximately 35-fold over background to $36.26(\pm 2.66)$ with Ad5/35s-LacZ and approximately 200-fold to $220.47(\pm 64.13)$ with Ad5s-LacZ. While significantly different than untreated cells, the values obtained with Ad5/35s-LacZ and Ad5s-LacZ are not statistically different ($p=0.0557$). In conclusion, each virus can infect A549 cells and express their LacZ constructs. This preliminary result was a proof of concept for the feasibility of the pilot project in mice.

Figure 4.1. Modified fiber viruses can infect A549 cells. A549 cells were infected with modified fiber Ads expressing LacZ constructs. Eighteen hours after infection, cells were collected, lysed, and the crude cell extract was assayed for β -gal using the GalactoStar™ System. Total protein in the crude cell extract was determined by Bradford assay. Relative luminosity units were normalized to the amount of protein found in the β -gal assay (RLU/ug). The logarithmic mean and standard deviation are graphed (n=2). Each letter represents a statistically different mean.



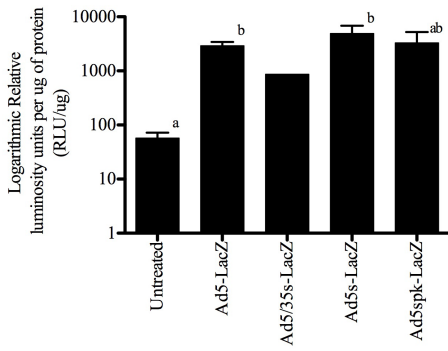
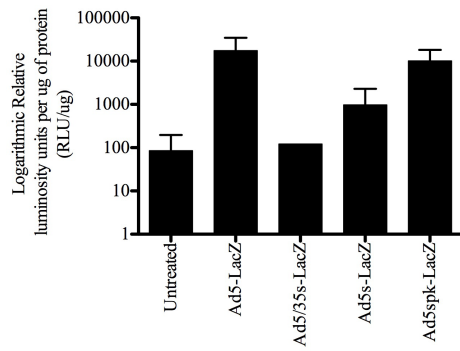
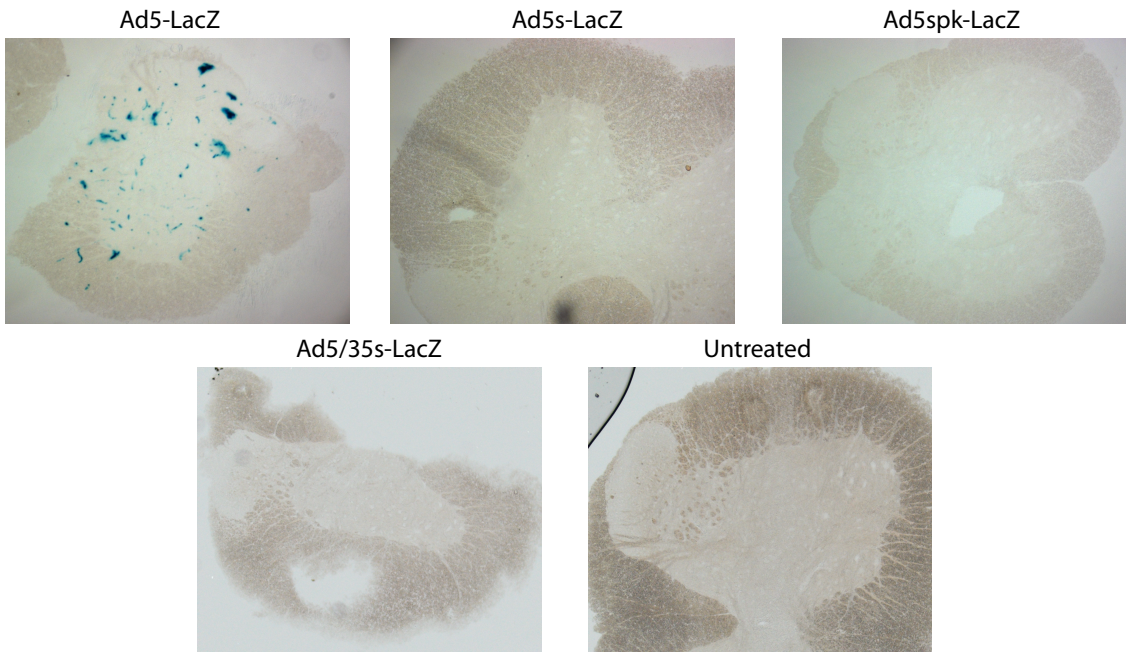
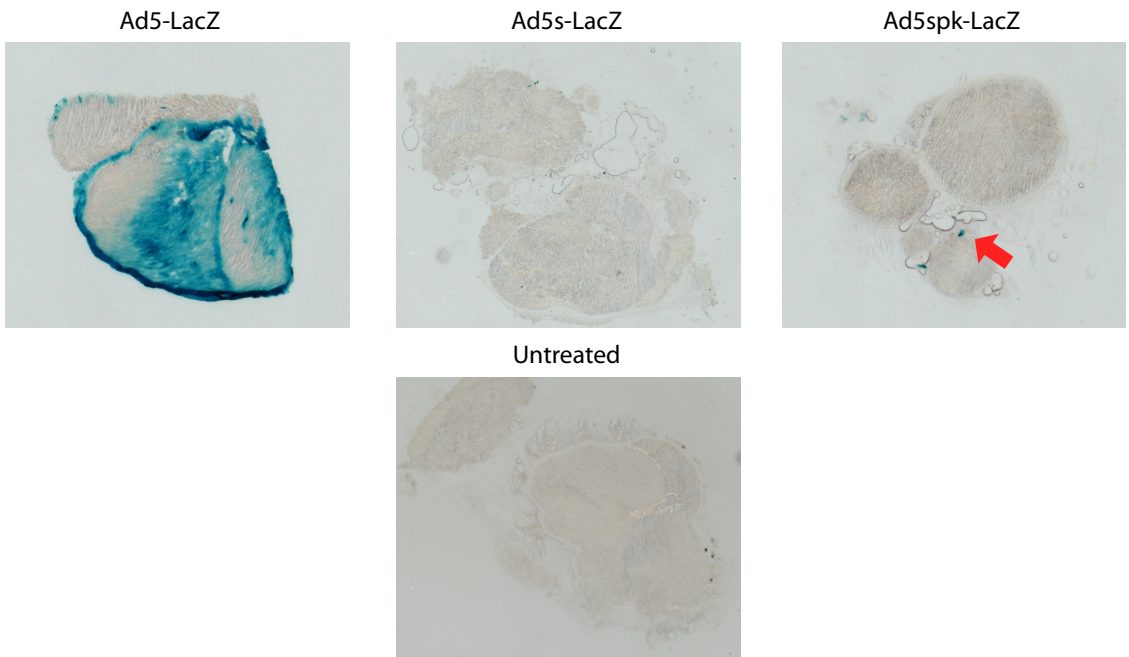
4.2.2 Wild-type (Ad5-LacZ) and polylysine modified wild-type knob (Ad5spk-LacZ) viruses can be detected in the spinal cords and sciatic nerves of mice given intravenous injections

We tested whether the route of administration of the viruses affected the efficacy of infection as well as the types of targeted tissues. During the first set of experiments, the viruses were given by a bolus injection in the tail vein. Viral quantity was estimated by β -gal luminescence assay in the liver and diaphragm and standardized to the total protein found in the luminescence assay, as determined by Bradford assay. The presence of the virus in the spinal cord and sciatic nerves was detected by dissection and staining of the tissues.

As can be seen in Figure 4.2 (Panel A), all four experimental viruses were detected in the liver. The livers from mice treated with the wild-type (Ad5-LacZ), the wild-type with short fiber (Ad5s-LacZ) and the polylysine modified wild-type knob (Ad5spk-LacZ) viruses, displayed significantly more luminescence than the livers from uninfected mice (respectively: $p=0.016$; $p=0.044$; $p=0.142$). There is an observable trend for significant detection in the liver of the mouse treated with Ad5/35s-LacZ. Unfortunately, two of the three mice given this formulation died as a result of the injection, thus leaving us with only one test subject. In the diaphragms of the mice given intravenous viral injections (Figure 4.2B), there is no significant difference in the detection of the β -gal luminescence between the uninfected and infected mice. Positive β -gal staining was only observed in the spinal cords from mice treated with wild-type (Ad5-LacZ) viruses (Figure 4.2C; $n=2$). On the other hand, positive staining was detected in the left sciatic nerve (Figure 4.2D) of mice treated with wild-type (Ad5-LacZ; $n=2$) as well as Ad5spk-LacZ ($n=2$) viruses.

In conclusion, shaft and knob modifications do not affect the amount of virus detected in the liver, but it appears that unmodified viruses are more easily detected in the spinal cord and neurons. Intravenous injections, although easy to adapt to human patients do not offer a good avenue for the delivery of these modified viruses. This is antithesis to our goals of improving targeting by modifying the virus fiber proteins.

Figure 4.2. Wild-type (Ad5-LacZ) and polylysine modified wild-type knob (Ad5spk-LacZ) viruses can be detected in the spinal cords and sciatic nerves of mice given intravenous injections. C57Bl/6 mice were given an intravenous injection of modified fiber Ads expressing LacZ constructs. Twenty-four hours later, the mice were euthanized and liver, diaphragm, spinal cord, and sciatic nerve were collected. Whole protein extract was obtained from the liver (Panel A) and diaphragm (Panel B) and assayed for β -gal using the GalactoStar™ System. Total protein in the crude cell extract was determined by Bradford assay. Relative luminosity units were normalized to the amount of protein found in the β -gal assay (RLU/ug). The logarithmic mean and standard deviation are graphed (n=1-3). Each letter represents a statistically different mean. The spinal cord (Panel C) and left sciatic nerve (Panel D) were dissected and stained for the presence of LacZ, represented by the blue staining.

A**B****C****D**

4.2.3 Viral constructs with the wild-type or modified (Ad5spk-LacZ) knobs, can readily infect muscle when directly injected into the tissue itself.

One of the major symptoms of SMA is the degeneration of the hind limb muscles, which is followed, in the more severe cases, by all skeletal trunk muscles. This is also a hallmark of other neuromuscular diseases such as Duchenne's Muscular Dystrophy (DMD). We postulated that the fiber modifications present in the viral constructs would affect the capability of the virus to infect muscle after an intramuscular injection. As with the tissues analyzed after intravenous injections, the luminescent values were normalized to the amount of protein used in the assay, as determined by Bradford assay.

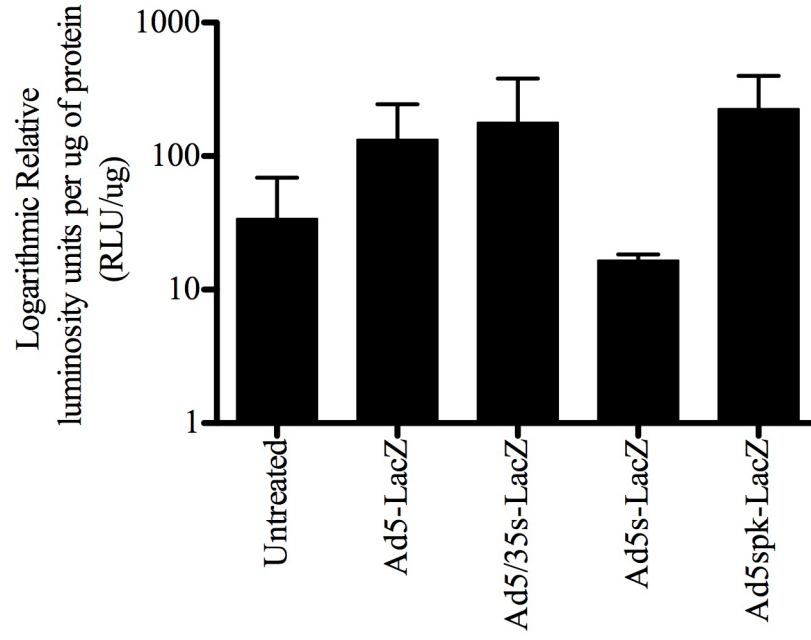
The three viruses that are based on the Ad5 knob serotype: wild-type knob protein (Ad5-LacZ), wild-type knob combined with a short shaft (Ad5s-LacZ), and polylysine modified wild-type knob (Ad5spk-LacZ); were all detected in the injected *Tibialis anterior* muscle (Figure 4.3B). The level of detection for these three viruses was approximately 1000-fold higher than the baseline luminescence muscle established in the muscle of uninfected mice (respectively: $p=0.028$, $p=0.0076$, $p=0.0046$). Furthermore, Ad5spk-LacZ was more abundant in the muscle than Ad5s-LacZ ($p=0.0035$). Ad5/35-LacZ was not detected above baseline levels of luminescence ($p=0.20$). On the other hand, there was no significant detection of luminescence in liver tissue following all the viral treatments (Figure 4.3A).

