

Exosomes: A novel biomarker and approach to gene therapy for spinal muscular atrophy

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Abstract

Spinal muscular atrophy (SMA) is a neuromuscular disease caused by reduced levels of the survival motor neuron (SMN) protein. SMA results in degeneration of motor neurons, progressive muscle atrophy, and death in severe forms of the disease. Currently, there is a lack of inexpensive, readily accessible, accurate biomarkers to study the disease. Furthermore, the current FDA approved therapeutic is neither 100 % effective nor accessible for all patients, thus more research is required. Tiny cell derived vesicles known as exosomes have been evaluated in an attempt to identify novel biomarkers for many disease states and have also shown therapeutic promise through their ability to deliver protein and nucleic acid to recipient cells. The research presented herein investigates whether (1) the level of SMN protein in exosomes isolated from the medium of cells, and serum from animal models and patients of SMA is indicative of disease, to serve as a biomarker for monitoring disease progression and therapeutic efficacy; (2) SMN-protein loaded exosomes can be utilized to deliver SMN protein to SMN-deficient cells; (3) adenoviral vectors are effective at creating SMN protein-loaded exosomes *in situ* for body wide distribution of SMN protein. This research has shown SMN protein is naturally released in extracellular vesicles, and the level of exosomal SMN protein is reflective of the disease state. Exosomes can also be modified to hold enhanced levels of SMN protein and deliver them to both the cytoplasm and nucleus of SMN-deficient cells. Furthermore, adenoviral vectors expressing luciferase-tagged *SMN1* cDNA, targeted to the liver, results in SMN protein-loaded exosomes and detectable luciferase activity, body-wide. Thus, exosomes present as an effective biomarker and potentially a novel approach to treat SMA.

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List of Abbreviations

Abbreviation	Word
293	Human embryonic kidney cells
AD	Alzheimer's Disease
Ad	Adenovirus
AdRP2014	Adenovirus expressing empty cassette
AdCre	Ad expressing Cre recombinase
AdF3SMN	Adenovirus expressing 3x Flag tagged SMN
AdFloxLuc	Ad expressing floxed stop luciferase
AdLucSMN	Adenovirus expressing Luciferase SMN
AdMyoDGFP	Ad expressing GFP tagged MyoD
AdSMN	Ad expressing flag-tagged SMN
AAV	Adeno-associated virus
ASO	Antisense oligonucleotide
ATP	Adenosine triphosphate
BMECs	Brain microvascular endothelial cells
CAR	Coxsackie adenovirus receptor
CMV	Cytomegalovirus
CNS	Central nervous system
CSF	Cerebral spinal fluid
E1, E2, E3, E4	Early region 1, 2, 3, 4
EGFR	Epidermal growth factor receptor
ESCRT	Endosomal sorting complexes required for transport
FDA	Food and Drug Administration
G3BP	Ras GTPase-activating protein-binding protein 1
GFP	Green fluorescent protein
HINES-2	Hammersmith Infant Neurological Examination 2
HdAd	Helper-dependent adenovirus
HIV	Human immunodeficiency virus
ILV	Intraluminal vesicles
Kb	Kilobases
Kd	Kilodelton
miRNA	Micro RNA
MOI	Multiplicity of infection
MVB	Multivesicular bodies
OTC	Ornithine transcarbamylase
pA	Polyadenylation site
PBMC	Peripheral blood mononuclear cells
PFU	Plaque forming units
P5	Post natal day 5
RBC	Red blood cells
RIPA	Radioimmunoprecipitation assay
RVG	Rabies Viral Glycoprotein
siRNA	Small interfering RNA
SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SMA	Spinal muscular atrophy
SMN	Survival motor neuron, telomeric
SMN2	Survival motor neuron gene, centromeric
SMN Δ 7	Survival motor neuron lacking exon 7
snRNP	Small nuclear ribonuclear protein
TA	Tibialis Anterior
TBI	Traumatic brain injury
TBST	Tris buffered saline tween
TIA-1/R	TIA1 Cytotoxic Granule Associated RNA Binding Protein
Unrip	unr-interacting protein
VPS4	Vacuolar protein sorting-associated protein 4
vp	Viral particles

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Chapter 1.0: Introduction

Spinal Muscular Atrophy

Genetics. Spinal muscular atrophy (SMA) is a monogenic, neurodegenerative disease that results in progressive wasting of the proximal muscles due to insufficient levels of survival motor neuron (SMN) protein. Often fatal in newborns, SMA is classified as a rare disease, with an estimated prevalence of 1-2 in 100,000, an incidence of 1 in 10,000 live births, and a carrier frequency of 1 in 50 [1]. This autosomal recessive disorder was first characterized in the early 1890s by Drs. Werdnig and Hoffman, in which a child displayed severe signs of muscle atrophy and loss of motor neurons [2]. Later described as SMA, diagnosed infants also presented a lack of motor control, paralysis of the diaphragm, breathing impairment, suffocation, and death within early life [3]. Currently, five subtypes have been described, type 0 through 4, presenting a range of phenotypes from respiratory distress and an inability to walk from birth, to individuals who maintain independence throughout life (Table 1) [2, 3].

Table 1.1 Characterization of the SMA disease subtypes

<i>Disease Type</i>	<i>SMN2 Copy No.</i>	<i>Age of Onset</i>	<i>Motor Milestone Achieved and/or Phenotype observed</i>	<i>Case %</i>	<i>Lifespan</i>
0	≥ 1	<i>In utero</i>	Severe hypotonia, immediate respiratory impairment, facial weakness	NA	< 3 months
1	1-3	< 6 months	Hypotonia present, unable to sit unaided. Spontaneous activity in the hands and feet.	50-60%	< 2 years
2	2-4	6-18 months	Sit unassisted, remain non-ambulant	30%	2 nd -3 rd decade
3	2-5	> 18 months	Learn to walk, but typically becomes wheelchair bound	10%	NA
4	≥ 4	> 30 years	Slow progression of proximal muscle weakness	1 %	NA

Approximately 95 % of SMN protein deficiency occurs due to *SMN1* homozygous deletion or mutation [4]. Fortunately, through evolution, humans alone have acquired a homologous copy of the *SMN1* gene, termed *SMN2* [5]. While these two genes differ by less than 1 % in their nucleotide sequence [6], a translationally silent, point mutation in *SMN2* (c.C840T) results in the formation of a non-functional splice variant, *SMN Δ 7*, producing full length SMN protein approximately 10-20 % of the time [7]. The *SMN Δ 7* protein is a truncated, unstable version of the full length SMN protein [8] which prohibits formation of protein complexes necessary for SMN to fulfill its biological duty [9]. Clinical epidemiology has shown *SMN2* copy number is roughly, positively correlated to SMN protein levels and an improved prognosis [10]. Thus, with disease phenotype negatively correlated to the level of SMN protein, it is hypothesized that increasing SMN protein levels may alleviate the SMA phenotype. However, the exact mechanism of pathogenesis resulting from SMN protein deficiency has yet to be elucidated and the lack of understanding of SMN's primary involvement in motor neuron pathogenesis makes it difficult to develop the necessary treatments for patients.

Complete loss of SMN is embryonic lethal [11]. Transgenic mice with their *Smn1* gene knocked out but provided 2 copies of the human *SMN2* transgene, develop normal numbers of motor neurons at birth. However, these motor neurons quickly degenerate and mice eventually succumb to illness by post natal day 5 (P5). In contrast, mice expressing 8 copies of the *SMN2* transgene, rescues the phenotype [12]. In mice, *Smn* mRNA has been documented in oocytes and while SMN protein is critical for embryo development and survival, *Smn*^{-/-} embryos still develop to the morula stage before undergoing massive cell death. Although the mechanism of action has not been elucidated, the initial survival of the embryos is likely the result of SMN protein transfer from the mother [11]. During development, SMN protein levels in rats are high and

tightly regulated. La Bella and coworkers identified SMN protein as early as embryonic day 14 (E14). Heightened levels were recorded in the spinal cord and brain, and were maintained for approximately 2 weeks after birth, before dropping significantly [13]. Examination of peripheral tissues such as muscle and liver displayed high levels of SMN protein during embryonic development, but similar to the brain, levels dropped significantly soon after birth [13]. Gemin2, a protein known to interact with SMN protein during the chaperoning of snRNA from the cytoplasm to the nucleus, was shown to have levels that were tightly regulated to the levels of SMN protein during development in both wildtype and *Smn*^{+/-} heterozygous mice. Yet, immunohistochemistry revealed the majority of SMN protein within neurons did not co-localize with Gemin2. Therefore, while SMN protein and Gemin2 may be bound by similar regulatory events, SMN's role in neurons is independent of the spliceosomal complex and its function during development occurs in a cell-specific manner [14]. In the severe mouse model of SMA (*Smn*^{-/-}; *SMN2*), *Smn2* transcripts were found to decline from E12.5 to E14.5. The drop was tightly associated with alterations in pre-mRNA splicing of affected mice, with errors in splicing demonstrating a positive correlation with disease severity [15]. This result further substantiates SMN's tight regulation and significance in development, while its reduction during pre-symptomatic stages leading to altered splicing transcripts suggests dysregulation of the spliceosome may be important in motor neuron development. In humans, *SMN* mRNA has been detected in spinal cords as early as 8 weeks into fetal development [16]. SMA type 1 fetuses, 16 weeks of age, demonstrate reduced levels of SMN protein in a variety of tissues including muscle, brain, and heart [17]. Using aged matched controls, there was a marked decrease in SMN protein levels when examining the different SMA disease states, with the most severe, type 1, showing the largest deficiency. While these studies show conflicting information as to whether

SMN gene expression declines during embryonic development or post-natally, this research highlights SMN protein's importance during development, specifically within the central nervous system (CNS).

Protein Function at Cellular Level. SMN's most established role is its involvement in the spliceosomal machinery with the assembly of small nuclear ribonucleoprotein particles (snRNP). Details arising about SMN's binding partners, such as the gemins 2-8, Sm proteins, and UnRip indicate SMN's importance for the assembly of snRNPs which ultimately form splicing complexes [15, 18-20]. Assembly of the SMN complex begins within the cytoplasm, where SMN self-oligomerizes, binds to Gemins, and UnRIP. With the help of the methyltransferase PRMT5 and pICln, Sm proteins are transferred from the methylsome to the SMN complex. From here, the SMN-Sm protein complex binds with Gemin5 and the desired snRNA, following which the 3'- and 5'- ends are processed [21]. The SMN protein complex is finally joined by several proteins, including snurportin and importin- β , to aid in the complex's movement into the nucleus via the nuclear pore [22]. Within the nucleus, the SMN complex interacts with coilin in membraneless organelles known as Cajal bodies. Here, the SMN complex dissociates, and snRNAs mature into their appropriate RNA-Protein complexes (snRNPs) which later migrate to the nucleolus to form the spliceosome complex [23, 24]. Reduction in SMN protein levels leads to reduced levels of snRNPs and alterations in the overall levels of individual snRNAs, leading to large defects in the minor spliceosome [25-27]. However, when splicing errors were assessed in the spinal cords of a severe mouse model of SMA (*Smn*^{-/-} *SMN2*; *SMN* Δ 7) at pre-, early- and late-symptomatic stages, significant changes in splicing were only identified in late stage mice. Accordingly, errors in splicing are likely secondary effects of disease pathology and not the cause [28]. Complementing this, splicing alterations in aged matched *Drosophila* comparing

wildtype and *Smn* null larvae during development showed a lack of significant difference in genome wide mRNA alterations with errors in stress transcripts only increasing with developmental arrest, suggesting alterations were due to locomotor defects and not locomotor defects arising from altered transcripts [29]. Interestingly, tissue wide splicing defects have been identified in SMN deficient mice [27], potentially suggesting alterations in motor neuron specific transcripts may contribute to their enhanced vulnerability.

Other reports detail SMN's involvement in the growth and development of motor neurons through SMN's ability to act as a chaperone in axonal transport of protein and RNA [30-32]. SMN protein has been identified in neuronal granules or ribonucleoprotein complexes that move bi-directionally in the cytoskeleton. However, the SMN Δ 7 protein demonstrated an inability to appropriately form granules or aid in active transport [33]. In the rat spinal cord, SMN protein is reported to be localized to the spinal dendrites and axons, especially during postnatal development [31]. While, decreased levels of *Smn* protein in mice results in reduced axon length, growth cone area, mRNA mislocalization, and a reduction in hnRNP R and β -actin, proteins important for neuronal development and path finding [32]. Furthermore, *Smn* knockdown in zebrafish results in defects in motor axon path finding, including truncated motor axons and increased branching [34]. HuD, a neuronal specific protein, has also been identified as a binding partner to *Smn* protein in zebrafish neuronal cells. The two proteins form a complex and bind with a variety of important neuronal mRNA including *actin* and *cpg15*, recognized for its role in the growth and development of spinal motor axons. Reduction of *Smn* protein results in lower *cpg15* levels, translating to reduced axonal path finding and synapse formation, however these defects could be reversed with the reintroduction of *Smn* or through

overexpression of HuD [30, 35, 36]. Thus, the reduction of SMN could lead to reduced local translation that severely impacts motor neurons growth and function.

SMN protein not only controls the movement of *actin* mRNA through neurons, but also regulates actin dynamics by its interaction with profilin-2, an actin binding protein. Reduction of SMN protein disrupts the ROCK/profilin-2 complex, likely due to the increased levels of unbound profilin-2 as the result of the absence of SMN protein. Consequently, the RhoA/ROCK pathway is up-regulated and the growth and maturation of neurons is stalled [37, 38]. Research later determined that increased profilin-2 levels led to significant decreases of an actin bundling protein, plastin 3. Plastin 3 has been suggested to be a disease modifier, as studies have reported heightened levels in asymptomatic, discordant SMA siblings [39]. Whereas, knockout of profilin-2 leads to significant increases of plastin 3, suggesting that SMN protein expression is tightly linked to profilin-2 and plastin 3 levels [37]. High ROCK activity also contributes to reduced myogenin and cofilin activity in skeletal muscle, leading to impairments in myoblast fusion and diminished muscle contraction due to impaired neuromuscular junction (NMJ) post-synapses [40]. Therefore, SMN deficiency leads to alterations in the RhoA/ROCK pathway, leading to impaired neuritogenesis, axon path-finding, and formation of the NMJ.

One of the lesser known roles for SMN protein, is its involvement in stress granule formation. Stress granules are assemblies of protein and RNA within the cytosol or nucleus (termed nuclear stress bodies), which form under stress (external chemicals, ultraviolet radiation, or cellular stress responses). These protein and RNA rich structures function to promote innate immune responses, control local translation, and clearance of failed RNA complexes in the process of translation [41]. Three main steps occur in the process of stress granule formation: 1. Translation is halted; 2. Proteins containing ‘prion like domains’ assemble together, supporting

protein aggregation, to form multimeric complexes with RNA, resulting in their liquid-liquid phase separation. As aggregation occurs more proteins are recruited to the complex to stabilize the stress granule core, and; 3. Signaling molecules are recruited to the complex. Stress granules can then be removed through either autophagy or disassembly by chaperones such as heat shock proteins [42]. SMN protein has been identified to be involved in the process of stress granule assembly, and interacts with other common stress granule ‘prion like’ proteins TIA-/R and G3BP [43]. Depletion of SMN protein results in a reduced ability to form stress granules. Interestingly, stress granule formation is dependent on the presence of exon 7 in SMN, as the truncated protein remains in the nucleus and fails to interact with proteins (TIA-1/R and G3BP) involved in stress granule formation [43, 44]. The reduction of stress granules in the absence of SMN protein increases cell death to known cell stressors, while over-expression of G3BP during SMN protein deficiency partially rescues this defect by increasing stress granule formation [44].

Protein Function in Peripheral Tissues. While the degeneration of motor neurons is seemingly the best characterized attribute of SMN protein deficiency, peripheral tissues such as muscle, heart, brain, testes, and bone also exhibit defects which contribute to the complexity of the disease (reviewed in [45]). Previously, the question as to whether treatment of peripheral tissues in patients with SMA, was necessary, was under heated debate. Initial work in mouse models of SMA demonstrated that selective expression of *Smn* in motor neurons was enough to correct the phenotype [46]. Contrary to this work, some studies have suggested restoration of full length SMN protein solely in the neurons does not fully rescue the phenotype in affected mice [47]. Transgenic mice with inducible *Smn* gene expression, revealed that long-term induction of *Smn* at P1 rescued the SMA pathology, but 70 % of the mice died within a month when induction was ceased. Notably, the NMJs appeared to be normal at the time of death, which

implies the absence of SMN protein has major implications on surrounding tissues, contributing largely to disease manifestation.

With the numerous roles SMN protein plays across various tissues, the question remains as to why reduction of a ubiquitous protein results in a disease primarily impacted by the loss of motor neurons. Several theories have been described to address this, including: (1) a higher requirement exists for SMN protein in motor neurons, in comparison to other tissues. For example, deletion of *Smn* within the muscle of SMN Δ 7 mice using a Cre-Lox system did not result in significant alterations in muscle strength or fiber size [48], while small increases in *Smn* protein within neurons significantly increased the lifespan and motor neuron count [46]. (2) SMN protein depletion negatively impacts the splicing of transcripts required for the integrity and maintenance of motor neurons. Motor neurons isolated from affected mice prior to detectable motor neuron pathology showed mRNA dysregulation in a cell specific manner, such as errors in *agrin* mRNA, a protein necessary for neuromuscular junction development [49]. (3) SMN protein is required for the appropriate localization and axonal transport of mRNA and protein required for the growth and development of motor neurons. Depletion of SMN protein results in the reduction and mislocalization of β -*actin* mRNA and protein, which is necessary for the growth and development of axons and growth cones [50]. Identifying the foundation of SMN's involvement in motor neuron development will aid in the advancement of direct therapeutic approaches which could prevent disease progression or manifestation, altogether.

Biomarkers for SMA. Currently available clinical outcome measures often lack sensitivity and are thus ineffective at demonstrating the efficacy of novel therapies. In SMA, clinical endpoints are very difficult to assess because neuromuscular diseases such as SMA are slowly progressive and clinical outcome measures (i.e. 6 minute walk test) are insensitive or

inappropriate for newborns or more severely affected non-ambulant patients [51]. One of the least-invasive methods to provide insight into disease severity and prognosis would be to identify a biomarker in blood that corresponds with the SMA disease state. To this end, several researchers have analyzed peripheral blood mononuclear cells (PBMC) for a variety of disease markers, including total *SMN* transcript level, relative full-length versus *SMNΔ7* mRNA transcript level, and SMN protein level [52-56]. Although these studies demonstrated a trend between motor function and several markers, no statistical correlation was observed. Thus, there is an urgent need for readily available, noninvasive biomarkers that can distinguish healthy and SMA disease states, and preferably one that provides information about therapeutic efficacy.

SMA Therapeutics. With the identification of the disease-causing gene, *SMN1*, combined with the evolutionary advantage of the *SMN2* gene, there are a variety of routes researchers can use to treat SMA. Gene therapy can be utilized to deliver a healthy copy of the *SMN1* gene using viral vectors [57, 58]. Correction of *SMN2* splicing can be attempted using small molecules, splice switching oligonucleotides, or antisense oligonucleotides (ASOs) to bind to the affected region and block splicing silencers or promote splicing enhancers [59-62]. Modification of the *SMN2* gene can be examined for increasing production of *SMN2* transcripts, ultimately leading to the translation of significantly more full length protein [59]. As SMN2 protein is relatively unstable and has a shorter half-life in comparison to its *SMN1* homologue [8, 63], methods are currently being employed to increase the stability of the SMN2 protein [8]. SMN-independent routes include neuro-protective agents to reduce neuro-degeneration, muscle stimulants to increase contractility and power, and anti-inflammatory agents to limit cell death [64-66].

As of December 2016, the FDA approved SMA's first therapeutic intervention known as Nusinersen, or by its market name, Spinraza [67]. Spinraza is an ASO used to correct the

splicing error in *SMN2* by binding to its pre-mRNA and blocking the formation of an intronic splicing silencer complex. This newly formed complex increases the level of full length *SMN2* mRNA, translating to significantly greater level of full length protein [68, 69]. Spinraza has been shown to increase the lifespan in responders, and the number of motor milestones reached when compared to natural history studies. Due to Spinraza's inability to cross the blood-brain barrier after systemic delivery, patients must receive the drug intrathecally [68]. Treatment begins with four loading doses, two weeks apart, followed by maintenance doses every four months, equating to a heavy financial burden of approximately 750,000 USD in the first year and 375,000 USD each following year. Complications include upper and lower respiratory infections, constipation, proteinuria (potentially renal toxicity), and an increased risk of bleeding complications [68, 70]. Reports from the phase 3 clinical trial ENDEAR, used to assess safety and efficacy in SMA type 1 patients from 37-42 weeks old, showed only half of treated patients achieved the next motor milestone [70]. While only 20 % (in comparison to 6 % in the placebo) achieved the next milestone in the phase 3 CHERISH clinical trial for late onset patients diagnosed after 6 months [71]. More recently, a side arm study with an extended treatment group of type 1 patients older than 6 months, with 2 or 3 *SMN2* copies, were provided Spinraza. A median improvement of 4 points was seen on the Hammersmith Infant Neurological Examination 2 (HINES-2) scale, which assesses motor milestones and neurological development, but this failed to reach statistical significance. The study noted that while some patients saw an improvement in their motor responses, there was also a worsening of respiratory function, potentially owing to either the slow action of the ASO to affect the respiratory system [72], or the inability of the ASO to cross the blood-brain barrier [73]. Concerns have also been raised regarding the quick FDA approval, suggesting that the HINES-2 and CHOP scales should not be primary outcomes due to the lack

of evidence providing a link to overall ventilation free survival, in addition to a lack of data for patients with type 2 or 3 SMA [74]. Thus, more research is required to develop novel therapies for non-responders, children who are unable to receive treatment until after 6 months of age, or those unable to receive intrathecal injections due to physical constraints such as scoliosis.

Another upcoming therapeutic for SMA, in its phase 3 trial is AVXS-101. Utilizing the gene replacement approach, AVXS-101 is a serotype 9, self-complementary AAV, which carries a healthy *SMN1* gene under a hybrid CMV enhancer and chicken- β -actin promoter [58, 75, 76]. The virus is delivered as a one-time administration, intravenously, at a dose of 1.1×10^{14} vg/kg. Patients are required to have no previous exposure to AAV9 or currently active viral infection. Results from the phase I clinical trial in which patients were provided a dose as high as 2×10^{14} vg/kg showed complications including raised levels of serum aminotransferases and aspartate aminotransferases, respiratory tract infections, and gastrointestinal issues [58]. Recent data following the phase I administration, 24 months post administration, indicated that all treated children were alive and did not require ventilation, compared to the 25 % chance of survival using the natural history study. Children also demonstrated dramatic improvements in their motor skills, including the ability to sit at a much earlier age, stand, or even walk.

With the current advancements in therapeutics and SMA's first approved treatment now available world-wide, many countries have begun to offer and implement newborn screening [77]. Research has continued to demonstrate that to minimize the developmental impact the disease has, patients must receive treatment as early as possible. Therefore, increasing awareness and support for parents could help remove SMA from being the leading cause of death by a genetic disease in newborns [78]. This became more evident in a recent pilot study for newborn screening when a type 1 newborn was identified and enrolled in the Spinraza clinical trial at

approximately 2 weeks of age. Due to the early treatment, the infant, who has currently had her first birthday, is asymptomatic and has met the motor milestones of a healthy child her age [77]. Presently, guidelines for diagnosed infants suggests that patients with 2 or 3 *SMN2* copies should receive immediate treatment, while those with one or greater than 3 *SMN2* copies are at the discretion of the physician [79]. Yet, these recommendations are conflicting, as research indicates early treatment, pre-symptomatically, leads to the greatest probability of rescuing and maintaining motor function. Additionally, the recognition that a non-linear relationship exists between disease state and *SMN2* copy number becomes more apparent in reports of children with greater *SMN2* copy numbers but a severe phenotype [10]. Thus, more research and continuous re-evaluation of the treatment plans surrounding newborn screening will be required to ensure that the quality of life and chance of survival is maximized in these patients.

While Spinraza and AVXS-101 have certainly paved the way for treatment options for patients with SMA, there are still several limitations. Not all patients are responding to treatment, others have antibodies to AAV which precludes them from participating, or perhaps physical limitations such as scoliosis which therefore inhibits the ability for a child to receive intrathecal injections [58, 70, 80]. The individual's *SMN2* copy number places a ceiling effect on the use of ASOs, thus limiting the amount of full-length protein produced [81]. ASOs are incapable of crossing the blood-brain barrier, thereby limiting its effect on either peripheral tissues if injected intrathecally, or on the CNS if injected systemically [61, 62, 73]. The titre being used for AAV is among the highest ever recorded, posing potential threats for immune responses and increased chance for integration events [82, 83]. Therefore, research into treatment options for patients with SMA is still necessary. From early mouse studies and now human trials, several aspects of the therapeutic development require optimization, (1) Defining the optimal window of

opportunity for rescuing motor neurons. Mouse and human trials have shown that the earlier a treatment can be provided, the more positive the result [71, 72, 75]. But, how early does an infant need to be treated to have the most optimal outcome? How late can a child be treated before there is no significant benefit in motor development or lifespan? (2) Is early treatment using SMN-dependent drugs enough to rescue the nervous system indefinitely? Or, will patients require treatment for the entirety of their lives? Although delivery of *SMN1*, or correction of *SMN2* splicing transcripts in motor neurons has shown to extend the lifespan of mouse models of SMA, correction in the CNS alone is not sufficient to rescue the phenotype [46, 61]. Yet, delivery of the *SMN1* gene or ASO systemically increases SMN protein levels, extends lifespan, improves motor neuron counts, neuromuscular junction physiology and motor function [60, 75, 84]. However, ASOs do not cross the blood-brain-barrier, therefore requiring invasive injections [73]. AAV vectors can integrate, increasing risk to cancer, and up to 70 % of the population hold pre-existing neutralizing antibodies to the AAV serotype [82, 85]. While small molecules such as valproic acid have lacked therapeutic benefit in clinical trials [86]. Thus, even with significant improvements, an un-invasive, low immunogenic therapeutic that can be delivered systemically and translate to a full rescue of the phenotype is still needed. Likewise, the inability to monitor disease status, progression, or therapeutic efficacy of SMN-dependent drugs, limits the capacity to predict disease outcome or accurately assess the effectiveness of treatments at the cellular level. Thus, identifying easily accessible, cost effective biomarkers are also in great need.

Exosomes

First described in 1983, exosomes were discovered when labeled transferrin within reticulocytes was found to be transported by intraluminal vesicles (ILV) within multivesicular bodies (MVB) formed by the endosome. These ILV were released into the extracellular milieu

after the MVB merged with the plasma membrane [87, 88]. However, it wasn't until 1987 when the term 'exosomes' was coined by Johnstone for the nano-sized vesicles being shed from reticulocytes during maturation [89]. In general, cells produce a variety of extracellular vesicles in addition to exosomes, such as microparticles and apoptotic bodies [90] (Table 1.2). Microparticles are large vesicles, up to 1 μm in diameter, formed through pinching of the plasma membrane, and contain an assortment of lipids, proteins, and RNA [91]. Apoptotic bodies are produced as the cell undergoes apoptosis and contain cellular waste, mitochondrial components, condensed DNA, and RNA [92]. Exosomes in particular, are nano sized particles, ranging from 30-150 nm in size, with a phospholipid bilayered membrane, formed within the endosome, and are naturally released and taken up by a variety of cell types. Exosomes have been identified to be released by all cells tested to date and the information stored can change, dependent on the cellular environment or external cues [90, 91].

Table 1.2 Characterization of the common extracellular vesicle subtypes

<i>Vesicle Type</i>	<i>Size</i>	<i>Biogenesis</i>	<i>Contents</i>	<i>Common Markers</i>
<i>Exosomes</i>	30 nm-150 nm	Exocytosis of MVB from the endosome. ILV form from inward budding of the lumen within the MVB using the ESCRT machinery.	mRNA, miRNA, lncRNA, protein, DNA	Tsg101 and Alix, CD63/CD9/CD81, flotillin
<i>Microparticles</i>	50 nm-1 μm	Budding from the plasma membrane in a Ca^{2+} dependent manner.	mRNA, miRNA, lncRNA, protein	CD40, Integrins,
<i>Apoptotic Bodies</i>	> 0.5 μm	Budding from the plasma membrane during the fragmentation phase of apoptosis	Cell organelles, genomic DNA	Histones, Annexin V

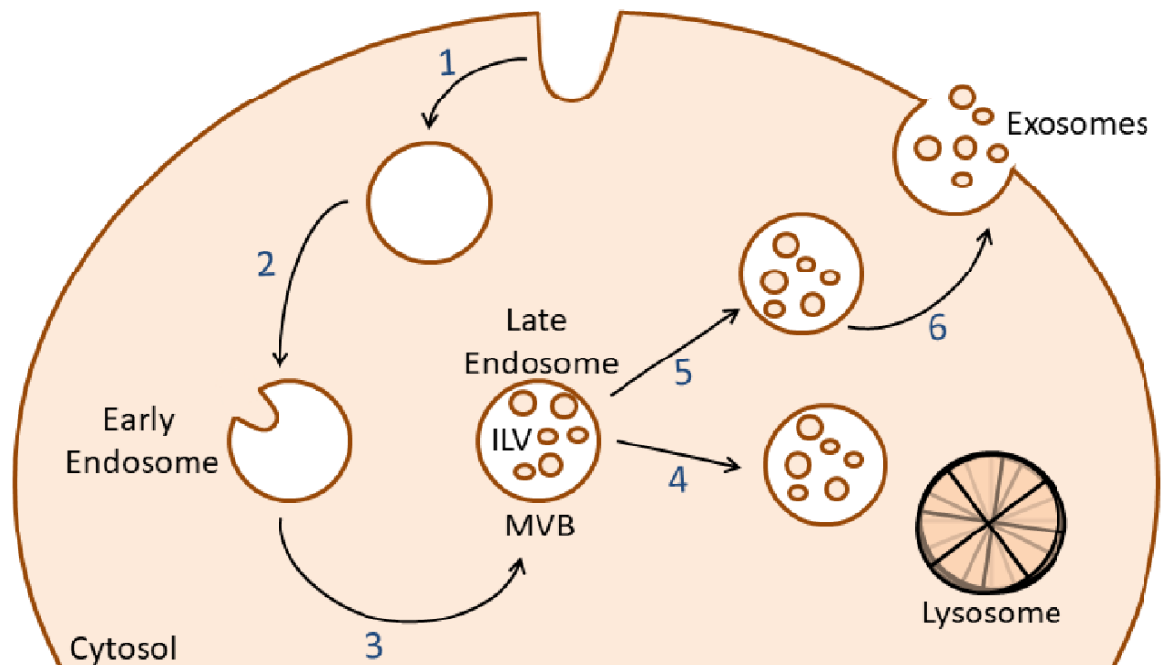


Figure 1.1 Exosome biogenesis. Exosomes are formed in a multi-step process which requires: 1. Endocytosis of the cellular membrane to form an endosome. 2. Cellular signals promote the addition of tetraspanins to the endosome, and the recruitment of the ESCRT complex. 3. As the ESCRT recruitment proceeds, nucleic acid and protein are loaded into the intraluminal vesicles (ILV), and then pinched off in an ATP-dependent process. 4. The multi-vesicular bodies (MVB) now merge with the lysosome for degradation and recycling of its contents, or 5 move towards the plasma membrane. With the help of additional proteins, the endosome merges with the plasma membrane and the ILVs are released as exosomes into the extracellular milieu [91, 93-99]

Biogenesis. Formed through a series of budding events within the endosome, exosomes are fashioned through inward pinching of the endosomal membrane (Figure 1), in pockets known as MVB. Formation of the ILV (internal exosomes) within the MVB relies on a series of events involving protein-protein interactions by the endosome sorting complex required for transport (ESCRT) complex. The ESCRT pathway allows for recruitment of proteins to help the endosomal membrane pinch off, in addition to sorting the appropriate protein or nucleic acid for exosomal loading [91]. These newly formed ILV hold nucleic acid (DNA or RNA), lipids, and protein [93-96]. The MVB, which hold the ILV, either merge with the lysosome for degradation,

or migrate to the plasma membrane for fusion and subsequent release of the ILV into the extracellular milieu [97]. The now, exosomes, can then be taken up by surrounding cells through a variety of mechanisms including endocytosis, fusion, macropinocytosis, and phagocytosis [98-100].

While exosomes and microparticles are very distinct in terms of their biogenesis, they both contain a dynamic range of lipids, protein, and RNA, while overlapping in size (exosomes 30-150 nm, microparticles 50 nm – 1 μ M) [101]. As current isolation methods are highly dependent on size exclusion/inclusion techniques (differential centrifugation, PEG based precipitation, tangential flow filtration, and size exclusion chromatography), achieving pure populations is nearly impossible and therefore all samples should be assumed a mix population [102]. To ensure exosomes are indeed present, the *International Journal for Extracellular Vesicles* recommends several measurements be used to characterize the vesicles isolated [103]. These measurements include providing at least three markers or measurements, which may include (but are not limited to): (1) protein markers: Alix (ESCRT machinery), Tsg 101 (ESCRT machinery), Flotillin (membrane bound signal protein), CD63, CD9, CD81 (tetraspanin proteins), and Hsp70 (concentrated in exosomes). [104] (2) Single vesicle characterization using methods such as electron microscopy or atomic force microscopy, and (3) functional studies, involving dose-response, contaminant examination, or exosome labeling. Using a combination of these methods will help limit variables and contamination within samples, in addition to minimizing variation.

Several recent reports have revealed the release of exosomal subpopulations by cells. Single exosome analysis using Raman spectroscopy, has shown that cells will release a heterogeneous population of exosomes, in terms of their lipid, protein, and nucleic acid content.

This result remained true across the 8 different cell lines tested [105]. Research in Oxford has identified two distinct densities of exosomes released from a single cell line. Further examination revealed the same two distinct subpopulations existed across multiple cell lines tested. Mass spectroscopy of these sub-populations revealed distinct protein compositions and RNA profiles. Moreover, delivery of these two exosome populations, independently, to the same cell line resulted in diverse gene expression patterns [106]. Similarly, Lasser et al. also identified two distinct exosome subpopulations, further characterizing them to show that RNA populations, such as the full length 18S and 28S RNA, existed in the low density exosomes, but not the high [107]. These results potentially indicate that subpopulations of exosome exist with distinctive roles, in which either, 1) each distinctive population of exosomes have individual communicative purposes for different recipients, or 2) multiple populations could be produced and utilized for both communicative and recycling purposes.

Biomarkers. Recognized for their heterogeneous contents, exosomes encompass a variety of RNA, lipids, and protein [90]. The content of exosomes is cell dependent [105, 108]. While the exchange of vesicles allows for transfer of information between cells, to promote biological changes in the recipient. These nano-sized particles have been identified in a variety of biological fluid including serum, cerebrospinal fluid (CSF), seminal fluid, breast milk, and saliva, making them extremely accessible for clinical diagnosis [108-112].

Sorting of protein and RNA into vesicles is a complex mechanism that is not currently, fully understood. ILV formation within the MVB involves the ESCRT machinery, which breaks down into 4 components, ESCRT-0, I, II, III, and IV, in addition to the AAA ATPase vacuolar protein associated protein 4 (VPS4) complex. ESCRT-0 gathers and ubiquitinates cargo, bringing them to the endosomal membrane to begin the formation of the ILV [113]. ESCRT-I also assists

in bringing together the ubiquitinated proteins and forms a link between ESCRT 0 and ESCRT-II. ESCRT II in turn binds to ESCRT III, in which the captured proteins are then de-ubiquitinated by an enzyme as they are sorted into the ILV. The vesicles are then pinched closed by the ESCRT complex. Upon completion, VPS4 promotes the dissociation of ESCRT from the membrane by providing it with ATP [113]. During this process cells can selectively sort desirable miRNA into their exosomes, resulting in differential expression patterns in comparison to the parental cell [114, 115]. Interestingly, exosomes from varying parental cell sources with the same diagnosis demonstrated higher correlation of mRNA content than when the mRNA content of exosomes are compared to the mRNA content of their parental cell [116]. Thus, cells can produce a varying degree of messages loaded into their exosomes depending on their current environment, and these messages can change upon exposure to stress [117]. This preferential loading has created unique signatures that allow for identification and classification of a variety of disease states including cancer, neurodegenerative disorders, and other illnesses, allowing for improved diagnosis and potentially determining a patient's prognosis [118-121].

Exosomes have been suggested to be beneficial biomarkers for a variety of genetic and acquired diseases. In particular, a vast amount of research has focused on using exosomes for cancer diagnosis. Numerous types of cancer utilize exosomes and microparticles for increasing self-proliferation through angiogenesis, creating and enhancing tumor microenvironments for metastasis, or evasion of the immune system [121, 122]. Patients with non-small-cell lung cancer have been shown to have significantly lower levels of miR-126 in serum (removed of exosomes), but significantly greater levels of miR-126 in exosomes at both early and late stages, in comparison to healthy controls. The ratio of miR-126 in serum to exosomes was significantly greater in late stage patients. *In vitro* studies demonstrated down regulation of miR-126

promoted angiogenesis in tumor cells, but exosomes with high levels of miR-126 prevented tumor growth, suggesting miR-126 could be a marker for tumor progression [121]. Another study showed miRNA-1246 significantly up-regulated in exosomes from aggressive prostate cancer when compared to non-aggressive prostate cancer and healthy exosomes. Interestingly, the miRNA-1246 was significantly down-regulated in the cancer cells themselves. Moreover, exosomal miRNA-1246 was shown to have 100 % specificity and 88 % sensitivity in diagnosing aggressive prostate cancers in patients [120]. But exosomes are not just useful for diagnosis; they also provide predictive qualities, in which exosomal surface integrins can approximate the tissue targeted for metastasis by the patient's specific tumor [123]. Thus, exosomes may help build better individualized treatment plans for cancer patients undergoing treatment.

More recently, exosomes have been identified as useful biomarkers for neurological conditions. Patients with traumatic brain injury (TBI) were found to release greater levels of vesicles in their CSF compared to healthy individuals. Examination of the vesicles' contents from TBI patients revealed previously identified markers of TBI, in addition to newly identified proteins involved in neuronal inflammation and cell death [124]. Individuals diagnosed with Alzheimer's disease (AD) were found to have high levels of miR-16 in their serum-derived exosomes that could predict nearly 90 % of cases, and the levels were significantly correlated to neuroimaging reflective of the disease state [125]. Another study examining serum-derived exosomes from AD patients and patients with frontotemporal dementia showed exosomes could be predictive of the disease state, prior to diagnosis, while still cognitively aware. Patients diagnosed with AD showed significantly high levels of tau, P-T181-tau, and P-S396-tau, and when assessed together, the sensitivity of AD diagnosis from aged matched controls was 96 %. There was also a 96 % sensitivity in distinguishing the disease state in the same patients, i.e.

cognitively aware, pre-AD, in comparison to their full dementia onset AD diagnosis. These predictive markers allowed for a remarkable predictive diagnosis of up to 10 years prior to disease onset [126]. Similarly, patients with Parkinson's disease were identified to have significantly elevated levels of a phosphorylated kinase, LRRK2, in their urinary exosomes in comparison to healthy controls and these levels were highly correlated to their disease state [119]. Patients with multiple sclerosis were revealed to have serum-derived exosomes containing unique miR signatures compared to healthy individuals. MS patient derived exosomes could be further analyzed to distinguish between relapsing-remitting and progressive disease states [118]. Together, these studies have shown the prognostic and diagnostic power exosomes have to predict, diagnose, and distinguish between disease states. The prediction value that exosomes provide prior to disease onset would open up a new realm of possibilities for therapeutics, disease outcomes, and disease management. Thus, exosomes may provide a useful means to predict and/or monitor diseases such as SMA, in addition to delineating their subtypes.

Therapeutics. In addition to holding unique protein or RNA signatures, exosomes are believed to be produced by cells as a method for information exchange. This exchange can occur through delivery of nucleic acid or protein, to elicit a biological change within the recipient [106]. Exosome communication likely occurs both within its immediate surroundings and across tissue types, as research has shown they have the potential to cross the blood-brain barrier [127-129]. Recent developments in the field have identified and manipulated unique protein signatures on the exosome's surface to allow for targeting of tissues, ultimately increasing their attractiveness as potential therapeutic delivery vehicles [129-131]. This natural process of 'telephone' offers scientists a unique opportunity to take advantage of an inherent biological process in order to deliver therapeutics in a safe, timely, and effective manner. The content

within the exosomes itself can also be modified to carry desirable RNA or protein using techniques such as transfection, stable cell lines, electroporation, and viral vectors [130-132].