As in the manner of the mice given intravenous injections of the viral therapeutics, the spinal cord and sciatic nerve were dissected and stained for the presence of LacZ. None of the sections analyzed demonstrated positive staining (Data not shown).

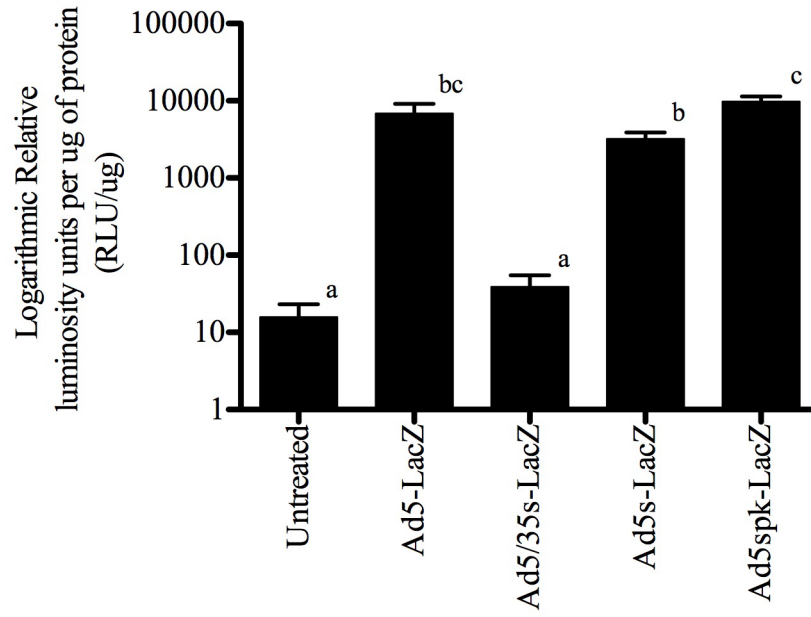
After intra-muscular injections, two of the modified viruses are detected with the same frequency as the wild-type virus in the muscle itself. It appears that the shaft length modifications have maintained the ability of the virus to infect mature muscle cells. This is important as intra-muscular delivery of a therapeutic bi-passes the liver and eliminates the problems of first-pass metabolism. Unfortunately, it appears that through this delivery method, none of the modifications tested improved the rate of infection above that of wild-type Ad.

Figure 4.3. Viral constructs with the wild-type or modified (Ad5spk-LacZ) knobs, can readily infect muscle when directly injected into the tissue itself. C57Bl/6 mice were given an intramuscular injection into the *Tibialis anterior* of modified fiber Ads expressing LacZ constructs. Twenty-four hours later, the mice were euthanized and liver and *Tibialis anterior* muscle were collected. Whole protein extract was obtained from the liver (Panel A) and muscle (Panel B), and assayed for β -gal using the GalactoStar™ System. Total protein in the crude cell extract was determined by Bradford assay. Relative luminosity units were normalized to the amount of protein found in the β -gal assay (RLU/ug). The logarithmic mean and standard deviation are graphed (n=2-3).

A



B



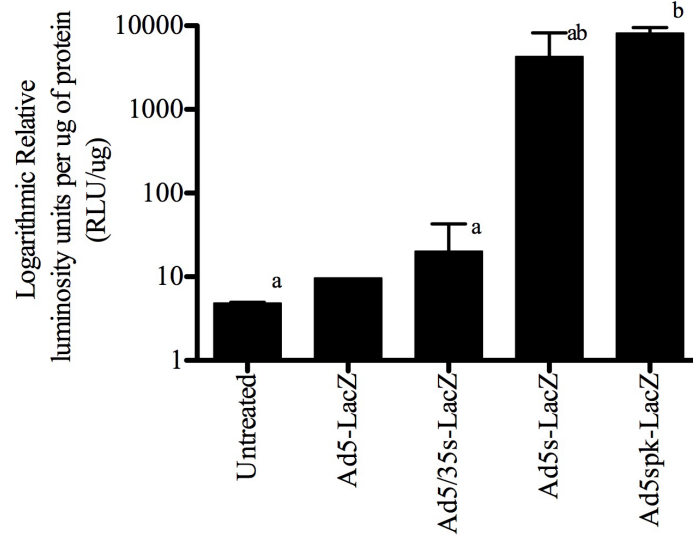
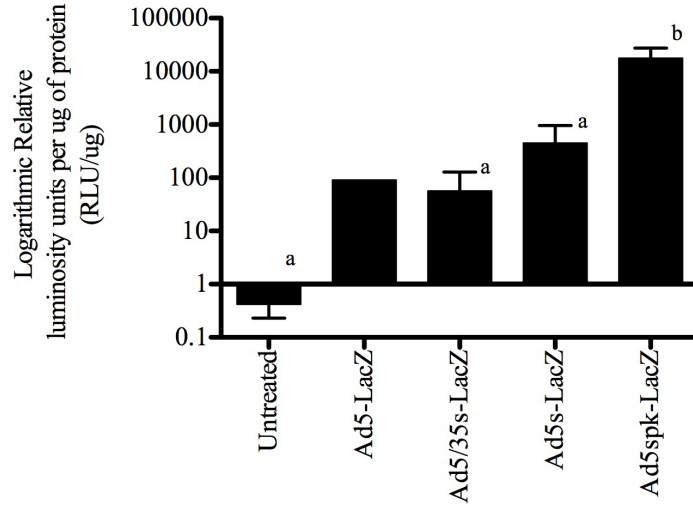
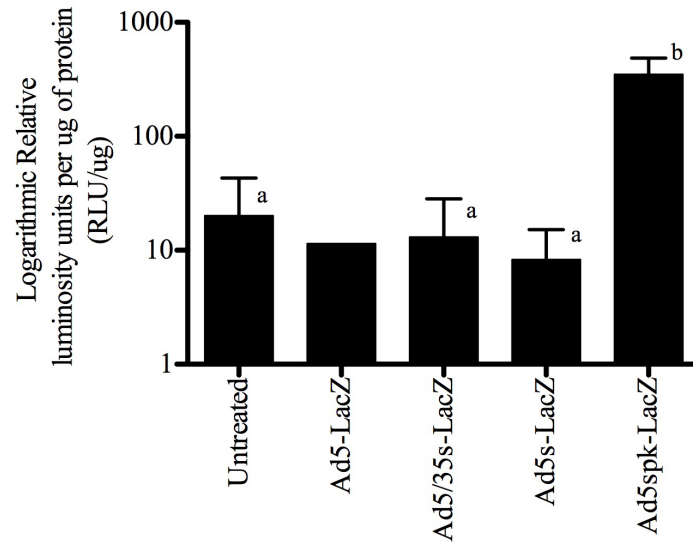
4.2.4 Addition of the polylysine tag to the knob protein improves infectivity when the virus is delivered by intra-peritoneal injection.

The third and final route of delivery tested was by intra-peritoneal injection. This method could be extremely important in potential DMD treatments if the therapeutic can easily infect the diaphragm, as this remains an important target. Twenty-four hours after the single injection, the mice were euthanized and the liver, diaphragm, *Tibialis anterior*, spinal cord, and sciatic nerve were collected. As before, the tissues were either analyzed for β -gal detection or LacZ staining. Spinal cord and sciatic nerve were collected and stained, but no positive results were obtained (Data not shown).

As can be seen in Figure 4.4 (Panel A), the virus with the polylysine tag added to its knob protein (Ad5spk-LacZ), is approximately 1000-fold more abundant than other viruses in the liver of the treated mice ($p=0.013$). The infectivity of Ad5s-LacZ also appears to be higher than the other regimens, but it lacks statistical significance when compared to the livers of uninfected mice ($p=0.24$). When analyzing the luminescence data from the diaphragms (Panel B), we observed that all the viruses could be detected. However, only Ad5spk-LacZ reached significance ($p=0.037$), while the values obtained for the other viruses were not statistically different than those obtained for the diaphragms of uninfected mice. Finally, these results are recapitulated in the *Tibialis anterior* muscle (Panel C). The luminescence measured in muscles from mice treated with Ad5spk-LacZ is approximately 100-fold higher than those from all other treatment regimens ($p=0.048$; when comparing to uninfected controls).

In the context of neurodegenerative diseases, the restoration of muscle function to the diaphragm is a major target of future therapies. Furthermore, intra-peritoneal injections are important therapeutic options for patients as they eliminate the pitfalls associated with first-pass metabolism. With this injection method, these results show that the combination of a shortened shaft and the polylysine knob modification improved the ability of the virus to infect the diaphragm and hind limb muscle. Ad5spk-LacZ has thus demonstrated immense potential as a viral construct for a potential therapeutic.

Figure 4.4. Addition of the polylysine tag to the knob protein improves infectivity when the virus is delivered by intraperitoneal injection. C57Bl/6 mice were given an intraperitoneal injection of modified fiber Ads expressing LacZ constructs. Twenty-four hours later, the mice were euthanized and liver, diaphragm, and *Tibialis anterior* muscle were collected. Whole protein extract was obtained from the liver (Panel A), diaphragm (Panel B), and muscle (Panel C), and assayed for β -gal using the GalactoStar™ System. Total protein in the crude cell extract was determined by Bradford assay. Relative luminosity units were normalized to the amount of protein found in the β -gal assay (RLU/ug). The logarithmic mean and standard deviation are graphed (n=1-3).

A**B****C**

4.3 Discussion

Our long-term goal is the development of viral vectors based on the Ad5 serotype for the targeting of specific tissues for the treatment of neuromuscular diseases, namely, Spinal Muscular Atrophy. Our laboratory has previously undertaken such endeavors (Poulin et al., 2010). The original strategy genetically modified the pIX capsid protein. The modifications encoded either a single-chain (scFv) or a single-domain (sdAb) antibody for the targeting of cells presenting a specific epitope. This strategy proved to be unsuccessful in regards to scFv as the fusion peptide is improperly folded. In contrast, the sdAb was successfully incorporated into the virion capsid and led to higher targeting of the cells presenting the proper epitope. We thus concluded that, although this method works well, it will be subject to the whims and wishes of the sdAb. A comprehensive understanding of all epitopes on the surface of different cell types, and the subsequent development of antibodies against those epitopes, is required for this method to become truly adaptable. For this study, we attempted to bypass these issues by using adenoviral protein sequences, from other viral subtypes, known to change the natural tropism of the virus, and incorporated them into the Ad fiber protein. If successful, this strategy would create viruses specifically curated to target a subset of tissues and/or cells.

Gene therapy is a promising approach for the treatment of incurable genetic diseases. It is important for the success of this method that viral vectors are delivered to specific tissues. Ad has long been considered an ideal vector for gene therapy due to its capability of packaging large transgenes and its relatively mild pathology. However, the tropism, or cell preference of wild-type Ad is not optimal for the treatment of SMA. This disease affects mainly α -motor neurons and skeletal muscle, and these cell types are not the primary targets

of Ad as they express very little CAR and αv -integrin receptors (Acsadi et al., 1994; Huard et al., 1995; Nalbantoglu et al., 1999). Additionally, Ad5 gene transfer is inefficient in a number of tissues, most importantly in the scope of this study: smooth muscle (Wickham et al., 1996) and brain tissue (Chillon et al., 1999). In conjunction with developing different vectors to target the appropriate cell types, we need to optimize the delivery method of the therapeutic. The most successful gene therapy strategy for SMA so far has been direct central nervous system (CNS) injections (Hua et al., 2011; Passini et al., 2011). Although it is feasible in mice, CNS injections are not a recommended method for use in human patients, especially if multiple injections are necessary. In this experiment, we chose to test three different methods. Each one has advantages and disadvantages that could all impact the efficacy of a future therapy.

Intravenous injection in the tail vein was chosen as the first injection method. This is a standard method of injection in mice and allows for large volumes to be administered. Furthermore it has been very successful in gene therapy strategies against SMA (Glascock, Osman, et al., 2012). In this study, intravenous delivery of self-complementary adeno-associated virus (sc-AAV) expressing SMN increased the lifespan of a severe mouse model ($SMN^{-/-}$; $SMN2^{+/+}$) 2-4 fold. The only difference with this study, and all others that were successful using intravenous injections, is that they used an scAAV9 vector instead of an Ad vehicle (Dominguez et al., 2011; Foust et al., 2010; Valori et al., 2010). In the case of an Ad injection, more than 80% of the virus is retained in the liver following this type of injection (Guo et al., 1996), but it will also be found in tissues expressing the appropriate CAR-receptors and αv integrins (Beatty & Curiel, 2012). This accounts for the success of gene therapy products whose main target is the liver either to restore a functional deficiency to

hepatocytes, or to use the organ as a protein production factory to produce large amounts of secreted protein. The luminosity assay performed on the livers of mice given an intravenous injection confirmed the high degree of infectivity for this organ. The three viruses that can target CAR, regardless of the length of the fiber, were all detected in abundance. Even the construct that targets CD46 (Ad5/35-LacZ) had a significant presence in the liver tissue when compared to the untreated mice. This result echoed the findings of the previous studies that claimed that a majority of virus administered by intravenous injections end up in the liver (Valori et al., 2010).