The development of exosomes into a tool for drug-delivery offers the medical field several advantages: (1) their natural half-life in circulation is currently estimated to be between 2-20 minutes [133, 134]. This likely occurs as exosomes are released and quickly taken up by tissues after passing through, and exiting circulation. Rapid delivery of a therapeutic would enhance bioavailability of small molecules with short half-lives, or therapeutic protein and nucleic acids that are at risk of attack by enzymes or immune cells. (2) Unlike viruses, small molecules, and macromolecules such as nucleic acids (antisense oligonucleotides, miRNA), exosomes may provide a means for crossing the blood-brain-barrier and therefore can be beneficial for targeting neuro-vasculature [129]. Intravenous injections into mice and zebrafish have led to exosome mediated therapeutic delivery to cells after crossing the blood-brain-barrier [130, 135]. Thus exosomes as drug delivery vehicles would alleviate many of the invasive methods currently used for drug delivery such as intracerebral or intrathecal injections, but could also provide a more efficient means for drug delivery when a therapeutic has multiple target organs. (3) Exosomes can be modified to carry multiple proteins of interest, while also expressing engineered surface-membrane proteins for tissue-specific targeting. Several research groups have used standard cloning techniques including electroporation and transfection for plasmid expression of well characterized exosome membrane ligands such as Lamp2 β fused to a 29 amino acid rabies viral glycoprotein peptide (RVG). The RVG peptide binds with high affinity to the nicotinic acetylcholine esterase receptor, allowing for CNS targeting [130, 131]. Exosomes can also be modified to target muscle, which also contains a blood-epithelial barrier, tough to penetrate. Researchers have tethered a muscle targeting peptide to CD63, a tetraspannin

highly expressed on exosomes. Exosomes expressing the muscle targeting peptide, injected into mice intravenously, led to uptake within the quadriceps, gastrocnemius, and triceps [136]. Thus, receptor specific sequences can be utilized for exosome engineering and tissue targeting. More intricately designed exosomes can even target cancerous cells. A group from Japan utilized knowledge of the epidermal growth factor receptor (EGFR) within breast cancer cells, and developed exosomes displaying a portion of the platelet derived growth factor receptor tethered to a peptide which specifically binds to the EGFR receptor. Systemically delivering these engineered exosomes (1 µg per week over 4 weeks), loaded with siRNA to mice, allowed for targeting of breast cancer cells, resulting in reduced cancer growth [137]. (4) Unlike viral vectors, several types of exosomes offer a non-toxic, immunologically inert delivery vehicle. For example, exosomes isolated from immature dendritic cells lack the major histocompatibility complex (MHC) and do not stimulate T cells upon injection [130, 138]. Additionally, an autologous source of exosomes can be used to minimize an immune response, creating a more personalized approach to therapeutic delivery. With these advantages in mind, exosomes offer a unique mechanism to systemically deliver therapeutic SMN protein to SMN-deficient cells found in both the periphery and CNS, potentially leading to the rescue of the SMA phenotype.

One of the first steps in choosing exosomes as a potential therapeutic delivery vehicle is determining the cell source from which to produce the exosomes. A common source for exosome production is 293 cells. These cells offer easy transfection to allow for engineering of exosomes for tissue targeting or carrying desired therapeutics [132, 139]. Protein analysis of 293 cell derived exosomes demonstrated these vesicles contained protein highly specific to tissues from platelets, placenta, fetal brain cortex, brain, liver, uterus, muscle, lungs, bone marrow, eyes, among other tissues. This result suggests 293-derived exosomes may have the potential to

interact and be taken up by a variety of cells in the body, making them desirable for treatment of multi-system disorders [95]. Dendritic cells have also posed a potential source for therapeutic exosomes. At the immature stage they lack MHC complexes, and therefore are useful to avoid immune responses. Dendritic cell derived exosomes also deliver to multiple sites *in vivo*, such as the liver, gastrointestinal tract, heart, muscle, and can cross the blood-brain barrier [130, 140]. The surfaces of dendritic cell-derived exosomes can be modified using transfection or electroporation of parental cells to express an exosomal membrane protein tethered to a targeting protein of interest, but the tissues they target are also heavily dependent on the route of injection [130, 140]. Red blood cells (RBC) have been identified as another potential cellular source for exosome production. These cells lack DNA, are largely abundant in the body, and O-negative universal donors are accessible at blood banks [141]. Intratumoral injections of exosomes isolated from RBCs transfected with therapeutic RNA, were shown to reduce breast cancer growth and spread in an xenograft breast cancer mouse model [141]. Intraperitoneal injection of RBC-derived exosomes carrying ASOs were shown to suppress acute myeloid leukemia progression in mice [141]. Importantly, exosome injection did not result in cytotoxicity. Mesenchymal stem cells (MSCs) have also been shown to be immunosuppressive by preventing B- and T- cell proliferation, and therefore are a great candidate to fight pro-inflammatory diseases [142]. Intravenously injected MSC-derived exosomes into a mouse model displaying a progressive form of multiple sclerosis were taken up in peripheral tissues such as the lungs, liver, and spleen. Most interestingly, these exosomes were also shown to cross the blood-brain barrier, delay brain atrophy, and promote re-myelination in this disease model [143]. Collectively, these studies suggest that exosomes can present as a non-toxic alternative for drug delivery.

Additionally, the cell source and the mode of injection greatly dictate exosome tropism, and thus must be considered when creating a drug delivery vehicle for treatment of a disease.

There are two main approaches for loading exosomes with a therapeutic of interest; these approaches are known as passive and active loading. Passive loading relies on allowing the therapeutic to naturally diffuse into the exosomes themselves, or having the therapeutic diffuse into the parental cell line, resulting in the release of therapeutic-loaded exosomes. In active loading, exosomes are incubated with an active reagent (i.e. antibodies, chemical permeabilizers, chemicals for direct conjugations) or an external force is placed on the exosome or cell to alter membrane integrity (i.e. sonication, electroporation, extrusion) [130, 144, 145]. Passive loading largely benefits from avoiding membrane disruption, but as it relies on the drug diffusing across the membrane, the level of therapeutic loaded is limited. Moreover, diffusion is often limited to particular therapeutics due to constraints on size, hydrophobicity, or chemical makeup [145, 146]. Active loading on the other hand increases loading efficiency, and offers a larger variety of therapeutics that can be loaded (i.e. siRNA, protein, small molecules, antibodies). However, active loading can be self-limiting in terms of the toxicity that is generated, often the result of cellular stress through membrane destabilization or production of RNA and protein aggregates, resulting in cell death [145, 147]. In the case of active loading, proteins that are naturally loaded into exosomes can be potentially up-regulated in the parental cell, to increase the level of protein placed within the exosomes [108, 148].

In the event the protein or RNA does not naturally load into exosomes, loading sequences can be examined. Several groups have identified ‘tags’, sequences of RNA or amino acids placed on the RNA or protein of interest, prompting them to be preferentially loaded into vesicles. A double tryptophan ‘ww’ tag is recognized by Ndfip1, a protein involved in exosome cargo

loading. Placing the double tryptophan tag on a Cre-recombinase prompted the release of exosomes loaded with the protein. When delivered intranasally, these exosomes crossed the blood-brain barrier, resulting in the delivery of the protein into neuronal tissue [149]. Other researchers utilized previous knowledge surrounding HIV infection, by taking advantage of the TAR-RNA-TAT interaction. TAT is a protein produced by HIV to allow for viral transcription, but in order for this to occur, it binds, with high affinity to TAR, an RNA stem loop that is found on the end of viral transcripts. Therefore, the TAR-RNA sequence was placed on a therapeutic miRNA, then the TAT binding peptide was bound to the Lamp2 β exosomal membrane protein to help increase loading into exosomes. The result was a 65-fold increase in miRNA loading into 293 cell-derived exosomes. However, while the result was a significant increase in RNA loading, there was a lack of desirable functional outcome when the exosomes were delivered to various recipient cell lines [132]. The lack of desirable effect may be the result of an inability to escape the endosome, or perhaps the target miRNA was bound to the surface of the vesicles, preventing the miRNA from being released into the cytoplasm and acting on its target.

Over the last decade many advances have been made with the development of exosomes as a therapeutic delivery vehicle. In 2011, a group from the University of Louisville demonstrated that intranasal delivery of exosomes could lead to their uptake in the brain to promote apoptosis and subsequently delay tumor growth when loaded with a Stat3 inhibitor [150]. Another major development of exosomal delivery in neurodegenerative diseases occurred in the same year from a group in Oxford, which demonstrated that therapeutic exosomes could not only be loaded with siRNA, but also delivered systemically to the CNS by engineering them to express the neuro-specific peptide RVG on the exosome's surface. Delivery of 150 μ g of exosomes led to knock down of a target protein in the CNS [130]. The RVG modification to the

exosome surface again proved useful when a group loaded genetically modified exosomes with siRNA directed against α -synuclein, a protein known to cause Parkinson's disease. When delivered systemically, these exosomes (150 μ g) crossed the blood-brain barrier and knocked down α -synuclein [131]. RVG labeled exosomes have also been investigated as a therapeutic delivery vehicle for treatment of morphine addiction. Intravenous injection in mice with 200 μ g of siRNA-loaded exosomes targeted against the mu opioid receptor resulted in siRNA detection in all regions of the brain, with the highest concentration in the cortex. SiRNA-loaded exosomes led to the functional knockdown of mu opioid receptor, which prevented morphine relapse in mice [151]. Cardiosphere-derived exosomes (20.64×10^7 particles) injected into the soleus of a mouse model of Duchenne muscular dystrophy (mdx), resulted in a decrease in inflammation and fibrosis, while increasing muscle regeneration. While systemic delivery of the same exosomes (10.32×10^9 particles) led to increased dystrophin protein in the heart, diaphragm, and soleus [152]. Exosomes expressing a muscle targeting peptide loaded with a phosphorodiamidate morpholino oligomer delivered systemically to mdx mice, resulted in a significant increase in dystrophin protein within the quadriceps, gastrocnemius, and triceps [136]. The inherent ability for cell-to-cell movement, in combination with the engineering potential of exosomes for tissue targeting or cargo loading, make exosomes extremely desirable as therapeutics for multi-system diseases such as SMA.

Adenoviral vectors

Originally labeled as the adenoid degenerating agent, Ad were viral agents isolated from the adenoids, found to be a newly discovered cause of respiratory illness in patients displaying respiratory infections [153]. Discovered in 1953 by Rowe and colleagues, it was not until 1956 that Enders coined the term 'Adenovirus', to designate a respiratory virus that infected both man

and animals, while also advising the sub categorization of this virus based on serotype [154]. Currently over 100 different characterized Ad family members exist, from mammalian to reptiles, with over 60 serotypes of mammalian Ad alone [155]. The development of Ad biology over the past 65 years has led to the understanding of important, fundamental concepts in cellular biology, including alternative splicing, DNA replication, and viral gene expression [156-158].

Ad transmission can occur through aerosol droplets from the mouth or nose, interaction with fecal matter, or fomites such as utensils, or clothing [155]. The resulting infection can translate to a respiratory illness with a wide spectrum of clinical characteristics. In the event of a mild infection, symptoms are similar to the common cold, while a more severe infection can be life-threatening in individuals with a weakened immune system, such the elderly, and those suffering from immune-compromised illnesses [155]. Currently, oral vaccines exist for serotypes 4 and 7, but these are largely provided to military personnel as the result of close living quarters which leads to rapid viral transmission [159].

Ad is a double stranded DNA virus that contains a non-enveloped protein coat, icosahedral in shape, and ranges in size from 70-100 nm. The protein coat, known as the capsid, surrounds a linear genome of approximately 36 kbp in length and is made of three main components known as (a) hexon, the facets on the surface to form the icosahedron; (b) penton base, protein which clusters at the vertices; and (c) fiber, a protein which extends from the pentons [160]. The terminal domains of the fiber are known as knobs. Knobs allow binding to cellular receptors, most commonly the coxsackievirus adenovirus receptor, or CAR. Upon binding, a conformational change occurs in prompting a second interaction at the penton base with cellular integrins to stimulate viral mediated endocytosis via clathrin-coated pits. Upon cell entry, fiber structures on the virus break down due to a change in pH that occurs from leaving the

extracellular milieu to the inside of the endosome. The change in virion structure results in the penton base interacting with the endosomal membrane, and due to the penton's toxic nature, the endosome disrupts, releasing the virus into the cytoplasm. Once in the cytoplasm, the virus interacts with microtubules, transporting it into the nucleus via nuclear pores. The virus finally disassembles, where its viral DNA binds to host cellular histones to initiate viral genome replication [161].

In addition to wildtype Ad, which contains all of its viral proteins to allow for replication, there exist three generations of non-replicating Ad vectors, useful for transgene expression in gene therapies. The first generation Ad is devoid of the early genes, E1 and E3, creating space for a 7.5 kb insertion. Removal of the E1 and E3 regions prevent viral replication as they function to block apoptosis, up-regulate Ad promoters, export Ad mRNA, and promote degradation of cellular proteins [162-165]. However, even with the removal of these genes, first generation Ad vectors still prompted an immune response [166]. Second generation Ad vectors were soon developed with the removal of the E2 and E4 regions, in addition to the original E1 and E3 regions. The E2 and E4 regions enhance viral replication, while also manipulating the cellular host by inhibiting host protein synthesis, increasing viral protein synthesis, and enhancing lytic infection [166-173]. Unfortunately, the use of second generation Ad vectors still led to quick loss of gene expression [170, 171]. The third generation of Ad vectors, also known as 'gutless' Ad, or helper-dependent Ad (hdAd), are completely void of viral proteins and only retain the 5' and 3' inverted terminal repeats and the packaging sequence. The elimination of viral proteins requires a hdAd to propagate the necessary components for gene transfer and viral replication *in vitro*. The removal of these genes adds many benefits for Ad vector-based gene

therapies, including increased cloning capacity, limited cytotoxicity, and long term gene expression [174, 175].

A major consideration for the application of Ad vectors in gene therapies is its low integration rate in comparison to other viral vectors. Ad integration into host cellular DNA has been reported at 10^{-3} to 10^{-5} plaque forming units per cell (cell line dependent) [176], while testing these vectors in mice has shown an integration rates of 1 for every 14880 transduced hepatocytes, approximately 0.0067 % [177]. Whereas viral vectors like AAV have average integration rates of 1 in every 100 to 1000 cells. The level of integration increases with increasing MOI, which results in insertions at DNA sites leading to hepatocellular carcinoma [82, 178, 179]. While a rate of 0.1-1.0% integration, dependent on the serotype, seems relatively low, this still accounts for approximately 30 or 300 million hepatocytes with a minimum of one integration event in the human liver [180, 181]. However, because Ad vectors are episomal, their transduction of cells results in transient gene expression, due to their DNA becoming diluted out over time, if not removed by the humoral immune response first. Similar to AAV or lentivirus, Ad also can infect both dividing and quiescent cells, making it a useful therapeutic tool for muscle and CNS disorders [182]. Nevertheless, a large portion of the population has been exposed to Ad5 at one point throughout their lifetime, currently estimated to be 37 % in the USA [183]. High levels of neutralizing antibodies to Ad5 were also identified in China, at approximately 77 % [184]. While, antibodies to Ad14 or Ad55 were around 25 % and 22 %, respectively [185]. Fortunately, because so many Ad serotypes exist, different Ad proteins can be examined and utilized for therapeutic development.

Ad's first rise to fame was due to an unfortunate death in a gene therapy trial for ornithine transcarbamylase (OTC) deficiency, a fatal, X-linked disease that results in high

mortality due to toxic buildup of ammonia [186]. The patient suffered a massive, systemic immune response resulting in his death 96 h after administration [187]. This unfortunate death was later determined to be the result of an accumulation of events including: failure to report toxicity in nonhuman primates from a pilot study; failure to report serious side effects in patients given a similar dose; and most importantly, the inclusion of Jesse as a clinical trial patient when his levels of ammonia surpassed the acceptable threshold for entry into the clinical trial [187, 188]. This tragic death led to the immediate halt of the clinical trial and general use of viral vector based gene therapy, in addition to the re-evaluation of Ad safety for use in humans. However, Ad rose again to the spotlight in 2003, when Ad was announced as the first gene therapy to be approved in the world. Gendicine, an Ad which carries a human p53 gene, was marketed for the treatment of head and neck cancer. As the majority of cancer patients possess a mutated p53 gene, Gendicine delivered p53 through intratumoral injections to promote apoptosis in tumor cells [189]. While there are currently no Ad based gene therapies approved by the FDA, Ad is the most commonly used viral vector for gene therapy in the world [190]. Presently, many groups are examining Ad based vectors for use in therapies including oncolytic therapies for cancer, to gene delivery for treatment of heart failure or refractory angina [191-193].

Utilization of Ad vectors for therapies provides many benefits for researchers. Ad vectors offer ample space for gene insertion and can accommodate up to 105 % of the wildtype genome length [194]. Ad preferentially accumulates in the liver [195], making it an excellent option for liver-based diseases such as liver carcinoma, transthyretin amyloidosis, non-alcoholic fatty liver disease, and alpha-1 antitrypsin deficiency. The large accumulation of Ad in the liver is the result of virus migrating through circulation and becoming trapped within sinusoids. Ad5 for example, is taken up by kupffer cells located in the sinusoidal walls and hepatocytes [196]. Ad vectors

have demonstrated to be successful at transducing a variety of cell types *in vivo*, in addition to the liver, including heart, lungs, gastrointestinal tract, spleen, kidneys [197, 198], while also being reported in the brain, pancreas, and muscle. However, another key advantage to Ad is its ability to have its fiber modified to alter tropism. Shortening the fibers on Ad5 has shown to reduce uptake by kupffer cells [199]. While other modifications, including mixing of Ad serotypes have improved infection and gene expression, by other cell types such as fibroblasts and dendritic cells [200, 201]. With the potential to manipulate Ad's tropism, its ability to be used as a replication defective therapy, and the wide range of cloning capacity, Ad vectors are both versatile and effective as a gene therapy for a variety of disorders.

Rationale

All cells tested to date release exosomes, and the content of these exosomes are dependent on host from which they were derived. Exosomes have been identified in many types of biological fluid including serum, CSF, breast milk, seminal fluid, and saliva. With the ease of access to these samples, the unique signatures that exosomes can provide, in addition to being protected from the harsh extracellular environment; exosomes offer an easily accessible, inexpensive means for assessing disease. Thus, exosomes may provide a unique biomarker to study the disease state, progression, and therapeutic efficacy for patients with SMA. Furthermore, cells naturally use exosomes as means for communication and their content can be released and taken up by an array of cell and tissue types. Exosomes also possess the unique ability to transverse the blood-brain barrier, in addition to their resistance to RNAses, and DNAses. Previous work has verified the therapeutic potential of exosomes to deliver their contents and elicit a biological change within the recipient cell. Therefore, exosomes also pose as

a novel therapeutic delivery vehicle for systemic delivery of therapeutic SMN protein to the CNS and peripheral tissues.

Hypothesis

Exosomes isolated from cell culture, animal models, and patients with SMA, will be an effective biomarker for monitoring the disease state; while exosomes loaded with SMN protein will serve as an effective therapeutic delivery vehicle to transport SMN protein to SMN-deficient cells.

Objectives and Aims

The objectives of my research are to (a) examine the potential of exosomes to aid as a biomarker or prognostic tool for SMA, and, (b) use exosomes as a delivery vehicle to carry therapeutic SMN protein to SMN-deficient cells. Specific aims to address these objectives include:

1. Examine the use of serum-derived exosomes as a potential biomarker or prognostic tool for SMA: Determine if exosomal SMN protein levels derived from SMA patients predict or change with the disease state
2. Examine the ability for SMN protein-loaded exosomes to deliver SMN protein to SMN deficient cells
3. Generate continuous, endogenous production, of SMN protein-loaded exosomes *in vivo* using Ad vectors expressing *SMN1* cDNA

Chapter 2.0: Survival Motor Neuron Protein is Released from Cells in Exosomes: A Potential Biomarker for Spinal Muscular Atrophy

Statement of contributions:

LAN, ERM, JKB, and RJP designed research studies.

LAN produced all of the figures in the following manuscript with exceptions of: Figure 2C,D produced by YD-R with the experiment performed by both LAN and YD-R; Figure 2B,C,D produced by ERM, Figure 7D produced by KLP and AMP with replicates done by LAN.

LAN, ERM, AMP, MT, KLP, YD-R conducted experiments and acquired data.

LAN, ERM, AMP, MT, KLP, YD-R, JKB, DB, RK and RJP analyzed data.

HJM, JWC, DB, and RK provided reagents.

LAN, ERM, AMP, MT, KLP, YD-R, JKB, HJM, JWC, DB, RK and RJP wrote and approved the manuscript.

Survival Motor Neuron Protein is Released from Cells in Exosomes: A Potential Biomarker for Spinal Muscular Atrophy

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ABSTRACT

Spinal muscular atrophy (SMA) is caused by homozygous mutation of the survival motor neuron 1 (*SMN1*) gene. Disease severity inversely correlates to the amount of SMN protein produced from the homologous *SMN2* gene. SMN protein is found naturally released in exosomes from all cell types examined. Fibroblasts from patients or a mouse model of SMA released exosomes containing reduced levels of SMN protein relative to normal controls. Cells overexpressing SMN protein released exosomes with dramatically elevated levels of SMN protein. Enhanced quantities of exosomes were observed in the medium from SMN-depleted cells, and in serum from a mouse model of SMA and a patient with Type 3 SMA, suggesting that SMN-depletion causes a deregulation of exosome release or uptake. The quantity of SMN protein contained in the serum-derived exosomes correlated with the genotype of the animal, with progressively less protein in carrier and affected animals compared to wildtype mice. SMN protein was easily detectable in exosomes isolated from human serum, with a reduction in the amount of SMN protein in exosomes from a patient with Type 3 SMA compared to a normal control. These results suggest that exosome-derived SMN protein may serve as an effective biomarker for SMA.

INTRODUCTION

With an occurrence of 1 in 10,000 live births and a carrier frequency of 1 in 40, spinal muscular atrophy (SMA) is the most common cause of death by a genetic disease in newborns [1,2]. This autosomal recessive disorder is characterized by the degeneration of α -motor neurons, resulting in progressive atrophy of skeletal muscle [3,4]. However, recent studies have shown that many different cell types and tissues also show impaired function in SMA [5-8]. SMA is caused by a deficiency in full length survival motor neuron (SMN) protein, due to homozygous deletion or mutation of the *SMN1* gene [9,10]. Complete loss of SMN protein results in embryonic lethality in mice [11], and likely also in humans [12,13]. However, humans have a second, homologous copy of the *SMN1* gene, termed *SMN2*. Although these two genes share greater than 99% nucleotide identity [14], a synonymous point mutation in the *SMN2* gene affects splicing of the pre-mRNA resulting in production of predominantly an mRNA lacking exon 7 [15]. The resulting SMN Δ 7 protein is relatively unstable and cannot perform all of the functions of the full length SMN protein [16-18]. Approximately 10% of the transcripts from the *SMN2* gene retain exon 7 and produce full length SMN protein [15]. Since the SMN locus is variably amplified in humans, the number of copies of the *SMN2* gene that a patient has can significantly influence the severity of the disease [19-21], as each additional copy of the *SMN2* gene brings a patient closer to wildtype levels of SMN protein [22]. While SMN protein has been implicated in intracellular processes including splicing [23,24], translational regulation [25], R-loop resolution [26], intracellular transport [27-29] and actin dynamics [30,31], the exact cause of SMA pathogenesis is currently unclear.

Very recently, an antisense oligonucleotide (ASO)-based therapy has been approved by the United States Food and Drug Administration (FDA) for treatment of SMA [32,33]. This ASO,

known as Nusinersen, or by its market name Spinraza, is also under review by Health Canada as well as the European Union, but is not yet approved in these jurisdictions. Nusinersen blocks an intronic splicing silencer in intron 7, thus promoting the inclusion of exon 7 in the *SMN2*-derived mRNA, resulting in greater production of full length SMN protein [34,35]. Recent results with gene therapy approaches to disease correction have also shown great promise in human clinical trials [36], while several other therapies have shown encouraging results in preclinical and clinical studies [37-39]. However, currently available clinical outcome measures often lack sensitivity to identify rapid responses to therapies, thereby necessitating long clinical trials particularly in patients with SMA type 2 or 3. Furthermore, clinical strength testing by MRC grading, 6 minute walk test or grip strength are not feasible for younger or more severely affected patients. Common motor outcome measurement tests in infants and children with SMA include Children's Hospital of Philadelphia Test of Neuromuscular Disorders (CHOP-INTEND), Hammersmith Functional Motor Scale (HFMS), HFMS-Expanded or Hammersmith Infant Neurological Examination (HINE) [40]. However, many of these tests have a certain level of subjectiveness to them.

One of the easiest and least-invasive methods to provide insight into disease severity and prognosis would be to identify a blood-based biomarker that accurately reflect the SMA disease state. To this end, several researchers have analyzed peripheral blood mononuclear cells (PBMCs) from patients with SMA for a variety of disease markers, including total *SMN* transcript level, relative full-length versus *SMNΔ7* mRNA transcript level, and protein level [41-45]. Although these studies demonstrated a trend between motor function and changes in several markers, no statistical correlation was observed. Following treatment of a mouse model of SMA with therapeutic ASO, Arnold et al. [46] did observe significant changes in several plasma-

derived biomarkers, suggesting that these may be of some value in monitoring therapeutic efficacy in patients. However, additional accurate and sensitive biomarkers are desired that more closely correlate with clinical phenotype or disease presentation in patients with SMA.

Cells produce a variety of extracellular vesicles, including exosomes, microparticles, and apoptotic bodies [47]. Exosomes are nano-sized particles that are approximately 30-100 nm in diameter and are involved in intercellular communication. Exosomes contain a heterogeneous mix of protein and nucleic acid which are reflective of the cell from which they were derived [48]. It is this unique feature that has prompted many research groups to examine whether exosomes can act as effective biomarkers for a diverse set of diseases, from cancer to neurological disorders [49-52]. In this study, we evaluated the SMN protein content of exosomes isolated from tissue culture and animal models of SMA, and performed an analysis regarding whether exosome-derived SMN protein may be an effective biomarker for SMA.

RESULTS

SMN protein is released from cells into the surrounding milieu in culture. As an initial test to determine if SMN protein was found extracellularly, we examined protein contained in TCA precipitates from a variety of cell lines. As shown in Figure 1A, all cell lines expressed varying levels of SMN protein within the cells. As expected, total cell lysates prepared from MEFs derived from the *Smn*^{2B/-} mouse, an intermediate model of SMA [53], showed barely-detectable levels of SMN protein. TCA precipitates of media from these cell lines revealed that SMN protein was indeed found in the extracellular milieu (Figure 2.1B). As expected, medium from MEF2B/- cells did not contain detectable levels of SMN protein. In short, SMN protein can be found extracellularly when expressed at significant levels in the host cell.

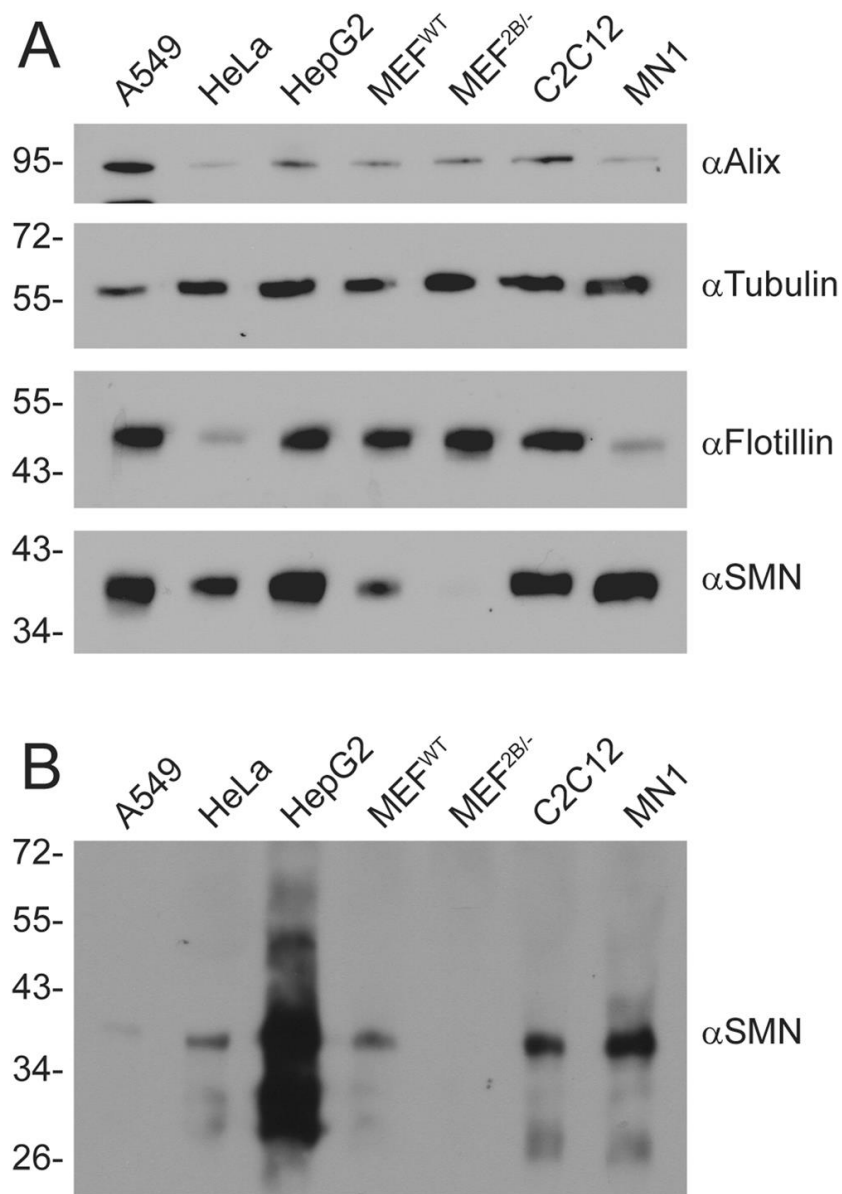


Figure 2.1 SMN protein is released from cells into the extracellular milieu. Various cell lines were plated in 35 mm dishes for 24 h. Media was removed, the cells lysed with protein loading buffer to analyse intracellular protein content (Panel A), and the media subjected to TCA precipitation to analyse extracellular protein content (Panel B). Equal volumes of protein sample were subjected to SDS-PAGE, transferred to a nylon membrane and probed by immunoblot with antibodies to Alix, tubulin, flotillin, or SMN. Data represents $n = 3$.

SMN protein is contained in extracellular vesicles derived from cells in culture.

SMN protein does not have an N-terminal secretory peptide, and would not be expected to be naturally secreted from cells. Cells release a variety of extracellular vesicles, including microparticles (0.1-1 μm) and exosomes (30-100 nm) [47]. These vesicles contain a mix of protein and nucleic acid characteristic of the host cell. To determine if SMN protein was released from cells in extracellular vesicles, we isolated microparticles and exosomes through differential centrifugation of medium from A549 cells, and subjected the samples to immunoblot for SMN protein. As shown in Figure 2A, the size distributions of particles obtained from the differential centrifugation protocol were consistent with the expected sizes for both the microparticle and exosome fractions. SMN protein was associated with both types of particles, as were the exosome and microparticle proteins Alix and TSG101 (Figure 2.2B). To confirm that the SMN protein was indeed contained within the exosomes, and not simply co-purifying with the particles, we performed electron microscopy using immunogold labeling to localize SMN protein within the particles. Extracellular vesicles of varying size were evident, some of which were labeled with the gold-tagged antibody (Figure 2.2C). We did not detect significant labeling outside of vesicles, suggesting that all the SMN protein in the extracellular vesicle purifications was contained within the vesicles, and not the result of co-purification of free protein. At higher magnification (Figure 2.2D), we clearly detected SMN protein within exosomes and microparticles. Thus, SMN protein is naturally released from cells in extracellular vesicles.

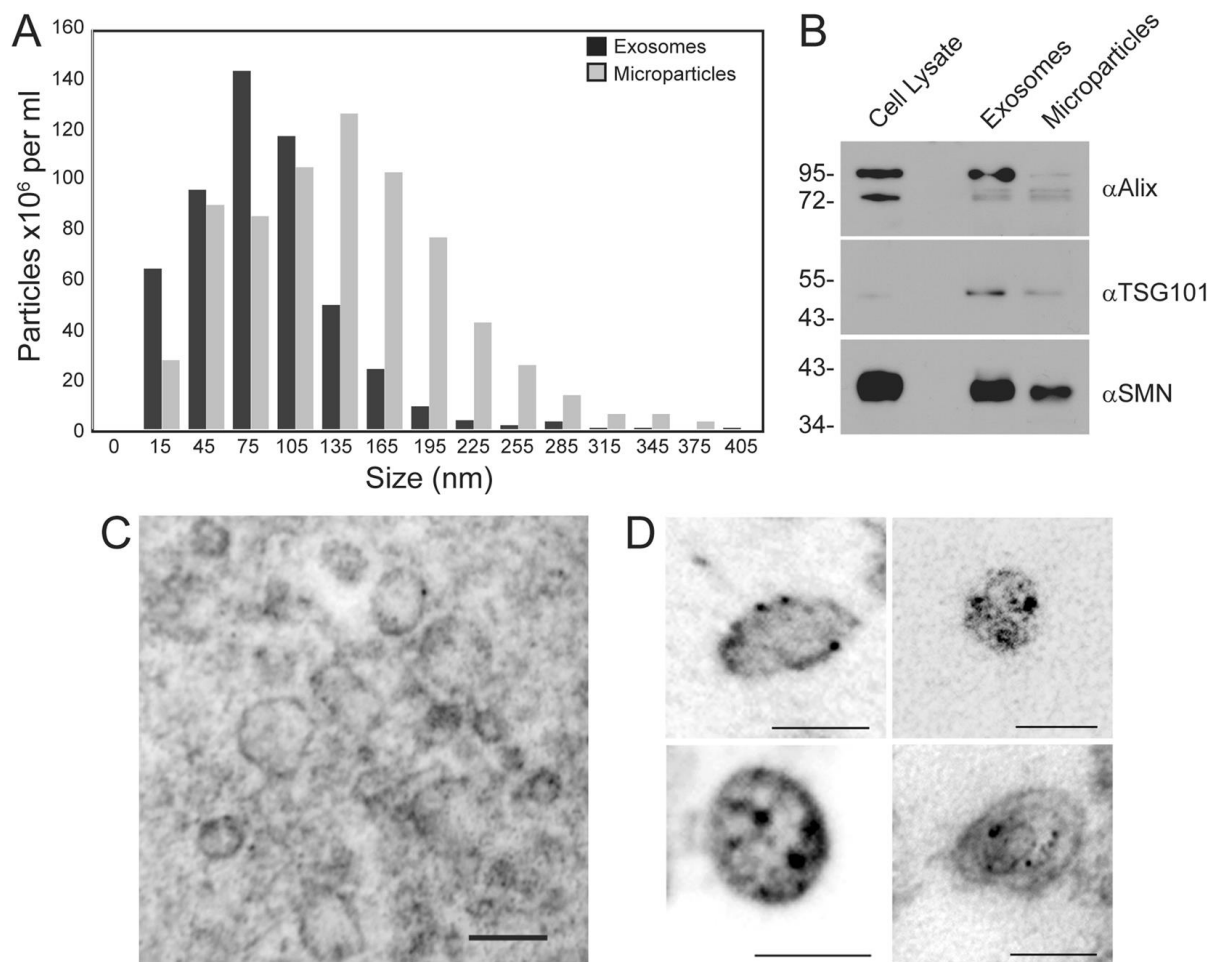


Figure 2.2 SMN protein is released from cells inside of extracellular vesicles. Panel A: Exosomes and microparticles were isolated from A549 cells, grown in medium supplemented with vesicle-depleted FBS, using differential centrifugation and their size profiles were determined using nanoparticle tracking analysis. Panel B: Protein from extracellular vesicles (5 μ g) was subjected to SDS-PAGE and the resulting immunoblot was probed for Alix, TSG101, and SMN. Data represents $n = 2$. Panel C: Immunogold labeling of SMN protein was used to demonstrate the presence of SMN protein within A549-derived exosomes, scale bar represents 100 nm. Panel D: Higher magnification of electron microscopy images showing SMN protein contained in exosomes, scale bar represents 100 nm. Four representative images are shown.

The quantity of SMN protein within exosomes correlates to the level expressed within the host cell. Research on exosomes represents a novel, emerging field in science due to the recent discovery of their involvement in RNA and protein transport between cells [47,54], including cells of the nervous system [55]. Proteins contained in exosomes often reflect the state of the host cell, and thus exosomes are being developed as biomarkers for a variety of disease states [56-58]. We therefore examined whether the quantity of SMN protein contained in exosomes correlated with the level expressed within the host cell using two different approaches. First, we developed an A549-based cell line that overexpressed a FLAG-tagged version of SMN protein, designated A549::SMN cells. The presence of the FLAG-tag causes a shift in the size of the protein, which appears as a doublet or an expanded area of reactivity on immunoblot with antibody to SMN protein (Figure 2.3A). Exosomes isolated from the media of the parental A549 and A549::SMN cell lines showed that significantly higher levels of SMN protein were found in exosomes from the over-expressing cell line, and FLAG-immunoreactivity was only detected in the exosomes derived from the FLAG-SMN-expressing cell line (Figure 2.3B). In a second experiment, A549 cells were infected with increasing quantities of AdSMN, which also expresses a FLAG-tagged SMN protein, and the intracellular and exosome-derived levels of SMN protein were examined 24 h later. As shown in Figure 3C, increasing the amount of AdSMN applied to the cells was accompanied by an increased quantity of SMN protein within cells, as detected by both antiSMN and anti-FLAG antibody. Exosomes isolated from AdSMN-infected cells showed a dramatic increase in the quantity of SMN protein relative to exosomes isolated from untreated cells (Figure 2.3D). Thus, the quantity of SMN protein contained within exosomes is reflective of the level within the host cell, at least for cells overexpressing the protein.

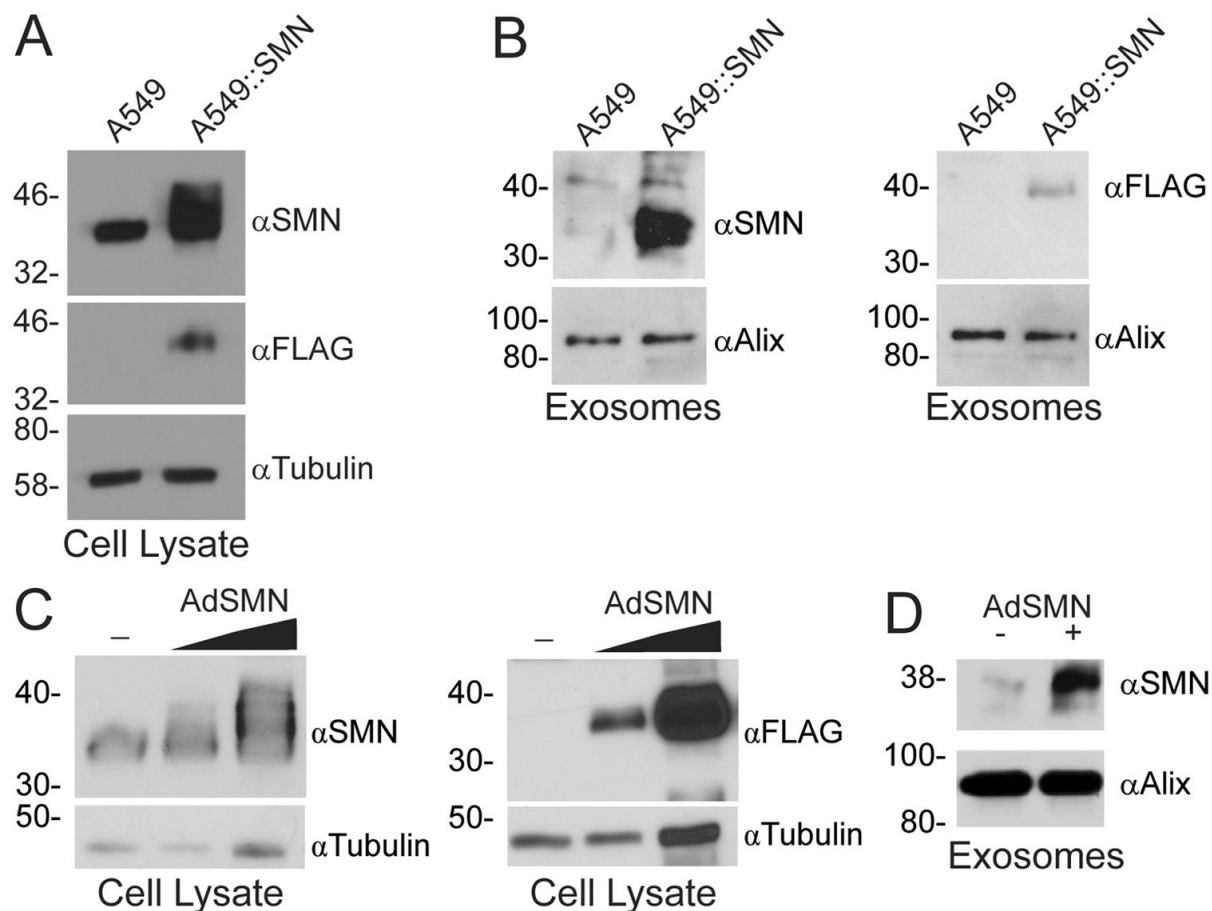


Figure 2.3. The quantity of SMN protein within exosomes reflects the level expressed within the cell from which they are derived. Panel A: A stable A549-based cell line that overexpresses a FLAG-tagged version of SMN protein was generated, designated A549::SMN cells, and the quantity of intracellular SMN protein was analysed by immunoblot using antibody to SMN and FLAG, relative to the parental A549 cell line. Equal protein loading was confirmed by probing the membrane with tubulin. Data represents $n = 3$. Panel B: Exosomes were isolated from medium of A549 or A549::SMN cells, 3 μ g of the resulting samples were separated by SDS-PAGE on duplicate blots, and the resulting immunoblots were probed for SMN and Alix (loading control), or FLAG and Alix. Data represents $n = 3$. Panel C: A549 cells were infected at an MOI of 10 or 100 with AdSMN (or mock infected) and, 24 h post-infection, crude cellular protein lysates were prepared and analysed by immunoblot on duplicate blots for SMN and tubulin (loading control), or FLAG and tubulin. Data represents $n = 3$. Panel D: A549 cells were infected with an MOI 50 and media was collected for exosome purification. Equal volume of each sample was separated by SDS-PAGE and analyzed by immunoblot for SMN and Alix (loading control). Data represents $n = 3$.