Acute toxicity of Ad5 based vectors is of significant concern for gene therapy strategies. Systemic injections of Ad5 serotypes can activate a severe innate immune response (Schnell et al., 2001), mediated by an interaction with epithelial cells (Lee et al., 2010). All six mice treated intravenously with Ad5-LacZ and Ad5spk-LacZ seemed to be affected by a reaction to the bolus. Within five minutes of the injection, they became lethargic and had difficulty breathing. They all recovered within thirty minutes, demonstrating that although a serious reaction was activated, it was not fatal. Going forward, it is imperative that the mice be closely monitored following the injections.

The second delivery method we tested was an intra-muscular injection. Along with affecting α -motor neurons, another important target of SMA are the hind limb muscles. Retrograde transport of the virus and/or gene product is a mechanism that would allow a therapy to treat both the muscles and the motor neurons in one injection. This method has been used before to introduce Brain-Derived Neurotrophic Factors (BDNF) to spinal neural cells (Uchida et al., 2012), by way of an intramuscular Ad injection. In our study, we identified significant gene transfer in the injected muscle from the viruses that interact with

CAR (Figure 4.3). Unfortunately, we were unable to observe any retrograde transport of the gene product into the sciatic nerve 24 hours after injection (data not shown). It is likely that the incubation time was too short. In the previous study by Uchida *et al.* (Uchida et al., 2012), the gene product was detected in the cervical neurons only four weeks after the initial intramuscular injection.

The final delivery method we chose is an intraperitoneal injection. According to Passini and Chang (Passini & Cheng, 2011), this method has not been successfully used in the development of a gene therapy SMA treatment strategy. It has, however, been used in the treatment of DMD. A single intraperitoneal injection of helper-dependant adenovirus (hdAd) containing the full-length dystrophin expression cassette, lessened respiratory distress in mice by restoring dystrophin levels in the diaphragm (Ishizaki et al., 2011). Thus, we added this injection method to our experiments to determine whether any of the viral constructs may eventually become fruitful in treating other diseases if they do not work for SMA. We found that adding the polylysine tag to the fiber knob dramatically increased the infectivity of the virus towards the diaphragm as well as the *Tibialis anterior*. This result is unprecedented with a vehicle based on an Ad serotype and is encouraging for therapies towards both DMD and SMA. Curiously, the hdAd used in the dystrophin study is also based on a gutted Ad5 serotype vector (Miyake et al., 1996), and readily infected muscle. The Ad5-LacZ vector used in our study was wholly inefficient following the intraperitoneal injection. The vector was not detected above background levels in the liver, diaphragm, or the *Tibialis anterior* muscle. This result needs to be taken with a grain of salt, as two of the three mice given Ad5-LacZ died shortly following the injection. As with the intravenous injection, they became lethargic and presented with obvious respiratory distress before dying. The mouse that

survived also became lethargic but recovered in a timely fashion. Following an autopsy, no apparent internal damage could be found to explain the observed symptoms. Unfortunately, the gene construct was not detected in either the spinal cord or the sciatic nerve twenty-four hours after injection. As is the case with the intra-muscular injections, it is possible that it would take up to four weeks for the construct to make its way into those tissues, especially through retrograde transport from the *Tibialis anterior* muscle.

Clearance studies have established that Ad5 viruses have a half-life of less than 2 minutes following an intravenous injection (Alemany, Suzuki, & Curiel, 2000). This phenomenon is caused by the sequestration of the virus by Kupffer cells in the liver (Kirn, Gut, & Gendrault, 1982). Since all our constructs are based on the serotype 5 Ad, we also found that a big fraction of the injected viruses were sequestered in the liver of the mice (Figure 4.2 A, 4.4A). This is likely a result of the fact that hepatocyte and blood factor X interactions were not ablated by the changes we made to the viral capsids in this study (Kalyuzhniy et al., 2008; Waddington et al., 2008). In these studies, the authors successfully prevented the uptake of the virus by the liver simply by changing the hypervariable region of the hexon capsid protein, from an Ad5 to an Ad48.

In the original study performed by Shayakhmetov *et al.* (2000) (Shayakhmetov & Lieber, 2000), the long shaft protein was more efficient at infecting three different cell types: 293s, Y79 (human retinoblastoma), and K562 (human erythroleukemia). They used three different cell types in order to vary the amount of CAR and αv -integrin receptors on the surface of the cells. In essence, the researchers found Ad5-LacZ was more efficient than Ad5s-LacZ *in vitro*. In figure 4.1, we recapitulated the same results in a fourth cell line, A549.

The Ad5/35 chimeric virus was first conceived by Shayakhmetov *et al.* (Shayakhmetov et al., 2000) in the hopes of targeting the vector towards cells that expressed the CD34 receptor. This receptor is generally associated with human hematopoietic stem cells (HSCs) (Shayakhmetov et al., 2000). The change in fiber increased the ability of the virus to infect CD34+ by approximately 50%, but only in the cells that also expressed α v-integrin. In our study, the change in fiber did not target this virus to our desired cell types. In fact, the results obtained with Ad5/35s-LacZ were almost identical to the results obtained with the untreated mice. This was to be expected as the liver, diaphragm, hind limb, spinal cord, and sciatic nerve do not express the CD34 cell receptor.

The addition of the pK tract to the knob of a small Ad9 fiber led to the highest increase of muscle targeting in this study (Figure 4.4B and C). This strategy meant that Ad5spk-LacZ was using both CAR-dependant and independant pathways for cellular internalization. The pK has been successfully added to viruses in the past in the hopes of targeting smooth muscle cells. In a study by Wickham *et al.* (Wickham et al., 1997), the addition of a 7 residues lysine moiety to the H-I loop of an Ad5 fiber, drastically improved the capability of the virus to infect muscle cells both *in vitro* and *in vivo*. The conclusions may not transfer adequately to this study as the authors used pigs as their test animals. In a second study, this time performed in mice, Bramson *et al.* (Bramson et al., 2004) increased the ability of the chimeric virus to infect muscle cells by adding a pK moiety to the fiber of an hdAd5 virus. As this virus is simply a traditional Ad5 that is completely gutted of it's viral genes, this is easily comparable to the structure of Ad5spk-LacZ.

4.4 Future Direction

Despite the positive results discovered in this study, it is only a first step in the development of a proper targeting virus. A follow-up study will be undertaken to determine whether varying the concentration of the vectors in the injections can modulate the virus rate of infection. The injections will be repeated only with three of the viruses: wild-type (Ad5-LacZ), short shaft protein (Ad5s-LacZ), and polylysine knob (Ad5spk-LacZ) viruses, as they proved to be the most successful. Furthermore, the results obtained with the intra-muscular injections were not satisfactory. Apart from good muscle tissue incorporation, we observed no transduction of the sciatic nerves or the spinal cord. In the scope of an SMA treatment, the motor neurons are the primary target (Goulet, Kothary, & Parks, 2013), thus the incubation period following intra-muscular injections will need to be extended in order to confirm these results. Additionally, our experiments did not allow us to detect the presence of the virus with the luminosity assay and stain at the same time. In other words, we know that the viruses can be detected in the diaphragm after intra-peritoneal injections, but we have no way to determine if the virus is evenly distributed or localized to certain cell-types. It will be important to verify this information before we can confirm that modifying the fiber proteins actually helps the virus to target certain tissues.

4.5 Conclusion

None of the fiber length and knob combinations tested helped in de-targeting the liver. The most significant result occurred following the intraperitoneal injections of Ad5spk-LacZ. The combination of a short fiber and polylysine motif dramatically improved the ability of this virus to target muscles (diaphragm and TA). This virus will garner significant interest going forward in the development of targeted therapy strategies for both DMD and SMA.

Reference list

- Acsadi, G., Jani, A., Massie, B., Simoneau, M., Holland, P., Blaschuk, K., & Karpati, G. (1994). A differential efficiency of adenovirus-mediated in vivo gene transfer into skeletal muscle cells of different maturity. *Hum Mol Genet*, 3(4), 579-584.
- Aleman, R., Suzuki, K., & Curiel, D. T. (2000). Blood clearance rates of adenovirus type 5 in mice. *J Gen Virol*, 81(Pt 11), 2605-2609.
- Anderton, R. S., Price, L. L., Turner, B. J., Meloni, B. P., Mitropant, C., Mastaglia, F. L., . . . Boulous, S. (2012). Co-regulation of survival of motor neuron and Bcl-xL expression: implications for neuroprotection in spinal muscular atrophy. *Neuroscience*, 220, 228-236. doi: S0306-4522(12)00660-4 [pii]
10.1016/j.neuroscience.2012.06.042
- Azzouz, M., Le, T., Ralph, G. S., Walmsley, L., Monani, U. R., Lee, D. C., . . . Mazarakis, N. D. (2004). Lentivector-mediated SMN replacement in a mouse model of spinal muscular atrophy. *J Clin Invest*, 114(12), 1726-1731. doi: 10.1172/JCI22922
- Baumer, D., Lee, S., Nicholson, G., Davies, J. L., Parkinson, N. J., Murray, L. M., . . . Talbot, K. (2009). Alternative splicing events are a late feature of pathology in a mouse model of spinal muscular atrophy. *PLoS Genet*, 5(12), e1000773. doi: 10.1371/journal.pgen.1000773
- Beatty, M. S., & Curiel, D. T. (2012). Chapter two--Adenovirus strategies for tissue-specific targeting. *Adv Cancer Res*, 115, 39-67. doi: 10.1016/B978-0-12-398342-8.00002-1
B978-0-12-398342-8.00002-1 [pii]
- Bergelson, J. M., Cunningham, J. A., Droguett, G., Kurt-Jones, E. A., Krithivas, A., Hong, J. S., . . . Finberg, R. W. (1997). Isolation of a common receptor for Coxsackie B viruses and adenoviruses 2 and 5. *Science*, 275(5304), 1320-1323.
- Bergelson, J. M., Krithivas, A., Celi, L., Droguett, G., Horwitz, M. S., Wickham, T., . . . Finberg, R. W. (1998). The murine CAR homolog is a receptor for coxsackie B viruses and adenoviruses. *J Virol*, 72(1), 415-419.
- Bevan, A. K., Hutchinson, K. R., Foust, K. D., Braun, L., McGovern, V. L., Schmelzer, L., . . . Kaspar, B. K. (2010). Early heart failure in the SMNDelta7 model of spinal muscular atrophy and correction by postnatal scAAV9-SMN delivery. *Hum Mol Genet*, 19(20), 3895-3905. doi: ddq300 [pii]
10.1093/hmg/ddq300
- Boon, K. L., Xiao, S., McWhorter, M. L., Donn, T., Wolf-Saxon, E., Bohnsack, M. T., . . . Beattie, C. E. (2009). Zebrafish survival motor neuron mutants exhibit presynaptic neuromuscular junction defects. *Human molecular genetics*, 18(19), 3615. doi: 10.1093/hmg/ddp310
- Bouri, K., Feero, W. G., Myerburg, M. M., Wickham, T. J., Kovesdi, I., Hoffman, E. P., & Clemens, P. R. (1999). Polylysine modification of adenoviral fiber protein enhances muscle cell transduction. *Hum Gene Ther*, 10(10), 1633-1640. doi: 10.1089/10430349950017635
- Bowerman, M., Anderson, C. L., Beauvais, A., Boyl, P. P., Witke, W., & Kothary, R. (2009). SMN, profilin IIa and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis. *Molecular and cellular neurosciences*, 42(1), 66. doi: 10.1016/j.mcn.2009.05.009