The quantity of SMN protein within exosomes correlates to the level expressed within the host cell in tissue culture models of SMA. We next examined two tissue culture models of SMA to determine if the quantity of SMN protein contained in exosomes also reflected that of the host cell. An examination of the intracellular levels of SMN protein in MEFs derived from the *Smn*^{2B/-} mouse model of SMA or wildtype mice showed that, as expected, there are significantly reduced levels of SMN protein in the SMA model (Figure 2.4A). Exosomes isolated from medium from these two cell lines also showed a significant reduction in the level of SMN protein contained within the exosomes released from MEF2B/- cells (Figure 2.4B), below the level of detection for this assay.

We next examined the intracellular and exosome-derived quantity of SMN protein in fibroblasts derived from a human patient with SMA or a carrier, relative to a fibroblast cell line from a normal control. As expected, the intracellular quantities of SMN protein within fibroblasts derived from carrier or type 1 SMA patients was considerably less than in fibroblasts derived from the normal control (Figure 2.4C). Indeed, the quantity of intracellular SMN protein in the fibroblasts from SMA patients was statistically significantly lower than both the control and carrier fibroblasts (Figure 2.4D). Exosomes isolated from the medium from these three cell lines showed a similar trend (Figure 2.4E), and a statistically significant difference was determined across the three groups (Figure 2.4F). Regression analysis showed that there was a high correlation between the genotype of the cell fibroblast cell line (control, carrier, SMA) and SMN protein levels ($R^2 = 0.922$, $p < 0.001$). Thus, the levels of SMN protein within exosomes reflects the disease or carrier state, at least in tissue culture models of SMA.

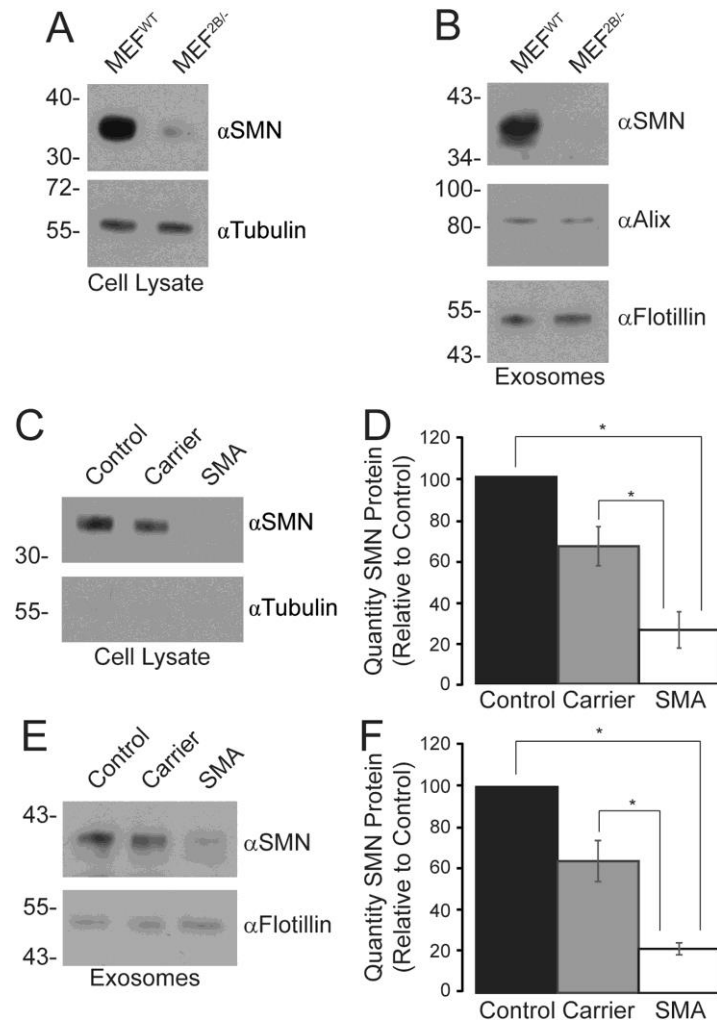


Figure 2.4. The quantity of SMN protein in exosomes reflects the intracellular levels in fibroblasts derived from a mouse model of SMA and patients with type 1 SMA. Panel A: Crude protein cell lysates (5 μ g) from MEFs derived from wildtype mice (MEF^{WT}) or *Smn*^{2B/-} mice (MEF^{2B/-}), a mouse model of SMA, were analysed by immunoblot for SMN and tubulin (loading control). Data represents $n = 3$. Panel B: Exosomes were isolated from the medium of MEF^{WT} and MEF^{2B/-}, 3 μ g of the resulting samples were separated by SDS-PAGE and analysed by immunoblot for SMN, Alix, and flotillin (loading control). Data represents $n = 3$. Panel C: Crude protein cell lysates (5 μ g) from normal, SMA carrier or SMA type 1 human fibroblasts were analysed by immunoblot for SMN and tubulin (loading control). Data represents $n = 3$, and is quantified in Panel D. Asterisks (*) indicates significant differences between groups, determined by Bonferroni post-hoc analysis ($p < 0.05$). Panel E: Exosomes were isolated from the medium of normal, carrier or SMA type 1 fibroblasts, 3 μ g of the resulting samples were separated by SDS-PAGE and analysed by immunoblot for SMN, Alix, and flotillin (loading controls). Data represents $n = 3$, and is quantified in Panel F. Asterisks (*) indicates significant differences between groups, determined by Bonferroni post-hoc analysis ($p < 0.05$).

Cell culture models of SMA showed elevated concentrations of exosomes in the medium. During studies evaluating SMN protein content in exosomes, we noted enhanced recovery of exosomes from the cell culture models of SMA relative to control cells, suggesting there was a greater accumulation of exosomes in the medium. To examine this phenomenon more closely, we used two approaches. First, we qualitatively compared by immunoblot exosome protein marker intensity after separating equal volumes of exosome sample isolated from medium from several models of SMA relative to their respective controls. Second, we used nanoparticle tracking analysis to determine the concentration of exosomes isolated from the two types of cells. For the MN1 motor neuron-like cells, analysis of equal volumes of isolated exosomes from culture medium showed that both Alix and flotillin exosomal markers were elevated in the MN1-kdSMN cell line that expresses reduced levels of SMN protein compared to the parental cell line (Figure 2.5A). Quantification of the concentration of exosomes in the medium samples showed that the MN1-kdSMN cell line contained almost 60 % more exosomes, which was statistically significant ($p < 0.05$) (Figure 2.5B). A similar trend was noted for MEF2B/- compared to MEFWT fibroblasts: MEF2B/- cells showed elevated signal intensity for the Alix and flotillin exosome markers by immunoblot, suggesting a higher concentration of exosomes (Figure 2.5C). Although this trend was also observed when the concentration of exosomes was determined by nanoparticle tracking analysis, it was not statistically significant (Figure 2.5D). Finally, exosomes isolated from the medium from fibroblasts from a patient with type 1 SMA showed significantly elevated signal intensity for exosomal markers Tsg101 and flotillin compared to fibroblasts (Alix was poorly detectable in these samples, data not shown) (Figure 2.5E). The elevated amount of exosomes isolated from the medium from type 1 SMA fibroblast, relative to normal control fibroblasts, was confirmed by nanoparticle tracking analysis

as almost a 3-fold enhancement in exosome concentration, which was statistically significant ($p < 0.05$) (Figure 2.5F). Of note, cells depleted of SMN protein can show a reduced growth rate [59, 60], and therefore a reduced number of cells may be present in the cultures depleted of SMN protein relative to their respective controls. Thus, the concentration of exosomes present in the medium from SMN-depleted cells shown in Figure 5 may be an underestimate. Taken together, these results suggest that cells with reduced levels of SMN protein show enhanced accumulation of exosomes.

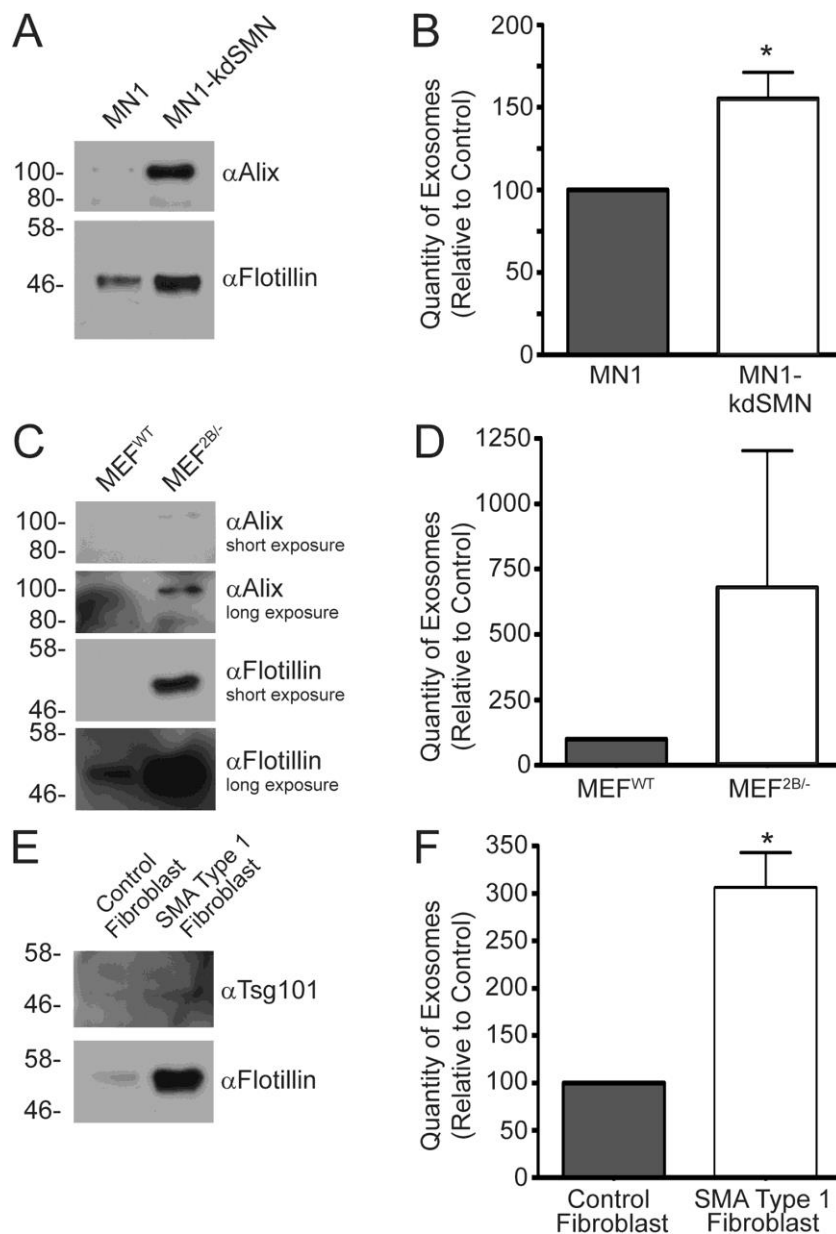


Figure 2.5. Tissue culture models of SMA show enhanced levels of exosomes in the medium. Exosomes were isolated from control (MN1, MEFWT, normal control human fibroblasts) and SMN protein-depleted cell culture models (MN1-kdSMN, MEF2B^{-/-} and SMA type 1 fibroblasts) using Exoquick. Panels A, C and E: Equivalent volumes of the isolated exosomes were separated by SDS-PAGE and analyzed by immunoblot for SMN, or exosome markers Alix, flotillin, or Tsg101. Panels B, D, and F: The concentration of exosomes in the samples was determined by nanoparticle tracking analysis. Data represents $n = 3-4$, and is presented as average particle concentration and standard deviation, normalized to the control for each cell type. An asterisk (*) indicates $p < 0.05$. Statistical analysis was performed using an unpaired Student's t-test. Exosomes were isolated from cells grown in medium supplemented with microvesicle-depleted FBS.

SMN protein levels in exosomes isolated from serum from a mouse model of SMA reflect the disease state. A useful biomarker of disease should not only be sensitive but also be able to be monitored using minimally invasive techniques. Attempts to develop a blood-based screen for SMA, using protein or RNA from PBMCs, have not been successful [41-44]. We asked whether exosomes isolated from serum from a mouse model of SMA reflected the disease state of the animal. We used a commercially available kit to isolate exosomes from the serum of three mice each of control, carrier *Smn*^{2B/+}, and affected *Smn*^{2B/-}, all on a C57BL/6 background. As shown in Figure 2.6A and 2.6B, the quantity of SMN protein contained in exosomes did indeed reflect the disease state of the mice – wildtype mice had high levels of SMN protein, heterozygous *Smn*^{2B/+} mice had intermediate levels of protein, and homozygous *Smn*^{2B/-} had low or undetectable levels of SMN protein. Consistent with the results with the tissue culture models of SMA, we also observed a greater recovery of exosomes in samples isolated from the mouse model of SMA relative to control animals (p<0.05) (Figure 2.6C). Interestingly, we also observed elevated levels of circulating exosomes in heterozygous *Smn*^{2B/+} mice, which was statistically significantly different from both affected and control mice. Taken together, these data suggest that analysis of SMN protein levels in serum-derived exosomes, or perhaps relative concentration of exosomes in serum, may represent a new biomarker for SMA.

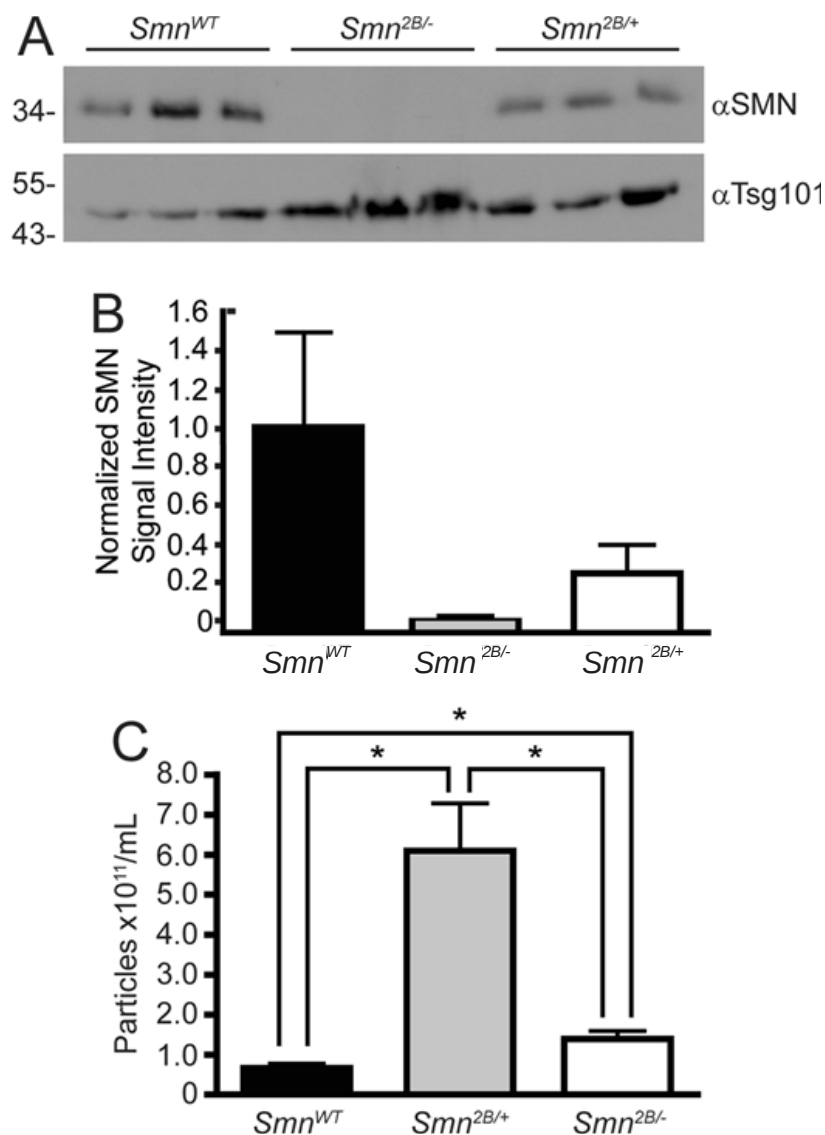


Figure 2.6. A mouse model of SMA shows enhanced levels of exosomes in serum, which contain a reduced quantity of SMN protein relative to wildtype mice. Panel A: Exosomes were isolated from control, $Smn^{2B/+}$ (carrier) and $Smn^{2B/-}$ (affected) mouse serum of a C57Bl/6 background using Exoquick. Equivalent volumes of exosomes were separated by SDS-PAGE and analysed by immunoblot for SMN and Tsg101 (loading control). Panel B: Densitometry analysis of the image in Panel A. The average normalized signal intensity is shown, and the error bars represent the range in signal intensity. Panel C: Exosome particle concentration was determined for the serum-derived exosomes from control, carrier and affected mice. Data represents $n = 6$, and is presented as average particle concentration and standard deviation. An asterisk (*) indicates $p < 0.05$. Statistical analysis was performed using a One-Way ANOVA followed by a Games-Howell t-test.

SMN protein is detected in human serum-derived exosomes. Lastly, we examined whether SMN protein could be detected in exosomes isolated from human serum. The protocol for isolating human serum-derived exosomes using Exoquick reagent yielded particles within the appropriate size range (Figure 2.7A), and which were of a size consistent with other studies [61]. Quantification of exosomes within the serum samples using nanoparticle tracking analysis showed that, as we observed in cells in culture and in a mouse model of SMA, there was a higher level of circulating exosomes in the serum sample from the patient with Type 3 SMA relative to the normal control (Figure 2.7B). Equal volumes of the serum-derived exosomes were next analyzed by immunoblot for the exosomal marker flotillin or SMN protein. Consistent with the higher concentration of exosomes in the serum sample from the Type 3 SMA patient, we observed a higher signal intensity for flotillin in the sample from the patient from SMA relative to the normal control (Figure 2.7C). When the SMN protein immunoblot signal was normalized to the loading control (flotillin), we detected a ~60% reduction in the relative amount of SMN protein contained in the serum-derived exosome sample from the patient with Type 3 SMA compared to normal control (Figure 2.7D). Taken together, these results suggest that analysis of the quantity of SMN protein isolated from serum-derived exosomes, or the concentration of serum-derived exosomes itself, may be a new method to monitor SMA disease or response to therapy in human patients.

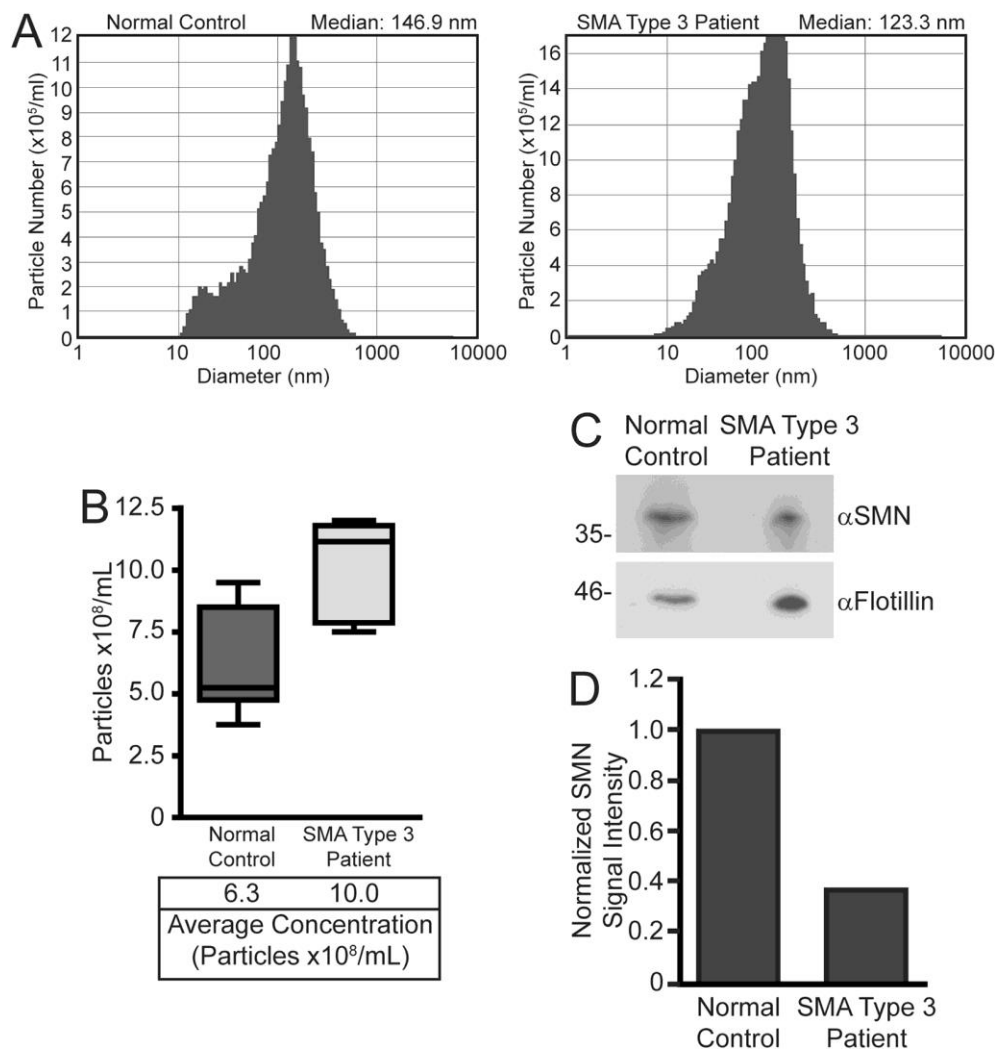


Figure 2.7. A patient with Type 3 SMA has an elevated level of exosomes in serum, which contained a reduced quantity of SMN protein relative to a normal control. Panel A: Particle diameter of human serum-derived exosomes isolated using Exoquick reagent was determined using nanoparticle tracking analysis for both a normal control (left panel) and a patient with Type 3 SMA (right panel). Panel B: The concentration of exosomes in serum samples from a patient with Type 3 SMA was compared to normal control human serum. The Zetaview ParticleMetrix system quantifies vesicles at 11 camera positions, which is represented in the boxplot. Panel C: Equal volume of serum-derived exosome samples from a patient with type 3 SMA or normal control were separated by SDS-PAGE and subjected to immunoblot analysis for exosomal marker flotillin (loading control) or SMA. Panel D: Densitometry analysis of the image in Panel C. SMN signal intensity is normalized to that of the flotillin loading control.

DISCUSSION

SMA is a debilitating neuromuscular disorder caused by reduced levels of full-length SMN protein. Approval of nusinersen, an ASO designed to promote retention of exon 7 in the mRNA transcript derived from the *SMN2* gene, by the Food and Drug Administration in the USA has provided the first effective therapy to treat SMA [32,33]. Several other approaches for the treatment of SMA are currently undergoing clinical testing, including the use of gene therapy to deliver a “good” copy of the *SMN1* cDNA [36,62,63], amongst others [37,38]. With these current advancements in SMA therapeutics, biomarkers that are accurate, sensitive and widely available are required to monitor disease advancement or the efficacy of available and emerging therapeutics.

Extracellular vesicles, including microparticles and exosomes, are comprised of lipids, RNA and protein, the specific constituents of which is dependent on the cells from which they are derived. Work by other groups have identified exosomes as useful biomarkers for neurological conditions such as Creutzfeldt-Jakob disease [51], and Parkinson’s disease [64], as well as prostate cancer [57], ovarian cancer [56], cardiac injury [65], kidney damage [58], and many more. We have shown that SMN protein is released from cells in both microparticles and exosomes (Figure 2.2). Moreover, the quantity of SMN protein within exosomes is reflective of the level expressed within the cell from which they are derived (Figures 2.3 and 2.4). Exosomes isolated from mouse and human SMA cell culture models demonstrated significantly lower SMN protein compared to controls (Figure 2.4). Interestingly, the data also indicates that there is an increase in the level of exosomes in medium from cells that have reduced levels of SMN protein (Figure 2.5). We also observed enhanced levels of circulating exosomes in serum samples from a mouse model of SMA and a human patient affected by SMA (Figure 2.6 and 2.7). Finally, we

showed that SMN protein could be readily detected in exosomes isolated from mouse or human serum (Figure 2.6 and 2.7), and that the quantity of SMN protein contained in the serum-derived exosomes was reflective of the genotype, or disease state, in a mouse model (Figure 6) and in patients (Figure 2.7) with SMA. Taken together, the results suggest that SMN protein content in exosomes, or the quantity of exosomes contained in the serum itself, may represent a novel biomarker for SMA.

Previous efforts to develop blood-based biomarkers for SMA were largely unsuccessful. Several researchers have analyzed PBMCs for a variety of disease markers, including total SMN transcript level, relative full-length versus SMN Δ 7 messenger RNA transcript level, and protein level [42,43,66-69]. Although several of these studies demonstrated a trend between motor function and several markers, no statistical correlation was observed. PBMC-based approaches can be significantly influenced by such things as underlying systemic infections that can alter SMN protein levels, likely due to the change in composition of immune cells circulating through the body [68,69]. Exosomes may offer a more precise, individualized and inexpensive tool for monitoring a patient's disease state. The data showing low quantities of SMN protein in serum-derived exosomes isolated from a mouse model of SMA, with carrier and wildtype mice showing correspondingly higher levels of the protein, suggest that this may serve as a useful biomarker for disease status in SMA.

Interestingly, using both cell culture and an animal model of SMA, in addition to preliminary work using patient samples, the data showed that SMN protein deficiency leads to impairment in exosome regulation, and enhanced levels of exosomes in the surrounding milieu relative to cells and mice expressing normal amounts of SMN protein (Figure 2.4, 2.5, 2.6, 2.7). This finding will need to be reproduced in a larger cohort of patients with SMA. The mechanism

behind this phenomenon is unclear. Reduced levels of SMN protein have been shown to reduce the release of synaptic vesicles [70], alter intracellular vesicle trafficking [28] and, more recently, endocytosis [71,72]. These deficiencies may be due to the impact of SMN proteins on actin dynamics within the cell [27,30,53]. Enhanced release of exosomes has been observed in other neurological disorders [73,74] and cancer [75,76]. Enhanced release of exosomes may be an attempt by the cell to “correct” the microenvironment inside the cell, and expel toxic or unwanted factors. Recent work in a mouse model of SMA has suggested that mitochondrial dysfunction occurs during the presymptomatic stage of the disease leading to heightened levels of oxidative stress [77], and oxidative stress can lead to enhanced release of exosomes in some cell types [78,79]. More recently, neurons from *Caenorhabditis elegans* were shown to release large (~4 μ m) membranebound vesicles called exophers containing misfolded protein and organelles, and stressed cells that release these factors survive better than those in which release is blocked [80]. These observations suggest that one function of exosomes and other extracellular vesicles may be to actively clear unwanted elements from the cell, to improve cell health. Alternatively, given the recent studies showing that cells depleted of SMN protein have decreased endocytosis [71,72], the increased levels of circulating exosomes could also be the result of impaired uptake, leading to their accumulation. Regardless, enhanced levels of extracellular vesicles from diseased tissues and cells in SMA tissue culture and animal models, and possibly in patients with SMA, may represent another viable biomarker of the disease state.

With the field of exosome biology consistently expanding, and novel findings on SMA pathology continuously arising, more research into how the SMA disease state alters exosome cargo, function and secretion is warranted. Future research should examine RNA and protein content within exosomes derived from both healthy and SMN-deficient cells to help elucidate

potential role(s) for exosome secretion in the SMA disease state. Moreover, several groups have shown that exosome content can be manipulated for therapeutic purposes [81-83], and therefore application of therapeutic exosomes may offer a unique strategy to deliver splicing modifiers or full length SMN protein to cells. Using two different approaches, we have shown that the quantity of SMN protein contained in exosomes can be dramatically enhanced (Figure 2.3), providing a relatively simple way to generate “therapeutic” exosomes that may be able to deliver the protein to diseased cells in patients.

In conclusion, we have shown that microparticles and exosomes released from a variety of cell types contain full length SMN protein. In particular, exosomes display SMN protein levels that are reflective of their parental cell or animal’s disease state. Furthermore, cell culture and animal models of SMA demonstrate enhanced levels of exosomes in medium and serum, respectively, in comparison to their healthy controls. Taken together, these results suggest that serum-derived exosomes from patients may offer a novel biomarker for SMA.

METHODS

Cell culture. 293 84,85, A549 (ATCC CCL 185, 86 and HeLa (ATCC CCL 2, 87) cells were grown in Minimum Essential Medium (MEM, Sigma, Oakville, ON) supplemented with 10% fetal bovine serum (FBS, Sigma), 2 mM GlutaMAX (Invitrogen, Burlington, ON), and 1X antimycotic-antibiotic (Invitrogen, Burlington, ON). HepG2 (ATCC HB-8065, 88), MN1 89, and C2C12 cells 90 cells have been previously described, and were grown in Dulbecco's Modified Eagle Medium (DMEM, Sigma) supplemented with 10% FBS, 2 mM GlutaMAX and 1X antimycotic-antibiotic. Mouse embryonic fibroblasts (MEFs) derived from wildtype (MEFWT) or the *Smn*^{2B/-} mouse strain (MEF^{2B/-}) were isolated as described previously [53], and were grown in DMEM supplemented as described above. An MN1-derived cell line with a stable knockdown of the *Smn* gene has been previously described [91], and was kindly provided by Dr. Jocelyn Cote (University of Ottawa). Control normal human patient fibroblasts (GMO8333), SMA carrier fibroblasts (GMO3814) and SMA Type 1 fibroblasts (GMO3813) were obtained from Coriell Cell Repository, and were also grown in DMEM supplemented as described above. All cell lines were cultured at 37°C and 5% CO₂. A549::SMN cells stably express an N-terminal FLAG-epitope tagged SMN protein, and was constructed as follows. pRP3129 contains the human cytomegalovirus (CMV) immediate early enhancer/promoter driving expression of a FLAG-tagged human *SMN1* cDNA linked to the hygromycin resistance gene through an internal ribosome entry site (IRES). A549 cells were transfected with pRP3129 using the Amaxa Cell Line Nucleofector Kit T (Lonza, Mississauga, Ontario), and pooled resistant cells were selected at 150 µg/ml hygromycin.

AdSMN is an early region 1 (E1) and E3-deleted vector containing an expression cassette comprised of the CMV immediate early enhancer/promoter driving expression of a flag-tagged *SMN1* cDNA and bovine growth hormone polyadenylation signal, and has been described previously [92,93]. AdSMN was propagated, purified and titered as previously described [94]. A549 cells were infected for 1 h at the indicated multiplicity of infection (MOI), and washed with phosphate buffered saline (PBS) immediately following infection to remove unattached virus. Infected cells were incubated in MEM lacking FBS for 24 h, and the media collected for exosome isolation as described below.

Trichloroacetic acid media precipitation. Cells were seeded on 35 mm plates and, 24 h later, 1 mL of conditioned media was collected in a microfuge tube and centrifuged at 16,000xg for 5 min. The cleared supernatant was added to 250 μ L of >99% trichloroacetic acid (TCA, Sigma, Oakville, Ontario), and incubated on ice for 1 h. The solution was centrifuged at 16,000xg for 5 min and the supernatant was discarded. The pellet was washed with 200 μ L of 100% ethanol and centrifuged at 16,000xg for 5 min. The resulting pellet was resuspended in 100 μ L 2x protein loading dye (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β -mercaptoethanol). Samples were stored at -20°C until the time of analysis.

Isolation of microparticles and exosomes. Extracellular vesicles were isolated from the medium of cultured cells at the indicated time points using one of two protocols. The method of exosome isolation for each experiment is indicated in the figure legend and in the text of the manuscript. Extracellular vesicles were typically isolated from cells grown for the indicated time in media lacking FBS or supplemented with microvesicle-depleted FBS, as indicated in the figure legend. For isolation of extracellular vesicles using differential centrifugation, conditioned

medium was initially centrifuged for 10 min at 500xg to remove cells and large debris. The resulting supernatant was subsequently centrifuged at 2,500xg for 10 min to obtain a pellet of apoptotic bodies, which was resuspended in PBS or RIPA buffer (150 mM NaCl, 1.0% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 50 mM Tris, pH 8.0). Microparticles were then pelleted from the supernatant through centrifugation at 20,000xg for 20 min at 4°C, and resuspended in PBS or RIPA buffer. Finally, exosomes were isolated using a 100,000xg centrifugation for 90 min at 4°C, and the resulting pellet was resuspended in PBS or RIPA buffer. Alternatively, exosomes were isolated using the commercially available Exoquick kit (System Biosciences, Mountain View, CA, EXOQ5A-1 for serum samples, EXOTC50A-1 for tissue culture media samples) according to the manufacturer's instructions. For all methods of isolation, the final pellet was resuspended in 100 µL of PBS or RIPA. Exosome protein concentration was determined using the Bradford assay (BioRad, Hercules, CA). Exosome size and particle concentration was determined using the Zetaview PMX110 Multiple Parameter Particle Tracking Analysis (ParticleMetrix, Meerbusch, Germany) in size mode. Vesicles were resuspended in 1x PBS and diluted to the working range of the system (106-109 particle/ml). Videos were captured and analyzed with the ZetaView software (version 8.02.28, Meerbusch, Germany) using 11 camera positions, a 2-second video length, and a camera frame rate of 15 fps (for microparticles) or 30 fps (for exosomes) at 21 °C.

Immunoblot analysis. Protein samples were isolated either directly in 2x protein loading dye, or PBS or RIPA buffer when protein quantification was required. For the latter samples, following Bradford protein quantification assay, the desired amount of protein was diluted in 2x protein loading buffer. Samples were heated for 5 min at 95°C, separated by 9 or 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (Millipore). The membrane was

blocked in 5% milk and probed with the following antibodies: rabbit anti-Alix (Sigma, Oakville, Ontario) at 1:1,000, mouse anti-Flag (Sigma, Oakville, Ontario) at 1:10,000 for cell lysates or 1:1,000 for exosomes, rabbit anti-flotillin-2 (Cell Signaling, MA) at 1:1,000, rabbit anti-Tsg101 (Cell Signaling, MA) at 1:1,000, mouse anti-SMN (BD Transduction, Mississauga, Ontario) or rabbit anti-SMN (Santa Cruz, Dallas TX) at 1:10,000 for cell lysates or 1:1,000 for exosomes, or mouse anti-Tubulin (EMD Millipore, Etobicoke, Ontario) at 1:10,000. Binding of the primary antibody was detected using a goat anti-mouse (BioRad, Hercules, CA) at 1:10,000, rabbit anti-goat (Sigma) at 1:10,000 or mouse anti-rabbit conformation specific (Cell Signaling, MA) at 1:1,000 secondary antibody conjugated to horseradish peroxidase, and visualized by chemiluminescent reaction (Pierce, Thermo Scientific). Signal intensities were quantified using Image J (version 1.51, developed by Wayne Rasband, NIH) or Image Studio Light (Licor, Lincoln, Nebraska), and normalized to the appropriate loading control.

Immunogold labeling for transmission electron microscopy. Exosomes were isolated from 12 ml of conditioned medium from A549 cells using the Exoquick method. The exosome pellet was fixed for 1 h at room temperature (RT) in 500 µl of Karnovsky's fixative (4% paraformaldehyde, 2% glutaraldehyde and 0.1 M sodium cacodylate in phosphate-buffered saline, pH 7.4). Exosomes were centrifuged at 20,000xg for 1 min and the supernatant removed. After fixation, the exosome pellet was subsequently washed 3x10 min in 0.1 M sodium cacodylate buffer. The fixed exosomes were permeabilized through incubation for 10 min in 500 µl of 0.1% Triton X100 in 0.1 M sodium cacodylate buffer. The exosome pellet was incubated in 500 µl of blocking buffer (5% goat serum in 0.1M sodium cacodylate buffer and 0.05% Triton x-100) for 30 min at RT. After centrifugation, the exosome pellet was incubated for 2 h at RT with primary antibody (purified mouse anti-SMN, BD Transduction Laboratories) diluted 1:10-1:20

in blocking buffer. Following the incubation period, the pellet was washed with 500 μ l of blocking buffer 3x10 min. The exosome pellet was incubated at RT with a goat polyclonal antibody to mouse IgG conjugated with 10 nm gold (Abcam) diluted 1:40-1:50 in blocking buffer for 1 h in the dark. Samples were washed with 500 μ l of 0.1 M sodium cacodylate buffer 3x10 min. Immunogoldlabelled exosomes were fixed with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer overnight, then washed with 0.1 M sodium cacodylate buffer 4x10 min. The exosomes were post-fixed with 1% osmium tetroxide in 0.1 M sodium cacodylate buffer for 60 min and washed in distilled water 3x5 min. Specimens were dehydrated in an ascending concentration of alcohol in water (30-50-70-85-95%) using molecular sieve type 3A (EMD) for 10 min each step followed by 10 min incubation in 100% ethanol. Exosomes were washed 10 min in 50% ethanol/50% acetone and then in 100% acetone for 10 min. Exosomes were incubated in 30% Spurr resin/acetone overnight (~15 h), 50% Spurr resin/acetone for 6 h, and in fresh 100% Spurr resin overnight on a rotating platform. Spurr resin was changed 3 times over the next 24 h period. The immunogold-labeled exosomes were finally embedded in fresh liquid Spurr resin, which was polymerized overnight at 70°C. The specimen embedded in the resin was cut with an ultramicrotome (80 nm sections). Ultrathin sections of exosomes were collected onto 200-mesh copper grids and stained with 2% aqueous uranyl acetate and with Reynold's lead citrate. These sections stained on grids were observed under a transmission electron microscope (Hitachi 7100) at 100,000x and 150,000x magnifications.

Animal Studies. The *Smn*^{2B/2B} mouse strain has been previously described 30,53,95,96. This mouse strain has a knock-in allele containing a 3 nucleotide substitution within the exon splice enhancer (ESE) of exon 7 of the mouse *Smn* gene, resulting in alternative splicing of the mouse *Smn* transcript and preferential production of *Smn* mRNA lacking exon 7. These mice

were maintained on a C57BL/6 background, and were bred and genotyped to identify affected *Smn*^{2B/-}, carrier *Smn*^{2B/+}, and wildtype littermates, as previously described 97. Experimental animals were cared for in the Animal Care Facility at the University of Ottawa, and kept in a conventional animal house with a constant room temperature of 24°C and a 12 h dark/light cycle, and with free access to food and water. Mice were monitored daily by animal facility professionals. Post-natal day 13 (P13) pups were anesthetized with Avertin (Sigma), blood collected by cardiac puncture, and processed with Exoquick (System Biosciences, EXOQ5A-1) to isolate exosomes from serum. Samples were stored at -80°C until analysed.