- Bowerman, M., Beauvais, A., Anderson, C. L., & Kothary, R. (2010). Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model. *Human molecular genetics*, *19*(8), 1468. doi: 10.1093/hmg/ddq021
- Bowerman, M., Murray, L. M., Beauvais, A., Pinheiro, B., & Kothary, R. (2011). A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy phenotype associated with a distinct neuromuscular junction pathology. *Neuromuscular disorders : NMD*, *22*(3), 263. doi: 10.1016/j.nmd.2011.09.007
- Bowerman, M., Shafey, D., & Kothary, R. (2007). Smn depletion alters profilin II expression and leads to upregulation of the RhoA/ROCK pathway and defects in neuronal integrity. *J Mol Neurosci*, *32*(2), 120-131. doi: JMN:32:2:120 [pii]
- Bowerman, M., Swoboda, K. J., Michalski, J. P., Wang, G. S., Reeks, C., Beauvais, A., . . . Kothary, R. (2012). Glucose metabolism and pancreatic defects in spinal muscular atrophy. *Ann Neurol*, *72*(2), 256-268. doi: 10.1002/ana.23582
- Boyer, J. G., Bowerman, M., & Kothary, R. (2010). The many faces of SMN: deciphering the function critical to spinal muscular atrophy pathogenesis. *Review Future Neurology*, *5*(6), 873-890.
- Bramson, J. L., Grinshtein, N., Meulenbroek, R. A., Lunde, J., Kottachchi, D., Lorimer, I. A., . . . Parks, R. J. (2004). Helper-dependent adenoviral vectors containing modified fiber for improved transduction of developing and mature muscle cells. *Hum Gene Ther*, *15*(2), 179-188. doi: 10.1089/104303404772679986
- Briese, M., Esmaeili, B., & Sattelle, D. B. (2005). Is spinal muscular atrophy the result of defects in motor neuron processes? *BioEssays : news and reviews in molecular, cellular and developmental biology*, *27*(9), 946. doi: 10.1002/bies.20283
- Burghes, A. H. (1997). When is a deletion not a deletion? When it is converted. *Am J Hum Genet*, *61*(1), 9-15. doi: S0002-9297(07)64269-4 [pii] 10.1086/513913
- Burghes, A. H., & Beattie, C. E. (2009). Spinal muscular atrophy: why do low levels of survival motor neuron protein make motor neurons sick? *Nat Rev Neurosci*, *10*(8), 597-609. doi: 10.1038/nrn2670 nrn2670 [pii]
- Burlet, P., Huber, C., Bertrand, S., Ludosky, M. A., Zwaenepoel, I., Clermont, O., . . . Lefebvre, S. (1998). The distribution of SMN protein complex in human fetal tissues and its alteration in spinal muscular atrophy. *Human molecular genetics*, *7*(12), 1927.
- Burnett, B. G., Munoz, E., Tandon, A., Kwon, D. Y., Sumner, C. J., & Fischbeck, K. H. (2009). Regulation of SMN protein stability. *Mol Cell Biol*, *29*(5), 1107-1115. doi: MCB.01262-08 [pii] 10.1128/MCB.01262-08
- Burnett, R. M. (1985). The structure of the adenovirus capsid. II. The packing symmetry of hexon and its implications for viral architecture. *J Mol Biol*, *185*(1), 125-143. doi: 0022-2836(85)90187-1 [pii]
- Cartegni, L., & Krainer, A. R. (2002). Disruption of an SF2/ASF-dependent exonic splicing enhancer in SMN2 causes spinal muscular atrophy in the absence of SMN1. *Nat Genet*, *30*(4), 377-384. doi: 10.1038/ng854 ng854 [pii]

- Cauchi, R. J. (2010). SMN and Gemins: 'we are family' ... or are we?: insights into the partnership between Gemins and the spinal muscular atrophy disease protein SMN. *Bioessays*, 32(12), 1077-1089. doi: 10.1002/bies.201000088
- Challberg, M. D., & Kelly, T. J., Jr. (1979). Adenovirus DNA replication in vitro. *Proc Natl Acad Sci U S A*, 76(2), 655-659.
- Chan, Y. B., Miguel-Aliaga, I., Franks, C., Thomas, N., Trulzsch, B., Sattelle, D. B., . . . van den Heuvel, M. (2003). Neuromuscular defects in a Drosophila survival motor neuron gene mutant. *Human molecular genetics*, 12(12), 1367.
- Chartier, C., Degryse, E., Gantzer, M., Dieterle, A., Pavirani, A., & Mehtali, M. (1996). Efficient generation of recombinant adenovirus vectors by homologous recombination in Escherichia coli. *J Virol*, 70(7), 4805-4810.
- Chillon, M., Bosch, A., Zabner, J., Law, L., Armentano, D., Welsh, M. J., & Davidson, B. L. (1999). Group D adenoviruses infect primary central nervous system cells more efficiently than those from group C. *J Virol*, 73(3), 2537-2540.
- Cho, S., & Dreyfuss, G. (2010). A degron created by SMN2 exon 7 skipping is a principal contributor to spinal muscular atrophy severity. *Genes Dev*, 24(5), 438-442. doi: 10.1101/gad.1884910
- 24/5/438 [pii]
- Christou, C., & Parks, R. J. (2011). Rational design of murine secreted alkaline phosphatase for enhanced performance as a reporter gene in mouse gene therapy preclinical studies. *Hum Gene Ther*, 22(4), 499-506. doi: 10.1089/hum.2010.171
- Chroboczek, J., Ruigrok, R. W., & Cusack, S. (1995). Adenovirus fiber. *Curr Top Microbiol Immunol*, 199 (Pt 1), 163-200.
- Coover, D. D., Le, T. T., McAndrew, P. E., Strasswimmer, J., Crawford, T. O., Mendell, J. R., . . . Burghes, A. H. (1997). The survival motor neuron protein in spinal muscular atrophy. *Human molecular genetics*, 6(8), 1205.
- Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Ronchi, D., . . . Comi, G. P. (2008). Neural stem cell transplantation can ameliorate the phenotype of a mouse model of spinal muscular atrophy. *J Clin Invest*, 118(10), 3316-3330. doi: 10.1172/JCI35432
- Corti, S., Nizzardo, M., Nardini, M., Donadoni, C., Salani, S., Ronchi, D., . . . Comi, G. P. (2010). Embryonic stem cell-derived neural stem cells improve spinal muscular atrophy phenotype in mice. *Brain*, 133(Pt 2), 465-481. doi: 10.1093/brain/awp318
- awp318 [pii]
- Coyne, C. B., & Bergelson, J. M. (2006). Virus-induced Abl and Fyn kinase signals permit coxsackievirus entry through epithelial tight junctions. *Cell*, 124(1), 119-131. doi: S0092-8674(05)01273-0 [pii]
- 10.1016/j.cell.2005.10.035
- Curiel, D. T. (1994). High-efficiency gene transfer employing adenovirus-polylysine-DNA complexes. *Nat Immun*, 13(2-3), 141-164.
- Curiel, D. T., Agarwal, S., Wagner, E., & Cotten, M. (1991). Adenovirus enhancement of transferrin-polylysine-mediated gene delivery. *Proc Natl Acad Sci U S A*, 88(19), 8850-8854.
- DiDonato, C. J., Ingraham, S. E., Mendell, J. R., Prior, T. W., Lenard, S., Moxley, R. T., 3rd, . . . Burghes, A. H. (1997). Deletion and conversion in spinal muscular atrophy

- patients: is there a relationship to severity? *Ann Neurol*, 41(2), 230-237. doi: 10.1002/ana.410410214
- DiDonato, C. J., Lorson, C. L., De Repentigny, Y., Simard, L., Chartrand, C., Androphy, E. J., & Kothary, R. (2001). Regulation of murine survival motor neuron (Smn) protein levels by modifying Smn exon 7 splicing. *Human molecular genetics*, 10(23), 2727.
- DiDonato, C. J., Parks, R. J., & 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(2), 179-188. doi: 10.1089/104303403321070874
- Dmitriev, I., Krasnykh, V., Miller, C. R., Wang, M., Kashentseva, E., Mikheeva, G., . . . Curiel, D. T. (1998). An adenovirus vector with genetically modified fibers demonstrates expanded tropism via utilization of a coxsackievirus and adenovirus receptor-independent cell entry mechanism. *J Virol*, 72(12), 9706-9713.
- Dominguez, E., Marais, T., Chatauret, N., Benkhalifa-Ziyyat, S., Duque, S., Ravassard, P., . . . Barkats, M. (2011). Intravenous scAAV9 delivery of a codon-optimized SMN1 sequence rescues SMA mice. *Hum Mol Genet*, 20(4), 681-693. doi: 10.1093/hmg/ddq514
- ddq514 [pii]
- Dubowitz, V. (1999). Very severe spinal muscular atrophy (SMA type 0): an expanding clinical phenotype. *European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Society*, 3(2), 49. doi: 10.1053/ejpn.1999.0181
- Ducommun, B., & Wright, M. (1989). Variation of tubulin half-life during the cell cycle in the synchronous plasmodia of *Physarum polycephalum*. *Eur J Cell Biol*, 50(1), 48-55.
- Fabry, C. M., Rosa-Calatrava, M., Conway, J. F., Zubietta, C., Cusack, S., Ruigrok, R. W., & Schoehn, G. (2005). A quasi-atomic model of human adenovirus type 5 capsid. *EMBO J*, 24(9), 1645-1654. doi: 7600653 [pii]
- 10.1038/sj.emboj.7600653
- Fan, L., & Simard, L. R. (2002). Survival motor neuron (SMN) protein: role in neurite outgrowth and neuromuscular maturation during neuronal differentiation and development. *Human molecular genetic*, 11(14), 1605.
- Foust, K. D., Wang, X., McGovern, V. L., Braun, L., Bevan, A. K., Haidet, A. M., . . . Kaspar, B. K. (2010). Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. *Nature biotechnology*, 28(3), 271. doi: 10.1038/nbt.1610
- Gaggar, A., Shayakhmetov, D. M., & Lieber, A. (2003). CD46 is a cellular receptor for group B adenoviruses. *Nat Med*, 9(11), 1408-1412. doi: 10.1038/nm952
- nm952 [pii]
- Gall, J., Kass-Eisler, A., Leinwand, L., & Falck-Pedersen, E. (1996). Adenovirus type 5 and 7 capsid chimera: fiber replacement alters receptor tropism without affecting primary immune neutralization epitopes. *J Virol*, 70(4), 2116-2123.
- Gavrilina, T. O., McGovern, V. L., Workman, E., Crawford, T. O., Gogliotti, R. G., DiDonato, C. J., . . . Burghes, A. H. (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(8), 1063-1075. doi: ddm379 [pii]
- 10.1093/hmg/ddm379

- Giberson, A. N., Davidson, A. R., & Parks, R. J. (2012). Chromatin structure of adenovirus DNA throughout infection. *Nucleic Acids Res*, *40*(6), 2369-2376. doi: 10.1093/nar/gkr1076
gkr1076 [pii]
- Glascock, J. J., Osman, E. Y., Wetz, M. J., Krogman, M. M., Shababi, M., & Lorson, C. L. (2012). Decreasing disease severity in symptomatic, *Smn*(-/-);*SMN2*(+/+), spinal muscular atrophy mice following scAAV9-SMN delivery. *Hum Gene Ther*, *23*(3), 330-335. doi: 10.1089/hum.2011.166
- Glascock, J. J., Shababi, M., Wetz, M. J., Krogman, M. M., & Lorson, C. L. (2012). Direct central nervous system delivery provides enhanced protection following vector mediated gene replacement in a severe model of spinal muscular atrophy. *Biochem Biophys Res Commun*, *417*(1), 376-381. doi: S0006-291X(11)02149-8 [pii]
10.1016/j.bbrc.2011.11.121
- Glasgow, J. N., Everts, M., & Curiel, D. T. (2006). Transductional targeting of adenovirus vectors for gene therapy. *Cancer Gene Ther*, *13*(9), 830-844. doi: 7700928 [pii]
10.1038/sj.cgt.7700928
- Gogliotti, R. G., Quinlan, K. A., Barlow, C. B., Heier, C. R., Heckman, C. J., & Didonato, C. J. (2012). Motor neuron rescue in spinal muscular atrophy mice demonstrates that sensory-motor defects are a consequence, not a cause, of motor neuron dysfunction. *J Neurosci*, *32*(11), 3818-3829. doi: 32/11/3818 [pii]
10.1523/JNEUROSCI.5775-11.2012
- Goulet, B., Kothary, R., & Parks, R. J. (2013). At the "Junction" of Spinal Muscular Atrophy Pathogenesis: The Role of Neuromuscular Junction Dysfunction in SMA Disease Progression. *Current Molecular Medicine*, *13*, 15.
- Graham, F. L. (1987). Growth of 293 cells in suspension culture. *J Gen Virol*, *68* (Pt 3), 937-940.
- Graham, F. L., Smiley, J., Russell, W. C., & Nairn, R. (1977). Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol*, *36*(1), 59-74.
- Grimm, D., Streetz, K. L., Jopling, C. L., Storm, T. A., Pandey, K., Davis, C. R., . . . Kay, M. A. (2006). Fatality in mice due to oversaturation of cellular microRNA/short hairpin RNA pathways. *Nature*, *441*(7092), 537-541. doi: nature04791 [pii]
10.1038/nature04791
- Guo, Z. S., Wang, L. H., Eisensmith, R. C., & Woo, S. L. (1996). Evaluation of promoter strength for hepatic gene expression in vivo following adenovirus-mediated gene transfer. *Gene Ther*, *3*(9), 802-810.
- Guse, K., Suzuki, M., Sule, G., Bertin, T. K., Tyynismaa, H., Ahola-Erkkila, S., . . . Lee, B. (2012). Capsid-modified adenoviral vectors for improved muscle-directed gene therapy. *Hum Gene Ther*, *23*(10), 1065-1070. doi: 10.1089/hum.2012.003
- Hammond, S. M., Gogliotti, R. G., Rao, V., Beauvais, A., Kothary, R., & DiDonato, C. J. (2010). Mouse survival motor neuron alleles that mimic *SMN2* splicing and are inducible rescue embryonic lethality early in development but not late. *PLoS one*, *5*(12), e15887. doi: 10.1371/journal.pone.0015887
- Hay, R. T. (2005). SUMO: a history of modification. *Mol Cell*, *18*(1), 1-12. doi: S1097-2765(05)01182-2 [pii]
10.1016/j.molcel.2005.03.012

- Hayhurst, M., Wagner, A. K., Cerletti, M., Wagers, A. J., & Rubin, L. L. (2012). A cell-autonomous defect in skeletal muscle satellite cells expressing low levels of survival of motor neuron protein. *Dev Biol*, *368*(2), 323-334. doi: S0012-1606(12)00307-7 [pii]
10.1016/j.ydbio.2012.05.037
- Hidaka, C., Milano, E., Leopold, P. L., Bergelson, J. M., Hackett, N. R., Finberg, R. W., . . . Crystal, R. G. (1999). CAR-dependent and CAR-independent pathways of adenovirus vector-mediated gene transfer and expression in human fibroblasts. *J Clin Invest*, *103*(4), 579-587. doi: 10.1172/JCI5309
- Hitt, M. M., Addison, C. L., & Graham, F. L. (1997). Human adenovirus vectors for gene transfer into mammalian cells. *Adv Pharmacol*, *40*, 137-206.
- Hsieh-Li, H. M., Chang, J. G., Jong, Y. J., Wu, M. H., Wang, N. M., Tsai, C. H., & Li, H. (2000). A mouse model for spinal muscular atrophy. *Nat Genet*, *24*(1), 66-70. doi: 10.1038/71709
- Hua, Y., Sahashi, K., Rigo, F., Hung, G., Horev, G., Bennett, C. F., & Krainer, A. R. (2011). Peripheral SMN restoration is essential for long-term rescue of a severe spinal muscular atrophy mouse model. *Nature*, *478*(7367), 123. doi: 10.1038/nature10485; 10.1038/nature10485
- Huang, S., Endo, R. I., & Nemerow, G. R. (1995). Upregulation of integrins alpha v beta 3 and alpha v beta 5 on human monocytes and T lymphocytes facilitates adenovirus-mediated gene delivery. *J Virol*, *69*(4), 2257-2263.
- Huard, J., Lochmuller, H., Acsadi, G., Jani, A., Holland, P., Guerin, C., . . . Karpati, G. (1995). Differential short-term transduction efficiency of adult versus newborn mouse tissues by adenoviral recombinants. *Exp Mol Pathol*, *62*(2), 131-143. doi: S0014-4800(85)71015-5 [pii]
10.1006/exmp.1995.1015
- Hubberstey, A. V., Pavliv, M., & Parks, R. J. (2002). Cancer therapy utilizing an adenoviral vector expressing only E1A. *Cancer Gene Ther*, *9*(4), 321-329. doi: 10.1038/sj.cgt.7700436
- Iannaccone, S. T. (2007). Modern management of spinal muscular atrophy. *J Child Neurol*, *22*(8), 974-978. doi: 22/8/974 [pii]
10.1177/0883073807305670
- Ishizaki, M., Maeda, Y., Kawano, R., Suga, T., Uchida, Y., Uchino, K., . . . Uchino, M. (2011). Rescue from respiratory dysfunction by transduction of full-length dystrophin to diaphragm via the peritoneal cavity in utrophin/dystrophin double knockout mice. *Mol Ther*, *19*(7), 1230-1235. doi: 10.1038/mt.2011.58
mt201158 [pii]
- Jakubczak, J. L., Rollence, M. L., Stewart, D. A., Jafari, J. D., Von Seggern, D. J., Nemerow, G. R., . . . Hallenbeck, P. L. (2001). Adenovirus type 5 viral particles pseudotyped with mutagenized fiber proteins show diminished infectivity of coxsackie B-adenovirus receptor-bearing cells. *J Virol*, *75*(6), 2972-2981. doi: 10.1128/JVI.75.6.2972-2981.2001
- Jodelka, F. M., Ebert, A. D., Duelli, D. M., & Hastings, M. L. (2010). A feedback loop regulates splicing of the spinal muscular atrophy-modifying gene, SMN2. *Hum Mol Genet*, *19*(24), 4906-4917. doi: ddq425 [pii]
10.1093/hmg/ddq425

- Kalyuzhniy, O., Di Paolo, N. C., Silvestry, M., Hofherr, S. E., Barry, M. A., Stewart, P. L., & Shayakhmetov, D. M. (2008). Adenovirus serotype 5 hexon is critical for virus infection of hepatocytes in vivo. *Proc Natl Acad Sci U S A*, *105*(14), 5483-5488. doi: 10.1073/pnas.0711757105
0711757105 [pii]
- Kariya, S., Park, G. H., Maeno-Hikichi, Y., Leykekhman, O., Lutz, C., Arkovitz, M. S., . . . Monani, U. R. (2008). Reduced SMN protein impairs maturation of the neuromuscular junctions in mouse models of spinal muscular atrophy. *Human molecular genetics*, *17*(16), 2552. doi: 10.1093/hmg/ddn156
- Kashima, T., & Manley, J. L. (2003). A negative element in SMN2 exon 7 inhibits splicing in spinal muscular atrophy. *Nat Genet*, *34*(4), 460-463. doi: 10.1038/ng1207
ng1207 [pii]
- Kirby, I., Davison, E., Bevil, A. J., Soh, C. P., Wickham, T. J., Roelvink, P. W., . . . Santis, G. (2000). Identification of contact residues and definition of the CAR-binding site of adenovirus type 5 fiber protein. *J Virol*, *74*(6), 2804-2813.
- Kirn, A., Gut, J. P., & Gendrault, J. L. (1982). Interaction of viruses with sinusoidal cells. *Prog Liver Dis*, *7*, 377-392.
- Kong, L., Wang, X., Choe, D. W., Polley, M., Burnett, B. G., Bosch-Marce, M., . . . Sumner, C. J. (2009). Impaired synaptic vesicle release and immaturity of neuromuscular junctions in spinal muscular atrophy mice. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, *29*(3), 842. doi: 10.1523/jneurosci.4434-08.2009
- Kotani, T., Sutomo, R., Sasongko, T. H., Sadewa, A. H., Gunadi, Minato, T., . . . Nishio, H. (2007). A novel mutation at the N-terminal of SMN Tudor domain inhibits its interaction with target proteins. *J Neurol*, *254*(5), 624-630. doi: 10.1007/s00415-006-0410-x
- Krasnykh, V., Dmitriev, I., Mikheeva, G., Miller, C. R., Belousova, N., & Curiel, D. T. (1998). Characterization of an adenovirus vector containing a heterologous peptide epitope in the HI loop of the fiber knob. *J Virol*, *72*(3), 1844-1852.
- Krasnykh, V. N., Mikheeva, G. V., Douglas, J. T., & Curiel, D. T. (1996). Generation of recombinant adenovirus vectors with modified fibers for altering viral tropism. *J Virol*, *70*(10), 6839-6846.
- Kwon, D. Y., Motley, W. W., Fischbeck, K. H., & Burnett, B. G. (2011). Increasing expression and decreasing degradation of SMN ameliorate the spinal muscular atrophy phenotype in mice. *Human molecular genetics*, *20*(18), 3667. doi: 10.1093/hmg/ddr288
- Le, T. T., Pham, L. T., Butchbach, M. E., Zhang, H. L., Monani, U. R., Coover, D. D., . . . Burghes, A. H. (2005). SMN Δ 7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Human molecular genetics*, *14*(6), 845. doi: 10.1093/hmg/ddi078
- Lee, B. H., Kushwah, R., Wu, J., Ng, P., Palaniyar, N., Grinstein, S., . . . Hu, J. (2010). Adenoviral vectors stimulate innate immune responses in macrophages through cross-talk with epithelial cells. *Immunol Lett*, *134*(1), 93-102. doi: 10.1016/j.imlet.2010.09.003
S0165-2478(10)00223-3 [pii]

- Lefebvre, S., Burglen, L., Reboullet, S., Clermont, O., Burlet, P., Viollet, L., . . . Zeviani, M. (1995). Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*, *80*(1), 155.
- Lefebvre, S., Burlet, P., Liu, Q., Bertrand, S., Clermont, O., Munnich, A., . . . Melki, J. (1997). Correlation between severity and SMN protein level in spinal muscular atrophy. *Nature genetics*, *16*(3), 265. doi: 10.1038/ng0797-265
- Li, E., Stupack, D., Bokoch, G. M., & Nemerow, G. R. (1998). Adenovirus endocytosis requires actin cytoskeleton reorganization mediated by Rho family GTPases. *J Virol*, *72*(11), 8806-8812.
- Li, E., Stupack, D., Klemke, R., Cheresh, D. A., & Nemerow, G. R. (1998). Adenovirus endocytosis via alpha(v) integrins requires phosphoinositide-3-OH kinase. *J Virol*, *72*(3), 2055-2061.
- Liu, H., Shafey, D., Moores, J. N., & Kothary, R. (2010). Neurodevelopmental consequences of Smn depletion in a mouse model of spinal muscular atrophy. *J Neurosci Res*, *88*(1), 111-122. doi: 10.1002/jnr.22189
- Liu, Q., & Dreyfuss, G. (1996). A novel nuclear structure containing the survival of motor neurons protein. *The EMBO journal*, *15*(14), 3555.
- Liu, Q., Fischer, U., Wang, F., & 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*(6), 1013-1021. doi: S0092-8674(00)80367-0 [pii]
- Lorson, C. L., Hahnen, E., Androphy, E. J., & Wirth, B. (1999). A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proceedings of the National Academy of Sciences of the United States of America*, *96*(11), 6307.
- Lorson, C. L., Strasswimmer, J., Yao, J. M., Baleja, J. D., Hahnen, E., Wirth, B., . . . Androphy, E. J. (1998). SMN oligomerization defect correlates with spinal muscular atrophy severity. *Nat Genet*, *19*(1), 63-66. doi: 10.1038/ng0598-63
- Lotti, F., Imlach, W. L., Saieva, L., Beck, E. S., Hao le, T., Li, D. K., . . . Pellizzoni, L. (2012). An SMN-dependent U12 splicing event essential for motor circuit function. *Cell*, *151*(2), 440-454. doi: 10.1016/j.cell.2012.09.012
S0092-8674(12)01115-4 [pii]
- MacLeod, M. J., Taylor, J. E., Lunt, P. W., Mathew, C. G., & Robb, S. A. (1999). Prenatal onset spinal muscular atrophy. *European journal of paediatric neurology : EJPN : official journal of the European Paediatric Neurology Societ*, *3*(2), 65. doi: 10.1053/ejpn.1999.0184
- Mailman, M. D., Heinz, J. W., Papp, A. C., Snyder, P. J., Sedra, M. S., Wirth, B., . . . Prior, T. W. (2002). Molecular analysis of spinal muscular atrophy and modification of the phenotype by SMN2. *Genet Med*, *4*(1), 20-26. doi: 10.109700125817-200201000-00004
- Maizel, J. V., Jr., White, D. O., & Scharff, M. D. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology*, *36*(1), 115-125. doi: 0042-6822(68)90121-9 [pii]
- Markowitz, J. A., Singh, P., & Darras, B. T. (2012). Spinal muscular atrophy: a clinical and research update. *Pediatr Neurol*, *46*(1), 1-12. doi: S0887-8994(11)00376-6 [pii]
10.1016/j.pediatrneurol.2011.09.001