Patient Serum Samples. A 29 year old male patient with SMA Type 3 (homozygous deletion of SMN1), and an age-matched male healthy control, gave informed consent to participate in the Care4Rare Canada research study, which was approved by the Children's Hospital of Eastern Ontario and Ottawa Health Science Network Research Ethics Board (OHSN-REB). Human blood was collected in BD red cap Vacutainer tubes, inverted several times, and left to coagulate for 30-45 min at RT. The clot was then removed by centrifugation at 1000xg for 10 min at 4°C. Serum was aliquoted into tubes and stored at -20 or -80°C. Exosome isolation continued with the Exoquick reagent (System Biosciences, Mountain View, CA, cat#EXOQ5A-1) as described above, and analyzed for exosome size and concentration using the ZetaView system or immunoblot as described above.

Statistical Analysis. Statistical analyses were performed using IBM SPSS Statistics 21. Paired/unpaired T-tests, ANOVA and regression analysis were completed with significance set to $p < 0.05$. Levene's test for variance was performed to determine the appropriate post-hoc analysis. Post-hoc analysis was performed using the Bonferroni t-test if there was homogeneity of variance, while other cases utilized Games Howell's post hoc test.

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Chapter 3.0: Exosomes are effective at delivering survival motor neuron protein to recipient cells

Statement of contributions:

LAN produced all of the figures in the manuscript

LAN and RJP designed research studies.

LAN, MOD, JDP, and ME conducted experiments and acquired data.

LAN, DB, RK and RJP analyzed data.

DB and RK provided reagents.

LAN, MOD, JDP, ME, DB, RK and RJP wrote and approved the manuscript.

Exosomes are effective at delivering Survival Motor Neuron protein to recipient cells.

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ABSTRACT

Spinal muscular atrophy (SMA) is an often lethal, genetic disorder, caused by reduced levels of survival motor neuron (SMN) protein. We have investigated whether exosomes, small nano-sized particles involved in intercellular communication, can be used to deliver full length SMN protein to recipient cells. Tissue culture cells engineered to express high levels of the SMN protein, or cells infected with an adenoviral (Ad) vector expressing SMN, released exosomes containing elevated levels of the protein. These SMN-containing exosomes efficiently delivered their cargo to a variety of cell types in culture, including SMA patient-derived fibroblasts. Adenovirus vector-based systemic delivery of a luciferase-SMN fusion reporter gene to mice resulted in high level expression of the fusion protein in the liver of animals, and production of circulating exosomes in blood that contained the protein. These results suggest that gene therapy targeted to the liver can be used to enable the production of a continual source of exosomes that circulate throughout the body, providing therapeutic protein to peripheral tissues; an approach that may be used to treat SMA or many other disorders.

INTRODUCTION

Spinal Muscular Atrophy (SMA) is the leading genetic cause of death in newborns, and affects about 1 in 10,000 live births worldwide [1, 2]. Approximately 95% of SMA cases are caused by homozygous mutation or deletion of the survival motor neuron 1 (*SMN1*) gene [3, 4], and patients with SMA are reliant on expression of the protein from the homologous *SMN2* gene. Unfortunately, a single nucleotide substitution in exon 7 causes the *SMN2* gene to produce primarily an alternatively spliced transcript, which generates a truncated protein that is less stable and cannot perform all of the functions of the full-length protein [5-8]. As the *SMN2* locus is variably amplified in humans, disease severity is roughly, inversely correlated to the number of *SMN2* copies, with each additional copy bringing the patient closer to levels of SMN protein seen in healthy individuals [9]. The hallmark of SMA is degeneration of α -motor neurons, resulting in progressive atrophy of the proximal skeletal muscle [10]. However, recent studies have shown that depletion of SMN also causes impaired function in many different cell types and tissues including within the immune, skeletal, and gastrointestinal systems [11-15].

A variety of strategies have been explored to treat SMA [16, 17] and the first approved therapeutic is now available [18]. Nusinersen, also known by its market name Spinraza, is an antisense oligonucleotide (ASO) that binds to the *SMN2* pre-mRNA and blocks an intronic splicing silencer in intron 7, thus promoting expression of the full-length protein [19]. However, Nusinersen appears to have a varying degree of effectiveness depending on the age of the patient, severity of disease, and the copy number of the *SMN2* gene [20]. The treatment is also invasive, requiring intrathecal delivery every few months. Recent results with adeno-associated virus (AAV)-mediated gene therapy for SMA have also shown great promise in human clinical trials [21], but requires surprisingly high doses of vector in order to achieve efficient delivery to the

central nervous system (CNS), which has raised some safety concerns [22].

Previously, we explored the use of exosomes as a potential biomarker for SMA [23]. Exosomes are nano-sized particles of 30-150 nm in diameter that are involved in intercellular communication [24]. These particles contain a heterogeneous mix of protein and nucleic acid which are reflective of the cell from which they were derived [25, 26]. We demonstrated that fibroblasts derived from patients or a mouse model of SMA released exosomes that contained reduced levels of SMN protein relative to healthy controls. Similarly, in a mouse model of SMA, SMN protein content in serum-derived exosomes correlated with the genotype of the animal, with progressively less protein in carrier and affected animals, respectively, compared to wildtype mice. SMN protein was also easily detectable in exosomes isolated from human serum, with a reduction in the amount of SMN protein contained in exosomes isolated from a patient with SMA compared to a normal control. Thus, analysis of SMN protein content in serum-derived exosomes may be an effective means to monitor response to either currently available treatments (i.e. Spinraza) or other SMN-dependent therapies under clinical evaluation.

Interestingly, we also showed that cells that over-expressed SMN protein through adenovirus (Ad)-mediated transduction of the *SMN1* cDNA released exosomes that contained very high levels of the protein. Since exosomes can be utilized as a drug delivery vehicle [27], and can naturally cross the blood-brain barrier to deliver functional therapeutic proteins or nucleic acids to cells of the CNS [28-30], SMN-loaded exosomes may be a potential novel therapeutic approach to treat SMA. In this study we explore the capability of exosomes to deliver SMN protein to recipient cells in cell culture and in mice.

MATERIALS AND METHODS

Cell culture. HepG2 cells (ATCC HB-8065), normal human control fibroblasts (GMO8333, Coriell Cell Repository), SMA type 1 fibroblasts (GMO3813, Coriell Cell Repository) and MN1 cells [31], were grown in complete DMEM (Sigma, Oakville, Ontario) containing 10% fetal bovine serum (FBS, Sigma, Oakville), 1X antimycotic-antibiotic (Invitrogen, Burlington, ON) and 2 mM L-glutamine (GlutaMAX, Invitrogen, Burlington, ON). An MN1-derived cell line with a stable knockdown of the *Smn* gene (MN1-kdSMN) has been previously described [32], and was kindly provided by Dr. Jocelyn Cote (University of Ottawa). Mouse embryonic fibroblasts (MEFs) derived from wildtype (MEF^{WT}) or an intermediate mouse model of SMA, the *Smn*^{2B/-} mouse [33] (MEF^{2B/-}), were grown in DMEM supplemented as above. 293 cells [34] were grown in Minimum Essential Medium (MEM, Sigma, Oakville, ON) containing 10% FBS, 2 mM GlutaMAX, and 1X antimycotic-antibiotic. All cell lines were cultured at 37°C and 5% CO₂.

A HepG2-based cell line stably expressing a 3xFlag-tagged SMN protein was generated as follows. The human *SMN1* cDNA was PCR amplified from pCD2-SMN [35] using synthetic oligonucleotides LN102F 5' gcg gct agc cgc cat gga cta caa aga cca tga cgg tga tta taa aga tca tga cat cga tta caa gga tga cga tga caa ggg agg ggg aat ggc gat gag cag cgg cgg c 3' and LN102R 5' gcg gtc gac tta att taa gga atg tga gca cct tcc ttc ttt ttg 3'. The resulting PCR product was digested with SalI and NheI, and cloned into SalI/NheI-digested pCI-neo. Positive clones were sequenced, and designated pLN102. An SpeI/MfeI fragment from pLN102 was used to replace an SpeI/MfeI fragment in pRP3129 [23], generating pLN103. Thus, pLN103 contains the human cytomegalovirus (CMV) immediate early enhancer/promoter driving expression of a 3xFlag-tagged human *SMN1* cDNA linked to the hygromycin resistance gene through an internal

ribosome entry site (IRES). pLN103 was transfected into HepG2 cells using Lipofectamine 2000 (Invitrogen, Burlington, Ontario). After transfection, cells were selected using 400 µg/mL hygromycin in complete DMEM, and used as a pooled population of stable clones.

Fibroblasts were converted to myotubes using an early region 1 (E1) and E3-deleted adenovirus (Ad) vector expressing MyoD under regulation by the CMV early enhancer/promoter and bovine growth hormone polyadenylation sequence. Fibroblasts were infected at a multiplicity of infection (MOI) of 1000 for 3 h in PBS, following which the cells were placed in growth medium (15% FBS, 1X antimyotic-antibiotic and 2 mM GlutaMAX). At 24 h post-infection (hpi), the medium was switched to differentiation medium (DMEM supplemented with 2% horse serum, 1X antibiotic-antimycotic, 2 mM GlutaMAX). Cells were allowed to differentiate for 5 days to generate myotubes. On the 5th day of differentiation, the cells were used for exosome delivery.

Virus constructs. The viruses used in this study were constructed using a combination of conventional cloning and bacterial RecA-mediated recombination [36] and were propagated and purified using standard techniques [37]. To generate an E1/E3-deleted Ad vector expressing the 3xFlag-SMN protein, an SpeI/NotI fragment from pLN102 was cloned into SpeI/NotI-digested pRP2645, an Ad left end shuttle plasmid, generating pLN105. pLN105 was recombined into an Ad genomic plasmid, pRP2014 [38], generating pLN107 and the resulting virus AdF3SMN.

To generate an Ad vector expressing the luciferase-SMN fusion protein, the firefly luciferase gene was PCR amplified from pGL4.23 using synthetic oligonucleotides LN115F 5' gcg gct agc cac cat gga aga tgc caa aaa c 3' and LN115R 5' gcg tct aga gag ccg ccc acg gcg atc ttg ccg ccc ttc 3'. The resulting PCR fragment was digested with NheI, and cloned into NheI-digested pLN105, generating pLN115. pLN115 was recombined with the Ad genomic plasmid

pRP2014 to generate pLN117 and the resulting virus AdLucSMN.

AdMA26 is an E1/E3-deleted Ad vector encoding the Cre recombinase under regulation by the murine CMV enhancer/promoter and simian virus 40 (SV40) polyadenylation sequence, and was generously provided by Dr. Frank Graham (McMaster University, CAN). AdMA26 is similar in structure to AdCre [39]. AdMA19 is an E1/E3-deleted Ad vector encoding a luciferase gene under regulation by the human CMV enhancer/promoter and SV40 polyadenylation sequence, but also contains a lox-stop-lox element between the promoter and luciferase gene [39]. Thus, AdMA19 expresses minimal luciferase until it is activated through Cre-mediated excision of the stop element, and is referred to as AdFloxLuc in this study. AdMA19 was a kind gift of Dr. Eric Kremer (Institut de Génétique Moléculaire de Montpellier, France). AdGFP has been described previously [40]. AdRP2014 has been described previously [38].

Exosome Isolation. HepG2 and HepG2-SMN cells (approximately 1×10^7 cells) were added to a 500 mL spinner flask containing 75 mL DMEM supplemented with 2% vesicle-depleted FBS (day 0), and an additional 75 mL of media was added on days 2 and 4. The spinner flask was then incubated for an additional 2 days before isolation of exosomes using differential centrifugation. Alternatively, exosomes were isolated from adherent HepG2 cells infected with AdF3SMN; 1×10^7 HepG2 cells were plated in 8-10 150 mm x 25 mm tissue culture plates, the following day, the cells were infected at an MOI of 10 for 1 h, washed three times with PBS, and incubated in complete DMEM containing vesicle-depleted FBS for 3-4 days. Media (20 mL per plate) was collected and cell debris was removed by centrifugation at $2,000 \times g$ for 20 min. The resulting supernatant was further cleared using a $0.2 \mu\text{m}$ filter containing an asymmetrical polyethersulfone membrane (Thermofisher Scientific, Nepean, Ontario). Collected filtrate was centrifuged at $100,000 \times g$ for 120 min at 4°C using a SW28 Ti rotor (Beckman Coulter,

Mississauga, Ontario). The resulting exosome pellets were resuspended in PBS, pooled, and re-centrifuged at 100,000 x g for 120 min at 4°C using a SW41 Ti rotor (Beckman Coulter, Mississauga, Ontario). The resulting pellet was resuspended in 100 - 200 µL of PBS and stored at -20°C, and used within a week to avoid substantial exosome loss [41-44].

Exosomes were also isolated using polyethylene glycol solution (PEG). HepG2 cells were plated at 2.7×10^6 cells in a 100 mm dish. Next day, the cells were infected with AdGFP, or AdLucSMN for 1 h at an MOI 10, or mock infected with PBS. Following infection, the cells were washed with PBS and 10 mL of DMEM with vesicle-depleted FBS was added for 24 h. Medium was then centrifuged at 3,000 x g for 20 min and the supernatant was added to a 16% w/v stock (2X) solution of PEG dissolved in dH₂O (BioUltra, mw: 8000, Sigma, Oakville Ontario) at a 1:1 ratio. The mixture was incubated on ice overnight. The following day, the mixture was centrifuged at 1,500 x g for 30 min and the pellets were resuspended in 100 µL of PBS. The cells from which the exosomes were isolated were also collected in 1 mL of Reporter Lysis Buffer (company) and stored at -20°C.

The size and concentration of the exosomes were determined using Particle Metrix ZetaView (Cell Guidance Systems, St. Louis, MO). ZetaView software (version 8.02.28, Meerbusch, Germany) was used to capture videos and analyze the particles using 11 camera positions, with videos taken for 2 seconds at a frame rate of 30 fps at 21°C. Exosome protein concentration was determined using the Bradford assay (BioRad, Hercules, CA). Exosome preparations yielded exosome stocks with a protein content of approximately 400 µg/mL for spinner flasks (initial culture volume of 225 mL), while AdF3SMN infections yielded typically 800-900 µg/mL of protein (initial medium volume 160-200 mL).

Exosome Delivery Experiments. Cells (fibroblasts, MN1 and myotube cells) were plated at 1×10^5 cells in 4-well plates in complete DMEM. The following day, the cells were switched to DMEM containing 2% vesicle-depleted FBS, 1% antimycotic-antibiotic and 2 mM GlutaMAX, and exosomes were added directly to the medium. The quantity of exosomes and incubation time are given in the figure legend for each experiment, and the total volume of medium in the 4-well plates was maintained at 150 μ L. At the end of the incubation period, medium was removed, and the cells were washed with 0.1% trypsin in PBS, followed by three washes with PBS, to remove exosomes that may have attached to the exterior cell surface but not entered the cells. The cells were either harvested immediately, or overlaid with complete medium for the duration of the experiment. The cells were collected at the indicated time points in protein loading dye (62.5 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 0.01% bromophenol blue and 5% β -mercaptoethanol).

Cycloheximide treatment of cells. Human SMA type 1 fibroblasts were plated at 6×10^4 cells in 4-well plates. Next day, the cells were incubated with 50 μ g/mL cycloheximide (CHX) for 2 h in medium containing 2% vesicle-depleted FBS. Purified exosomes (30 μ g), isolated from HepG2-SMN cells, were then added to the CHX-supplemented media for an additional 2 h, after which the cells were washed with 0.1% trypsin in PBS, three additional washes of PBS, and the cells collected in protein loading dye.

Immunofluorescence analysis. Cells were seeded in 3 or 4 well slide chambers at 4×10^5 or 2×10^5 cells/well, respectively. Next day, cells were switched to DMEM supplemented with 2% vesicle-depleted FBS, and incubated with exosomes (100 μ g) isolated from AdF3SMN-infected HepG2 cells for 3 h in a total volume of 250 μ L or 150 μ L, respectively. The cells were then washed with 0.1% trypsin in PBS and three additional washes of PBS, overlaid with

DMEM supplemented with vesicle-depleted FBS, and the cells were incubated for an additional 21 h. Cells were washed with PBS, fixed with 4% paraformaldehyde in PBS and blocked for 60 min with 10% donkey serum, 1% BSA, and 0.1% Triton X-100 in PBS. Cells were incubated with primary mouse or rabbit anti-SMN (1:250, BD Transduction, Mississauga, Ontario), mouse anti-Flag (1:250, Sigma, Oakville, Ontario) or mouse anti-tubulin or rabbit anti-tubulin (1:10000, EMD Millipore, Etobicoke, Ontario) overnight at 4°C. The cells were then incubated in the dark with Alexa Fluor anti-mouse-IgG 594 (1:5000, Invitrogen) or anti-rabbit-IgG 488 (1:5000, Invitrogen) for 1 h. Hoechst was added for 10 min at 1 µg/mL, and the cells were mounted with Dako solution (Agilent, Mississauga, Ontario). The cells were imaged with a Zeiss LSM8800 AxioObserverZ1 mot with AiryScan Confocal Microscope using λ_{ex} 488 nm and λ_{ex} 594 nm, and processed using ZEN lite and Bitplane-Imaris microscopy image analysis software.

Cellular Fractionation. HepG2, HepG2-SMN, wildtype fibroblasts or SMA type 1 fibroblasts (1×10^6) were seeded in 60 mm dishes and, next day, switched to DMEM supplemented with 2% vesicle-depleted FBS, and incubated with exosomes (200 µg) isolated from AdF3SMN-infected HepG2 cells for 3 h in a total volume of 500 µL. The cells were then washed with 0.1% trypsin in PBS and three additional washes of PBS, overlaid with DMEM supplemented with vesicle-depleted FBS, and incubated for an additional 21 h. The cells were fractionated according to Challberg and Kelly [45] and assessed for endogenous SMN protein and Flag-tagged SMN protein within the cytoplasm and nucleus. Experiments utilizing CHX prior to fractionation were plated at 1.1×10^6 cells in a 10 cm dish, and included a 2 h pre-incubation of CHX at 50 µg/mL prior to exosome addition. Exosomes (200 µg) were then added to the cells for 3 h, washed with 0.1% trypsin in PBS, followed by three additional washes with PBS. Cells were then overlaid with DMEM supplemented with vesicle-depleted FBS and 50

µg/mL of CHX for an additional 5 h. Cellular lysates were then collected and fractionated.

Immunoblot analysis. Samples in protein loading dye were heated for 5 min at 95°C, separated by 9, 12 or 15% SDS-PAGE and transferred to a polyvinylidene difluoride membrane (EMD Millipore, Etobicoke, Ontario). The resulting membrane was blocked in 5% skim milk in PBS and the membrane was probed with the following antibodies: rabbit anti-Alix (Sigma, Oakville, Ontario) at 1:1,000, mouse anti-Flag at 1:10,000 for cell lysates or 1:1,000 for exosomes, rabbit anti-flotillin-2 (Cell Signaling, MA) at 1:1,000, rabbit anti-Tsg101 (Cell Signaling, MA) at 1:1,000, mouse anti-SMN or rabbit anti-SMN at 1:10,000 for cell lysates or 1:1,000 for exosomes, or mouse anti-tubulin at 1:10,000, rabbit anti-histone H3 (Cell Signaling, MA) at 1:5,000). Binding of the primary antibody was detected using secondary antibodies conjugated to horse radish peroxidase, including goat anti-mouse (1:10,000, BioRad, Hercules, CA) and goat anti-rabbit (1:10,000, Sigma) and visualized by chemiluminescent reaction (Pierce, Thermo Scientific). Signal intensities were quantified using Image J (developed by Wayne Rasband, NIH) and normalized to the appropriate loading control. Alternatively, secondary antibodies were fluorescently labeled (IRDye 1:10,000 anti-mouse 680RD and IRDye 1:10,000 anti-rabbit 800CW, LI-COR) and developed using the Odyssey CLx and visualized using Image Studio 4.0.

Animal Studies. All animal experiments were approved and performed according to the Animal Care and Veterinary Services guidelines at the University of Ottawa. *Study 1.* Six week old female C57BL/6 mice (Jackson Laboratories) were injected through the tail vein with 100 µL of PBS or 5×10^{12} vp/kg of either AdGFP, AdF3SMN or AdLucSMN. At the indicated time points, mice were anesthetised with isofluorane for blood collection by cardiac puncture. Tissues were also harvested, snap frozen in liquid nitrogen, and stored at -80°C until further processing.

The blood samples were incubated at room temperature for 30 min to promote coagulation, and centrifuged at 1000 x g for 10 min to collect the serum. The resulting serum was centrifuged at 20,000 x g for 10 min to remove microparticles, and the exosomes isolated using Exoquick reagent (EXOQ5A-1, System Biosciences, Palo Alto, CA) according to the manufacturer's instructions using the overnight protocol. Harvested tissues were frozen in liquid nitrogen, crushed using a mortar and pestle, weighed, and combined with Reporter Lysis Buffer (Promega, Fitchburg, WI) at a ratio of 10 times the volume per weight of tissue (i.e. mL buffer per mg of tissue). The tissue homogenate and exosomes were then subjected to two rounds of freeze-thaw, and centrifuged at 12,000 x g for 10 min to remove debris. The supernatants were collected and 10 μ L of the supernatant from the tissues or 20 μ L of exosomes were added to 100 μ L of luciferase assay reagent (Luciferase Assay System, Promega). Samples were assayed in triplicate, and luminescence quantified using a 20/20n luminometer (Turner Biosystems). Data was normalized to protein level as determined by Bradford assay (BioRad). *Study 2.* Five to six week old female C57BL/6 (Jackson Laboratories, Stock number 000664), and Albumin-Cre mice (Jackson laboratories, Stock number 003574) were injected with 100 μ L of PBS or 5×10^{12} vp/kg of either AdFloxLuc or AdLucSMN through the tail vein. Tissues and blood were processed at days 3 and 5 as described above.

Statistical Analysis. Statistical analyses were performed using IBM SPSS Statistics 21 and Graph Pad Prism 7.0. One-way and two-way ANOVAs were completed with significance set to $p < 0.05$. Homogeneity of variance across sample sets were tested for using Levene's test to determine the appropriate post-hoc analysis. If Levene's test passed ($p > 0.05$) Bonferroni or Tukey post hoc test was used, while a failure of Levene's test ($p < 0.05$) resulted in the use of Games Howell's post hoc test.

RESULTS

Engineering cells to release exosomes containing high levels of SMN protein. We have previously reported that SMN protein is naturally found in exosomes isolated from both cell culture and serum samples of mice and humans [23]. Importantly, we revealed that the level of SMN protein contained within the exosomes reflected that of the cell and these levels could be enhanced through stable plasmid transfection or transient expression from an Ad vector. Since exosomes are naturally involved in intercellular communication, and can deliver their internal contents to recipient cells, our observations suggest that we may be able to develop SMN protein-loaded exosomes as a potential therapeutic.

As SMN protein within the exosomes is dependent on the cell from which they were derived [23], and the level of vesicles released also varies from cell to cell [46], we tested a number of common cell lines for their natural level of SMN protein expression and also the amount of SMN protein released in the medium as a surrogate measure of vesicles. These experiments revealed that the HepG2 human hepatocellular carcinoma cell line naturally released high amounts of SMN protein [23]. We used HepG2 cells to generate a cell line that stably expressed a 3xFlag-tagged version of the full-length SMN protein. The presence of the 3xFlag-tag on SMN causes a shift in the protein from approximately 36 kDa to 40 kDa. As shown in Figure 3.1A, analysis of crude cellular protein lysates showed that HepG2-SMN cells expressed the tagged version of the protein at the same level as the endogenous protein. Analysis of exosomes isolated from the medium from HepG2-SMN cells showed that the Flag-tagged protein was efficiently loaded into exosomes, again at a level approximately equal to the endogenous protein (Figure 3.1B). We also examined the ability of HepG2 cells to support expression from an Ad vector expressing SMN protein, and showed that both the cells (Figure

3.1C) and exosomes derived from the infected cells (Figure 3.1D) exhibited elevated levels of the exogenously-expressed SMN protein. As expected, our exosome isolation protocol produced particles of the expected size (Supplemental Figure 3.1A-D), with HepG2 exosomes averaging a median diameter of $110 \text{ nm} \pm 3.3 \text{ nm}$, HepG2-SMN exosomes $102 \text{ nm}, \pm 4.7 \text{ nm}$, and HepG2 infected AdF3SMN exosomes $107 \text{ nm} \pm 1.8 \text{ nm}$ (Supplemental Figure 3.S.1D). Thus, overexpression of SMN protein in HepG2 cells led to the release of exosomes containing high levels of the SMN protein.

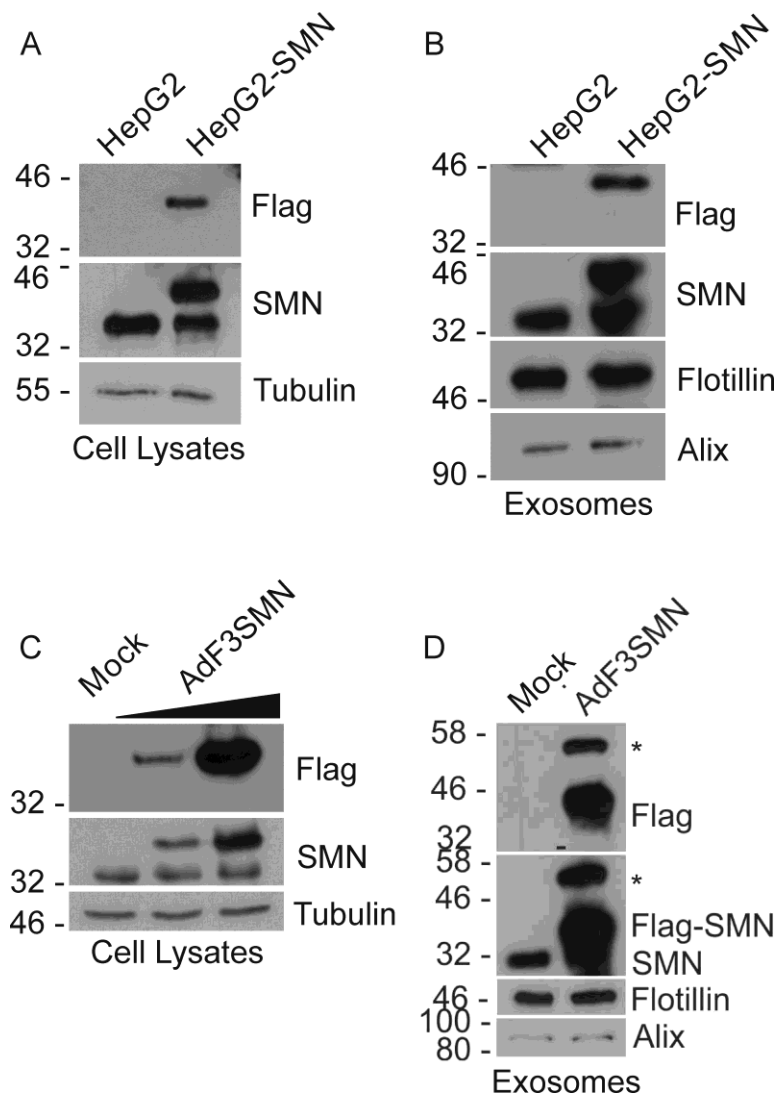


Figure 3.1. Over-expression of SMN using stable cells or Ad vectors results in SMN-loaded exosomes. Panel A: A HepG2 cell line that stably expressed a 3X Flag-tagged SMN protein was generated, designated HepG2-SMN, and examined for the level of expression of SMN protein by immunoblot, using tubulin as a loading control. Data represents $n = 3$. Panel B: Exosomes were isolated from medium from HepG2 or HepG2-SMN cells, 5 μ g of the resulting samples were separated by SDS-PAGE, and the resulting immunoblot was probed for Flag, SMN, and Flotillin and Alix (loading control). Panel C: HepG2 cells were infected with AdF3SMN at MOI 10, MOI 100, crude cellular protein extracts were prepared, separated by SDS-PAGE and probed for SMN, Flag and tubulin (loading control). Panel D: HepG2 cells were infected at an MOI of 100 with AdF3SMN, exosomes were isolated 24 h later, and 5 μ g of the samples were analyzed by immunoblot. Three aliquots of each sample were applied on the same gel and probed for three separate proteins, Flag, SMN, Alix and Flotillin (loading controls). Asterisks indicate non-specific bands.

SMN protein-loaded exosomes associate with SMN deficient fibroblasts in a dose- and time-dependent manner. We next examined whether exosomes could be used to deliver SMN protein to recipient cells, using human fibroblasts isolated from a patient with type 1 SMA as our recipient cell line. These patient-derived fibroblasts have 2 copies of the *SMN2* gene and, in our hands, exhibit approximately 30% SMN protein compared to a healthy control [23]. Varying quantities of SMN protein-loaded exosomes derived from the HepG2-SMN cell line were applied to the fibroblasts for 2 h, at which point the cells were briefly treated with trypsin to remove any exosomes lightly bound to the surface of the cell. Cell lysates were collected immediately and total protein from the cells were examined for the presence of the Flag-tagged exogenous SMN protein. As shown in Figure 3.2A, there appeared to be a dose-dependent increase in association or uptake of the Flag-tagged SMN protein. There also appeared to be a transfer of the endogenous, un-tagged SMN protein, as the signal for endogenous protein also increased with increasing quantities of applied exosomes. We also examined the timing of association: fibroblasts were treated with 30 µg of SMN-loaded exosomes for varying time, and the quantity of SMN protein associated with the cells determined by immunoblot. For this experiment, the exosomes remained in the medium for the duration of the experiment until each sample was harvested and, once again, the cells were briefly treated with trypsin prior to harvesting the protein samples. We observed a continual increase in protein association with increasing incubation time, peaking at 2-3 h after delivery (Figure 3.2B). Exosomes were also efficient at delivering SMN protein to MEFs isolated from *Smn*^{2B/-} mice, a less severe mouse model of SMA [47], MN1 motor neuron-like cells that were knocked down for SMN protein, and SMA type 1 human fibroblasts converted into myotubes (Supplemental Figure 3.S.2). Thus, addition of SMN protein-loaded exosomes is an efficient means to transfer the protein to a

variety of cell types.

The half-life of full-length SMN protein is reported to be between 4 to 8 h [7, 8, 48]. To determine if exosome-derived Flag-tagged SMN protein has a similar half-life, we delivered HepG2-derived exosomes to fibroblasts derived from an SMA type 1 patient for 2 h, after which the cells were washed, medium replaced, and protein samples harvested at varying time points for analysis by immunoblot. Crude protein extracts were harvested at 0 h (immediately after exosome removal), and every 3 h, up to 15 h post removal, separated by SDS-PAGE and examined for the presence of Flag-tagged SMN protein. The level of Flag-tagged SMN protein within the treated cells remained elevated for at least 3 h, in which it then showed a significant decrease, dropping to 50% of the original levels by 6 h after exosome removal (Figure 3.2C). This data suggests that the half-life of the Flag-tagged SMN protein delivered in exosomes is similar to that of the endogenous protein.

Exosomes carry a variety of proteins and nucleic acids, including mRNA [49]. Recent studies have suggested that many of these mRNA are fragments, derived largely from the 3' untranslated region of genes [50]. Regardless, it is possible that the Flag-tagged SMN protein that we see in our exosome-treated cells is due to delivery of mRNA and *de novo* translation of the protein in the recipient cells, rather than delivery of the SMN protein itself. To investigate this possibility, we examined exosome-mediated protein transfer in cells pretreated with cycloheximide (CHX), a strong inhibitor of protein synthesis. CHX was added to the cells 2 h prior to exosome addition, and the drug was maintained in the medium during exosome treatment for the subsequent 2 h incubation. To confirm the effectiveness of CHX treatment, we monitored the stability of cyclin D, a protein involved in cell cycle regulation that has a reported half-life of only a few hours [51]. As expected, CHX treatment led to a dramatic reduction in the

level of cyclin D (Figure 3.2D). Treatment with CHX had no impact on the amount of Flag-tagged SMN protein associated with the cells. Thus, the Flag-SMN protein signal associated with the cell is from the SMN protein contained within the exosomes and not due to *de novo* synthesis from delivered mRNA. Taken together, this data indicates that addition of SMN protein-loaded exosomes to cells leads to a dose- and time-dependent association of exogenous SMN protein with the cells.

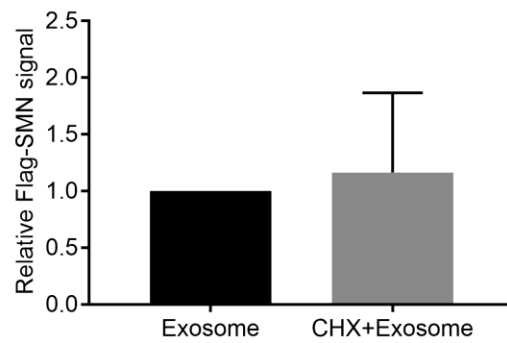
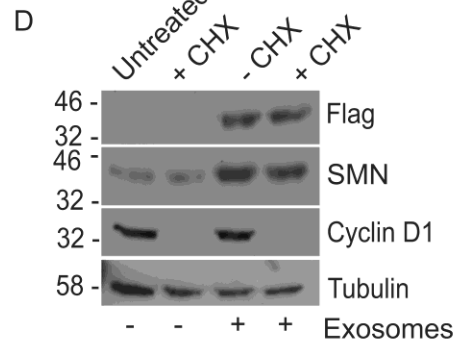
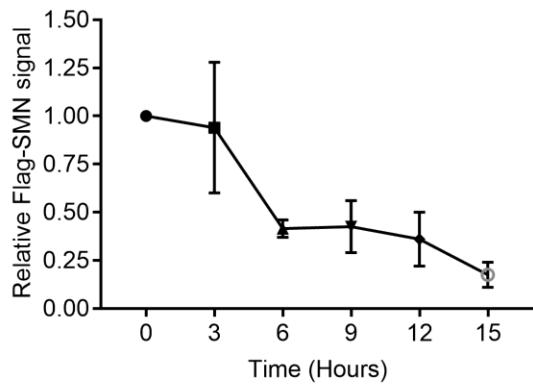
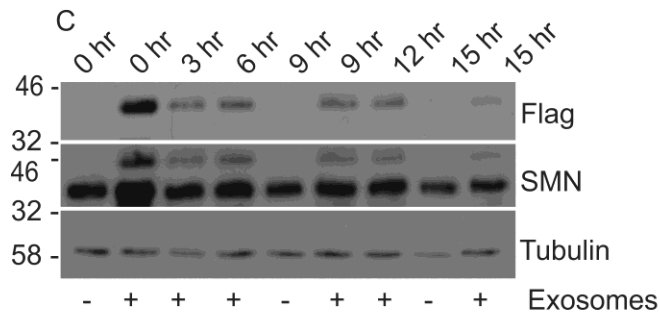
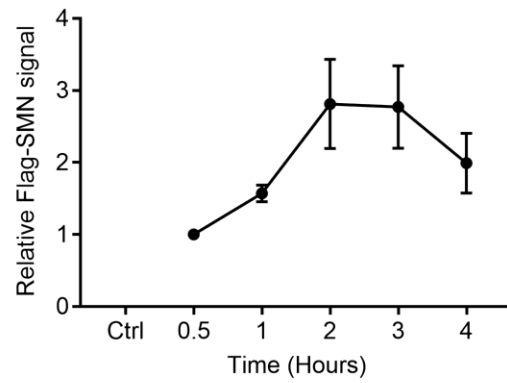
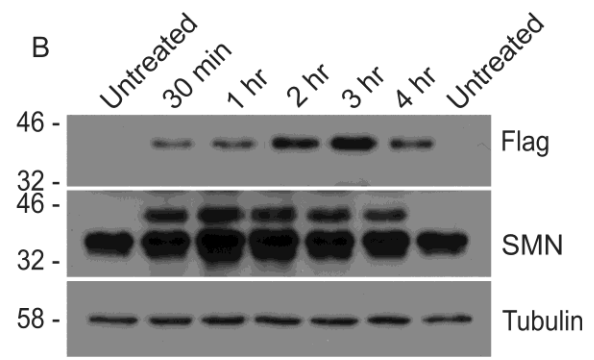
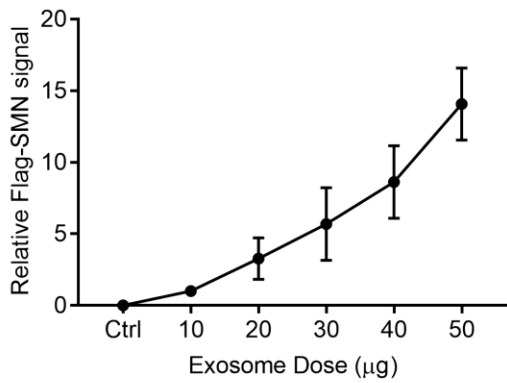
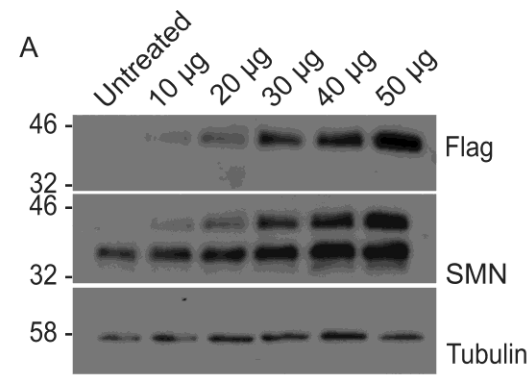


Figure 3.2. HepG2-SMN-derived exosomes associate with SMA type 1 fibroblasts. Panel A: Exosomes isolated from the medium of HepG2-SMN cells were added to SMA type 1 fibroblasts at increasing dose over 2 h, and association of the SMN protein was assessed by immunoblot. Flag signal was quantified using Image J, normalized to the tubulin signal, and plotted relative to the Flag signal seen at 10 μ g/mL. Data represents n = 3. Panel B: To assess the timing of exosome association, SMN-loaded exosomes (30 μ g) were applied to SMA type 1 fibroblasts and incubated for increasing time. Association of the SMN protein was once again assessed by immunoblot. Flag signal was quantified using Image J, normalized to the tubulin signal, and plotted relative to the Flag signal seen at 30 minutes. Data represents n = 3. Panel C: To assess the half-life of the delivered SMN protein, exosomes (30 μ g) prepared from HepG2 cells were applied to SMA type 1 fibroblasts for 2 h, washed, and the cells overlaid with vesicle-depleted medium. The cells were harvested at varying time points, separated by SDS-PAGE and examined for the presence of SMN protein and tubulin (loading control). Flag signal was quantified using Image J, normalized to the tubulin signal, and plotted relative to the Flag signal seen at 0 h. Data represents n = 2. Panel D: SMA type 1 fibroblasts were pre-incubated with cycloheximide (CHX) and then treated with HepG2-SMN-derived exosomes in the presence of CHX-supplemented media for an additional 2 h. Cells were washed, lysates were prepared and analysed by immunoblot for Flag, SMN, Cyclin D1 (CHX control), and tubulin. Flag signal was quantified using Image J, normalized to the tubulin signal, and plotted relative to the Flag signal seen when exosomes were applied to fibroblasts, without CHX addition. Data represents n = 3.

Flag-tagged SMN protein delivered by exosomes is found in the cytoplasm and nucleus of the recipient cell. SMN protein is ubiquitously expressed, and naturally found in both the cytoplasm and nucleus of the cell. Our data has shown that SMN protein delivered by exosomes can associate with recipient cells, but we have not yet demonstrated that the protein is taken into the cells. We therefore examined localization of the exosome-delivered protein in recipient cells by confocal microscopy. Fibroblasts from a patient with type 1 SMA were exposed to PBS or 100 µg of SMN protein-loaded exosomes for 3 h, and processed for microscopy 21 h later. Analysis of the z-stacks with Fiji Image J showed SMA type 1 fibroblasts incubated with SMN-loaded exosomes had significantly increased SMN and Flag staining in comparison to SMA type 1 fibroblasts treated with PBS (Figure 3). Examination of the images using Bitplane-Imaris, also confirmed an enhanced positive Flag signal within fibroblasts incubated with SMN-loaded exosomes in comparison to those incubated with PBS. This positive signal was found in both the nucleus and the cytoplasm of the SMA type 1 fibroblasts (Supplemental video), suggesting the SMN-loaded exosomes were indeed taken up and not just associating on the cell surface.