- Martin, Y., Valero, A., del Castillo, E., Pascual, S. I., & Hernandez-Chico, C. (2002). Genetic study of SMA patients without homozygous SMN1 deletions: identification of compound heterozygotes and characterisation of novel intragenic SMN1 mutations. *Hum Genet*, *110*(3), 257-263. doi: 10.1007/s00439-002-0681-y
- Martinez-Hernandez, R., Soler-Botija, C., Also, E., Alias, L., Caselles, L., Gich, I., . . . Tizzano, E. F. (2009). The developmental pattern of myotubes in spinal muscular atrophy indicates prenatal delay of muscle maturation. *J Neuropathol Exp Neurol*, *68*(5), 474-481. doi: 10.1097/NEN.0b013e3181a10ea100005072-200905000-00003 [pii]
- Mathias, P., Wickham, T., Moore, M., & Nemerow, G. (1994). Multiple adenovirus serotypes use alpha v integrins for infection. *J Virol*, *68*(10), 6811-6814.
- McAndrew, P. E., Parsons, D. W., Simard, L. R., Rochette, C., Ray, P. N., Mendell, J. R., . . . Burghes, A. H. (1997). Identification of proximal spinal muscular atrophy carriers and patients by analysis of SMNT and SMNC gene copy number. *American Journal of Human Genetics*, *60*(6), 1411. doi: 10.1086/515465
- McGovern, V. L., Gavrulina, T. O., Beattie, C. E., & Burghes, A. H. (2008). Embryonic motor axon development in the severe SMA mouse. *Human molecular genetics*, *17*(18), 2900. doi: 10.1093/hmg/ddn189
- Meier, O., Boucke, K., Hammer, S. V., Keller, S., Stidwill, R. P., Hemmi, S., & Greber, U. F. (2002). Adenovirus triggers macropinocytosis and endosomal leakage together with its clathrin-mediated uptake. *J Cell Biol*, *158*(6), 1119-1131. doi: 10.1083/jcb.200112067
jcb.200112067 [pii]
- Meister, G., Buhler, D., Pillai, R., Lottspeich, F., & Fischer, U. (2001). A multiprotein complex mediates the ATP-dependent assembly of spliceosomal U snRNPs. *Nat Cell Biol*, *3*(11), 945-949. doi: 10.1038/ncb1101-945
ncb1101-945 [pii]
- Melki, J. (1997). Spinal muscular atrophy. *Current opinion in neurology*, *10*(5), 381.
- Mentis, G. Z., Blivis, D., Liu, W., Drobac, E., Crowder, M. E., Kong, L., . . . O'Donovan, M. J. (2011). Early functional impairment of sensory-motor connectivity in a mouse model of spinal muscular atrophy. *Neuron*, *69*(3), 453-467. doi: 10.1016/j.neuron.2010.12.032
S0896-6273(10)01080-9 [pii]
- Michael, S. I., Hong, J. S., Curiel, D. T., & Engler, J. A. (1995). Addition of a short peptide ligand to the adenovirus fiber protein. *Gene Ther*, *2*(9), 660-668.
- Miguel-Aliaga, I., Culetto, E., Walker, D. S., Baylis, H. A., Sattelle, D. B., & Davies, K. E. (1999). The *Caenorhabditis elegans* orthologue of the human gene responsible for spinal muscular atrophy is a maternal product critical for germline maturation and embryonic viability. *Hum Mol Genet*, *8*(12), 2133-2143. doi: ddc247 [pii]
- Millino, C., Fanin, M., Vettori, A., Laveder, P., Mostacciuolo, M. L., Angelini, C., & Lanfranchi, G. (2009). Different atrophy-hypertrophy transcription pathways in muscles affected by severe and mild spinal muscular atrophy. *BMC Med*, *7*, 14. doi: 10.1186/1741-7015-7-14
1741-7015-7-14 [pii]

- Missias, A. C., Chu, G. C., Klocke, B. J., Sanes, J. R., & Merlie, J. P. (1996). Maturation of the acetylcholine receptor in skeletal muscle: regulation of the AChR gamma-to-epsilon switch. *Developmental biology*, *179*(1), 223. doi: 10.1006/dbio.1996.0253
- Miyake, S., Makimura, M., Kanegae, Y., Harada, S., Sato, Y., Takamori, K., . . . Saito, I. (1996). Efficient generation of recombinant adenoviruses using adenovirus DNA-terminal protein complex and a cosmid bearing the full-length virus genome. *Proc Natl Acad Sci U S A*, *93*(3), 1320-1324.
- Miyazawa, N., Leopold, P. L., Hackett, N. R., Ferris, B., Worgall, S., Falck-Pedersen, E., & Crystal, R. G. (1999). Fiber swap between adenovirus subgroups B and C alters intracellular trafficking of adenovirus gene transfer vectors. *J Virol*, *73*(7), 6056-6065.
- Mizushima, S., & Nagata, S. (1990). pEF-BOS, a powerful mammalian expression vector. *Nucleic Acids Res*, *18*(17), 5322.
- Monani, U. R., Coover, D. D., & Burghes, A. H. (2000). Animal models of spinal muscular atrophy. *Human molecular genetics*, *9*(16), 2451.
- Monani, U. R., Lorson, C. L., Parsons, D. W., Prior, T. W., Androphy, E. J., Burghes, A. H., & McPherson, J. D. (1999). A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Human molecular genetics*, *8*(7), 1177.
- Monani, U. R., McPherson, J. D., & Burghes, A. H. (1999). Promoter analysis of the human centromeric and telomeric survival motor neuron genes (SMNC and SMNT). *Biochim Biophys Acta*, *1445*(3), 330-336. doi: S0167-4781(99)00060-3 [pii]
- Munsat, T. L., & Davies, K. E. (1992). International SMA consortium meeting. (26-28 June 1992, Bonn, Germany). *Neuromuscular disorders : NMD*, *2*(5-6), 423.
- Muqit, M. M., & Feany, M. B. (2002). Modelling neurodegenerative diseases in Drosophila: a fruitful approach? *Nat Rev Neurosci*, *3*(3), 237-243. doi: 10.1038/nrn751
nrn751 [pii]
- Murakami, M., Ugai, H., Belousova, N., Pereboev, A., Dent, P., Fisher, P. B., . . . Curiel, D. T. (2010). Chimeric adenoviral vectors incorporating a fiber of human adenovirus 3 efficiently mediate gene transfer into prostate cancer cells. *Prostate*, *70*(4), 362-376. doi: 10.1002/pros.21070
- Murray, L. M., Beauvais, A., Bhanot, K., & Kothary, R. (2012). Defects in neuromuscular junction remodelling in the Smn(2B⁻) mouse model of spinal muscular atrophy. *Neurobiol Dis*, *49C*, 57-67. doi: S0969-9961(12)00306-3 [pii]
10.1016/j.nbd.2012.08.019
- Nakai, M., Komiya, K., Murata, M., Kimura, T., Kanaoka, M., Kanegae, Y., & Saito, I. (2007). Expression of pIX gene induced by transgene promoter: possible cause of host immune response in first-generation adenoviral vectors. *Hum Gene Ther*, *18*(10), 925-936. doi: 10.1089/hum.2007.085
- Nalbantoglu, J., Pari, G., Karpati, G., & Holland, P. C. (1999). Expression of the primary coxsackie and adenovirus receptor is downregulated during skeletal muscle maturation and limits the efficacy of adenovirus-mediated gene delivery to muscle cells. *Hum Gene Ther*, *10*(6), 1009-1019. doi: 10.1089/10430349950018409
- Navascues, J., Bengoechea, R., Tapia, O., Casafont, I., Berciano, M. T., & Lafarga, M. (2008). SUMO-1 transiently localizes to Cajal bodies in mammalian neurons. *J Struct Biol*, *163*(2), 137-146. doi: S1047-8477(08)00122-6 [pii]

10.1016/j.jsb.2008.04.013

Nelson, J. E., & Kay, M. A. (1997). Persistence of recombinant adenovirus in vivo is not dependent on vector DNA replication. *J Virol*, *71*(11), 8902-8907.

Nemerow, G. R., & Stewart, P. L. (1999). Role of alpha(v) integrins in adenovirus cell entry and gene delivery. *Microbiol Mol Biol Rev*, *63*(3), 725-734.

Ogino, S., Gao, S., Leonard, D. G., Paessler, M., & Wilson, R. B. (2003). Inverse correlation between SMN1 and SMN2 copy numbers: evidence for gene conversion from SMN2 to SMN1. *Eur J Hum Genet*, *11*(9), 723. doi: 10.1038/sj.ejhg.5201032

5201032 [pii]

Parsons, D. W., McAndrew, P. E., Iannaccone, S. T., Mendell, J. R., Burghes, A. H., & 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*(6), 1712-1723. doi: S0002-9297(07)61616-4 [pii]

10.1086/302160

Passini, M. A., Bu, J., Richards, A. M., Kinnecom, C., Sardi, S. P., Stanek, L. M., . . . Cheng, S. H. (2011). Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Science translational medicine*, *3*(72), 72ra18. doi: 10.1126/scitranslmed.3001777

Passini, M. A., Bu, J., Roskelley, E. M., Richards, A. M., Sardi, S. P., O'Riordan, C. R., . . . Cheng, S. H. (2010). CNS-targeted gene therapy improves survival and motor function in a mouse model of spinal muscular atrophy. *J Clin Invest*, *120*(4), 1253-1264. doi: 10.1172/JCI41615

41615 [pii]

Passini, M. A., & Cheng, S. H. (2011). Prospects for the gene therapy of spinal muscular atrophy. *Trends Mol Med*, *17*(5), 259-265. doi: 10.1016/j.molmed.2011.01.002

S1471-4914(11)00003-7 [pii]

Pastore, L., Morral, N., Zhou, H., Garcia, R., Parks, R. J., Kochanek, S., . . . Beaudet, A. L. (1999). Use of a liver-specific promoter reduces immune response to the transgene in adenoviral vectors. *Hum Gene Ther*, *10*(11), 1773-1781. doi: 10.1089/10430349950017455

Paushkin, S., Gubitz, A. K., Massenet, S., & Dreyfuss, G. (2002). The SMN complex, an assemblysome of ribonucleoproteins. *Current opinion in cell biology*, *14*(3), 305.

Pearn, J. (1978). Incidence, prevalence, and gene frequency studies of chronic childhood spinal muscular atrophy. *Journal of medical genetics*, *15*(6), 409.

Pearn, J. (1980). Classification of spinal muscular atrophies. *Lancet*, *1*(8174), 919.

Pellizzoni, L., Charroux, B., & Dreyfuss, G. (1999). SMN mutants of spinal muscular atrophy patients are defective in binding to snRNP proteins. *Proc Natl Acad Sci U S A*, *96*(20), 11167-11172.

Pellizzoni, L., Kataoka, N., Charroux, B., & Dreyfuss, G. (1998). A novel function for SMN, the spinal muscular atrophy disease gene product, in pre-mRNA splicing. *Cell*, *95*(5), 615-624. doi: S0092-8674(00)81632-3 [pii]

Pellizzoni, L., Yong, J., & Dreyfuss, G. (2002). Essential role for the SMN complex in the specificity of snRNP assembly. *Science*, *298*(5599), 1775-1779. doi: 10.1126/science.1074962

298/5599/1775 [pii]

- Philipson, L., & Pettersson, R. F. (2004). The coxsackie-adenovirus receptor--a new receptor in the immunoglobulin family involved in cell adhesion. *Curr Top Microbiol Immunol*, 273, 87-111.
- Poulin, K. L., Lanthier, R. M., Smith, A. C., Christou, C., Risco Quiroz, M., Powell, K. L., . . . Parks, R. J. (2010). Retargeting of adenovirus vectors through genetic fusion of a single-chain or single-domain antibody to capsid protein IX. *J Virol*, 84(19), 10074-10086. doi: JVI.02665-09 [pii]
10.1128/JVI.02665-09
- Poulin, K. L., Tong, G., Vorobyova, O., Pool, M., Kothary, R., & Parks, R. J. (2011). Use of Cre/loxP recombination to swap cell binding motifs on the adenoviral capsid protein IX. *Virology*, 420(2), 146-155. doi: S0042-6822(11)00407-7 [pii]
10.1016/j.virol.2011.09.004
- Prior, T. W., Swoboda, K. J., Scott, H. D., & Hejmanowski, A. Q. (2004). Homozygous SMN1 deletions in unaffected family members and modification of the phenotype by SMN2. *Am J Med Genet A*, 130A(3), 307-310. doi: 10.1002/ajmg.a.30251
- Reddy, V. S., Natchiar, S. K., Stewart, P. L., & Nemerow, G. R. (2010). Crystal structure of human adenovirus at 3.5 Å resolution. *Science*, 329(5995), 1071-1075. doi: 10.1126/science.1187292
329/5995/1071 [pii]
- Rein, D. T., Volkmer, A., Beyer, I. M., Curiel, D. T., Janni, W., Dragoi, A., . . . Breidenbach, M. (2011). Treatment of chemotherapy resistant ovarian cancer with a MDR1 targeted oncolytic adenovirus. *Gynecol Oncol*, 123(1), 138-146. doi: 10.1016/j.ygyno.2011.06.007
S0090-8258(11)00445-8 [pii]
- Roberts, D. F., Chavez, J., & Court, S. D. (1970). The genetic component in child mortality. *Archives of Disease in Childhood*, 45(239), 33.
- Rocconi, R. P., Zhu, Z. B., Stoff-Khalili, M., Rivera, A. A., Lu, B., Wang, M., . . . Makhija, S. K. (2007). Treatment of ovarian cancer with a novel dual targeted conditionally replicative adenovirus (CRAd). *Gynecol Oncol*, 105(1), 113-121. doi: S0090-8258(06)00895-X [pii]
10.1016/j.ygyno.2006.10.057
- Rodrigues, N. R., Owen, N., Talbot, K., Ignatius, J., Dubowitz, V., & Davies, K. E. (1995). Deletions in the survival motor neuron gene on 5q13 in autosomal recessive spinal muscular atrophy. *Hum Mol Genet*, 4(4), 631-634.
- Roelvink, P. W., Kovesdi, I., & Wickham, T. J. (1996). Comparative analysis of adenovirus fiber-cell interaction: adenovirus type 2 (Ad2) and Ad9 utilize the same cellular fiber receptor but use different binding strategies for attachment. *J Virol*, 70(11), 7614-7621.
- Roelvink, P. W., Lizonova, A., Lee, J. G., Li, Y., Bergelson, J. M., Finberg, R. W., . . . Wickham, T. J. (1998). The coxsackievirus-adenovirus receptor protein can function as a cellular attachment protein for adenovirus serotypes from subgroups A, C, D, E, and F. *J Virol*, 72(10), 7909-7915.
- Roelvink, P. W., Mi Lee, G., Einfeld, D. A., Kovesdi, I., & Wickham, T. J. (1999). Identification of a conserved receptor-binding site on the fiber proteins of CAR-recognizing adenoviridae. *Science*, 286(5444), 1568-1571. doi: 8017 [pii]

- Romanczuk, H., Galer, C. E., Zabner, J., Barsomian, G., Wadsworth, S. C., & O'Riordan, C. R. (1999). Modification of an adenoviral vector with biologically selected peptides: a novel strategy for gene delivery to cells of choice. *Hum Gene Ther*, *10*(16), 2615-2626. doi: 10.1089/10430349950016654
- Ross, P. J., Kennedy, M. A., Christou, C., Risco Quiroz, M., Poulin, K. L., & Parks, R. J. (2011). Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J Virol*, *85*(8), 3950-3958. doi: JVI.01787-10 [pii] 10.1128/JVI.01787-10
- Ross, P. J., & Parks, R. J. (2009). Construction and characterization of adenovirus vectors. *Cold Spring Harb Protoc*, *2009*(5), pdb prot5011. doi: 2009/5/pdb.prot5011 [pii] 10.1101/pdb.prot5011
- Rossoll, W., Kroning, A. K., Ohndorf, U. M., Steegborn, C., Jablonka, S., & 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*(1), 93-105.
- Rouget, R., Vigneault, F., Codio, C., Rochette, C., Paradis, I., Drouin, R., & Simard, L. R. (2005). Characterization of the survival motor neuron (SMN) promoter provides evidence for complex combinatorial regulation in undifferentiated and differentiated P19 cells. *Biochem J*, *385*(Pt 2), 433-443. doi: 10.1042/BJ20041024 BJ20041024 [pii]
- Rudnik-Schoneborn, S., Heller, R., Berg, C., Betzler, C., Grimm, T., Eggermann, T., . . . Zerres, K. (2008). Congenital heart disease is a feature of severe infantile spinal muscular atrophy. *J Med Genet*, *45*(10), 635-638. doi: jmg.2008.057950 [pii] 10.1136/jmg.2008.057950
- Ruggiu, M., McGovern, V. L., Lotti, F., Saieva, L., Li, D. K., Kariya, S., . . . Pellizzoni, L. (2012). A role for SMN exon 7 splicing in the selective vulnerability of motor neurons in spinal muscular atrophy. *Mol Cell Biol*, *32*(1), 126-138. doi: 10.1128/MCB.06077-11 MCB.06077-11 [pii]
- Saban, S. D., Silvestry, M., Nemerow, G. R., & Stewart, P. L. (2006). Visualization of alpha-helices in a 6-angstrom resolution cryoelectron microscopy structure of adenovirus allows refinement of capsid protein assignments. *J Virol*, *80*(24), 12049-12059. doi: JVI.01652-06 [pii] 10.1128/JVI.01652-06
- Salazar-Gruoso, E. F., Kim, S., & Kim, H. (1991). Embryonic mouse spinal cord motor neuron hybrid cells. *Neuroreport*, *2*(9), 505-508.
- Sanchez, G., Dury, A. Y., Murray, L. M., Biondi, O., Tadesse, H., El Fatimy, R., . . . Cote, J. (2013). A novel function for the survival motoneuron protein as a translational regulator. *Hum Mol Genet*, *22*(4), 668-684. doi: 10.1093/hmg/dds474 dds474 [pii]
- Schnell, M. A., Zhang, Y., Tazelaar, J., Gao, G. P., Yu, Q. C., Qian, R., . . . Wilson, J. M. (2001). Activation of innate immunity in nonhuman primates following intraportal administration of adenoviral vectors. *Mol Ther*, *3*(5 Pt 1), 708-722. doi: 10.1006/mthe.2001.0330 S1525-0016(01)90330-2 [pii]

- Schrank, B., Gotz, R., Gunnensen, J. M., Ure, J. M., Toyka, K. V., Smith, A. G., & 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*(18), 9920-9925.
- Shababi, M., Habibi, J., Ma, L., Glascock, J. J., Sowers, J. R., & Lorson, C. L. (2012). Partial restoration of cardio-vascular defects in a rescued severe model of spinal muscular atrophy. *J Mol Cell Cardiol*, *52*(5), 1074-1082. doi: S0022-2828(12)00034-X [pii] 10.1016/j.yjmcc.2012.01.005
- Shafey, D., Boyer, J. G., Bhanot, K., & Kothary, R. (2010). Identification of novel interacting protein partners of SMN using tandem affinity purification. *J Proteome Res*, *9*(4), 1659-1669. doi: 10.1021/pr9006987
- Shafey, D., Cote, P. D., & Kothary, R. (2005). Hypomorphic Smn knockdown C2C12 myoblasts reveal intrinsic defects in myoblast fusion and myotube morphology. *Exp Cell Res*, *311*(1), 49-61. doi: S0014-4827(05)00384-8 [pii] 10.1016/j.yexcr.2005.08.019
- Shayakhmetov, D. M., Li, Z. Y., Ternovoi, V., Gaggar, A., Gharwan, H., & Lieber, A. (2003). The interaction between the fiber knob domain and the cellular attachment receptor determines the intracellular trafficking route of adenoviruses. *J Virol*, *77*(6), 3712-3723.
- Shayakhmetov, D. M., & Lieber, A. (2000). Dependence of adenovirus infectivity on length of the fiber shaft domain. *J Virol*, *74*(22), 10274-10286.
- Shayakhmetov, D. M., Papayannopoulou, T., Stamatoyannopoulos, G., & Lieber, A. (2000). Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector. *J Virol*, *74*(6), 2567-2583.
- Shpargel, K. B., & Matera, A. G. (2005). Gemin proteins are required for efficient assembly of Sm-class ribonucleoproteins. *Proc Natl Acad Sci U S A*, *102*(48), 17372-17377. doi: 0508947102 [pii] 10.1073/pnas.0508947102
- Stoff-Khalili, M. A., Rivera, A. A., Stoff, A., Michael Mathis, J., Rocconi, R. P., Matthews, Q. L., . . . Curiel, D. T. (2007). Combining high selectivity of replication via CXCR4 promoter with fiber chimerism for effective adenoviral oncolysis in breast cancer. *Int J Cancer*, *120*(4), 935-941. doi: 10.1002/ijc.22338
- Strasswimmer, J., Lorson, C. L., Breiding, D. E., Chen, J. J., Le, T., Burghes, A. H., & Androphy, E. J. (1999). Identification of survival motor neuron as a transcriptional activator-binding protein. *Hum Mol Genet*, *8*(7), 1219-1226. doi: ddc135 [pii]
- Sumner, C. J., Kolb, S. J., Harmison, G. G., Jeffries, N. O., Schadt, K., Finkel, R. S., . . . Fischbeck, K. H. (2006). SMN mRNA and protein levels in peripheral blood: biomarkers for SMA clinical trials. *Neurology*, *66*(7), 1067-1073. doi: 01.wnl.0000201929.56928.13 [pii] 10.1212/01.wnl.0000201929.56928.13
- Sun, Y., Grimmmler, M., Schwarzer, V., Schoenen, F., Fischer, U., & Wirth, B. (2005). Molecular and functional analysis of intragenic SMN1 mutations in patients with spinal muscular atrophy. *Hum Mutat*, *25*(1), 64-71. doi: 10.1002/humu.20111
- Tao, N., Gao, G. P., Parr, M., Johnston, J., Baradet, T., Wilson, J. M., . . . Fawell, S. E. (2001). Sequestration of adenoviral vector by Kupffer cells leads to a nonlinear dose response of transduction in liver. *Mol Ther*, *3*(1), 28-35. doi: 10.1006/mthe.2000.0227

S1525-0016(00)90227-2 [pii]

Todd, A. G., Morse, R., Shaw, D. J., McGinley, S., Stebbings, H., & Young, P. J. (2010). SMN, Gemin2 and Gemin3 associate with beta-actin mRNA in the cytoplasm of neuronal cells in vitro. *Journal of Molecular Biology*, *401*(5), 681. doi: 10.1016/j.jmb.2010.06.058

Todd, A. G., Shaw, D. J., Morse, R., Stebbings, H., & Young, P. J. (2010). SMN and the Gemin proteins form sub-complexes that localise to both stationary and dynamic neurite granules. *Biochemical and biophysical research communications*, *394*(1), 211. doi: 10.1016/j.bbrc.2010.02.158

Tomko, R. P., Xu, R., & Philipson, L. (1997). HCAR and MCAR: the human and mouse cellular receptors for subgroup C adenoviruses and group B coxsackieviruses. *Proc Natl Acad Sci U S A*, *94*(7), 3352-3356.

Uchida, K., Nakajima, H., Hirai, T., Yayama, T., Chen, K., Guerrero, A. R., . . . Baba, H. (2012). The retrograde delivery of adenovirus vector carrying the gene for brain-derived neurotrophic factor protects neurons and oligodendrocytes from apoptosis in the chronically compressed spinal cord of twy/twy mice. *Spine (Phila Pa 1976)*, *37*(26), 2125-2135. doi: 10.1097/BRS.0b013e3182600ef7

Valori, C. F., Ning, K., Wyles, M., Mead, R. J., Grierson, A. J., Shaw, P. J., & Azzouz, M. (2010). Systemic delivery of scAAV9 expressing SMN prolongs survival in a model of spinal muscular atrophy. *Science translational medicine*, *2*(35), 35ra42. doi: 10.1126/scitranslmed.3000830

Waddington, S. N., McVey, J. H., Bhella, D., Parker, A. L., Barker, K., Atoda, H., . . . Baker, A. H. (2008). Adenovirus serotype 5 hexon mediates liver gene transfer. *Cell*, *132*(3), 397-409. doi: 10.1016/j.cell.2008.01.016

S0092-8674(08)00116-5 [pii]

Wan, L., Battle, D. J., Yong, J., Gubitz, A. K., Kolb, S. J., Wang, J., & 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*(13), 5543-5551. doi: 25/13/5543 [pii]

10.1128/MCB.25.13.5543-5551.2005

Wickham, T. J., Mathias, P., Cheresch, D. A., & Nemerow, G. R. (1993). Integrins alpha v beta 3 and alpha v beta 5 promote adenovirus internalization but not virus attachment. *Cell*, *73*(2), 309-319. doi: 0092-8674(93)90231-E [pii]

Wickham, T. J., Segal, D. M., Roelvink, P. W., Carrion, M. E., Lizonova, A., Lee, G. M., & Kovesdi, I. (1996). Targeted adenovirus gene transfer to endothelial and smooth muscle cells by using bispecific antibodies. *J Virol*, *70*(10), 6831-6838.

Wickham, T. J., Tzeng, E., Shears, L. L., 2nd, Roelvink, P. W., Li, Y., Lee, G. M., . . . Kovesdi, I. (1997). Increased in vitro and in vivo gene transfer by adenovirus vectors containing chimeric fiber proteins. *J Virol*, *71*(11), 8221-8229.