To complement our immunofluorescence studies, cellular fractionation was performed on exosome-treated cells. Initial fractionation experiments were performed using HepG2 and HepG2-SMN cells to determine the localization of endogenous SMN and the Flag-tagged SMN proteins (Figure 3.4A). Endogenous SMN and Flag-tagged SMN were found in both the cytoplasm and nucleus. Following, SMA type 1 fibroblasts were infected with AdF3SMN for 24 h and fractionated into their cytoplasmic and nuclear fractions to assess the distribution of the Flag-tagged SMN by viral translation (Figure 3.4B). Expression of the Flag-tagged SMN in the SMA type 1 fibroblasts shows a distribution pattern similar to cells stably expressing the Flag-tagged SMN cDNA (Figure 3.4A). However, multiple banding is seen within the Ad infected

fibroblasts, potentially owing to alternative splicing events, previously identified with Ad constructs [52]. Next, SMA type 1 fibroblasts or wildtype fibroblasts were incubated for 3 h with either PBS or exosomes containing the 3xFlag-tagged SMN protein (isolated from HepG2 cells that had been treated with AdF3SMN), washed, then fractionated 21 h later (Figure 3.4C). For wildtype fibroblasts, SMN is equally distributed among the nuclei and cytoplasmic fractions, while the SMA Type 1 fibroblasts incubated with PBS show overall a reduced level of SMN protein within the cytoplasm and nuclei when normalized to their loading controls (tubulin or H3). SMA type 1 fibroblasts incubated with SMN-loaded exosomes revealed Flag-tagged SMN protein within both the cytoplasm and the nucleus, whereas this signal was absent in SMA type 1 fibroblasts incubated with PBS. To ensure the identified Flag tag was indeed due to exosome delivery and not the result of residual viral protein expression, a third fractionation experiment was performed in which SMA type 1 fibroblasts were pre-incubated with CHX for 2 h, then supplemented with DMEM containing exosome depleted FBS, CHX and SMN-loaded exosomes for 3 h. Following exosome incubation, fibroblasts were washed and overlaid with DMEM with exosome depleted FBS and CHX for an additional 5 h, to prevent Ad viral expression but allow for exosome uptake. SMA type 1 fibroblasts incubated with CHX and SMN-loaded exosomes produce a Flag tag in both the nucleus and cytoplasm, albeit the Flag signal was much greater in the nucleus. The signal intensity is not significantly different from treatment with exosomes alone, thus confirming the presence of Flag is due to exosome uptake (Figure 3.4D). Thus, exosomes can be used to deliver SMN protein into recipient cells, and that protein localizes to both the cytoplasm and nucleus of treated cells.

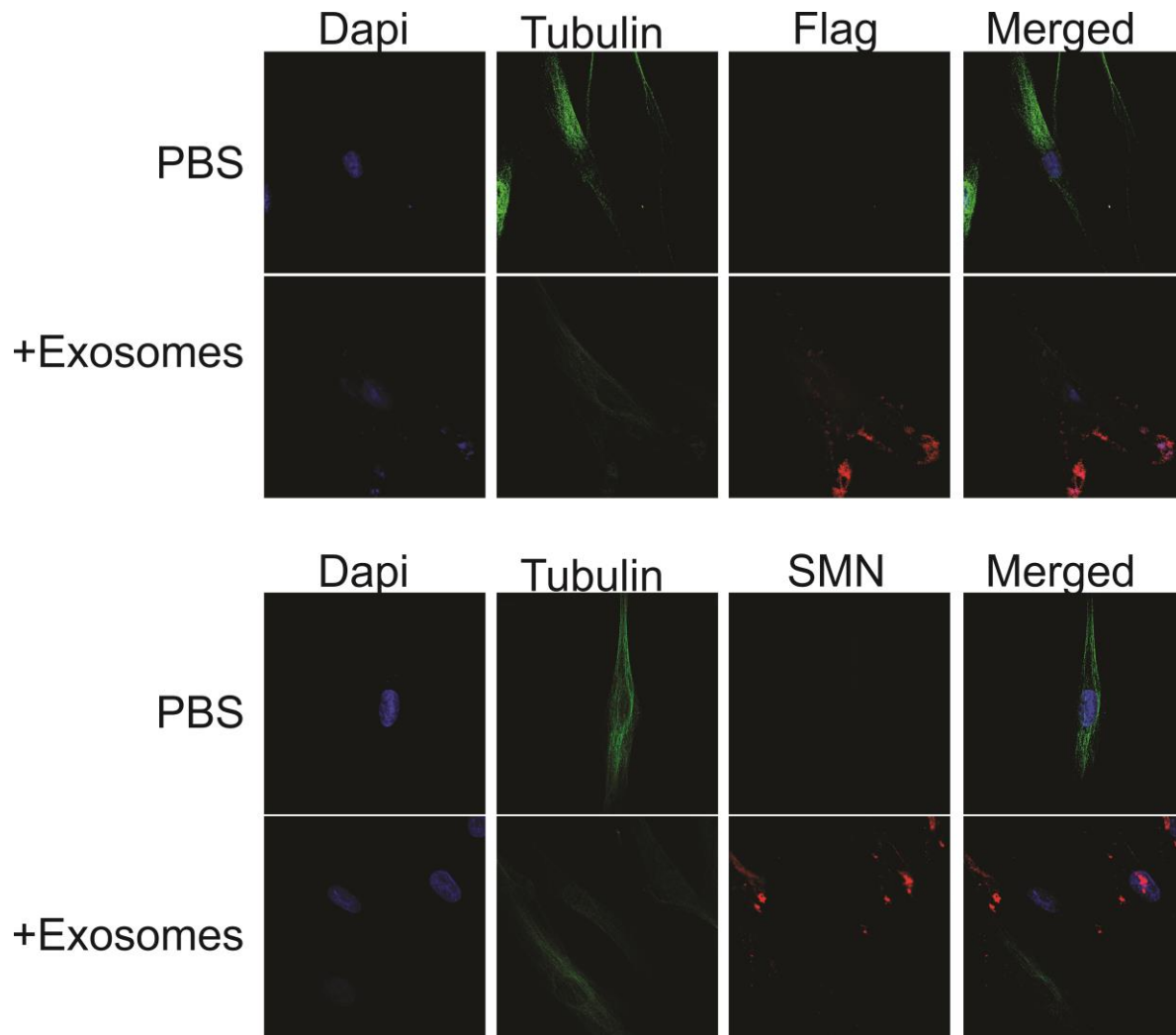


Figure 3.3. SMN protein delivered by exosomes localizes to both the cytoplasm and nucleus of treated cells, as assessed by confocal microscopy. Exosomes isolated from HepG2-SMN cells (100 μ g) were added to human SMA type 1 fibroblasts for 3 h, washed, and fresh media replaced. The cells were incubated for an additional 21 h, washed again with PBS, fixed, and analyzed by indirect immunofluorescence confocal microscopy for Flag-tagged SMN and tubulin. Each image was visualized under a 63x, 1.4A objective and had produced 55-70 z-stacks at 0.1 μ M per slice. Each panel represents the average fluorescence of the 20 middle z-stacks.

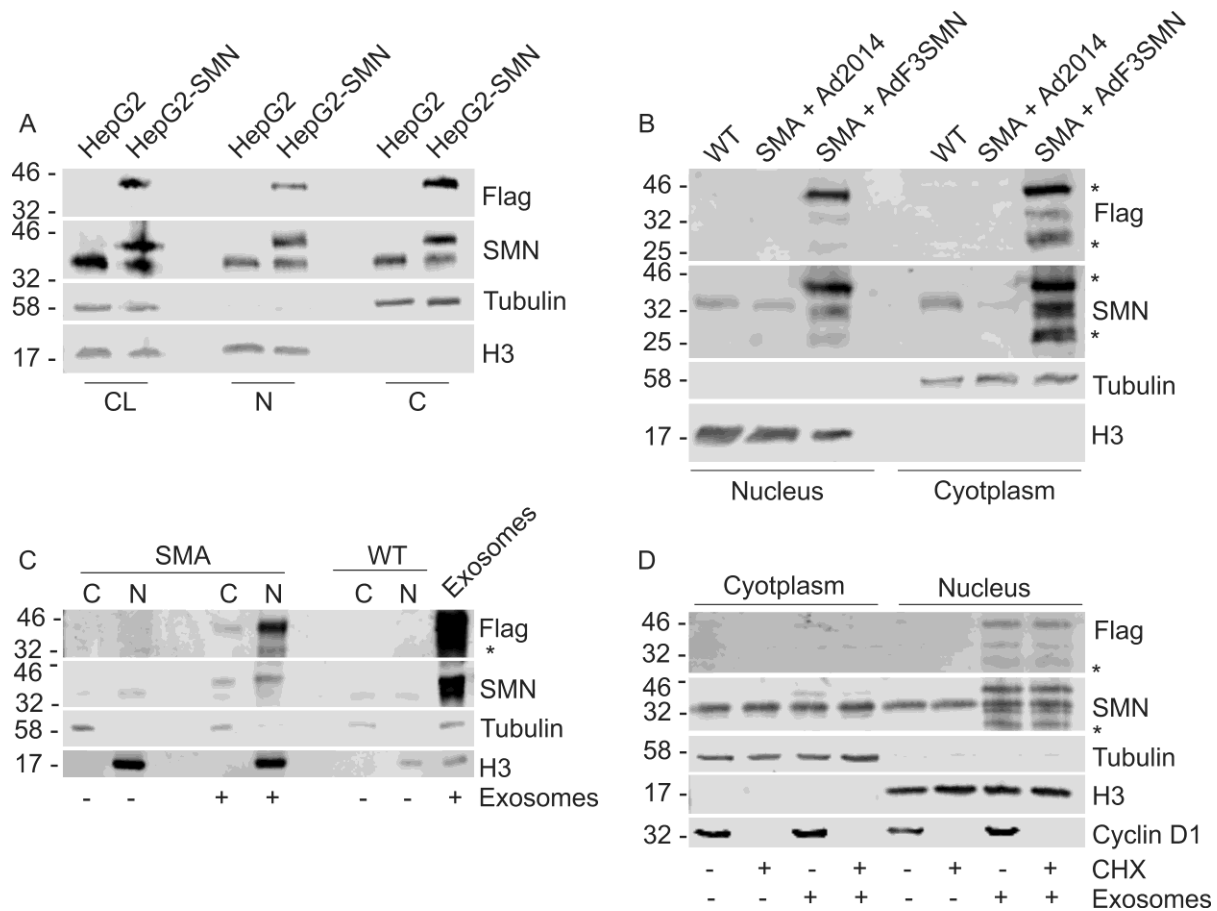


Figure 3.4. SMN protein delivered by exosomes localizes to both the cytoplasm and nucleus of treated cells, as assessed by cell fractionation. Panel A: HepG2 or HepG2-SMN cells were fractionated to prepare cytoplasmic and nuclear fractions. Equal protein quantity of each fraction was analyzed by immunoblot for the overall level of SMN protein, Flag-tagged SMN protein, as well as tubulin (cytoplasmic control protein) and histone H3 (nuclear control protein). Panel B: SMA type 1 fibroblasts were infected with AdF3SMN at MOI 100 for 24h. Cells were fractionated into cytoplasmic and nuclear fractions with equal protein analyzed as in Panel A. treated Panel C: SMA type 1 fibroblasts were treated with SMN-loaded exosomes (200 µg, prepared from AdF3SMN-infected HepG2 cells) or PBS, subjected to cell fractionation 24 h later, and analyzed as in Panel A. In parallel, untreated wildtype human fibroblasts were also analyzed for the natural distribution of SMN protein. Panel D: SMA type 1 fibroblasts were pre-treated with 50 µg/mL of CHX or PBS for 2 h, then washed and replaced with media containing PBS, 50 µg/mL CHX, or SMN-loaded exosomes (200 µg, prepared from AdF3SMN-infected HepG2 cells) or a combination of these conditions. Cells were fractionated 8 h later, and analyzed as in Panel A Asterisks indicate modified spliced forms of Flag-tagged SMN protein. As seen in previous publications [17].

Delivery of an Ad vector encoding human *SMN1* to mice allows for production of SMN-loaded exosomes *in vivo*. While the use of exosomes to deliver therapeutic proteins or nucleic acid shows much promise, there still remain many technical challenges to their use, not the least of which is the difficulty in producing the large quantities necessary for animal or human studies. We have shown the SMN protein delivered through HepG2-derived exosomes has a half-life similar to the endogenous protein, approximately 6 h (Figure 3.2C). Thus, to maintain SMN protein at a near-physiological level, patients would likely require daily injections of the SMN protein-loaded exosomes. Perhaps a more efficient way to provide a continual supply of exosomes would be to produce them *in situ*. Since liver hepatocytes are efficient at generating exosomes that can be found circulating throughout the body [53, 54], we explored whether a gene therapy approach could be used to engineer the liver to release exosomes containing high quantities of SMN protein.

We generated an Ad vector encoding a luciferase-SMN fusion protein under regulation by the high-activity CMV enhancer/promoter to be used in our *in vivo* studies, designated AdLucSMN. Following systemic delivery *in vivo*, the vast majority of Ad vector transduces the liver [55, 56], which can give rise to very high levels of therapeutic transgene expression for the lifetime of a mouse [57], or at least 7 years in non-human primates [58]. As a preliminary evaluation of this vector, HepG2 cells were transduced with AdLucSMN or control vector (AdGFP) at an MOI of 10, or treated with PBS, and both the cells and exosomes were isolated from the medium and examined for luciferase activity 24 hpi. As expected, cells treated with PBS or AdGFP showed only low, background levels of luciferase activity, while cells treated with AdLucSMN showed 3-log higher levels of luciferase activity (Supplemental Figure 3.S.3A). Similarly, exosomes isolated from HepG2 cells infected with AdLucSMN had 3-log

higher level of luciferase activity compared to exosomes isolated from fibroblasts infected with AdGFP or treated with PBS (Supplemental Figure 3.S.3B). Thus, the luciferase-SMN fusion protein is readily detected in exosomes isolated from cells treated with AdLucSMN.

We next examined whether systemic delivery of an Ad vector to mice encoding a luciferase-SMN fusion protein could lead to exosomes in the blood that contained the LucSMN protein. Adult C57BL/6 mice were treated with PBS or 5×10^{12} VP/kg of AdLucSMN by tail vein injection, and 3 or 5 days later, the liver and blood were collected and assayed for luciferase activity. Delivery of AdLucSMN resulted in high-level luciferase activity in the liver of mice, reaching almost 5-log above background at both time points (Figure 3.5A). Significant luciferase activity was also noted in exosomes isolated from the serum of these animals, again reaching over 5-log above mice treated with PBS (Figure 3.5B). Thus, systemic delivery of AdLucSMN to mice leads to the presence of circulating exosomes containing the fusion protein in the serum.

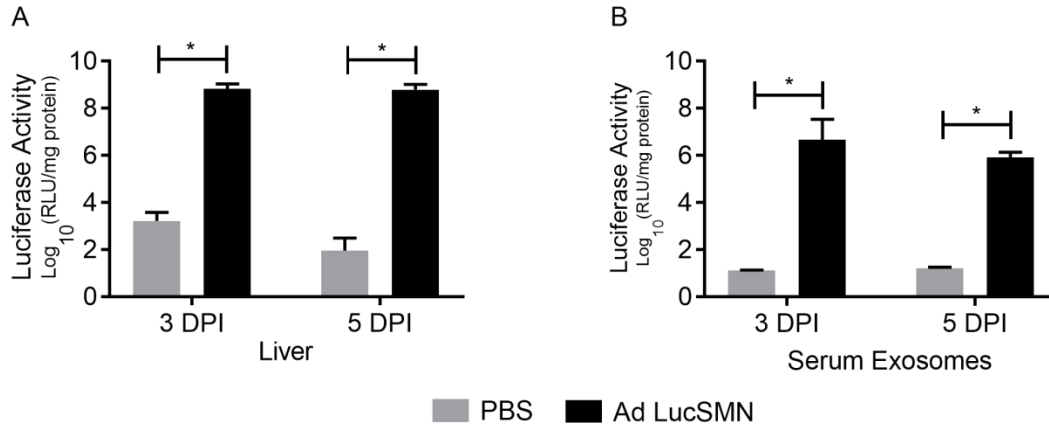


Figure 3.5. Luciferase activity in vivo after systemic delivery of AdLucSMN. Panel A and B: Adult C57BL/6 mice were injected through the tail vein with 5×10^{12} vp/kg of AdLucSMN or PBS and assessed for luciferase activity at 3 or 5 dpi in the liver (Panel A) or exosomes (Panel B). Values were transformed into log scale and assessed for statistical significance using a two-way ANOVA with significance set to $p < 0.05$ and indicated with an asterisk. Data are presented on a linear scale, $n = 3$.

Using gene therapy to engineer the liver to release therapeutic exosomes. To determine if the circulating exosomes containing luciferase-SMN protein were produced from liver, we took advantage of transgenic mice that express the Cre recombinase under regulation by the liver-specific albumin promoter [59], and an Ad vector containing a Cre-activatable luciferase cassette [39]. In this approach, Ad expressing a floxed luciferase reporter would be delivered systemically to mice by intravenous injection, resulting in high-level uptake of the vector by liver hepatocytes. Expression of the Cre recombinase within the hepatocytes would cause excision of the floxed stop element in this virus leading to activation of the luciferase gene expression only in this tissue [59]. If detected, serum-derived exosomes containing luciferase activity would have originated from the liver. As an initial test this system, we infected MEFs from wildtype mice with an MOI of 10 or 100 with either AdCre or AdFloxLuc alone, or a combination of the two viruses. Infection of MEFs with AdCre resulted in only background levels of luciferase activity (Figure 3.6A). Infection with AdFloxLuc alone led to a 10- and 100-fold increase in luciferase expression above background at an MOI of 10 and 100, respectively, suggesting that AdFloxLuc provided a low level of leaky expression even in the absence of Cre activation, possibly due to inefficient stop codon usage in the stop element. However, infection with both AdCre and AdFloxLuc led to a further almost 50- and 200-fold increase in luciferase activity within the cells, suggesting this system would be effective at analyzing liver-derived expression in the Albumin-Cre mice.

Albumin-Cre or the parental C57BL/6 mice were injected with PBS or 5×10^{12} VP/kg of AdFloxLuc through the tail vein, following which liver and blood were isolated 3 or 5 days later. Delivery of AdFloxLuc to C57BL/6 mice resulted in a modest increase in luciferase activity in the liver of these animals likely due to leaky expression from this Ad vector (Figure 3.6B,D).

However, systemic delivery of AdFloxLuc to Albumin-Cre mice resulted in an over 4-log increase in luciferase expression within the liver at both day 3 and day 5 post vector delivery (Figure 3.6B,D). Importantly, Albumin-Cre mice treated with AdFloxLuc resulted in an over 2-log increase in luciferase activity in serum-derived exosomes, whereas no significant difference was observed in C57BL/6 mice ($p < 0.05$, Figure 3.6C,E). As our previous research showed greater levels of exosomes circulating in animal models of SMA and patients with SMA [60], and other groups have reported impairments in endocytosis with depletion of SMN [61, 62], we decided to examine the levels of exosomes in serum, after treatment. Interestingly, circulating levels of exosomes were found to be not statistically different across treatment groups, suggesting the use of a non-replicating Ad, nor over-expression of Luc or SMN lead to alterations in endocytosis (Supplemental Figure 3.S.4). These results indicate that high-level expression of SMN-fusion protein in the liver of mice led to the release of exosomes containing the fusion protein into blood.

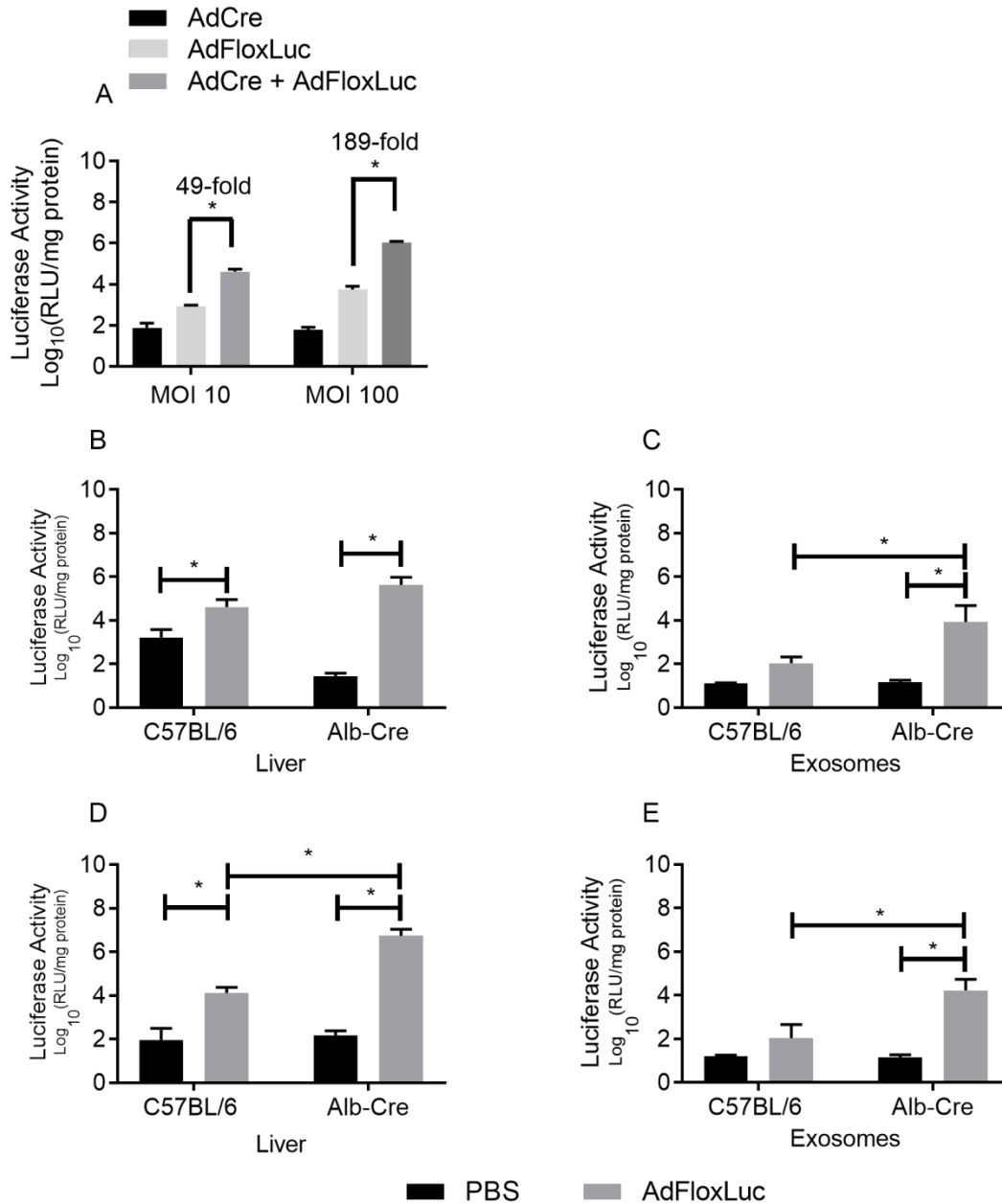


Figure 3.6. Gene therapy can be used to induce the liver to produce therapeutic exosomes *in vivo*. Panel A: Wildtype MEF cells were infected with either AdCre, AdFloxFuc or both viruses at MOI 10 or 100 and assessed for luciferase activity 24 hpi. MEFs infected with both viruses had significantly enhanced luciferase activity compared to MEFs treated with AdFloxFuc or AdCre virus alone. Panels B-E: Adult C57BL/6 mice and Albumin-Cre mice were injected through the tail vein with 5×10^{12} vp/kg of AdFloxFuc or PBS. Livers (Panels B, D) and serum-derived exosomes (Panels C, E) were assessed for luciferase activity at 3 or 5 dpi. Values were transformed into log scale and assessed for statistical significance using a two-way ANOVA with significance set to $p < 0.05$ and indicated with an asterisk. Data are presented on a linear scale, $n = 3$.

To determine if systemic delivery of AdLucSMN resulted in detectable luciferase activity in peripheral tissues, we again treated C57BL/6 mice by tail vein injection with PBS or 5×10^{12} VP/kg of AdGFP (control), AdF3SMN, or AdLucSMN, at 6 weeks of age, and isolated blood and a variety of tissues at 1, 2 or 3 days post injection. As expected, we detected very high level activity of the luciferase-SMN protein in the liver of mice treated with AdLucSMN (Figure 3.7A), and in exosomes isolated from serum (Figure 3.7B). Similar to the previous study, there was no significant difference in the levels of circulating exosomes across treatment groups (Supplemental Figure 3.S.5). We also observed significant luciferase-SMN activity in all other tissues examined, averaging about 3-log above background (Figure 3.7C-G). Importantly, we observed significant levels of luciferase-SMN in the spinal cord of AdLucSMN treated mice (Figure 3.7C). These data provide support for the idea that the liver can be used as a production factory to generate SMN-loaded exosomes that circulate throughout the body to provide the therapeutic protein body-wide.

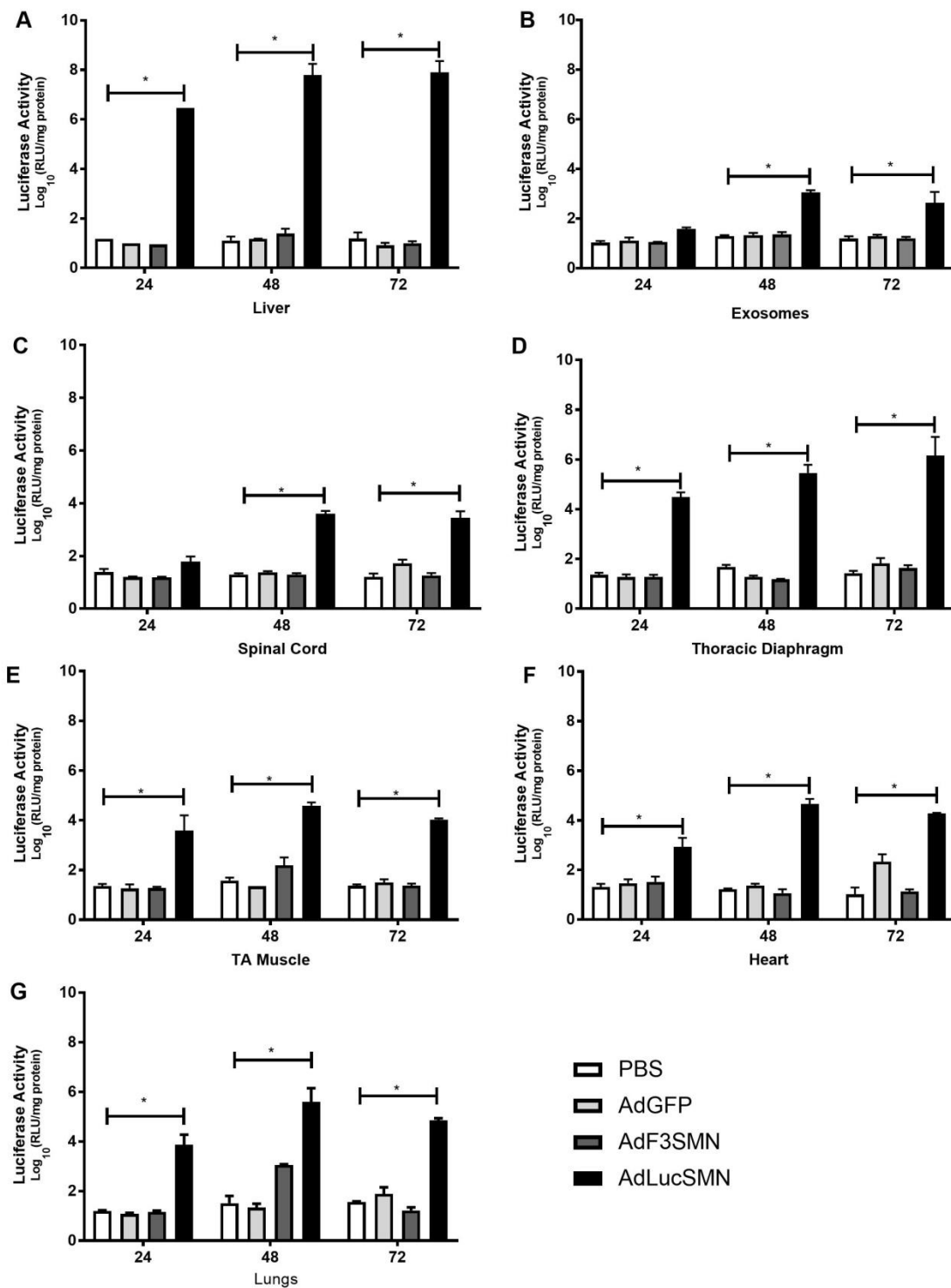


Figure 3.7. Ad-mediated expression of luciferase-SMN protein in the liver of mice results in circulating exosomes containing the protein and detectable levels of the protein in peripheral tissues. Adult C57BL/6 mice were injected through the tail vein with PBS or 5×10^{12} vp/kg of AdGFP, AdF3SMN or AdLucSMN, and tissues and serum-derived exosomes were assessed for luciferase activity 24, 48 and 72 hpi. Values were transformed into log scale and assessed for statistical significance using a two-way ANOVA with significance set to $p < 0.05$ and indicated with an asterisk. Luciferase activity was assessed in the liver (A), exosomes (B), spinal cord (C), thoracic diaphragm (D), TA muscle (E), heart (F), lungs (G). Data are presented on a linear scale, $n = 3$.

DISCUSSION

Exosomes are gaining increasing attention as biomarkers of disease and as potential therapeutic delivery vehicles. Exosomes are particularly attractive for treatment of diseases of the CNS, due to their ability to cross the blood-brain barrier [28, 63]. Likely due to SMN protein's natural ability to be loaded into exosomes [60], we have shown that we can easily increase the quantity of SMN protein in exosomes simply by over-expressing the protein in either a stable cell line or through delivery of an Ad vector encoding the *SMN1* cDNA (Figure 3.1). Exosomes purified from the medium of these cells were able to be taken up by recipient cells, and deliver the SMN protein into the interior of the cell (Figure 3.2-5). To circumvent the need for repeat administration of purified exosomes, we demonstrated that delivery of an Ad vector expressing an SMN fusion protein to the liver of mice resulted in circulating exosomes containing the liver-derived SMN protein (Figure 3.6-7). Importantly, we also detected the luciferase-tagged SMN protein in all peripheral tissues examined, including the spinal cord. However, it is important to note that it is unclear if the activity in peripheral tissues was due to (1) uptake of luciferase-SMN protein containing exosomes by these tissues, or (2) primary uptake of the AdLucSMN vector by these tissues. Biodistribution studies have shown that approximately 80% of Ad delivered systemically transduces the liver, the lungs and spleen receive about 10-fold less, and other tissues about 1- to 2-log lower [55, 56]. This pattern of transduction does resemble our pattern of luciferase expression in the various tissues of AdLucSMN-treated mice (Figure 3.7). Following systemic delivery, Ad vectors can cross the blood-brain barrier [64], but very inefficiently [55, 65]. Overall, the Cre-activatable vector AdFloxLuc provided luciferase expression levels that were approximately 2-log lower than AdLucSMN, which reduced its sensitivity and precluded its use to definitively determine if

expression in peripheral tissues was derived from systemically-circulating exosomes originating from the liver. We are currently generating a more active version of AdFloxFoxLuc to address this point.

Our work has shown that we can induce the mouse liver to release exosomes containing elevated levels of the SMN protein; however, we have yet to determine whether this level is therapeutic. SMA is a disease caused by reduced levels of the SMN protein, and even a small increase in the cellular levels of the protein may provide a benefit in patients. In the *Smn*^{2B} mouse model of SMA, *Smn*^{2B/2B} mice have ~36% of wildtype levels of SMN protein in the spinal cord and these mice are phenotypically normal, while *Smn*^{2B/-} mice that have ~25% or less of wildtype levels of SMN protein in the spinal cord have a significantly reduced lifespan (~28 days) and exhibit many of the hallmarks of SMA disease [47]. Thus, as little as a 10% difference in SMN protein levels in the spinal cord (and likely other tissues) is the difference between phenotypically normal and diseased mice. SMN protein-loaded exosomes produced in the liver may be able to provide the small boost in SMN protein required for disease correction in all affected tissues.

One advantage to engineering the liver to release SMN protein-containing exosomes is that these therapeutic particles can circulate throughout the body, and are available for uptake by all tissues. Increasing evidence has shown that SMA may be a disease of more than just motor neurons. Numerous studies have now shown in mouse models of SMA that there are primary defects in skeletal muscle [66-68], heart [69-71], autonomic and enteric nervous systems [13, 15], bone [12] and reproductive systems [72] as well as impaired metabolic/endocrine function [73, 74], and these deficiencies may contribute to the disease state (reviewed in [75]). Thus, therapies may only be completely effective if they restore SMN protein levels systemically.

Interestingly, a significant proportion of both intrathecally-delivered Nusinersen [20, 76] and systemically-injected AAV-SMN [22] transduces the liver (and likely many other tissues), suggesting that at least part of the beneficial effects of these therapeutics may be due to “off-target” transduction or even production and redistribution of SMN-loaded exosomes in other tissues similar to what we have described herein.

Although the recent very promising results in human clinical trials using gene therapy to treat SMA utilized an AAV-based vector, we chose to use an Ad-based vector for our studies for a number of reasons. We wished to achieve very high level of SMN protein expression in the liver of animals and, although largely anecdotal, Ad is generally considered to provide much higher levels of transgene expression than AAV. A few published studies have provided quantitative data to support this idea. A direct comparison of self-complementary AAV8 *versus* Ad using the same expression cassette consisting of the liver-specific LP1 promoter driving expression of UGT1A1 in the Gunn rat showed that a 30-fold greater dose of the AAV vector was required to achieve the same level of expression as Ad [77]. Similarly, in a study looking at vector delivery to the heart, a 10-fold higher dose of AAV2-lacZ was required to provide the same level of expression as Ad-lacZ [78]. Importantly, we showed that in tissue culture, Ad can easily mediate supraphysiological levels of SMN protein expression with no apparent adverse effect on the cells [17]. Although no gene therapy vector is completely safe, Ad has a reduced tendency to integrate into the host genome compared to AAV, decreasing the likelihood of cellular gene activation or inactivation. While difficult to compare between studies, published reports have suggested that up to 0.1% of the AAV vector delivered to liver *in vivo* undergoes integration [79], whereas this number is in the range of 0.006% for Ad [80]. AAV vector integration has been linked to induction of hepatocellular carcinoma [81] although some studies

have not observed this link [82]. Integration has also been observed in human muscle biopsies from patients receiving AAV-mediated therapy for lipoprotein lipase deficiency [83]. Given that a lower dose is required to achieve high-level expression, and the reduced frequency of integration in the liver, Ad may still have some advantages over AAV for treatment of SMA.

In conclusion, we have shown that exosomes are efficient at delivering SMN protein to recipient cells *in vitro*, and that a gene therapy approach can be used to convert the liver into a SMN-loaded exosome production factory. These exosomes are free to circulate throughout the body and are available for uptake by all peripheral tissues. Thus, we have developed a novel approach (liver-directed, gene therapy-induced production of therapeutic exosomes) that may be an effective therapy for treating SMA and many other disease states.

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SUPPLEMENTARY FIGURES

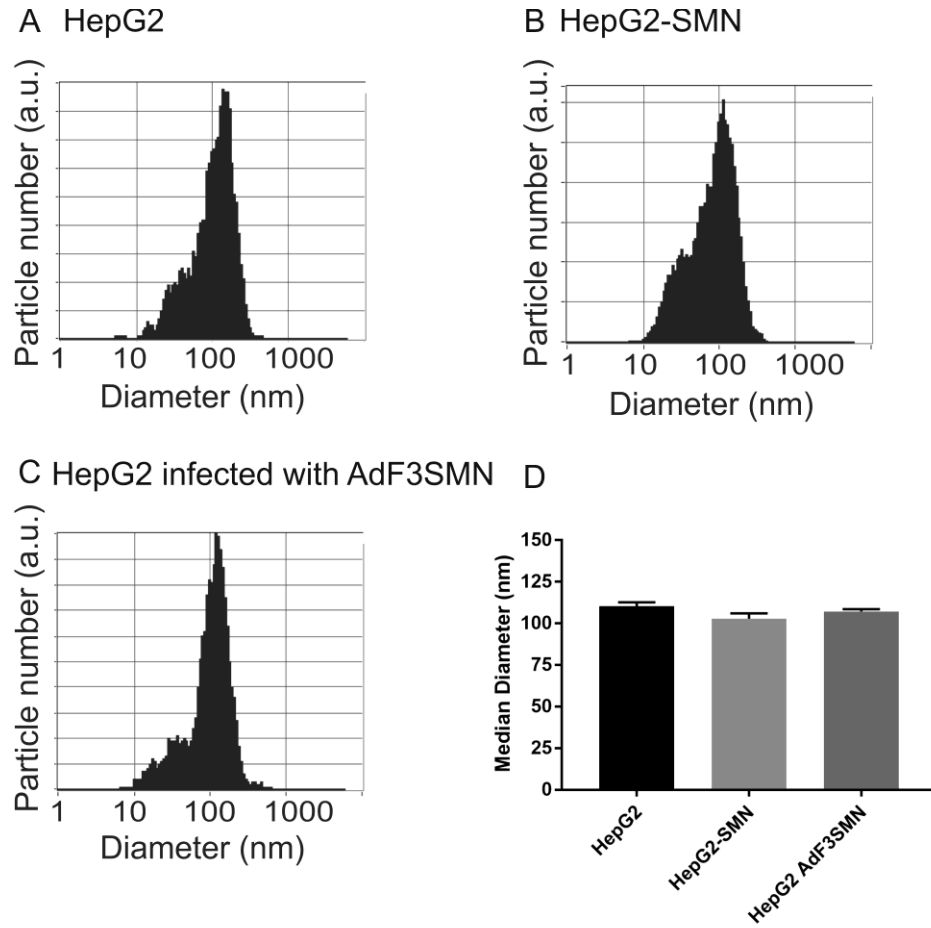


Figure 3.S.1. Representative size distribution profiles of exosomes isolated from (A) HepG2 cells, (B) HepG2-SMN cells, and (C) HepG2 cells infected with AdF3SMN. (D) Average median diameter of exosomes isolated from HepG2, HepG2-SMN, and HepG2 cells infected with AdF3SMN. Data represents $n = 3$.