Wilber, A., Frandsen, J. L., Wangenstein, K. J., Ekker, S. C., Wang, X., & McIvor, R. S. (2005). Dynamic gene expression after systemic delivery of plasmid DNA as determined by in vivo bioluminescence imaging. *Hum Gene Ther*, *16*(11), 1325-1332. doi: 10.1089/hum.2005.16.1325

Wirth, B., Brichta, L., Schrank, B., Lochmuller, H., Blick, S., Baasner, A., & Heller, R. (2006). Mildly affected patients with spinal muscular atrophy are partially protected

- by an increased SMN2 copy number. *Human genetics*, 119(4), 422. doi: 10.1007/s00439-006-0156-7
- Wood, M., Perrotte, P., Onishi, E., Harper, M. E., Dinney, C., Pagliaro, L., & Wilson, D. R. (1999). Biodistribution of an adenoviral vector carrying the luciferase reporter gene following intravesical or intravenous administration to a mouse. *Cancer Gene Ther*, 6(4), 367-372. doi: 10.1038/sj.cgt.7700090
- Young, P. J., Le, T. T., Dunckley, M., Nguyen, T. M., Burghes, A. H., & Morris, G. E. (2001). Nuclear gems and Cajal (coiled) bodies in fetal tissues: nucleolar distribution of the spinal muscular atrophy protein, SMN. *Experimental cell research*, 265(2), 252. doi: 10.1006/excr.2001.5186
- Young, P. J., Le, T. T., thi Man, N., Burghes, A. H., & Morris, G. E. (2000). The relationship between SMN, the spinal muscular atrophy protein, and nuclear coiled bodies in differentiated tissues and cultured cells. *Experimental cell research*, 256(2), 365. doi: 10.1006/excr.2000.4858
- Zapletalova, E., Hedvicakova, P., Kozak, L., Vondracek, P., Gaillyova, R., Marikova, T., . . . Fajkusova, L. (2007). Analysis of point mutations in the SMN1 gene in SMA patients bearing a single SMN1 copy. *Neuromuscul Disord*, 17(6), 476-481. doi: S0960-8966(07)00084-3 [pii] 10.1016/j.nmd.2007.03.003
- Zhang, H., Xing, L., Rossoll, W., Wichterle, H., Singer, R. H., & Bassell, G. J. (2006). Multiprotein complexes of the survival of motor neuron protein SMN with Gemins traffic to neuronal processes and growth cones of motor neurons. *The Journal of neuroscience : the official journal of the Society for Neuroscience*, 26(33), 8622. doi: 10.1523/jneurosci.3967-05.2006
- Zhang, H. L., Pan, F., Hong, D., Shenoy, S. M., Singer, R. H., & Bassell, G. J. (2003). Active transport of the survival motor neuron protein and the role of exon-7 in cytoplasmic localization. *J Neurosci*, 23(16), 6627-6637. doi: 23/16/6627 [pii]
- Zhang, Z., Lotti, F., Dittmar, K., Younis, I., Wan, L., Kasim, M., & Dreyfuss, G. (2008). SMN deficiency causes tissue-specific perturbations in the repertoire of snRNAs and widespread defects in splicing. *Cell*, 133(4), 585. doi: 10.1016/j.cell.2008.03.031
- Zou, J., Barahmand-pour, F., Blackburn, M. L., Matsui, Y., Chansky, H. A., & Yang, L. (2004). Survival motor neuron (SMN) protein interacts with transcription corepressor mSin3A. *J Biol Chem*, 279(15), 14922-14928. doi: 10.1074/jbc.M309218200 M309218200 [pii]
- Zubieta, C., Schoehn, G., Chroboczek, J., & Cusack, S. (2005). The structure of the human adenovirus 2 penton. *Mol Cell*, 17(1), 121-135. doi: S109727650400752X [pii] 10.1016/j.molcel.2004.11.041

Curriculum Vitae

Education

- 2010 – Present **Master's in Science: Microbiology and Immunology**
- Ontario Graduate Studies (OGS) Bursary (2011-2012)
 - University of Ottawa Excellence Scholarship (2011-2012)
 - University of Ottawa Admission Scholarship (2010)
- 2005 – 2010 **Baccalaureate in Science with Specialization in Biopharmaceutical Science – Genomics Option (Co-op)**
- *Cum Laude*
 - Completed four Co-op terms
 - CGPA: 7.8 DGPA: 8.8
 - University of Ottawa entrance scholarship (2005-2006)
- Thesis title: Developmental Programming by Resistant Starch Has Limited Effects in Weaning Wistar Rats

Work Experience

- June 2013 **Research Assistant**
- Present Agriculture and Agri-Food Canada, Ottawa, Ontario
- Day to day maintenance and upkeep of the Canadian Collection of Fungal Cultures
 - Fulfilment of client requests regarding the procurement and deposition of samples from the collection.
- December 2012 **Laboratory Technician**
- April 2013 Department of Biochemistry, University of Ottawa, Ottawa, Ontario
- In charge of solutions, equipment and day-to-day activities of the second year undergraduate introductory biochemistry course (BCH 2333/2733).
 - Taught and supervised second year students in basic biochemistry techniques.
 - Managed lab demonstrators (Graduate student) to ensure that the labs were taught properly and in the right timeframe.

June 2010 **Graduate Student – Masters in Science**

November 2012 Department of Regenerative Medicine, Ottawa Hospital Research
Institute, Ottawa, Ontario

- Developing a gene therapy strategy using adenoviral vectors for the
treatment of Spinal Muscular Atrophy.

September 2012 **Lab demonstrator – BCH 3756**

December 2012 Department of Biochemistry, University of Ottawa, Ontario

- Guided and taught third year students basic molecular biology skills.
Graded student’s lab reports and oral presentations.

Januray 2012 **Marker – BCH 4125**

April 2012 Department of Biochemistry, University of Ottawa, Ontario

- Marked exams for a fourth year undergraduate course in cell cycle
regulation.

September 2010 **Teaching assistant – BPS 4900**

April 2011 Department of Biology, University of Ottawa, Ontario

- Led weekly class discussions and student presentations on recent
papers in the field of molecular biology and biopharmaceuticals.

January 2011 **Lab demonstrator – BCH 2733**

April 2011 Department of Biochemistry, University of Ottawa, Ontario

- Guided and taught second year students basic lab skills in
biochemistry. Corrected and graded student’s lab reports.

September 9th 2009 **Honour Student**

April 17th 2010 Dietary Carbohydrates, Health Canada, Ottawa, Ontario

- Took part in a study analyzing foetal programming of obesity in rats. Performed necropsies, glucose analysis of serum as well as measured the level of gene and protein expressions in liver, colon, stomach, brain and adipose tissues for 3 week old pups.

April 27th 2009 **Laboratory Technician – Co-op Student**

September 8th 2009 Dietary Carbohydrates, Health Canada, Ottawa, Ontario

- Analyzed the effects of resistant starch diets on colonic gene expression in rats with dietary induced obesity. The quantification of lipid metabolism genes was achieved with quantitative real-time PCR (RT-PCR). Protein levels were also quantified by Western blotting and with ELISA kits.

September 2nd 2008 **Laboratory Technician – Co-op Student**

December 19th 2008 Dietary Carbohydrates, Health Canada, Ottawa, Ontario

- Measured the level of expression of genes linked to dietary lipid metabolism in the liver of rats predisposed to develop dietary obesity when challenged with high and low glyceamic index diets. Achieved with quantitative real-time PCR (RT-PCR) and semi-quantitative PCR (qPCR; using 18s RNA as a standard). Proteomic analysis methods (Western immunoblotting) were also used to quantify the level of proteins of select genes that showed no difference in the transcriptome.

January 7th 2008 **Laboratory Technician – Co-op Student**

August 29th 2008 Environmental Microbiology, Agriculture and Agri-Food Canada – Experimental Farm, Ottawa, Ontario

- Researched, modified and established new protocols for the isolation and

growth of *Arcobacter spp.* as well as develop a new solid growth media. Part of a pilot project aimed at determining the level of contamination of the South Nation River by fecal bacteria from field runoff. Created a frozen isolate bank and confirmed the species and genus of the isolates by multiplex-PCR (mPCR).

Skills

- Communication
- Fully bilingual in both French and English
 - Presented several posters at conferences and symposiums as intra-departmental podium talks.
 - Taught several different laboratories to undergrad students at the University of Ottawa.
- Information Technologies
- Advanced knowledge of Microsoft Office
 - Designed primers and researched sequences using the NCBI and IDT tools: Entrez, blastn, PrimerQuest, OligoAnalyze, Spidey, ORF finder.
 - Highly developed skills with Photoshop, Illustrator, GraphPad, and Endnote.
- Laboratory skills
- Molecular Biology: Cloning, sequencing, bacterial expression of recombinant proteins, western, ELISA, qPCR, RT-PCR, PCR, luciferase assays, immunofluorescence and confocal imaging, Protein purification through His-tag and anion exchange columns.
 - Cell culture: infection, transfection, growth of suspension and adherent cells, virus preparation and purification.
 - Animal experiments: Daily upkeep of rat cages and food allocation. Oral glucose tolerance test and daily weighing. Decapitations and collection of whole trunk blood, body fats, liver, intestines and its contents, and the brain. Intra-muscular, intraperitoneal, and intra-vascular injections of viral vectors in mice. Collection of spinal cord, sciatic nerve, diaphragm, liver and tibialis anterior. X-gal tissue stains and B-gal luminosity assays on collected mice tissues.
- Hobbies
- Men's league hockey – OSMHL since 2011
 - Competitive and recreational ultimate frisbee – OCUA, Firebird, and University of Ottawa Geegees – Since 2008

Publications

1. Goulet, BB., McFall, ER., Wong, CM., Kothary, R., and Parks, RJ. (2013). "Supraphysiological expression of survival motor neuron protein from an adenovirus vector does not adversely affect cell function". *Biochemistry and Cell Biology*. 91: 1-13.
2. Goulet, BB., Kothary, R., and Parks, RJ. (2012). "At the "Junction" of Spinal Muscular Atrophy Pathogenesis: The Role of Neuromuscular Junction Dysfunction in SMA Disease Progression". *Current Molecular Medicine*., 13: 1-15.
3. Aziz, AA., Kenney, LS., Goulet, B, and El-Sayed, AA. (2009). "Dietary Starch Type Affects Body Weight and Glycemic Control in Freely Fed but Not Energy-Restricted Obese Rats". *J. Nutr.*, 139: 1881-1889.

Posters

1. Goulet, B., Kothary, R., and Parks, RJ. 2011. Development of Gene Therapy for Spinal Muscular Atrophy. 10e Colloque de l'Association de Thérapie Génique du Québec, November 18th, 2011. Quebec City, Quebec, Canada.
2. Goulet, B., Kothary, R., and Parks, RJ. 2011. Development of Gene Therapy for Treatment of Spinal Muscular Atrophy (SMA). OHRI Research Day, November 2011. Ottawa, Ontario, Canada.
3. Goulet, B., Kothary, R., Parks, RJ. 2011 Development of Novel Gene Therapy Strategy for the Treatment of SMA. 15th Annual International Spinal Muscular Atrophy Research Group Meeting, June 23-25th, 2011. Orlando, Florida, United States. Abstract 70B.
4. Goulet, BB., and Parks, RJ. 2011. Gene Therapy for Treatment of Spinal Muscular Atrophy. BMI Poster Day, May 19th 2011. University of Ottawa, Ottawa, Ontario, Canada.
5. Goulet, B., Kothary, R., and Parks, RJ. 2010. Gene Therapy for Treatment of Spinal Muscular Atrophy (SMA). OHRI Research Day, November 2010. Ottawa, Ontario, Canada.
6. Lanthier, M., Moreau, G., Cloutier, M., Goulet, B., Nossova, N., Lachapelle, N., Najem, D., Lapen D.R., Topp, E. 2010. Antibiotic Resistance of *Arcobacter butzleri*

- isolates from the South Nation River Watershed, Ontario, Canada. 2nd American Society for Microbiology Meeting on Antimicrobial Resistance in Zoonotic and Foodborne Pathogens in Animals, Humans and the Environment, June 8-11, 2010. Toronto, Ontario. Abstract B78.
7. Goulet, B., Roberts, J., Kenney, L., Raju, J., and Aziz, A. (2009) « Inflammatory Responses to a High Resistant Starch Diet and Energy Restriction in Rats with Diet-Induced Obesity ». Health Canada Science Forums, Ottawa, Canada, November 16-17 2009. Abstract 1.12.
 8. Aziz, A., Goulet, B, Kenney, L, Cockell, K., and Griffin P. (2009) «Comparative effects of amylose vs amylopectin starch on lipid metabolism in rats with dietary-induced obesity. 17th European Congress on Obesity». Amsterdam, The Netherlands, May 6-8 2009 (poster).
 9. Lanthier, M., Lapen, DR., Wilkes, G., Cloutier, M., Goulet, B., Nossova, N., and Topp, E. (2009) “Isolation and Characterization of *Arcobacter spp.* From the South Nation Watershed, Ontario Canada.” 59th Annual Meeting of the Canadian Society of Microbiologists, June 15-18 2009, Montréal, Québec. Abstract AE37.
 10. Lanthier, M., Lapen, DR., Wilkes, G., Cloutier, M., Goulet, B., Nossova, N., and Topp, E. (2009) “Isolation and Characterization of *Arcobacter spp.* From the South Nation Watershed, Ontario Canada.” 109th General Meeting of the American Society of Microbiology, May 17-21 2009, Philadelphia, Pennsylvania. Abstract Q-170.