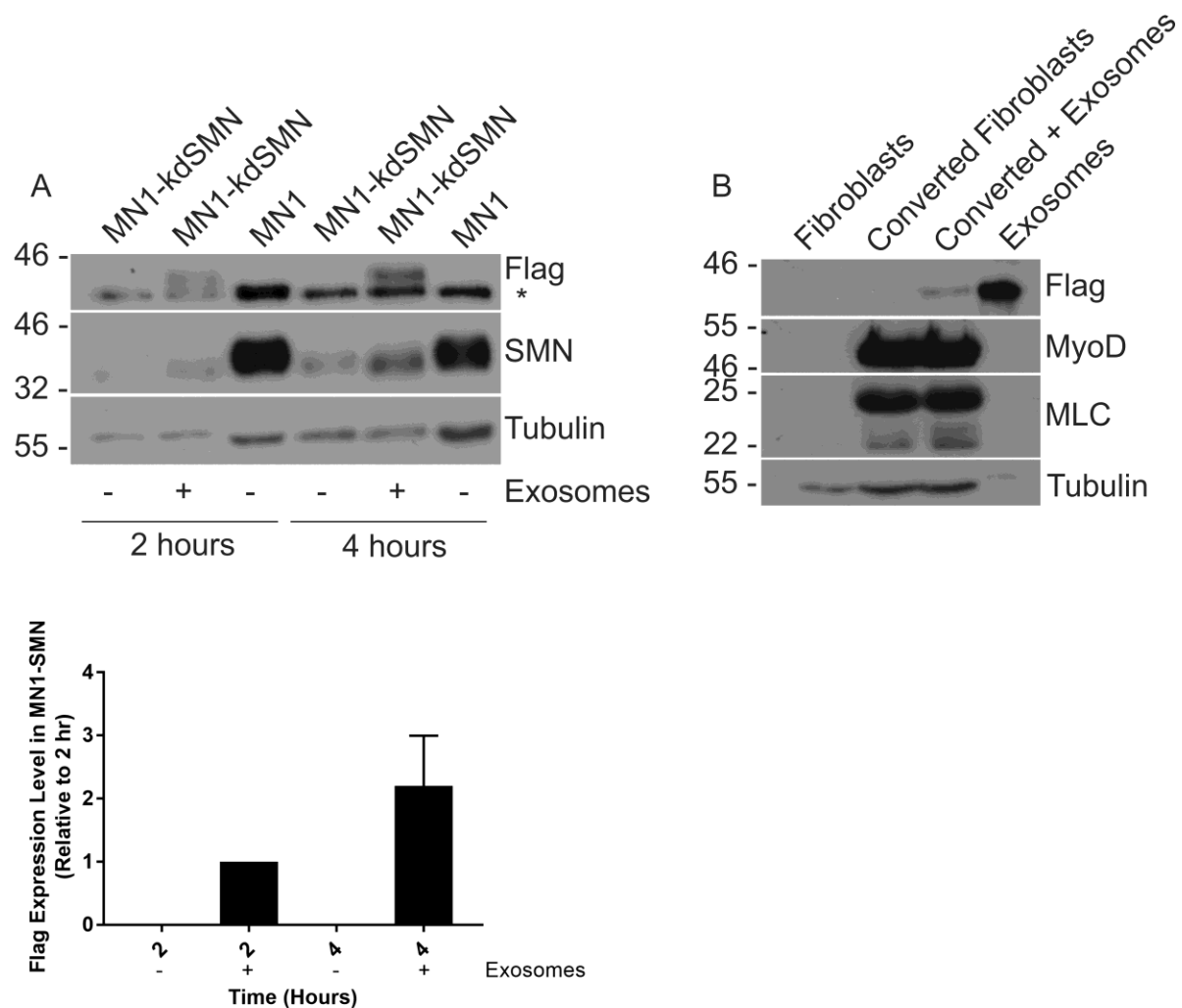


Figure 3.S.2. Panel A. HepG2-SMN derived exosomes associate with SMN deficient motor neuron like cells. Exosomes isolated from HepG2-SMN (50 μ g) were added for 2 or 4 h to a 4-well confluent plate of MN1-SMN cells. Wildtype MN1 cells were used as a positive control. MN1-SMN cells were washed with 0.1% trypsin in PBS post addition and lysates were prepared and analyzed by immunoblot for Flag, SMN and tubulin. Asterisk indicates non-specific band under the Flag signal. Flag signal increases from 2 to 4h. Flag signal was normalized to tubulin and plotted against time, with the Flag expression graphed relative to the Flag signal seen at 2 h. Data represents $n = 3$. Panel B. HepG2-SMN derived exosomes associate with SMN deficient myotubes. SMA type 1 fibroblasts were converted into SMA type 1 myotubes by infecting fibroblasts with an Ad vector expressing MyoD and switched to differentiation medium. Exosomes isolated from HepG2-SMN (150 μ g) were added for 2 h to a 6 cm confluent plate of SMA type 1 fibroblasts converted myotubes. Myotubes were washed with 0.1% trypsin in PBS and lysates were immediately prepared and analysed for Flag, MyoD and myosin light chain (MLC). Data represents $n = 3$.

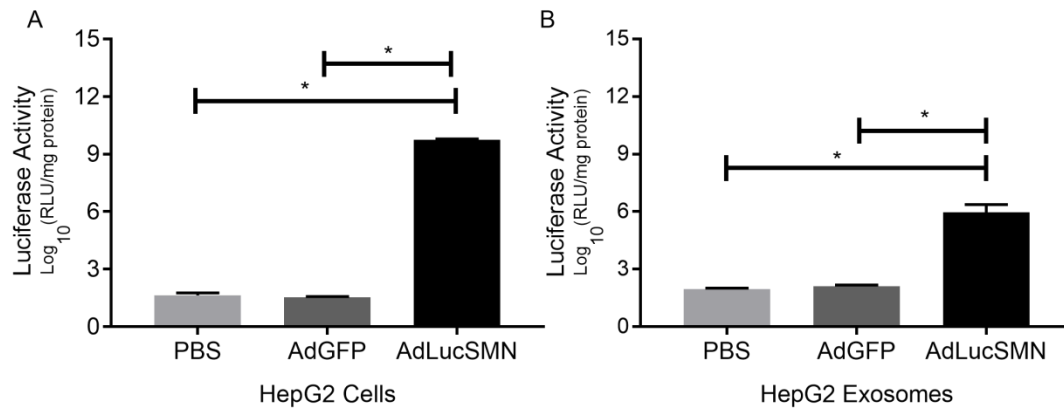


Figure 3.S.3. Luciferase-SMN protein expressed from an Ad vector is loaded into exosomes in cell culture. Panel A and B: HepG2 cells were infected with Ad vectors encoding GFP (AdGFP) or a luciferase-SMN fusion protein (AdLucSMN) at an MOI of 10, or treated with PBS. Twenty-four h later, exosomes were isolated from the medium and protein collected from the cells, and analyzed for luciferase activity. Data represents $n = 3$.

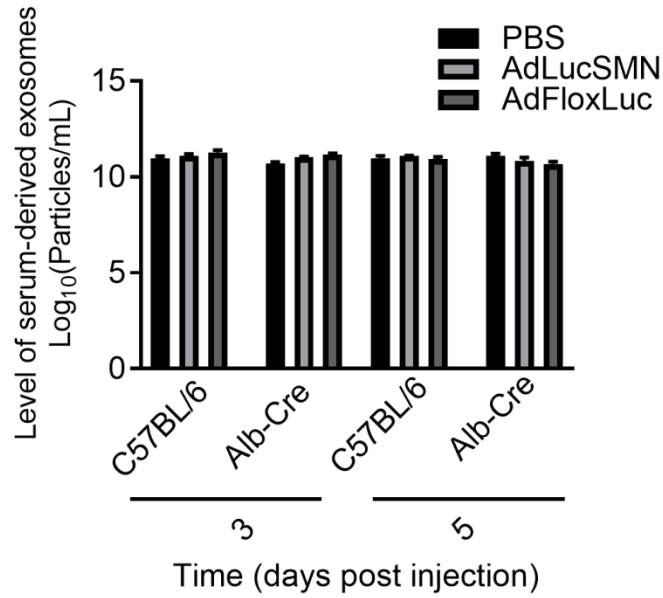


Figure 3.S.4. Circulating levels of exosomes do not change over 5 days, after delivery of a replicating deficient Ad expressing Luc or SMN. Circulating levels of serum-derived exosomes are not altered by E1 E3 deleted Ad vectors expressing LucSMN or AdFloxLuc. Mice were injected with PBS or 5×10^{12} vp/kg of AdLucSMN or AdFloxLuc and exosomes were collected 3 or 5 dpi. Values were transformed into log scale and assessed for statistical significance using a two-way ANOVA with significance. Overall levels of circulating exosomes were significantly different between the WT and Cre mice ($p = 0.0475$). However, levels of exosomes across groups were not significantly different.

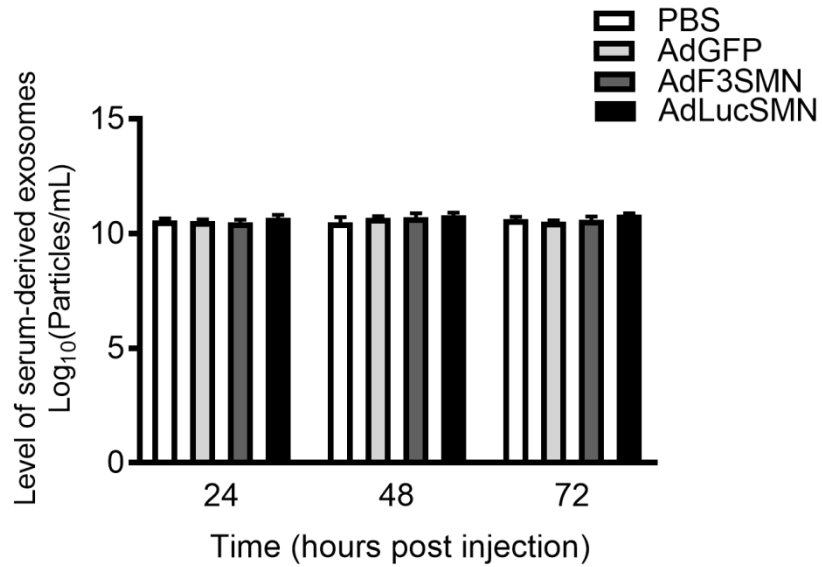


Figure 3.S.5. Circulating levels of exosomes do not after change after delivery of a replicating deficient Ad expressing SMN. Delivery of SMN using E1 E3 deleted Ad does not significantly alter circulating level of serum-derived exosomes in adult WT mice. Mice were injected by IV with PBS or 5x10¹²vp/kg of AdGFP, AdF3SMN, or AdLucSMN and examined for levels of exosomes at 24, 48, 72 hpi. Values were transformed into log scale and assessed for statistical significance using a two-way ANOVA with significance. Ad delivery did not result in significantly different levels of circulating serum-derived exosomes across groups.

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Chapter 4.0 Discussion

Biomarkers. The research presented herein is the first to demonstrate that exosomes isolated from the medium of various *in vitro* models, and serum from mice and humans, contain SMN protein. SMN protein within these vesicles is highly dependent on the disease state of the cell, animals or person from which they were derived (Figures 2.3, 2.4, 2.6, 2.7). Cells, animals, and patients with reduced SMN protein levels show lower levels of exosomal SMN protein, compared to healthy controls. A novel link between SMN protein and the level of circulating vesicles was also identified, where low SMN protein levels showed greater levels of vesicles in circulation (Figures 2.5, 2.6, 2.7). Previous attempts to quantify SMN mRNA or protein in PBMCs and whole blood, to distinguish between SMA disease states have failed. This failure is attributed to the drastic patient to patient variation, making it difficult to distinguish one disease type from another (type 1 vs type 2) or mild disease states from healthy individuals (type 3 vs healthy) [52-54, 202]. Using exosomes, preliminary data suggests one can distinguish healthy individuals from patients with a mild form of SMA using exosomal SMN protein levels (Figure 2.7). Further investigation will be required to examine differences in exosomal SMN protein levels between severe types, (type 1 vs type 2), and mild states (types 3 vs 4).

As exosomes can be readily extracted from serum and this process does not pose a barrier with greater disease severity, unlike intrathecal injections in patients with scoliosis, levels of exosomal SMN protein present as a suitable biomarker for assessing the SMA disease state. The discovery of low levels of SMN protein coinciding with enhanced levels of circulating exosomes in comparison to healthy controls, could also serve a secondary readout for the disease state. Previous research has shown impairments in endocytosis when *smn1* is knocked down in *C. elegans* or in severe mouse models of SMA, suggesting perhaps, exosome release is not altered,

but instead uptake is reduced [203, 204]. With the research presented herein and the previous knowledge of endocytosis impairments, evidence suggests that exosome levels may be a secondary indicator of the disease state.

To determine if circulating levels of exosomes could also pose as a potential prognostic marker to predict disease state, serum derived exosomes were assessed in a less severe model of SMA, *Smn*^{2B/-}, at varying ages. The *Smn*^{2B/-} mice are the result of a tri-nucleotide substitution within the exon 7 which results in missplicing of the *Smn* gene to produce *Smn* mRNA lacking exon 7, while still producing full length Smn protein approximately 15 % of the time [205]. The result of the shift from full length to largely $\Delta 7$ *Smn* mRNA, causes weight loss around P12, a loss of motor neurons, increased NMJ pathology and muscle atrophy by P16, with a significant decrease in weight (approximately 50 %), decrease in motor function, and a median survival around 28 days [205, 206]. Thus, serum-derived exosomes were quantified from *Smn*^{2B/-} and their heterozygous control *Smn*^{2B/+} mice, at pre- (p6), early- (p13), and late- (p18) symptomatic stages of disease progression [205, 206]. At p6, affected mice are indistinguishable from healthy, and therefore are genotyped to confirm their disease state. *Smn*^{2B/-} mice at p13 display early symptomatic features such as stunted tails, shorter ears, and begin to show slower growth, but do not present with neuromuscular defects. Late stage (p18) *Smn*^{2B/-} mice show necrosis in their extremities, body tremors, trouble righting themselves, and significantly smaller weights, while also presenting neuromuscular defects [206]. Quantification of serum-derived vesicles using two methods (Zetaview and Exocet) show no significant differences between affected and non-affected mice at pre-symptomatic stage, however there was a significant difference identified at late stage (Appendix Figure A.1). Additionally, greater levels of circulating particles were found to have a significant positive correlation with age and disease progression in the *Smn*^{2B/-} mice.

These results suggest that the increase in circulating exosomes is the result of disease progression and may be a good indicator of the current disease state, but do not predict disease outcome. Future work should examine the impact SMN-dependent therapeutics has on this phenotype.

While Zetaview and Exocet demonstrate the same trend when comparing affected versus non-affected mice, Zetaview potentially overestimates the amount of exosomes in comparison to Exocet by approximately 2 orders of magnitude at each age. There is also a significant difference in estimation in the amount of vesicles circulating in *Smn*^{2B/+} and *Smn*^{2B/-} at p18, in which Zetaview assumes there is an 18-fold change, while Exocet only assumes a 2-fold change (Appendix Table A.1). Exocet quantifies particles based on acetylcholine esterase, an enzyme concentrated in exosomes, in which its activity has been shown to have a positive, linear correlation with exosome concentration [207]. One hypothesis to support Exocet's results is the potential for serum-derived 'exosome' resuspensions to contain contaminating particles. Low density lipoprotein (LDL), is a particle similar in size to exosomes, averaging around 100 nm and these have been reported in samples using a variety of techniques for exosome isolation [208]. LDL would also fail to demonstrate acetylcholine esterase activity, which could account for the large discrepancy between the Exocet and Zetaview data. Furthermore, while LDL has yet to be measured in SMA patients, it has been previously reported to be elevated in patients with similar neuromuscular disorders such as ALS or spinal bulbar muscular atrophy [209, 210]. While these results point out the importance of using multiple parameters for quantification, and the impact contaminating particles could have on interpretation of results, they may have also revealed a potentially newly, unidentified characteristic in patients with SMA, high LDL. As total cholesterol and high LDL can be a predictor of cardiovascular health, an area in which SMA patients are already impacted, future work should assess lipid metabolism in these patients,

and their levels of circulating cholesterol, in comparison to healthy, aged-matched controls. Conversely, Exocet may be underestimating the level of exosomes present. This could arise if diseased animals have impairments in the ESCRT machinery, or exosome loading process, potentially suggesting inefficient loading of acetylcholine esterase in SMA-derived exosomes, thus making acetylcholine esterase an inefficient method for exosome quantification.

Therapeutics. Using non-denaturing conditions, SMN protein within exosomes was found to be, in part, in its native form (Appendix Figure A.3). Further examination of HepG2 derived exosomes, show SMN protein to be bound to previously identified protein partners, Gemin2 and Gemin3 (Appendix Figure A.4). Together, this data suggests a portion of SMN protein is loaded into exosomes correctly folded, and perhaps as a complex, enhancing the likelihood that exosomal SMN protein is functional and can be utilized to deliver therapeutic SMN protein to SMN-deficient cells. The work presented here exploited two techniques to enhance SMN protein levels in exosomes; the creation of a stable cell line, and the use of a replication defective Ad vector expressing *SMN1* cDNA (Figure 2.4, 3.1). The resulting SMN protein-loaded exosomes could be delivered to a variety of SMN-deficient cell lines in a dose- and time- dependent manner, including patient derived SMA type 1 fibroblasts and motor neuron like cells knocked down for *SMN* (Figures 3.2, 3.S2). Delivery of SMN protein-loaded exosomes resulted in SMN protein levels correctly localized to the cytoplasm and nucleus (Figure 3.3, 3.4). Lastly, a unique approach to gene therapy for treatment of SMA was identified, wherein Ad vectors expressing therapeutic Luciferase-tagged *SMN1* directed to the liver, could produce SMN protein-loaded exosomes *in situ*, resulting in SMN-loaded exosomes in circulation and luciferase activity throughout the body (Figures 3.5, 3.6, 3.7).

Exosomes are utilized for cell-to-cell communication [90]. Their recent development as drug delivery vehicles has allowed researchers to revisit previously developed therapeutics for neurological disorders, due to their capacity to cross the blood-brain barrier [129]. This work shows Ad delivery to the liver results in transgene expression in the target organ, serum-derived exosomes, spinal cord (Figure 3.5, 3.6 3.7), brain (Figure A.5), and muscle (Figure 3.7, A.5). While a variety of tissue types can take up exosomes, especially when delivered systemically, the

potential for exosomes to cross the blood-brain barrier in healthy conditions, without modification, has been debated. An *in vitro* study modeling the blood-brain barrier using brain microvascular endothelial cells (BMECs) argues against natural exosomal crossing [127]. BMECs form tight junctions, preventing the transport of molecules, except for the typical nutrients or hormones used for growth and communication [127]. In this study exosomes were labeled with a luciferase reporter and added to the luminal chamber in a transwell lined with BMECs, and assessed for their ability to transfer to the abluminal chamber. In normal conditions exosomes could not cross the transwell. However, when the BMECs were stimulated to mimic a stroke-like condition by TNF α activation, there was a significant increase in exosomes crossing the BMEC layer in an endocytosis-dependent manner [127]. In contrast, radiolabeling of exosomes derived from RBCs, injected intravenously into mice, demonstrated that exosomes were able to cross the blood-brain barrier, independent of stroke like conditions. But, similar to the previous studies, it was confirmed that exosome uptake could be enhanced when the blood-brain barrier was disrupted using lipopolysaccharide (LPS), a pro-inflammatory, neurodegenerative compound. Authors attributed exosome uptake to also be the result of endocytosis [128]. To increase the crossing of exosomes into the CNS, researchers have utilized the neuro-targeting peptide, RVG, tethered to the exosomal membrane protein Lamp2 β . Utilization of these modified exosomes has led to greater uptake by neurons, while also leading to functional outcomes, such as the knockdown of neuro-specific proteins in the CNS [130, 131, 139, 151]. Exosome research has also shown that not only is uptake significantly increased in the CNS following stroke like conditions, but functional benefits can be seen such as neuro-regeneration and reduction of inflammatory markers, when delivering therapeutics [211-213]. These results indicate that while exosomes may naturally cross the blood-brain barrier,

modification of the surface proteins enhances their delivery to the CNS, as does disruption of the blood-brain barrier.

As exosomes may possess the unique ability to cross the blood-brain barrier naturally and this uptake can be enhanced by engineering neuro-targeting peptides on the exosome's surface, these vesicles may provide benefits by encapsulating already established therapeutics. Exosomes could enhance efficacy of a therapeutic in patients where the blood-brain barrier is fully formed. For example, when older type 1 patients (>6 months) received Spinraza, the result was only a mild, but not statistically significant benefit in the HINES-2 scale. Furthermore, many patients still experienced progressive respiratory failure [72]. The authors suggest the benefit to peripheral tissues may take longer to be identified, or more likely, the inability of the ASO to cross the blood-brain barrier results in an insufficient coverage to alter adequate levels of *SMN2* splicing in the periphery. This issue can be addressed with the use of exosomes, as the neuro-targeting peptides could enhance crossing of the therapeutic to the blood-brain barrier using systemic delivery, while still allowing for exosome uptake by peripheral tissues, thus being a potentially useful surface modality in the future development of exosome based therapeutics for SMA.

While many groups utilize active loading techniques such as transfection, electroporation, and sonication [130, 131, 144], the work herein utilized a replication incompetent Ad to actively load exosomes. Nevertheless, because treatment for SMA would likely require daily injections, producing the quantity of exosomes necessary for animal studies would be inefficient. As Ad preferentially targets the liver, and the liver produces ample amounts of exosomes, this vector was examined for its ability to express SMN protein within the liver, in hopes to achieve SMN protein-loaded exosomes in circulation [198]. Delivery of Ad systemically, expressing *LucSMN*

cDNA, results in transgene expression in the liver, serum-derived exosomes, spinal cord, muscle and a variety of peripheral tissues. A similar technique was utilized by Kojima *et al.*, where 293 cells were engineered to express both the neuro-targeting RVG peptide and a luciferase reporter tethered to the exosomal membrane protein CD63. The 293 cells were also transfected with a plasmid expressing a triad of proteins (syndecan-4, STEAP3, NadB) to increase exosome production (up to 40-fold) and *catalase* mRNA, to combat neuro-inflammation. These super-modified 293 cells were then encapsulated within a polymer that was transplanted subcutaneously into mice. The polymer allowed for continuous exchange of exosomes, but prevented cells from escaping into circulation. Exosome presence was confirmed in the brain, spleen, and liver, and importantly resulted in reduced levels of pro-inflammatory proteins in the CNS, while also preventing neuronal cell death [139]. This novel, *in situ*, dual approach to gene therapy presented in this thesis, utilizes Ad vectors and exosomes, and results in systemic delivery of SMN protein. Future work will require testing with a more sensitive reporter while minimizing the leaky transgene expression. Leaky transgene expression could be minimized using an enhanced stop signal or potentially using the FLEX Cre model [214]. The FLEX model initially places the gene inverted and utilizes two loxP pairs that are different from one another. One pair of loxP sites are oriented in opposing directions to invert the gene in the correct direction, and one pair of loxP sites surrounding the first loxP sites which face the same direction ultimately resulting in the excision of the first loxP sites, preventing further re-inversion .

While the notion of enhanced therapeutic delivery of SMN protein to a variety of tissue types is exciting, it also brings about the question of its impact on reproduction, especially if children with SMA are surviving longer and wish to have children of their own. Notably, Ad vectors have the added benefit of being extremely low risk of female germline transduction.

Previous work has shown Ad to be inefficient at infecting oocytes through either direct ovary injection or by particle bombarding oocytes prior to implantation [215]. Systemic delivery of Ad via left ventricular cavity injection in mice, using a testis-specific promoter, has shown Ad also incapable of infecting the male germline [216]. Furthermore, even when infection of spermatogonial stem cells was achieved in cell culture, then implanted back into infertile mice using micro-insemination, no evidence of Ad DNA was observed in the offspring, suggesting Ad was incapable of integrating the germline [217]. In contrast to this, lentivirus has shown to be an efficient viral vector for the production of transgenic chickens, mice, and pigs, due to their high efficiency at transferring the transgene to their offspring [218-220]. Results surrounding AAV germline transmission have not been as clear. A recent study in mice found AAV9/1 was able to cross the blood-testes barrier resulting in genetically modified spermatogonial stem cells, however the offspring did not carry the AAV genome [221]. Another study demonstrated that direct testicular injection of AAV serotypes 2, 5, 8, 9, and rh10 also did not result in spermatogonial cell infection [222]. Meanwhile, other reports have indicated both AAV and lentivirus are effective at transducing germline stem cells in pigs. Furthermore, when sperm testing positive for the transgene was used for *in vitro* fertilization, both AAV and lentivirus resulted in transgenic embryos, suggesting that both viruses were capable of germline transgene transmission [223]. Likewise, *in vitro* fertilization in goats using AAV transduced germ cells from males, were shown to result in 10 % of the offspring expressing the transgene [224]. Therefore, Ad vectors may provide a safe alternative to other viral vectors, for SMA patients wishing to one day have a family and may be concerned about the potential hazards of germline integration in their children.

Future Studies

The continuous study and advancement of biomarkers within the field of SMA is crucial. To ensure that exosomal SMN protein levels are consistent and accurate representations of disease progression, future work should take care when extracting samples to make note of the patient's age, disease state, gender, and the time the patient's blood was extracted. These parameters identified must be indicated as disease modifiers may exist in females (or males), SMN protein levels may change with age, and more recent evidence has shown circadian rhythm alters *SMN* gene expression [53, 54, 56, 225, 226]. In the event that exosomal SMN protein levels are indicative of disease state, they may also be utilized as a biomarker to determine efficacy of SMN-dependent therapeutics. The currently available FDA approved therapeutic, Spinraza, is given intrathecally and corrects splicing of the *SMN2* transcript, in order to create more full length SMN protein [68, 80]. Currently, the only way to measure drug efficacy is to examine the ASO half-life in serum or CSF of surviving patients, or SMN mRNA and protein levels in the spinal cord of deceased patients, or lastly, wait until a motor milestone is or is not achieved [68, 80]. Thus, serum-derived exosomal SMN protein levels should be assessed before and after treatment with Spinraza to examine the ability of exosomal SMN protein levels to indicate therapeutic efficacy. To assess the prognostic capability of exosomes, patient blood and CSF would be withdrawn before the first round of treatments and during each maintenance injection to determine if there are changes in exosomal SMN protein levels. Ideally, SMN protein levels within exosomes would increase with treatment and the levels of SMN protein within serum-derived exosomes would not be significantly different from the exosomal SMN protein levels within the CSF. Thus, serum-derived exosomes would be easily accessible and a virtually pain-free tactic to follow therapeutic efficacy. Most importantly, exosomal SMN

protein levels would be a pre-indication of improved functional outcomes to follow, as outlined by HINES-2 scores. Additionally, with the ability to follow disease progression, or therapeutic efficacy using serum-derived exosomes, one could also utilize exosomes as a way to monitor and adjust patient dosing. As the level of exosomal SMN protein changes with therapeutic intervention, exosomes can be utilized as a readout to determine whether the patient requires more (or less) therapeutic, thus providing another novel, theranostic approach.

While, exosomal SMN protein levels, nor the levels of circulating exosomes themselves, have shown to be a predictive biomarker for the SMA disease state, other approaches may be utilized to identify predictive biomarkers centered around the use of serum-derived exosomes. Several research groups have identified protein and RNA markers that are indicative and predictive of progressive diseases such as MS and AD [118, 125, 126]. Hence, the use of proteomics and RNAseq could potentially aid in identification of other novel SMA prognostic biomarkers. Serum-derived exosomes isolated from healthy, carrier, and SMA neonates for differences in protein and RNA, using mass spectroscopy and RNA sequencing (RNAseq), respectively, could reveal novel prognostic indicators. Labeling serum-derived exosomes with iTRAQ would allow for a non-bias, quantifiable approach to examine exosomal protein by liquid chromatography-mass spectroscopy [227]. Proteomics could be complemented with RNAseq, as many miRNA have been previously identified in SMA to be altered, including miRNA-9, miRNA-206, miRNA-132, and miRNA-183 [228, 229]. Thus, exosomes pose the ability to aid as a novel biomarker for the disease state, with the capability of acting as a biomarker to determine therapeutic efficacy of SMN-dependent therapeutics. Further research into exosomes using mass spectroscopy and RNAseq could allow for the identification of

prognostic markers or novel biomarkers, aiding in the ability to predict disease outcomes and novel treatment targets for patients with SMA.

As treatment continues to occur largely after the onset of muscle weakness, and thus motor neuron degeneration has already begun, many patients will not see full benefits from the available therapeutics. SMN transcripts and protein levels have been shown to peak during development, with the greatest levels found in the CNS. SMN protein levels drop soon after birth, suggesting SMN plays important roles in development [13, 16, 17]. Accordingly, if affected children could be identified as early as possible through newborn screening, thus the future of SMA therapeutics may involve *in utero* therapies could be developed to create the most impactful outcome in the developing CNS. Nevertheless, this poses many challenges, as the therapy needs to be both impactful but also safe for the mother and developing embryo. Some studies noted that very early delivery of Ad at the morula stage (approximately embryonic day 2, E2) can result in embryonic transgene expression, but it also reduced the numbers of blastocysts [230]. While, intraplacental injections later in development (E12.5 days in mice) resulted in approximately 82 % viable embryos brought to term, a level within the acceptable range for normal gestational loss. Importantly, this later injection also led to significant transgene expression which persisted until P1 [231]. High transgene expression was also identified in cardiac and lung tissues, both of which show impairments in type 1 patients [70, 72, 232, 233]. In another study, a transuterine intraperitoneal injection *in utero* at E15 of Ad resulted in transgene expression within the brain of P2 mice, while also achieving 90% embryo viability [234]. Long term studies have shown Ad injection at E15 can persist for a month post-natally, without eliciting an immune response. When the same mice received a second Ad injection at 3 months of age, there was a further significant increase in transgene expression that was not

significantly different from mice not previously exposed to Ad. Both groups of mice developed a similar humoral response, irrespective of receiving Ad *in utero*. However, a third administration of Ad at 6 months did not result in significant transgene expression [235]. Thus, while trepidation exists surrounding the idea of *in utero* injections, these studies suggest embryonic delivery of a replication defective Ad is safe and results in transgene expression which continues post-natally. Furthermore, a second Ad injection can be provided post-natally to achieve significant transgene expression for a second time. Thus, the development of Ad vectors expressing therapeutic SMN for *in utero* therapy could prevent early, irreversible disease in the CNS and peripheral tissues. But, the addition of the second injection post-natally could also allow for maintenance of SMN protein levels, potentially leading to phenotypic correction and increased survival in severe SMA patients.

While Ad systems provide good transgene expression within the liver [236], they can evoke potentially harmful immune responses [187] and gene expression is limited to a few weeks [237, 238]. Therefore, future development of Ad based SMN-therapeutics could be enhanced through the use of hdAd systems. HdAd, a third generation Ad vector, lacks all viral protein coding sequences and allows for long term gene expression with minimal immune response could provide a more suitable gene therapy approach for long term expression of SMN protein within the liver [175]. Previous research using hdAd vectors have shown sustained transgene expression for up to 7 years post injection, in a non-human primate [175]. Thus, the hdAd would be a great alternative as a co-therapy to exosomes, or as a ‘booster shot’ post-natally to allow for long term, systemic expression of SMN protein.

Another potentially useful strategy is the combinatorial therapeutic approach which utilizes both exosomes and viral vectors injected simultaneously. Interestingly, vesicles have

been shown to associate with AAV after infection in cell culture, these same vesicles could then be isolated and used to enhance AAV uptake in cells not naturally privy to AAV infection [239, 240]. AAV2 associated exosomes expressing GFP delivered to adult C57BL/6 mice by intravitreal injection led to greater GFP levels in comparison to AAV2 injection (2×10^9 vg) alone. GFP levels produced by the delivery of exosome associated virus were greater at both 2 and 4 weeks post injection by approximately 4- and 3.3-fold, respectively. Exosomes aided in deeper penetration in the retinal layers, and more cell types expressing GFP [241]. Similar results were seen with Ad associated exosomes, in which exosomes pre-incubated with Ad were shown to have significantly enhanced uptake in neuronal stem cells deficient in CAR, in comparison to Ad alone [242]. In contrast to these studies, AAV9 associated exosomes delivered intravenously into mice had similar efficiency to virus alone (1.5×10^{11} gc) at crossing the blood-brain barrier. However, the transduction profile of cells in the CNS was greater when using exosome associated virus [243]. Most interesting, delivery of AAV8 (5×10^8 gc), a vector known to have issues crossing the blood-brain barrier, was significantly enhanced when isolated with exosomes and injected intravenously in mice. The combination of AAV and exosomes led to a 15-fold increase in gene expression in comparison to AAV8 alone [243]. Overall these results suggest virus associated exosomes leads to potentially greater transgene expression due to increased tropism, the result of the virus ‘piggy-backing’ on exosome-dependent endocytosis. Thus, virus-associated exosomes may be an effective strategy for enhancing the level of transgene expression and the diversity of tissues expressing the desired transgene for multi-system disorders like SMA.

Chapter 5.0 Conclusion

Aim 1: The content of exosomes are dictated from the cell which they are derived. SMN protein is found within exosomes and alterations in SMN protein correlate with alterations in exosome levels. Thus, the levels of SMN protein and the concentration of exosomes themselves are both dependent on the disease state. These attributes suggest exosomes to be a suitable biomarker for SMA and potentially a novel biomarker to follow therapeutic efficacy of SMN-dependent therapies.

Aim 2: Exosomes can be loaded with SMN protein and successfully delivered to the cytoplasm and nucleus of a variety of cell types, including SMN-deficient cell lines, suggesting exosomes are excellent candidates as therapeutic delivery vehicles to transport SMN protein to SMN-deficient cells.

Aim 3: Producing large quantities of therapeutic exosomes for daily dosing in animals or patients poses a technical challenge. Conversely, a gene therapy approach involving IV injection of Ad vectors expressing *SMN1* cDNA results in SMN protein-loaded exosomes in serum, in addition to SMN protein expression in peripheral tissues. Utilization of Ad vectors for continuous, endogenous production of SMN protein-loaded exosomes may provide a novel way to treat the SMA disease state.

This research has set the fundamental groundwork for developing a more precise, individualized tool for monitoring disease state, in addition to a new therapeutic option to treat this devastating disorder. The development of exosome based diagnostics and therapies are helping to build a future towards a more theranostic approach in healthcare.

Introduction and Discussion References

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Appendix

Statistical analysis of the Zetaview data using a two-way ANOVA indicates a strong interaction between age and disease state ($p = 0.0003$), whereas exosome levels indicated by the Exocet kit has suggested a significant effect of disease ($p = 0.02$) and age ($p = 0.0005$), but these are independent of each other (interaction $p = 0.15$). This suggests that using Zetaview, age and disease are significant factors in exosome release that rely on each other, i.e. as you age, disease progresses, and therefore more exosomes are released. However, according to the Exocet kit, age and disease state do not rely on each other for exosome release, in other words, aging alters exosome release, as does having SMA, but they occur independent of one another.

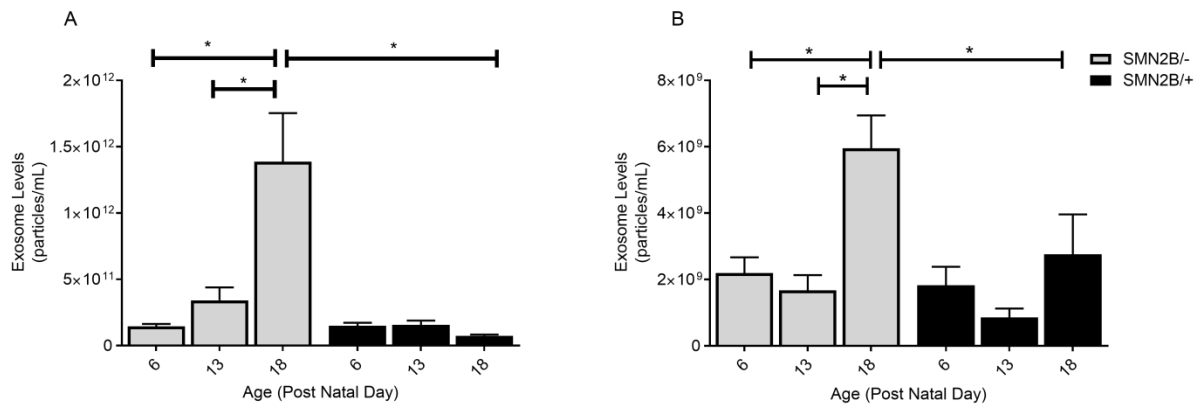


Figure A.1. Serum-derived extracellular vesicle levels are age and disease dependent in a less severe mouse model of SMA. Vesicles isolated from equivalent volumes of serum from *Smn*^{2B/-} and *Smn*^{2B/+} mice at pre- (p6), early (p13), and late (p 18) disease state as determined by Zetaview (Panel A) and Exocet (Panel B). A two-way ANOVA was used to assess statistical significance of age and disease on levels of circulating vesicles. Statistical analysis of the Zetaview data set indicates a significant interaction between age and disease state, while the Exocet data shows significant main effects for age and disease, with no significant interaction. Sample sizes range from $n = 4$ to $n = 8$, using a statistical significance for post-HOC analysis set to $p = 0.05$, indicated by an asterisk.

	Zetaview Particle Tracker			Exocet		
Age (Post Natal Day)	<i>Smn</i> ^{2B/-}	<i>Smn</i> ^{2B/+}	Fold Change (^{2B/-} vs ^{2B/+})	<i>Smn</i> ^{2B/-}	<i>Smn</i> ^{2B/+}	Fold Change (^{2B/-} vs ^{2B/+})
6 (Pre-symptomatic)	1.46E+11	1.50E+11	0.97	2.19E+09	1.83E+09	1.20
13 (Early-symptomatic)	3.41E+11	1.56E+11	2.18	1.68E+09	8.58E+08	1.95
18 (Late-symptomatic)	1.39E+12	7.33E+10	18.92	5.96E+09	2.76E+09	2.16

Table A.1. Levels of serum-derived extracellular vesicles increases with disease state in *Smn*^{2B/-} mice. Levels of circulating vesicles in serum of mice at varying ages in affected (*Smn*^{2B/-}) and non-affected (*Smn*^{2B/+}) mice as determined by Zetaview particle tracker and the commercially available, Exocet kit. Serum-derived exosomes were isolated at pre-symptomatic (p6), early symptomatic (p13) and late symptomatic (p18) disease states. Significantly greater levels of vesicles in affected mice at late stage, and levels of vesicles increase in affected mice with disease progression. In comparison to Zetaview, Exocet estimates a lower level of particles per mL, most prominently seen at late stage symptomatic.

Cells release a variety of vesicles, such as exosomes. Exosomes have been shown to have two distinct subpopulations, based on density [83-85]. These exosomes were found to have distinct protein and RNA cargo or cargo that varied in their level of protein or RNA. It is likely that subpopulations exist to allow for communication between several cell types at once, or potentially a mix between a communicative and a recycling role. To get a better idea of SMN protein's function in exosomes, the protein's state (naïve vs denatured) was investigated. Exosomes subpopulations were purified using a highly stringent isolation technique involving a combination of centrifugation, tangential field flow filtration and size-exclusion chromatography. The combination of these techniques minimizes aggregation and removes a large portion of the soluble protein left behind. The resulting subpopulations were loaded onto a dot blot and probed for SMN, Flag, and Tsg101 (exosome marker). SMN protein and Flag-tagged SMN were found in the majority of fractions, and detectable in non-denaturing conditions, suggesting that native SMN protein is present within these exosomes.

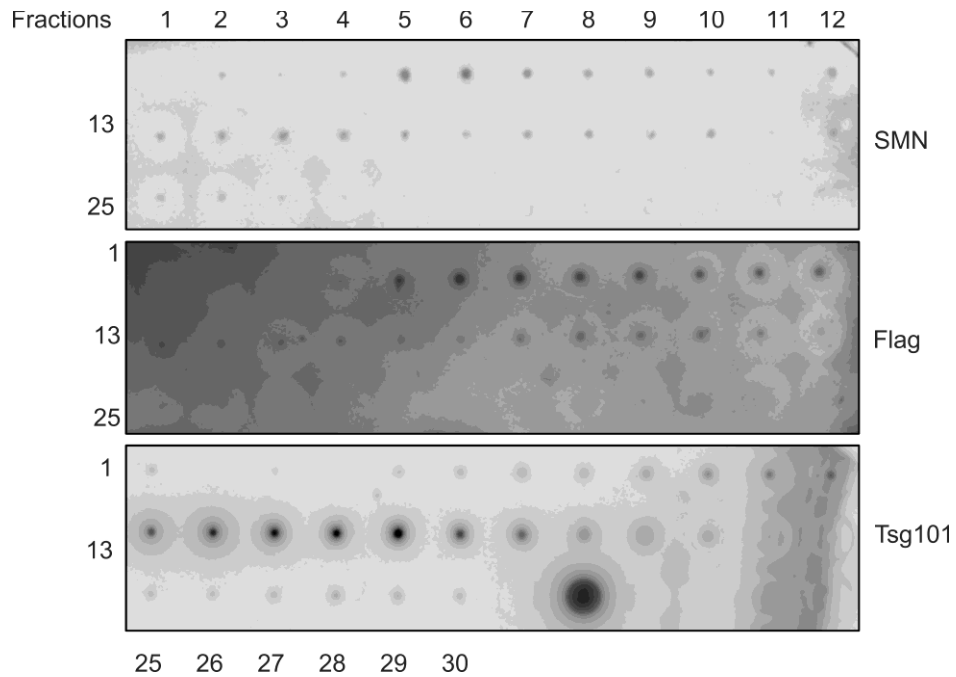


Figure A.2. Exosomal SMN protein is found in its native form. 293 cells stably expressing the 3X flag-tagged *SMN cDNA* was isolated using a combination of differential centrifugation, tangential flow filtration, and size-exclusion chromatography into 30 fractions. Fractions were loaded into a dot blot, and ran onto a membrane using a light vacuum, then probed for SMN, Flag, and Tsg101 (exosomal marker). Data was generated in Dr. Matthew Wood's laboratory in Oxford.

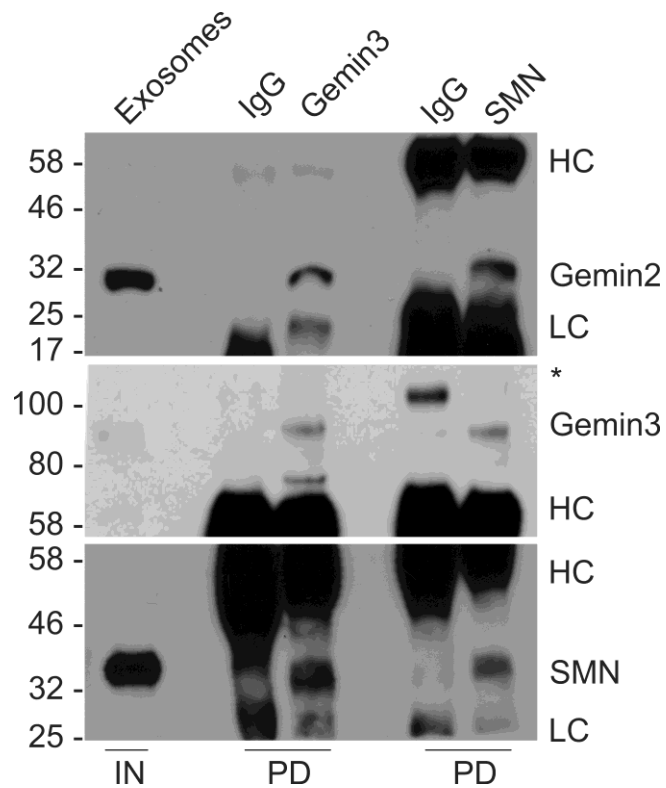


Figure A.3. Exosomal SMN protein is found in a protein complex. Protein extracts from HepG2-derived exosomes were isolated, separated by SDS-PAGE and examined for potential interactions with known protein partners Gemin2 and Gemin3. The input lane (IN) shows the presence of SMN protein, Gemin2, and Gemin3, while the pull down lanes (PD) demonstrate SMN protein within HepG2-derived exosomes is interacting with Gemin2 and Gemin3.

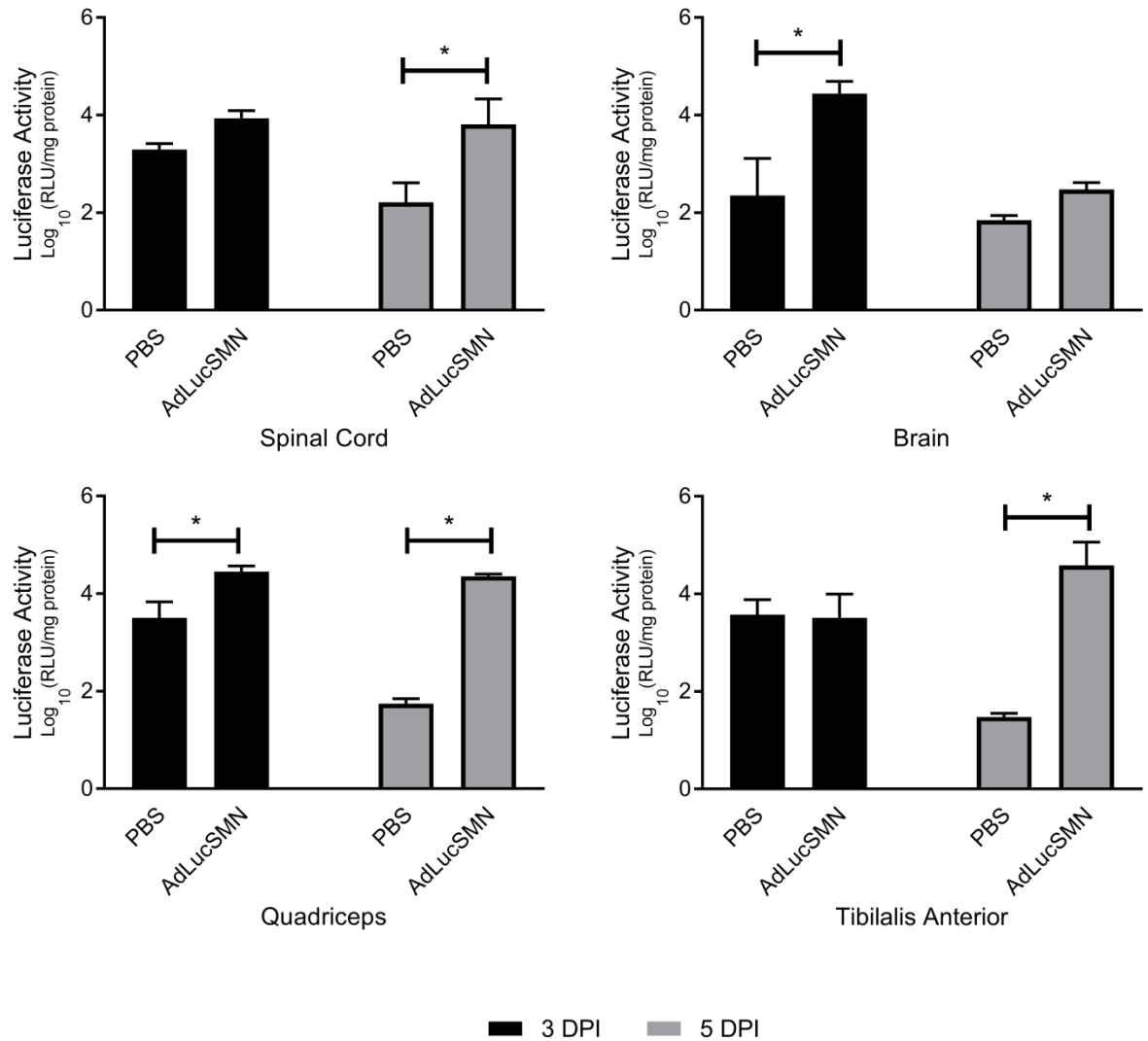


Figure A.4. Delivery of Ad LucSMN leads to transgene activity in CNS and muscle. Adult C57BL/6 were delivered PBS or 5×10^{12} vp/kg of AdLucSMN by tail vein injection. Tissues were harvested at either 3 or 5 dpi. A two-way ANOVA was used to assess statistical significance between time and treatment in each tissue. Significance is indicated using an asterisk with $p < 0.05$. AdLucSMN delivery to the liver results in LucSMN-loaded exosomes, in addition to Luc activity in the spinal cord, brain, quadriceps, and tibialis anterior.

Review: Spinal Muscular Atrophy: More than a Disease of Motor Neurons?

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Abstract: Spinal muscular atrophy (SMA) is the most common genetically inherited neurodegenerative disease resulting in infant mortality. SMA is caused by genetic deletion or mutation in the survival motor neuron 1 (SMN1) gene, which results in reduced levels of the survival of motor neuron (SMN) protein. SMN protein deficiency preferentially affects α -motor neurons, leading to their degeneration and subsequent atrophy of limb and trunk muscles, progressing to death in severe forms of the disease. More recent studies have shown that SMN protein depletion is detrimental to the functioning of other tissues including skeletal muscle, heart, autonomic and enteric nervous systems, metabolic/endocrine (e.g. pancreas), lymphatic, bone and reproductive system. In this review, we summarize studies discussing SMN protein's function in various cell and tissue types and their involvement in the context of SMA disease etiology. Taken together, these studies indicate that SMA is a multi-organ disease, which suggests that truly effective disease intervention may require body-wide correction of SMN protein levels.

Keywords: Gene Therapy, Motor Neuron, Neuromuscular Disease, Spinal muscular atrophy, Survival Motor Neuron, Therapeutics,

1. INTRODUCTION

Proximal spinal muscular atrophy (SMA) is an autosomal recessive disorder that affects 1 in 6,000-10,000 live births [1]. SMA results from reduced levels of full-length survival motor neuron (SMN) protein caused by mutations in the survival motor neuron 1 (SMN1) gene. While SMN protein is ubiquitously expressed, the hallmark of the disease is degeneration of α -motor neurons in the brain stem and spinal cord. This progressive degeneration leads to weakness of bulbar, trunk and limb muscles, and in severe forms of the disease, paralysis [2-4], progressing to respiratory failure, and ultimately death [5]. SMA causes more deaths in infants than any other genetic disorder [3, 6]. Currently, there is no cure, while palliative care utilizes respiratory support, nutritional management, and physiotherapy to aid in relieving disease symptoms.

Primarily considered a disease of motor neurons, accumulating evidence suggests that SMA also affects a variety of other tissues. Studies have now shown that reduced levels of SMN protein causes deficiencies in skeletal muscle, heart, autonomic and enteric nervous systems, impaired metabolic/endocrine function (e.g. pancreas), bone and reproductive systems, and these deficiencies may contribute to the disease state. With improved assistive

technology, and with novel therapies currently in clinical trials, patients with SMA are living longer. Thus, this recently identified “secondary” tissue involvement needs to be more closely examined to evaluate the clinical impact in SMA. Furthermore, therapies may only be completely effective if they restore SMN protein levels body-wide to achieve a complete reversal or amelioration of the disease state. In this review, recent findings in SMN protein function and its involvement in peripheral tissues are discussed, with the implication that SMA is more than just a disease of motor neurons.

2. SPINAL MUSCULAR ATROPHY

2.1 SMA Disease Types.

SMA is clinically heterogeneous, with patients classically divided into one of five types, dependent on age of onset, and peak motor function achieved (Table 1) [7]. However, it is now recognized that SMA presents as more of a continuum of phenotypes rather than discrete types. Type 0 is the most severe form of the disease which progresses during pregnancy, reducing fetal movement and development, leading to respiratory distress at birth and a life expectancy of less than 6 months. Type 1, also known as Werdnig-Hoffmann disease, presents before six months of

age, with notable muscle atrophy and an inability to control head movement. These patients are unable to sit up on their own, and life expectancy is less than three years [8]. In type 2, onset occurs between 6 and 18 months of age and, while these patients are able to sit independently, they never achieve the ability to walk. Life expectancy is between 10 and 40 years. Type 3, also known as Kugelberg-Welander disease, presents after 18 months of age and can result in patients losing the ability to walk in adulthood, while others achieve the ability to walk without assistance throughout life. The mildest form of SMA, type 4, produces symptoms of muscle weakness in adulthood but individuals retain the ability to walk. Types 3 and 4 are not generally associated with a reduced life expectancy [9].

Table 1, Clinical types of spinal muscular atrophy.

SMA Type	Age of onset	Diagnostic features	Life-span	SMN2 copies
Type 0	Prenatal	Reduced movement <i>in utero</i> . Respirator required immediately at birth	> 2 mo.	1
Type I (Werdnig-Hoffman disease)	< 6 mo.	Poor muscle tone and lack of movement · Never able to sit.	> 3 years	1-2
Type II	< 18 mo.	Cannot sit or walk unaided	10-40 years	2-3
Type III (Kugelberg-Welander disease)	>18 mo.	May lose the ability to walk in their adulthood	Normal	3-4
Type IV	Adulthood	Mild proximal muscle weakness	Normal	>4

2.2 Genetic basis of SMA.

Proximal SMA, which accounts for ~95% of patients diagnosed with all forms of SMA, is caused by homozygous deletion or mutation of the survival motor neuron 1 (*SMN1*) gene, found on chromosome 5q in band 13 [10, 11]. The functional loss of *SMN1* results in a deficiency of SMN protein. However, SMN protein is not completely absent in affected patients due to the presence of *SMN2*, a highly homologous gene that differs from *SMN1* by five nucleotides [10]. One of the differing nucleotides in *SMN2* results in a point mutation, a cytosine to thymidine transition at position +6 of exon 7 that significantly impacts function of this gene [12]. Although this nucleotide transition is silent in terms of the amino acid encoded, the change promotes an alternative mRNA splicing event that leads to the exclusion of exon 7 from approximately 90% of *SMN2* transcripts [13]. The protein generated from this incomplete transcript, termed SMNΔ7, has a truncated C-terminus [12, 14] and is relatively unstable, with a half-life of only 2-3 hours in comparison to the 4-8 hours for full-length SMN protein [15-17]. The SMNΔ7 protein is unable to perform the biological functions of the full-length SMN protein. Several research groups have attempted to elucidate the molecular mechanism responsible for the differences in splicing between *SMN1* and *SMN2*, with these efforts largely focused on identifying cis-acting elements regulating the efficiency of exon 7 inclusion. Exon splicing enhancers (ESE) have been identified within exon 7 [18, 19], including at least one whose activity may be affected by the cytosine to thymidine transition within this exon [20]. It has also been suggested that the nucleotide transition may lead to the formation of an exonic splice silencer [21, 22]. Several elements have also been identified in the intronic sequences flanking exon 7 [23-26]. All of these elements serve as potential therapeutic targets for therapies designed to enhance inclusion of exon 7 in the *SMN2* mRNA transcript (discussed below).

Importantly to the disease phenotype, some full-length SMN protein is generated from the 10% of *SMN2* transcripts that naturally include exon 7 [13]. No patients have been identified with deletions or functional loss of both *SMN1* and *SMN2* [10], suggesting that complete loss of these genes results in embryonic lethality [27, 28]. Since the *SMN* locus is variably amplified in humans, an important modifier of SMA disease severity is the gene copy number of *SMN2*. In patients with increased *SMN2* copy number, an additive effect occurs in which the combined full-length transcript derived from each copy of the gene brings the patient closer to the levels of SMN present in an unaffected person [29-31]. Thus, *SMN2* gene copy number is a rough predictor of disease type and prognosis [32, 33] (Table 1). Other genetic modifiers of SMA disease pathogenesis have also been identified, including *SERF1A* and *PLS3* [34-36].

3. SMN PROTEIN AND PUTATIVE FUNCTION

3.1 SMN protein.

SMN protein is ubiquitously expressed in the cytoplasm and nucleus of all cell types [37, 38], and has many protein and RNA binding partners. The full-length 38 kDa SMN protein self-associates via amino acids encoded by exon 2b [39] and through a 30-amino acid domain encoded by exon 6 [14]. Of note, the protein generated from the SMN Δ 7 transcript is unable to self-oligomerize [12, 14, 15]. Following self-association, nuclear full-length SMN is recruited into a macromolecular complex containing Gemin proteins 2 through 8 [40], which form discrete foci termed “gems” in the nucleus [37]. SMN protein level and gem copy number in the nucleus are both inversely correlated with disease severity [31]. The cellular localization of the complex

Table 2, Functions of SMN protein

appears to be influenced by its interaction with unr-interacting protein (Unrip), which directs localization of SMN protein to the cytoplasm and is absent in nuclear gems [41].

3.2 Functions of SMN protein.

Functions attributed to SMN protein are summarized in Table 2.

Function	SMA Disease Manifestation
Molecular Function	
Small nuclear ribonucleoprotein (snRNP) assembly and pre-mRNA splicing	Reduced snRNP assembly and altered splicing pattern of many genes in brain and spinal cord [42, 43]
Translational Regulation	Altered protein translation (e.g. CARM1 [44])
R-loop resolution	Alterations in cellular gene expression and splicing [45]
Active transport	Altered transport of β -actin mRNA to motor neuron growth cones [46]; altered transport of Golgi-associated secretion vesicles [47]
Actin dynamics	Inappropriate activation of the RhoA/ROCK pathway [48, 49],
Biological Function	
Motor neuron outgrowth and axonal pathfinding	Impaired motor neuron connections [50] [51, 52]
Neuromuscular junction function and maturation	Abnormal endplate morphology, endplate denervation, neurofilament accumulation and disturbed function, impaired astrocyte function [53-55]
Skeletal muscle development	Impaired satellite cell differentiation [56]; muscle weakness and delay in expression of mature proteins [57-59]; induction of proteasomal degradation or autophagy [60]
Cardiac function	Bradycardia, dilated cardiomyopathy, decreased contractility [61-63].
Pancreas development	Reduced β cell numbers leading to defective glucose metabolism [64, 65]
Liver function	Altered fatty acid metabolism [66]; altered expression of IGFALS leading to reduced stability of IGF-1 and stunted growth [67]; Immature, disorganized sinusoids, and increased levels of iron deposition, immature RBC and megakaryocytes [68]
Spleen	Reduced size, lack of cellular proliferation and increased cellular apoptosis, increased fibrosis, reduced levels of lymphocytes, increased levels of megakaryocytes [69]
Autonomic nervous system	Cardiac defects and bradycardia [61-63]; vasodilation defects [70, 71]

Gastrointestinal system	Severe diarrhea, submucosal edema present early within the disease state and, reduced number and altered morphology of the villi [72];Enteric nervous system defects (constipation, reduced colon motility and intestinal transit, reduced gastric emptying) [73]
Skeletal/bone system	Lower bone density, increased fractures, increased numbers of osteoclasts, increased osteoclast activity, reduced osteoblast differentiation [74-78]
Reproductive System	Reduced size of testis, impaired spermatogenesis, reduced male fertility, altered transcriptome and splicing regulation during spermatogenesis [79]

3.2.1 Assembly of snRNPs and pre-mRNA splicing.

The best characterized role of SMN protein is its involvement in the assembly of small nuclear ribonucleic particles (snRNPs) [80], complexes composed of small nuclear RNA (snRNA) and Sm proteins that form the spliceosome [81, 82]. Depletion of SMN from a cell results in decreased snRNP assembly [80], and a correlation exists between snRNP assembly impairment and disease severity in mouse models of SMA [83]. The role of SMN in snRNP assembly may have direct consequences for pre-mRNA splicing [84], leading to alterations in protein isoform expression, likely in a cell-type specific manner [42]. Samples from brain, spinal cord, and kidney of a mouse model of SMA displayed widespread aberrant splicing at the peak of disease progression, and these differed between tissues [42, 84-86]. Such observations provide a possible link between a general SMN protein function and a tissue-specific disease. However, exon-array analysis of mouse models of SMA, across three time points, pre-, early-, and post-symptomatic, indicated that little aberrant splicing occurred until the post-symptomatic stage [87], suggesting that splicing defects observed were not the cause, but instead were the result of the SMA disease. Nevertheless, the possibility remains that the few transcripts that show incorrect splicing at earlier time points may be important for SMA pathogenesis.

Recent RNA sequencing studies in the Taiwanese mouse model of SMA, which displays an average lifespan of approximately 10-11 days, showed decreased minor spliceosomal snRNP activity, in which U12-dependent introns were retained in tissues such as the spinal cord and muscle [43]. Aberrant splicing was identified in genes important in regulating axonal growth, neuronal migration, neuronal development, transportation of vesicles along tunneling nanotubes, chromatin remodeling, DNA damage response, cytoskeleton reorganization, and cell growth.

3.2.2 Translational regulation.

In addition to regulating protein expression at the mRNA level, SMN may also have a role in translational repression. Upregulation of the arginine methyltransferase CARM1 occurs in the spinal cords of mouse models of SMA and in patient-derived fibroblasts [44]. Consistent with a previously established regulatory pathway in motor neurons [88], SMN appears to inhibit translation of CARM1 mRNA, likely

through its interaction with HuD, a poly(A) mRNA binding protein which binds the 3' untranslated region of the CARM1 mRNA [44]. Recently, CARM1 has been implicated in nonsense-mediated decay, and a specific subset of mRNA targets that are dependent on CARM1 for degradation and are also misregulated in SMA have been identified [89].

3.2.3 R-loop resolution.

A recent study showed that SMN may be involved in resolution of R-loops that are naturally formed at the transcription terminal region of some genes. R-loops occur in regions that are particularly high in G-C content, when the nascent transcript behind elongating RNA polymerase II hybridizes with the DNA template strand [90]. The RNA/DNA hybrid prevents reannealing of the template and non-template DNA strand, giving rise to the three-stranded R loop. Failure to resolve such R-loops can lead to genome instability due to these regions being susceptible to DNA damaging agents or nucleases. The R-loop may also physically interfere with normal DNA replication or transcription. SMN binds the symmetrically dimethylated carboxy-terminal domain of the RNA polymerase II subunit POLR2A [45], and also binds senataxin, a helicase necessary for R-loop resolution [91]. Interestingly, mutations in senataxin are associated with juvenile amyotrophic lateral sclerosis (ALS4 [92]), suggesting that defects in the R-loop resolution pathway (SMN or senataxin) may be a common contributing mechanism to these neurodegenerative disorders.

3.2.4 Active transport and actin dynamics in motor neurons.

With no clear connection between the housekeeping functions of SMN protein and SMA disease pathogenesis, some researchers have focused on other putative functions of SMN protein, which may explain the specific degeneration of α -motor neurons. Fan and Simard established that SMN protein localized in growth cones during differentiation of murine embryonal teratocarcinoma cells into neurons [93]. This localization was later determined to be exon 7 dependent, further implicating axon growth abnormalities in SMA pathogenesis [50]. Cytoplasmic SMN protein complexes are found in actively transported granules which contain gemin proteins but not Sm proteins [94], suggesting

that this function of SMN protein is independent from its role in snRNP assembly. Instead, cytoplasmic SMN appears to associate with heterogeneous nuclear ribonucleoprotein R, (hnRNP-R) which in turn binds β -actin mRNA [46, 95]. Cytosolic SMN protein also associates with the coatamer protein Cop- α and is found in Golgi-associated secretion vesicles [47]. These observations suggest that SMN granules originating from the Golgi associate with hnRNP-R and β -actin mRNA, and travel to the growth cones of axons to allow for localized β -actin synthesis and axon extension. In fact, depletion of SMN protein results in decreased levels of hnRNP-R and β -actin along the axons and in growth cones [46]. Reduction of SMN protein levels may affect trafficking of mRNA along axons, which could lead to incomplete extension and improper polarization of neuronal axons. In vitro studies interfering with granule transport from the Golgi caused growth cone defects similar to those observed in SMN knockdown cells [47]. Previous work has suggested that the Tudor domain of SMN interacts with HuD, which may allow SMN to participate in transporting other mRNAs to axons [96]. Depletion of SMN was found to inhibit motor axon outgrowth and pathfinding in a zebrafish model of SMA [51, 52], though no such axonal defects were observed in a mouse model of SMA [97]. Thus, the data from animal models appears to conflict regarding whether or not axonal transport defects contribute to disease pathogenesis.

In addition to its role in active transport to axons, SMN appears to have important functions in maintaining cytoskeletal integrity of neurons [48, 98]. SMN depletion causes increased availability of profilin IIa, a protein which is likely sequestered by SMN in neurons. The increase in free profilin IIa results in abnormally high levels of the ROCK/profilin IIa complex, which in turn activates the RhoA/ROCK actin remodelling pathway [98] resulting in cytoskeletal instability. Consistent with this model of disease, treatment of *Smn*^{2B/-} mice with the ROCK inhibitors Y-27632 or Fasudil led to an average survival prolongation of greater than 100 and 300 days, respectively, compared to the normal lifespan in these animals of 10-30 days [49, 99].

Taken together, these studies illustrate the many functions of SMN protein. However, further investigation is required to determine which of these functions is responsible for SMA pathogenesis so that appropriate therapeutic targets can be identified.

4. CELL TYPES AFFECTED IN SMA

The ubiquitous expression of SMN implies that deficiency of the protein may have other consequences that are not immediately obvious in patients with SMA with severe phenotypes due to their short life expectancy. In humans, SMN protein starts to be expressed in embryos 5.3 weeks into gestation and is found at high levels in most tissues including skeletal muscle, spinal cord, spleen, heart, kidney, liver, and lungs [100]. The expression pattern is similar across all stages of embryonic development. Using an snRNP assembly assay, researchers have shown that

SMN activity could be detected as early as E16, with highest activity in the spinal cord and brain, even though similar levels of SMN expression were found in most tissues [101]. In the rat, SMN expression in tissues other than the spinal cord and brain, decreases substantially after embryogenesis [102], although the SMN activity assay showed that SMN activity decreased 10-fold at p10 and p21, respectively, for these two tissues [101]. Kothary and coworkers identified a decline in *Smn* transcripts from E12.5 to E14.5, in addition to alterations in pre-mRNA splicing within embryos and neonates of phenotypic mice, and these splicing alterations correlate with disease severity [85]. These studies suggest that high levels of SMN protein are required in many tissues early during development, but this requirement is reduced for most tissues after embryogenesis is complete. Examination of *SMN2* levels demonstrated that the contribution of *SMN2* to the pool of full-length SMN protein was greater in muscle and kidney compared to the spinal cord [103], suggesting that *SMN2* may be more effective at preventing the disease phenotype in peripheral tissues than in α -motor neurons. Considering that peripheral tissues appear to have lesser need for SMN protein, *SMN2* may be able to compensate for the deletion of *SMN1* in these tissues, particularly in patients with a higher *SMN2* copy number.

4.1 Motor neurons.

The vast majority of research delving into the pathogenesis of SMA has focused on the effect of reduced levels of SMN protein on motor neuron development and function. Muscle atrophy in patients with SMA is caused by a loss of innervation to the muscle, but an important question is whether there is a failure to correctly establish initial innervation or if there is a failure to maintain these connections after they are successfully initiated. In the severe *Smn*^{-/-};*SMN2* model, there is a lack of innervation of the intercostal muscles as early as E18.5, suggesting that axon outgrowth and NMJ formation simply had not occurred [97]. However, this SMA mouse model is very severe and likely does not reflect the disease state in the majority of patients afflicted with SMA. Moreover, for other muscle types analysed, motor neurons were able to grow and form NMJs, indicating that, in general, low levels of SMN does not affect early motor neuron development and innervation of muscle [97].

Although NMJs are initially formed at a relatively normal frequency in mouse models of SMA, NMJ defects occur very early during disease progression [53]. In *Smn*^{-/-};*SMN2* mice, widespread and early breakdown of the NMJ was observed as early as P2, and endplates were significantly vacant when compared to control littermates at P5/6, the end-stage of the disease in this model. In the less severe *SMN Δ 7* mouse model, similar results were obtained, although the level of denervation varied between muscle groups. For example, in this model, the transversus abdominis (TVA) muscle was significantly denervated at P7 compared to control littermates, whereas the levator auris longus (LAL) muscle demonstrated a much lower degree of

endplate denervation. Similar results have been seen in other studies [104], and in the *Smn*^{2B/-} intermediate mouse model of SMA [105, 106]. Taken together, these studies indicate that in mouse models of SMA, innervation of muscle occurs at a relatively normal efficiency, but the NMJs are not maintained.

Structural defects have been observed at both the pre- and post-synaptic regions of the NMJ. Several studies have reported aberrant accumulation of neurofilaments at the NMJ during very early stages of disease progression [53, 104, 105, 107]. At the post-synaptic motor end plate, there appears to be a general lack of efficient maturation. Specifically, the foetal form of acetylcholine receptors continues to be expressed at later stages of NMJ development, whereas control animals transition to the adult form [54, 108]. In *SMNΔ7* mice, motor endplates are on average half the size of control littermates [109], which was also observed in the *Smn*^{2B/-} mice [105]. Finally, development of the classic pretzel-like shape of the NMJ was significantly delayed in *SMNΔ7* mice, and fewer perforations were observed [54, 110]. Thus, reduced levels of SMN are associated with significant impairment in NMJ maturation. Not surprisingly, these structural defects are associated with functional deficits. In mouse models of SMA, the NMJ's capacity to transmit electrical stimulus are significantly reduced compared to wild type controls, which is attributed to reduced synaptic vesicle density [111]. This reduced vesicle density appears to be due, in part, to a reduction in fast axon transport of key proteins involved in synaptic vesicle function [112]. Although the majority of NMJ studies have been performed exclusively in mouse models of SMA, motor neurons derived from SMA patient-specific induced pluripotent stem cells (iPSCs) demonstrate significantly impaired clustering of the acetylcholine receptor (AChR) [113], suggesting defects may also occur in patients. Collectively, these studies clearly illustrate that reduced levels of SMN significantly impact motor neuron structure and function.

Impaired motor neuron health in SMA may also be impacted by the effects of reduced levels of SMN in astrocytes, a cell type that normally functions to support neuron development and synapse formation [55]. Astrogliosis, which can inhibit axon regeneration, was evident in end-stage mouse models of SMA and in post-mortem patient spinal cords. Importantly, restoration of SMN specifically in astrocytes of these mice increased lifespan, improved motor unit function, and normalized NMJ defects. Thus, reduced levels of SMN may affect motor neuron function and survival both directly and indirectly.

4.2 Skeletal muscle.

SMN deficiency affects skeletal muscle function due to both direct effects within the cell and impairment caused by denervation. Comparison of skeletal muscle from type 1 patients with SMA to age-matched controls demonstrated that myotubes from SMA patient samples were smaller and less well-organized, suggesting that muscle growth and

maturation is delayed in patients with severe SMA [114]. The effect of SMA on the skeletal muscle system is not uniform, and the muscle groups that are affected by the disease and those that are spared are similar in both humans and mouse models of SMA [104, 115, 116]. MRI data from SMA type 3 patients demonstrated significant deterioration in the gluteus maximus and triceps brachii that was positively correlated to disease progression over an 8-year period [116]. Analysis of muscle in two different mouse models of SMA (*Smn*^{-/-}; *SMN2* or *SMN2B/-*) suggest that atrophy occurs through either induction of proteasomal degradation or autophagy, and the pathway invoked may be linked to the disease severity [60].

Deficits in SMN directly impact muscle cell function. Knockdown of SMN expression in C2C12 myoblasts resulted in reduced proliferation (without affecting the rate of cellular death), defects in myoblast fusion, and malformed myotubes [117]. SMN deficiency also affects myogenesis, in the form of aberrant initiation of the differentiation program and reduced efficiency of fusion of myoblasts into myotubes [56-59]. Reduced SMN levels also leads to muscle weakness in pre-symptomatic mice, with a delay in the appearance of mature isoforms of the ryanodine receptor 1, coinciding with decreased levels of sodium channel Na_v1.4 and calcium pump SERCA1a, all of which are necessary for muscle contraction [57]. Thus, the skeletal muscle phenotype of SMA appears to be a primary symptom, which is compounded by defects in the NMJ and the death of motor neurons.

4.3 Heart.

Several studies have described cardiac muscle defects in mouse models of SMA [61-63]. At P14, *SMNΔ7* mice display bradycardia and develop dilated cardiomyopathy with a decrease in heart muscle contractility [61]. These defects are thought to be due to aberrant autonomic signaling, and may not be a direct result of reduced levels of SMN in the heart. However, studies in patients with severe SMA disease suggest that SMN may play an important role in heart development, since three of four type 0 patients had congenital heart defects [118]. This observation is supported by *in vivo* studies where structural and functional abnormalities were found in the hearts of mice from two SMA models [61, 119, 120]. However, a retrospective analysis of 37 patients with type 2 or type 3 SMA revealed that all but two patients had normal heart function. The remaining two patients had dilation of the left ventricle likely caused by coronary heart disease and hypertension [121]. Similar results were obtained in a more recent study of 157 patients with type 2 or 3 SMA disease [122]. These observations suggest that compensation by *SMN2* is sufficient to prevent defects in the heart of patients with higher copy numbers, but insufficient in the most severe patients.

4.4 Autonomic and enteric nervous systems.

In addition to its effect on the CNS, deficiency of SMN may also have consequences for the autonomic nervous system (ANS). Some of the observed cardiac defects, such as bradycardia and blood pressure fluctuations, appear to be the result of ANS dysfunction [70, 123]. While examining defective hearts in mouse models of SMA, Didonato and coworkers noted reduced innervation of the heart in the diseased mice compared to age-matched littermates [120]. Other conditions observed in patients with SMA which implicate ANS damage include unusual vasodilation responses to cold and necrosis of fingers and toes [70, 71]. Similar ANS abnormalities have been observed in patients with type 1 SMA, suggesting that compensation by *SMN2* in the ANS is not adequate in patients with severe forms of SMA. Like cardiac tissues, the ANS appears to be largely unaffected in patients with type 2, 3, or 4 SMA.

Reduced levels of SMN also lead to defects in the enteric nervous system (ENS), which contributes to dysfunction of the gastrointestinal tract. In severe mouse models of SMA, mice present with severe diarrhea, submucosal edema early in the disease state, and reduced number and altered morphology of the villi that line the small intestine [72]. Selective Cre-mediated loss of mouse SMN in the CNS and ENS in an *SMNΔ7* background caused constipation, reduced colonic motility and slowed intestinal transit [73]. Importantly, these same gastrointestinal issues were still present in mice in which SMN expression had been restored in the CNS, clearing showing that the observed defects were due to disrupted ENS function. The prospect of ENS and ANS damage caused by SMN deficiency suggests that SMN protein may be essential to other neurons as well, but possibly to a lesser degree.

4.5 Metabolic and Endocrine defects.

SMN protein may also play an important role in regulating metabolic function. Plasma levels of the fatty acids dodecanoic acid and tetradecanoic acid in patients with severe SMA were significantly higher compared to healthy controls or infants with similarly debilitating denervation disorders [66]. This result indicates that aberrant fatty acid metabolism was not due to denervation or muscular atrophy alone. Patients with SMA with severe phenotypes also exhibited dicarboxylic aciduria when fasted, and yielded urine fatty acid profiles similar to those observed in patients with mitochondrial disorders. Autopsy of some patients with severe SMA revealed fatty vacuolization of the liver, which is characteristic of fatty acid oxidation disorders [66]. Patients with SMA with less severe disease exhibited normal fatty acid metabolism [66].

Reduced levels of SMN protein also affect proteins involved in the insulin-like growth factor 1 (IGF1) pathway in the liver [67]. In the *Smn^{-/-};SMN2* severe mouse model of SMA, although IGF1 mRNA levels were unchanged in affected and non-affected controls, IGF-binding-protein acid labile subunit (IGFALS), a protein involved in stabilizing circulating IGF1, was markedly reduced in affected mice, leading to reduced levels of circulating IGF1 [67]. IGF1 is a

polypeptide protein hormone with molecular structure comparable to insulin, essential for early childhood growth. Dysregulation of the IGF1 signaling pathway has also been observed in biopsies from type I patients with SMA [124]. This observation may help explain why affected pups in mouse models of SMA are typically smaller than unaffected littermates.

Work in the Taiwanese mouse model of SMA, a fairly severe model of SMA, demonstrated livers with defects in iron deposition and megakaryopoiesis, while size and myelopoiesis remained largely unaffected. Altered color was also noted, with increased levels of red blood cells. Importantly, these defects could be rescued in late symptomatic mice with treatment using morpholino antisense oligonucleotides to increase SMN protein levels [68].

Pancreatic defects were observed in the *Smn^{2B/-}* model, specifically an excess of glucagon coupled with decreased levels of insulin [64]. Examination of the pancreatic islets revealed an abnormally high number of glucagon-producing α cells and a diminished representation of insulin-producing β cells, resulting in fasting hyperglycemia and glucose resistance in the mice [64]. The abnormalities observed in the murine pancreata were reflected in autopsy samples from patients with severe SMA, some of whom had a recorded history of glucose resistance [64]. Heterozygous *Smn^{+/-}* mice display defects in pancreas development and aberrant glucose metabolism, in the absence of any SMA-like disease, suggesting *SMN* may represent a diabetes susceptibility locus [65, 125].

4.6 Lymphatic System.

New research is emerging about the effect of SMN depletion on tissues within the lymphatic system such as the spleen. Work by Parson's group has shown the Taiwanese mouse model of SMA, displays a smaller spleen relative to its body weight with altered morphology. In addition to the increased fibrosis, there is a lack of cellular proliferation with increased levels of apoptosis, decreased levels of lymphocytes with greater numbers of megakaryocytes. Further assessment of spleens from SMA type 1 patients also demonstrated morphological changes including reduced size, a lack of lymphocytes with increased levels of megakaryocytes [69]. With the spleen a key component of the immune system, future research should address potential complication of SMN depletion on innate and adaptive immunity.

4.7 Skeletal System.

Frequently overlooked, the skeletal system is often involved in patients with SMA, and does not appear to be simply due to the weakened state or lack of weight bearing in these patients. Patients with type I disease have significantly lower bone mineral density (BMD) than age-matched controls [74], with similar results observed in

patients of SMA types 2 and 3 [75]. As expected, patients who are non-ambulatory have significantly lower BMD than ambulatory patients with SMA [75], suggesting BMD could be correlated to disease state, or possibly degree of ambulation. Importantly, patients with SMA types 2 and 3, both ambulatory and non-ambulatory, were also found to have significant increases in fractures of their forearms, hands, femurs and lower legs [76] compared to controls. More recently, children with SMA types 2 and 3 were revealed to have greater rates of vertebral fractures, higher levels of bone resorption, and lower BMD [77]. Surprisingly, 7 of 30 SMA children had previously undiagnosed vertebral fractures, suggesting this may be an under-reported aspect of SMA disease [77].

To understand if SMN protein deficiency specifically contributed to skeletal weakness, the *Smn*^{-/-};SMN2 mouse model was examined, and showed a significant decrease in bone volume, density and trabecular number, in comparison to healthy controls [78]. Analysis of vertebrae demonstrated that affected mice have thin and porous cortical bone and thin trabeculae. Poor bone structure was the result of a substantial increase in the number of bone resorbing cells (osteoclasts) and bone resorbing activity. A significant decrease in bone matrix and bone differentiation markers including osteocalcin, osteopontin and osterix, were also noted. Further examination of skeletal defects at the molecular level using bone marrow cultured from the *Smn*^{-/-};SMN2 mouse model indicated that SMN protein depletion leads to bone loss through increased formation and activity of osteoclasts due to stimulation of the RANK receptor signaling pathway [78]. Collectively, these human and mouse studies indicate that patients with SMA experience significant bone abnormalities and that SMN proteins plays an important role in controlling osteoclast differentiation and activity, which likely also contributes to SMA morbidity.

4.8 Reproductive System.

The majority of SMA mouse models, severe and intermediate, do not reach an age for reproduction, and therefore reports on the effects of SMN deficiency on male and female fertility are not possible. However, an intermediate mouse model of SMA, *Smn*^{c/c} which produces approximately 25-50% of WT SMN protein levels, was used to examine fertility of SMN deficient male and female mice [79]. While female mice largely do not present with any reproductive abnormalities, males had significantly smaller testis, and impairments in spermatogenesis and reproduction. SMN-deficient males exhibited an approximate 10-fold reduction in sperm count, degenerated seminiferous tubules, and differential expression and/or splicing of over 3,700 genes within the testes. Further analysis revealed increased levels of apoptosis and DNA fragmentation within the testes of *Smn*^{c/c} mice. Interestingly, while SMN protein expression in *Smn*^{c/c} mice was reduced by approximately 50%, relative to WT mice, within the CNS, there was no significant difference in SMN expression between WT and the SMA mouse model in the testes. These findings suggest there is a

relatively high requirement for SMN protein for the appropriate functioning of the male reproductive system and that SMN deficiency leads to an aberrant reproductive phenotype. These observations have not been reported in humans, but are likely under assessed.

In summary, the view that SMA is a disease of α -motor neurons is perhaps an oversimplification. An increasing number of studies have clearly shown that reduced levels of SMN compromise the function of other tissues including a variety of neuronal cell types, muscle, liver, pancreas, bone and reproductive system. With patients with SMA now living longer due to improved assistive technology, deficiencies in these other tissues and organs need to be more closely examined to evaluate their impact on the clinical course of SMA.

5. PROSPECTS FOR THERAPY

5.1 Emerging therapies.

The genetic basis and phenotypic effects of SMA allow for three areas of focus for therapeutic development. The first method to achieve correction is simply to re-introduce a functional copy of the *SMN1* gene. Second, therapeutics can be targeted towards the *SMN2* gene, in an attempt to increase the amount of full-length transcript and SMN protein produced from this gene. Lastly, the downstream damage caused by SMN deficiency can be addressed by protecting affected tissues. Within each of these strategies, there exists a wide variety of therapeutics in development, and previous reviews have presented excellent overviews of these approaches [126-128]. Several therapeutic strategies that have shown particular promise in human clinical trials are discussed below.

Although early studies have shown that gene therapy had the potential to correct SMN deficiency in tissue culture and animal models of SMA [129-131], it is the utilization of adeno-associated viral (AAV) vectors that have demonstrated real promise [2, 132-134]. Positive preclinical results led to a Phase I/II clinical trial in children with SMA Type I, using a self-complementary AAV based on serotype 9 (sc-AAV9) encoding a full-length copy of the *SMN1* cDNA [135]. Preliminary results from this study showed that the vector, AVXS-101, appears to be improving motor function within subjects, while also proving to be generally safe and well-tolerated in the nine patients studied to date [136].

The presence of the *SMN2* gene in patients with SMA raises the possibility of targeting the *SMN2* mRNA transcript to manipulate its splicing to include exon 7 and thus translate a fully functional protein. Antisense oligonucleotides (ASOs) have shown promise by targeting an intronic splicing silencer in intron 7, to produce full length SMN in animal models of SMA [132, 137, 138]. An ASO developed by Ionis Pharmaceuticals (formally Isis Pharmaceuticals) and Biogen Idec was well-tolerated and appeared to improve motor function in some of the treated

patients in a Phase I clinical trial [2, 139]. In the follow-up open-label Phase II study of children with SMA, Ioniis observed a time- and dose-dependent increase in muscle function scores in children treated with multiple-doses of their lead ASO, IONIS-SMN_{Rx} [140]. No severe adverse events were reported. IONIS-SMN_{Rx} efficacy is currently being assessed in a Phase III clinical trial [141].

Non-SMN targeted therapies have been developed to help mitigate the downstream effects of SMN deficiency. Two such therapeutics are Olesoxime, a neuroprotectant developed by Roche pharmaceuticals, and CK-2127107, developed by Cytokinetics for improving muscle function. Olesoxime is a molecule with a cholesterol-like complex, which inhibits apoptosis in neurons by interacting with the mitochondrial permeability transition pore to prevent the efflux of cytochrome c [142]. As SMN depletion results in motor neuron degeneration, blocking apoptotic pathways may delay the phenotypic onset. In 2014, a phase II clinical trial of Olesoxime in SMA type 2 and 3 patients yielded very promising results, with neuroprotective effect lasting over 2 years (n = 165) [143]. CK-2127107 acts to slow the release of calcium from the troponin C complex, ultimately enhancing muscle contraction, and increasing force and slowing fatigue [144]. In January 2016, Cytokinetics announced the start of a Phase II trial with CK-2127107 for patients with SMA.

5.2 CNS versus systemic SMN restoration.

As more functions for SMN are discovered across a wide variety of tissues, concerns are raised as to whether restoration of SMN will be required in many, or all, tissues, rather than solely in the CNS. Experiments using transgenic mice have shown that selective expression of SMN in motor neurons can correct the phenotype in mouse models of SMA [145]. However, other studies have shown that selective restoration of SMN in neurons has only a limited effect in a severe model of SMA [146], suggesting other tissues are crucially involved in disease pathogenesis. Transgenic mice with doxycycline-induced SMN expression revealed that long-term induction of SMN at P1 rescued the phenotype, but 70% of the mice died within a month once the induction was ceased. Importantly, the NMJs of these animals appeared to be normal at the time of death, which implies that the absence of SMN in another tissue was ultimately the cause of death [147].

Conflicting results concerning the need for CNS versus peripheral tissue restoration of SMN protein have been observed in studies of novel therapeutics for SMA. In the SMN Δ 7 model, intracerebroventricular (ICV) delivery of sc-AAV9-SMN1 showed that the average survival was dramatically increased in comparison to intravenous injection, with a median survival of 10, 30 and 200 days for untreated, intravenous and intracranial injected, respectively [148]. Similarly, a single ICV injection of a therapeutic ASO in SMN Δ 7 mice led to an increase in median survival from 15 to 112 days, with a normalization of many phenotypic measures, including weight, righting response time and grip

strength [149]. The same ASO administered peripherally was less effective than the ICV injection, and the combination of the two resulted in no added benefit over ICV alone. In contrast, a second study showed that a single ICV, two subcutaneous (on days P0 and P3), or a combination of these treatments led to a median extension of survival to 16, 108 or 173 days, respectively, demonstrating that systemic delivery is very efficacious, and even necessary, for recovery from the SMA phenotype [67]. Differences between the studies was attributed to increased permeability of the blood brain barrier in neonatal mice which may have allowed the ASO to pass into the CNS after systemic delivery in the latter study [149]. Using systemic delivery of a therapeutic ASO in combination with a CNS delivery of a “decoy” ASO that neutralized activity of the therapeutic ASO in the CNS [150], peripheral SMN protein levels were restored enough to correct the SMA phenotype, suggesting rescue of the motor neurons was a cell non-autonomous event in mice. Collectively, these studies demonstrate the complexity of the disease, as it still remains unclear as to whether restoration of SMN within the CNS versus peripheral tissues is required.

5.3 Therapeutic window.

Researchers have attempted to identify the temporal requirements for SMN protein in order to achieve disease correction. A mouse line containing human SMN2 and a floxed murine *Smn* allele that can be inactivated following Cre-mediated recombination was crossed with mice encoding a ubiquitously expressed tamoxifen-inducible Cre^{ER} transgene to generate a population of mice that could be depleted of SMN protein at varying times of development [151]. Reducing SMN protein in neonatal mice resulted in an SMA-like phenotype. However, depletion of SMN protein in mice after P17 had little effect, although mature animals depleted of SMN eventually exhibited evidence of selective neuromuscular pathology that was heightened by traumatic injury. These results suggest that enhanced SMN protein levels are required during early development, until full maturation of the NMJ has occurred, and also during NMJ regeneration in injured muscle. Using a mouse in which SMN could be selectively induced through administration of doxycycline, induction of expression of SMN protein until P28, followed by 1 month removal, resulted in no morphological or electrophysiological abnormalities at the NMJ and no overt motor phenotype [147]. Using a different mouse model of SMN-inducible expression, post-symptomatic mice, even those with a severe phenotype, could be rescued by induction of SMN expression [152]. However, mice in which the NMJ pathology had significantly progressed failed to respond to induction. These studies collectively suggest that high level expression of SMN is required during early development and prior to NMJ maturation, with perhaps lower levels required to maintain proper NMJ function.

An early requirement for SMN protein in motor neurons to correct the disease state has also been supported by studies examining a variety of therapeutic approaches. ASOs that

rescue the phenotype in mouse models of SMA must be administered *in utero* or immediately upon birth to achieve a maximal beneficial effect [67, 138, 149]. Similarly, studies utilizing the scAAV9-SMN1 showed that vector administration must occur very soon after birth to achieve a therapeutic benefit and extend survival [133]. The therapeutic window for patients with severe SMA is also believed to be early and narrow in order to prevent the rapid degeneration of the α -motor neurons and other affected tissues. These observations suggest that early detection of SMA is essential to optimize therapeutic benefits, likely at the pre-symptomatic stage [2, 153, 154]. Although medical and other costs associated with caring for patients with SMA is thought to be approximately \$1 billion per year in the United States [155-158], the cost for population-based carrier and perinatal screening for SMA is likely too high to be a viable approach at this time [159]. As technology and therapeutic development progresses, and the cost of screening for SMA declines, it will be important to re-address the need for SMA screening.

CONCLUSION

Spinal muscular atrophy causes tremendous suffering – physical, emotional, and financial – to the patient and their family. Although often considered a disease of motor neurons, accumulating evidence suggests that the disease affects a wide range of tissues. Several studies have proposed that motor neuron death is a cell non-autonomous event, and restoration of SMN in peripheral tissues is equally as important, and in some

studies even more important, than restoration in motor neurons. Moreover, available data from preclinical studies also suggests that SMN protein restoration is required early during the disease, before widespread loss of motor neurons has occurred. Several therapeutics have reached advanced phases of clinical trial, suggesting that new treatment options may be on the horizon. Therapeutics providing systemic delivery of SMN during early development will likely maximize a positive clinical outcome and lead to more efficient therapies to treat this devastating disease.

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Book Chapter 1. Adenovirus Biology and Development as a Gene Delivery

Vector

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1.1 Abstracts. Adenovirus (Ad)-based vectors are one of the most commonly used gene delivery vehicle in molecular biology and gene therapy applications. For over 60 years, researchers have studied the basic biology of Ad, unraveling the subtle, yet profound, interactions between the virus and the host. With our increased understanding of the biology of the virus, we have refined the function of Ad as a gene delivery tool, and tailored it to disease-specific applications. For example, removal of all viral genes from the vector, generating helper-dependent Ad, can result in life-long correction of genetic defects in mouse models of human disease, whereas oncolytic Ads contain more modest modifications of the viral genome that allows them to specifically replicate in, and kill, cancer cells. In this review, we will discuss the development and evolution of Ad as a gene therapy vector.

1.2 Introduction. Adenovirus (Ad) vectors are used extensively in molecular biology applications to achieve high-level gene expression of a desired transgene in mammalian cells. Ads are also the most commonly used delivery vehicles in gene therapy applications. As of July 2015, 22 % ($n = 506$) of all human gene therapy clinical trials used Ad vectors to deliver a therapeutic gene of interest, for a variety of indications [1]. The first commercially available gene therapeutic, an Ad-based vector expressing p53 for treatment of head and neck cancer, became approved for use in China in October 2003. This same vector is now in advanced stage clinical trials in the United States and other countries around the world for the treatment of a variety of cancers, including head and neck, lung, and ovarian cancer. Ad has progressed as a favored delivery vehicle, despite the significant notoriety Ad achieved in the popular press due to the death of a patient entered in a clinical trial for treatment of ornithine transcarbamylase deficiency in 1999. This death was directly attributed to a systemic inflammatory response due to the Ad vector that was administered. This event raised questions regarding whether Ad should be used in gene therapy applications and, more broadly, whether we should reconsider the rationale and validity of gene therapy as an experimental therapeutic. Regardless of this perceived major setback to the field of gene therapy, research on Ad and its use in clinical trials continues.

The goal of this review is to provide more of a historical perspective of the development of Ad as a gene delivery tool. We will briefly highlight a number of significant milestones (Figure 1), and describe how the identification of Ad vector limitations spurred their further development and refinement.

1.3 Adenovirus Biology.

Ads were first discovered in the early 1950's as novel viral agents associated with respiratory

ailments in human patients [2, 3]. Their name derives from the original source of tissue from which the prototype member was isolated, the adenoids. Since that time, over 100 family members have been identified and characterized, from a wide variety of mammalian and avian species, in addition to reptiles and amphibians. The discovery that some Ads are tumorigenic in rodents [4, 5] stimulated intensive research into the physiology, genetics and molecular biology of Ads which has continued over the past 60 years. These studies have given us a great deal of information about DNA replication and control of gene expression of the virus, and have provided significant insight into these processes in the host cell itself. For example, alternative splicing, a ubiquitous process in mammalian cells, was first identified in Ad [6, 7]. It is this broad knowledge of Ad virus biology that laid the foundations for the later development of Ads as gene transfer vectors.

Of the human Ads, serotype 2 (Ad2) and 5 (Ad5), both of subclass C, are the most extensively characterized (reviewed in [8]). Their genomes have been sequenced, and are ~95 % identical at the nucleotide level, with a similar arrangement of transcriptional units. Unless otherwise indicated, the remainder of this review will focus on Ad5 (with information gleaned for Ad2 assumed to also be valid for Ad5).

1.3.1 Ad virion structure. All Ads have the same general structural characteristics: an icosahedral, non-enveloped capsid (~70 to 100 nm in diameter) surrounding a nucleoprotein core containing a linear double-stranded genome (~30-40 kbp) (Figure 2) [9]. The atomic structure of the outer Ad capsid has been refined to a significant degree, however the inner nucleoprotein core does not appear to have an ordered structure [10-14]. The Ad capsid is composed of 8 polypeptides, named in order of decreasing size. Hexon (a trimer of protein II) assembles into a sheet-like structure called the “group-of-nine”, which forms the 20 facets

of the icosahedron. Protein III clusters into groups of five (known as pentons) at the vertices of the icosahedron, from which extend trimers of protein IV, known as fiber. These proteins which make up the major capsid are supported by 5 minor capsid proteins (IIIa, IVa2, VI, VIII, and IX). Within the viral capsid, the viral DNA associates with three basic proteins, V, VII and Mu (μ), which function to neutralize the charge on DNA, permitting its tight packing within the virion. Protein V is postulated to create a shell and coat the protein VII-DNA complex [15, 16]. Protein VII functions similar to that of cellular protamines, in that it is responsible for wrapping and condensing the viral DNA [17] with the help of pre-Mu [18]. Ad-encoded proteases can reverse this process and relax the viral DNA nucleoprotein structure by cleavage of pre-Mu prior to entering the nucleus [19]. Once inside the nucleus, remodeling of the nucleoprotein structure containing the Ad DNA must occur, as the tightly packed DNA structure is inhibitory to viral transcription [20, 21], and its relaxation allows for efficient gene expression and DNA replication [22].

1.3.2 The Ad genome. The length of the wildtype Ad5 genome is approximately 36 kb, yet the capsid can accommodate DNA up to 105 % of the wildtype genome length [23]. However, increasing the genome size to this upper limit tends to lead to instability and can result in spontaneous rearrangement of the genome to reduce the size of the DNA closer to wildtype length. This observation has important implications to the design of Ad vectors with large transgenes [24]. No lower limit for DNA packaging has been identified, and genomes as small as 9 kb have been found packaged into virions [25]. Nonetheless, similar to large genomes, small genomes tend to rearrange or multimerize to bring the overall size of the genome closer to wildtype Ad [26, 27].

Genes encoded by Ad are classified as early or late, depending on whether they are

expressed before or after DNA replication (Figure 3) [28]. The early regions E1A, E1B, E2, E3, and E4 are the first regions transcribed and encode proteins involved in activating transcription of other viral regions and altering the cellular environment to promote viral production. The E1A proteins induce mitogenic activity in the host cell and stimulates the expression of other viral genes [8]. The E2 proteins mediate viral DNA replication, while E3 and E4 proteins alter host immune responses and cell signaling, respectively [29, 30]. Activation of the major late promoter (MLP) following the start of virus DNA synthesis, allows expression of the late genes which encode primarily virion structural proteins. These late regions (L1–L5) are transcribed from an alternatively spliced transcript. Recently, it was shown that the regions encoding the L4-22K and -33K proteins are initially expressed at low levels from a novel promoter located within the L4 region [31], and these proteins are responsible for fully activating the MLP [32]. There are also four small products produced at intermediate/late times of infection, including the structural protein IX (pIX), and the IVa2 protein that helps package viral DNA into immature virions [10]. The late products, VA RNA I and II, inhibit activation of the interferon response, impede cellular micro-RNA processing, and may influence expression of host genes [33, 34]. The viral DNA also contains the origins of replication (the inverted terminal repeats [ITR], ~100 bp located at both the left and right end of the genome) and the packaging sequence (~150 bp, located immediately adjacent to the left ITR).

Perhaps somewhat surprisingly, our knowledge of genes encoded by the virus is still expanding. In 2007, Tollefson et al. [35] identified a new open reading frame (ORF) that they termed U exon protein (UXP), which was located between the fiber coding sequence and E3. UXP is expressed from a unique promoter during late stages of infection, and may

play a role in virus DNA replication or RNA transcription [35, 36]. A recent study using deep cDNA sequencing identified many new alternatively spliced transcripts originating from the Ad genome [37], suggesting that there may be many undiscovered, new or altered polypeptides produced by Ad in the infected cell.

1.3.3 Ad infection. In most cells, Ad5 infection initiates with the fiber knob domain binding to the primary receptor for Ad5 on the cell surface, termed the Coxsackie-Adenovirus receptor (CAR) [38, 39]. However, it should be noted that different serotypes have different cell surface receptors and these can be used to “naturally” alter Ad vector infection to enhance uptake by specific cell types [40]. After initial binding, the Ad5 penton interacts with a secondary receptor comprised of $\alpha_v\alpha_3$ or $\alpha_v\alpha_5$ integrins [41]. The efficiency with which Ad binds to and enters cells is directly related to the level of the primary and secondary receptors found on the cell surface [42, 43]. More recent studies have suggested that some Ad serotypes, including Ad5, can enter cells using heparin sulfate proteoglycans as an alternative receptor, either through direct binding to sequences in the Ad fiber shaft [44], or bridged through interaction of Ad with various blood factors or the complement component C4-binding protein [45-47].

Upon systemic delivery in most species, human Ad5 preferentially accumulates in the liver [48-50]. This preferential uptake is due in part to the physical architecture of this tissue, as Ad becomes trapped in the liver sinusoids and fenestrations [51, 52]. However, in the liver, Ad shows a nonlinear uptake by hepatocytes - at low doses, little Ad is taken up by hepatocytes whereas at higher doses disproportionately more is taken up [53]. Initially, Ad tends to be preferentially taken up by Kupffer cells within the liver, and it is only after the receptors on these cells are saturated that the virus is available to infect hepatocytes. Uptake

of Ad by Kupffer cells leads to death of the cells [54]. Consequently, enhanced hepatocyte transduction can be achieved *in vivo* using two sequential vector injections (the first with an irrelevant Ad vector that saturates and kills the Kupffer cells, allowing the second therapeutic vector to effectively transduce hepatocytes [53]) or through pre-treatment with Kupffer cell-killing compounds such as clodronate liposomes [53, 55].

Ad5 is taken up by hepatocytes using a rather unique mechanism. The Ad5 hexon protein interacts with coagulation factor X which provides a bridging interaction for binding to heparin sulphate proteoglycans expressed on the surface of hepatocytes, allowing for internalization [46, 47, 56, 57]. However, Ad vectors containing mutations in the hexon protein that prevent its interaction with factor X still localize to the liver, although uptake is dramatically reduced [47, 58]. These results suggest that physical constraints, other than receptor binding, contribute significantly to vector bio-distribution.

Internalization of Ad occurs through endocytosis, triggered by the penton-integrin interaction [41]. Acidification of the endosome alters the Ad capsid structure, allowing for the release of protein VI from the inner capsid. Protein VI possesses membrane lytic activity [59], and mediates rupture of the endosome and release of the virus [59-63]. The virion translocates to the nucleus along the microtubule network [62], during which time there is a sequential disassembly of the Ad virion and, as a final step, the Ad hexon remains outside the nuclear membrane while the DNA bound to protein VII passes into the nucleus [64-66]. Viral DNA replication and assembly of progeny virions occur within the nucleus of infected cells, and the entire life cycle takes approximately 24-36 hr, providing an output of approximately 10^4 virions per cell for wildtype virus. In humans, Ads are not associated with neoplastic disease, and only cause relatively mild, self-limiting illness in immunocompetent

individuals; primarily respiratory illnesses, keratoconjunctivitis, or gastroenteritis (depending on the serotype) [67]. For a more comprehensive discussion of Ad biology, the reader is referred to other excellent reviews [8, 13, 68].

1.4 First-generation Ad vectors

1.4.1 The first Ad vectors. The first suggestion that Ad could be used to express foreign genes in mammalian cells came with the identification of spontaneous recombinants between Ad and simian virus 40 (SV40) [69, 70], in which large regions of the Ad genome, mainly the E3 and E4 transcription units, were replaced with SV40 sequences. One of these recombinants, Ad2+D2, produced large quantities of T antigen (fused to an Ad structural protein) from the MLP, which allowed for the purification of large quantities of a T antigen-related protein that retained biological activity. Due to the loss of essential E4 functions, Ad2+D4 could only be propagated in the presence of wildtype helper virus, usually present in at least a 10-fold excess. To ensure that Ad2+D2 was maintained in the mixed virus culture, the viruses were propagated in COS cells, which are only permissive for Ad replication in the presence of T antigen, thus providing a strong selection for maintenance of Ad2+D2. Due to the difficulty in maintaining stable vector stocks in the absence of helper virus, the use of this type of vector was limited.

1.4.2 E1-complementing cell lines. In 1973 Graham and Van der Eb [71] developed the calcium phosphate technique for introducing DNA into mammalian cells. This technique allowed for the recovery of infectious virus from purified DNA of Ads and other viruses, in addition to providing a relatively efficient method to transform any DNA into almost any cell type *in vitro*. Using this technique, they were also able to map the transforming genes of Ad5 (*i.e.* the E1 region) [72, 73]. In 1977, Graham et al. [74] reported the isolation and

characterization of a human embryonic cell line transformed by Ad5 after transfection with sheared viral DNA. These cells were originally designated 293-31 cells, because Graham sequentially numbered all his experiments, this was his 293rd experiment, and the transformed colony appeared in experimental dish 31. Now simply known as 293 cells, they have nucleotides 1-4344 of the Ad5 genome inserted in chromosome 19 [75], and express both the E1A and E1B transcription units. The pIX transcription unit is also present in these cells, however, they do not express this protein at a detectable level [76]. Although 293 cells were derived after transfection of human embryonic kidney cells (giving them the common "HEK" designation), the cell line actually expresses many protein markers consistent with a neuronal lineage [77]. This and other data has led to the speculation that 293 cells may not be derived from kidney cells, but rather from rare neuronal lineage cells present in human kidney cultures [77, 78]. A separate study suggested that the 293 cell line transcription profile was more consistent with being derived from adrenal lineage [79].

Regardless of their cell type of origin, because 293 cells complement Ads with mutations or deletions in E1, these cells have proven to be extremely useful for researchers interested in production of Ad vectors expressing foreign genes. The E1 region of Ad could now be replaced with a gene of interest, and the resulting vector could be easily propagated in the E1-complementing 293 cell line. The significance of this was two-fold: first, Ad vectors could now be generated that were not dependent on the presence of a helper virus, so very pure preparations of the vector could be produced and, second, these E1-deleted vectors can infect many different cell lines with the efficiency of wildtype Ad and express high levels of the gene of interest. However, since the vectors do not replicate in the absence of E1 functions, cells not expressing E1 (*i.e.* virtually all mammalian cells) can be transduced

with the Ad vector and maintained for a period of time without cell death due to virus replication. Not only are 293 cells used for generation of Ad vectors, but they are also frequently used as the base cell line for production of adeno-associated virus and retrovirus/lentivirus vectors, and because of a number of other useful properties, 293 cells have become one of the most commonly utilized mammalian cell lines in molecular biology.

1.4.3 The problem of replication competent Ad (RCA). Although the 293 cell line is extremely useful for production of Ad vectors, it does have one limitation: E1-deleted Ad vectors propagated on 293 cells can recombine with the E1 sequences contained within the cell line, thus transferring E1 to the vector and yielding replication competent Ad (RCA) [80, 81]. The presence of RCA, essentially wildtype virus, in vector stocks is undesirable, and was a serious concern and obstacle in early gene therapy clinical trials with Ad. As a result, new E1-complementing cell lines have been generated which contain little or no sequence overlap with the E1-deleted vector [82-84]. Although these new cell lines do prevent RCA formation, some reports have shown that other E1+, but replication-defective, recombinant viruses can be generated in these cell lines [85, 86]. These recombinants contain and express E1 but lack other essential viral sequences, and thus cannot replicate by themselves. However, co-infection of the recombinant E1+ "virus" and the E1-deleted vector results in cross-complementation, permitting virus replication. The E1+ viruses likely arise through recombination between very short stretches of homology between the vector and the cell line, or through non-homologous recombination events, and clearly illustrates how adept viruses are at circumventing replication blocks. Although the formation of RCA (and other less-well defined recombinant viruses) appears to be an inevitable consequence when using Ad vectors, this has not prevented Ads rise to the forefront of gene delivery vectors.

1.4.4 Methods for generation of E1-deleted Ad vectors. One of the bi-products of Ad DNA replication is the formation of covalently closed circles of full-length Ad genomic DNA [87]. This observation was followed by a study showing that, if an antibiotic resistance gene and bacterial origin of replication were inserted in the otherwise wildtype Ad genome, these circles could be recovered and propagated in bacteria, and are capable of producing infectious virus when re-introduced into appropriate mammalian cells [88]. Other methods for cloning infectious viral DNA in bacterial plasmids involved insertions of linear Ad genomes into plasmid DNA, from which the viral DNA must be released by restriction enzyme digestion in order to produce infectious virus following transfection [89]. These advances facilitated wide-scale manipulation of the Ad genome using common cloning techniques. However, the large size of these plasmids (typically 30-40 kbp) makes standard cloning procedures difficult. As a result, several methods have been developed in order to simplify the process of viral construction, typically taking advantage of the recombination pathways present in prokaryotic and eukaryotic cells (reviewed in [90]). One of the earliest systems for creating first-generation Ad employed the natural recombination pathway in mammalian cells following the transfection of two DNAs: 1) a plasmid containing the left hand portion of the virus (including the packaging signal and 5' ITR) with the desired transgene expression cassette replacing the E1 sequences and, 2) the right hand portion of the virus genome (either purified virus DNA, digested with appropriate restriction endonucleases to remove the packaging signal, or a plasmid containing a circularized genome) (reviewed in [91]). Recombination between homologous Ad sequences on the two DNAs leads to the formation of recombinant viral genomes. The efficiency of this system has been improved 100-fold by using a site-specific recombinase (Cre or FLP) to mediate recombination between the two plasmids instead of the natural homologous recombination pathways in

mammalian cells [92-94].

Alternatively, recombinant Ad DNA molecules can be generated using the highly efficient homologous recombination machinery present in RecA⁺ bacteria [95, 96]. In this system, bacteria are transformed with two plasmids: 1) a shuttle plasmid, similar to the one used for rescue in mammalian cells and, 2) a circularized Ad genome with a deletion of the entire left-end region of the virus, including the 5' ITR, packaging signal, and E1 region. Recombination between homologous sequences in these two plasmids occurs through the *E. coli* RecA recombination pathway. Appropriate recombinant genomes are then screened by identifying bacterial clones with the correct plasmid. Once the correct plasmid is identified and amplified, the viral DNA must be released from the plasmid by cleavage with an appropriate restriction enzyme and transfected into an E1-complementing cell line, where it will generate the desired recombinant Ad. The simplicity of these systems, and the fact that all of the necessary reagents are commercially available, has made the generation and use of Ad vectors a common technique in many laboratories that do not necessarily specialize in virological techniques.

1.4.5 E1-deleted adenoviruses in gene therapy. Which gene therapy study was the first to use an Ad-based vector to deliver a therapeutic gene *in vivo* is somewhat debatable, and may largely depend on the definition of "gene therapy". In 1990, a study was published in one of the first issues of the journal *Human Gene Therapy* that described the use of an Ad vector to deliver the gene encoding ornithine transcarbamylase (OTC) to the Spf-ash strain of mice, which express reduced levels of the OTC protein [97]. Normal levels of hepatic OTC were reached in approximately 25 % of these animals, and expression persisted for 2 months. One animal continued to show protein expression and a phenotypic effect for over 1 year. These

results suggested that long term correction of a genetic defect could be achieved using Ad-mediated delivery of a therapeutic gene. This study was followed over the next few years by a flurry of high-profile studies describing the use of Ad vectors in animal models to deliver a variety of genes to a range of tissues (for a review of these early studies, see [98]).

Since Ad2 and Ad5 are respiratory viruses, several of these early gene therapy studies explored Ad-mediated delivery of a therapeutic gene to the lungs of mice, rats, and non-human primates [99-103]. The resulting data provided the rationale for proposing the first Ad-mediated human gene therapy clinical trials using the virus to deliver the cystic fibrosis transmembrane conductance regulator (CFTR) gene to patients with cystic fibrosis [104-106], with 3 protocols receiving formal approval in late 1992. In general, these studies showed that low dose (5×10^7 IU and 2×10^9 pfu) administration of an Ad vector was well tolerated and that some evidence of phenotypic correction was noted [107, 108]. However, therapeutic gene expression from the Ad vector was only transient. Ad has now been used in over 450 clinical trials world-wide to explore therapies for a variety of genetic and acquired diseases (Table1).

1.4.6 Limitations of E1-deleted Ad vectors. Throughout the 1990's, Ad continued to be used prominently in many gene therapy-based studies. However, much of the data suggested that E1-deleted Ad had a significant limitation: gene delivery was accompanied by strong anti-Ad immune response [109-111]. Systemic delivery of Ad vectors to mice results in immediate activation of innate immunity, and this response seems to be solely dependent on the Ad capsid and/or the infection process, since viral gene expression or replication is not required [112]. It appears that almost every aspect of the Ad entry process is under scrutiny by the innate immune system. This includes binding of Ad to CAR [113] or integrins [114];

within the endosome, detection of capsid-bound blood factors by TLR4 [115] or the viral DNA by TLR9 [116, 117]; and rupture of the endosome by Ad [118]. Once in the cytoplasm, the Ad DNA can be detected by DNA-dependent activator of IFN-regulatory factors (DAI) [119] or the nucleotide oligomerization domain (NOD)-like receptors [120]. These events activate several signal transduction pathways that culminate in the production and release of a plethora of cytokines and chemokines, such as IP-10, MIP-2, MCP-1, RANTES, IL-8, IL-6, IL-12, IFN- γ , and TNF- α [112, 120, 121]. Many of these same responses have been noted after Ad injections into non-human primates, and humans [122]. Within hours of induction, many of the chemokine/cytokine molecules act to recruit neutrophils, macrophages, and T-lymphocytes (including cytotoxic T-lymphocytes, CTL) [121, 123]. The presence of these cells correlates with the onset of acute toxicities, such as rises in hepatocyte derived transaminases (AST, ALT) and pan-hepatitis within the serum, leading to an immune-mediated loss of the Ad transduced cells [124-126]. Delivery of large doses of Ad vector to primates results in significant transduction of the liver, but this is also accompanied by hepatic inflammation, thrombocytopenia, and hematological indications of disseminated intravascular coagulation [127-129]. Primates can become moribund after a high dose vector administration (1.2×10^{13} virus particles/kg), while administration of vector at a 10-fold lower dose results in no symptoms [129]. Taken together, these data clearly show that activation of the innate immune response by Ad vectors can have serious deleterious consequences that limit vector efficacy and can significantly affect the health and safety of the host.

In addition to innate immunity, E1-deleted Ad induce adaptive immunity, classically shown by the formation of anti-Ad CTL and antibodies in treated animals [130, 131]. Formation of adaptive immunity coincides with a continual production of some of the

inflammatory cytokines and chemokines noted during the early innate response [121, 132]. It is believed that the long-term inflammatory response is due, at least in part, to low level expression of viral proteins from the vector backbone [133]; however, anti-Ad CTL can also be generated in the absence of viral gene expression [134]. Although transgene expression can be extended by several months using T-cell deficient animals, implicating acquired immunity as a major limiting factor in long-term gene expression [131, 135, 136], other studies showed that Ad can persist long term, even in the presence of a robust cellular response to the virus [137]. In addition to the CTL response, high dose intravenous injection of Ad vectors results in activation of the humoral immune response and the generation of high-titer, neutralizing anti-Ad capsid antibodies that prevent transgene expression following re-infection with the same serotype of Ad vectors [138-140]. Anti-Ad antibodies have also been elicited in human studies [141], although not in all cases [108]. More recently, Ads have been shown to activate the complement pathway [142, 143], which can also lead to a virus induced inflammatory response. Ad activation of the various components of the host immune response ultimately limits their ability to provide long-term therapeutic gene expression, which is required for correction of most genetic diseases. However, E1-deleted Ads have proven valuable in applications directed towards cancer, where, in many cases, only short term expression is desired since once the tumor is eradicated, the vector is no longer necessary.

1.5 Multiply-attenuated Ads. Although the early inflammation and innate immune responses to Ad vector are a consequence of interaction of the Ad capsid proteins with the infected cell, long-term inflammation and toxicity is likely a result of low-level viral gene expression. To further reduce expression of viral genes, many researchers have developed Ad

vectors with deletions of other essential genes (reviewed in [144]). For example, viruses have been generated with additional mutations or deletions in the E2 [145-148] or E4 [124, 149-154] regions, both of which are required for normal viral replication. These vectors, termed “second-generation” Ad vectors, must be propagated in cell lines which complement both E1 and the second missing function, and are generated using similar methods as described for first-generation Ads. Results using second-generation Ads have been somewhat mixed, ranging from no improved function [146, 154] to significantly improved, long-term transgene expression and reduced immunogenicity and toxicity [125, 155-157] compared to vectors with only E1-deleted. One interesting observation arising from analysis of transgene expression from E4-deleted vectors is the influence that this region can have on the persistence of expression of transgenes controlled by viral promoters contained in these vectors (*e.g.* cytomegalovirus immediate-early enhancer/promoter). Vectors deleted of most or all of E4 showed reduced transgene expression over time, which was not accompanied by a loss of vector DNA [158, 159]. Subsequently, it was determined that the Ad E4 open reading frame 3 (E4ORF3) was able to prevent viral promoter down-regulation, which can occur over time in transduced cells [160], and this appears to be a generalized phenomenon for viral, but not cellular, promoters contained in Ad vectors. Inclusion of an E4ORF3 expression cassette in plasmid constructs also resulted in an improved duration of transgene expression *in vivo* [161].

Perhaps the best example of the utility of a second generation Ad is from Amalfitano and co-workers [162-164], who showed that administration of an E1/E2B (Ad DNA polymerase)-deleted vector encoding human acid- α -glucosidase (GAA) to GAA-knockout mice or in a quail model of GAA-deficiency could result in systemic correction of muscle

glycogen storage disease. In these experiments, a single injection of the vector resulted in efficient uptake of the virus by hepatic cells, and GAA proenzyme produced from the hepatic “protein factories” was secreted into the serum and, subsequently, taken up by skeletal and cardiac muscle. Although second-generation Ads are easier to generate than fully-deleted Ad vectors (see below), they have not gained wide spread use because of their inconsistent performance and the only marginal increase in cloning capacity over first-generation Ads.

1.6 Helper-dependent Ads. An alternative approach to eliminating the complications of the immune response brought upon by the use of the Ad vectors would be to completely remove all viral protein coding sequences from the vector backbone, giving rise to fully deleted or helper-dependent Ad vectors (HDAd). Such vectors must be propagated in the presence of a helper virus which provides all of the required functions for HDAd vector replication and packaging. Early studies suggested that HDAd were capable of delivering a therapeutic transgene to cells; however, problems with vector production meant that these stocks were contaminated with large quantities of the helper virus [165-169]. In 1996, two independent research groups developed the Cre/loxP system for propagating HDAd [170, 171]. This system utilized an E1-deleted helper virus with the viral DNA packaging sequence flanked by loxP sites such that, upon infection of a 293-derived cell line that stably expressed the Cre recombinase, the packaging signal was excised thus rendering the helper virus DNA unpackageable. Removal of the packaging element does not interfere with replication or viral gene expression from this virus, or its ability to co-replicate with and support the packaging of the HDAd genome. The problem of RCA formation was circumvented by designing the helper virus to contain a relatively large E3 insert that prevented packaging of E1+ derivatives [170]. Utilizing this system, it was possible to produce large quantities of

relatively pure HDAd. Similar systems have been developed utilizing the FLP recombinase [172, 173], and the Cre/lox system has now been adapted to non-adherent spinner culture for very large scale HDAd vector production [174]. Most HDAd vectors in current use are generated using this latter system.

HdAd have consistently demonstrated distinct advantages over E1-deleted Ad vectors (reviewed in [175-178]). Numerous studies have shown very long-term gene expression in both mice and non-human primates with HDAd, typically complemented with reduced toxicity and immune reaction compared to E1-deleted Ad. Perhaps the best early example of the potential of HDAd was provided by Kim *et al.* [179] who showed that a single injection of a HDAd encoding apolipoprotein E (HDAd-ApoE) lead to a significant reduction in blood cholesterol and prevented formation of atherosclerotic lesions in ApoE-deficient mice for the life of the animal (2.5 years). Studies in non-human primates have shown that HDAd can persist and express a transgene for greater than 7 years [180], the longest that any gene transfer vector has been followed.

Since HDAd have a cloning capacity of approximately 36 kbp, they can accommodate additional "options" to further improve vector function. These include large upstream regulatory regions to achieve tissue specific expression [181-184], or gene regulatory systems to achieve on/off transgene expression [185-187]. "Hybrid" HDAd vector systems have also been produced that combine the high transduction efficiency of Ad vectors with elements from other vector systems that permit stable persistence of vector DNA within the transduced cell. Examples of these hybrid systems include utilizing the integrative machinery of adeno-associated virus (AAV) [188, 189] or retrovirus/lentivirus [190, 191], transposition [192], or episomal maintenance [193]. Also, since the HDAd genome is

packaged into capsid proteins provided by the helper virus, genetically identical HDAd have been generated using helper viruses based on different serotypes (*e.g.* Ad2, Ad5 or Ad6). Sequential use of HDAd based on different serotypes allows for evasion of neutralizing antibodies in previously immunized animals, thereby allowing HDAd vector readministration [194, 195]. Alternatively, helper viruses with altered tropism have been used to enhance HDAd transduction of target tissues [196, 197]. Taken together, these studies indicate that HDAd are a very versatile platform to achieve long-term gene expression *in vivo*.

The HDAd genome should be constructed between approximately 27 and 36 kb to accommodate upper and lower size constraints of the DNA for efficient packaging into the Ad capsid [26]. Since many therapeutic transgenes are smaller than this optimal size, non-coding "stuffer" DNA must be included in the vector, preferably derived from eukaryotic sources [198-200]. The question addressed in several studies was whether the inclusion of large contiguous regions of chromosomal DNA would alter the frequency of vector insertion, which is normally considered very low for wildtype or E1-deleted Ad. In one study [201], inclusion of a 27.4 kb fragment of DNA derived from the X chromosome into a HDAd did not lead to integration of the vector by homologous recombination. Rather, low-frequency integration occurred by non-homologous events, resulting in almost perfect insertion of the entire HDAd (*i.e.* in many cases the vector ends remained intact). Random integration did occur at a slightly higher frequency for the HDAd compared to a control E1-deleted Ad (166 versus 26 neo-resistant colonies per 10^6 cells for HDAd-neo and E1-deleted Ad-neo, respectively). However, integration of an entire genome of an E1-deleted vector could be slightly deleterious or toxic to the host cells, possibly explaining the enhanced frequency of recovery of clones transformed by HDAd-neo compared to a first generation vector. In a

second study, HDAd were used to target gene correction of the hypoxanthine-guanine phosphoribosyl transferase (HPRT) locus in mouse embryonic stem cells [202]. The frequency of homologous recombination between the vector and the locus was relatively high (0.2% with an 18.6 kb stretch of homologous sequence), however the frequency of non-homologous integration was over 10-fold higher. The non-homologous insertion sites were distributed in both coding and non-coding DNA, which is in contrast to retrovirus, lentivirus and AAV vectors which tend to integrate within active genes [203, 204]. It should be noted that Ad and HDAd vectors seem to persist in cycling cells much better than would have been predicted based on their normally episomal location [205], suggesting that the virus has evolved a mechanism to remain associated with the nucleus even during cell division.

At least part of the reason that HDAd perform better than E1-deleted Ad is due to their improved immunological profile. In contrast to E1-deleted Ad, which stimulates both an early and late inflammatory response, HDAd do not induce inflammation at late times after delivery *in vivo* [133], likely due to the lack of viral gene expression from the vector backbone. However, the early inflammatory response against HDAd can be substantial and lethal [206]. Systemic delivery of 5.6×10^{12} vector particles per kg to non-human primates resulted in 50% hepatocyte transduction, accompanied by relatively mild, acute toxicity which resolved within 24 hr [206]. At a two-fold higher dose (1.1×10^{13} vector particles per kg), 100% of the liver was transduced; however, there was severe and lethal acute toxicity. This is perhaps not surprising, since this early inflammatory response is driven solely by the infecting capsid, and does not require viral gene expression. Fortunately, early inflammation from HDAd can be reduced somewhat through coating the virus with masking agents, such as polyethylene glycol [207, 208], suggesting that even this limitation to HDAd can be

overcome.

1.7 Oncolytic Ad for cancer-directed therapy. Although one arm of Ad vectorology has focused on trying to create stealth-type vectors that provide minimal insult during gene delivery, other researchers have focused upon creating Ad that actively replicate in certain tissues of the host. Oncolytic or conditionally replicating Ad (CRAd) are designed to replicate in cancer cells, but not normal tissue. Perhaps the best characterized CRAd is Addl1520, also known as ONYX-015, which is deficient for the E1B-encoded 55 kD protein [209, 210]. During infection with wildtype Ad, one of the functions of proteins encoded within the E1B region is to sequester p53, and promote its degradation, thus preventing p53 activation at the G2 checkpoint, which might lead to premature cell death (thereby causing premature termination of the virus lifecycle) [211]. E1B also aids in viral replication by forming a complex with E4 open reading frame 6 (ORF6) and allowing for the accumulation and transportation of viral mRNA, while inhibiting host mRNA transportation and translation [212, 213]. In the absence of E1B 55 kD, Ad cannot replicate in normal cells; however, since p53 is mutated in almost half of all tumors [214], the virus will replicate in, and kill, tumor cells, but not spread to adjacent normal tissue [215-217]. CRAd are becoming very popular for cancer-directed therapy, and are evolving and maturing in their design (reviewed in [218-220]). For example, the E1A coding region in these vectors can be placed under regulation by a tumor-specific promoter, thereby increasing their specificity for replication in a particular tumor type [221]. Alternatively, "armed" CRAd can be generated through inclusion of a therapeutic transgene in the vector, such as a cytokine, to enhance immune reaction to the tumor cell, or a cell suicide gene such as the herpes simplex virus thymidine kinase gene (HSV TK, used in combination with gancyclovir administration) or p53 [218].

The promising results observed for ONYX-015 in preclinical studies spurred its movement into phase I and II trials, either as a lone therapeutic or in combination with radiation or chemotherapy [220, 222]. Unfortunately, one of the main biotechnology companies exploring this mode of cancer therapy, Onyx Pharmaceuticals, closed its therapeutic virus program in 2003 and, with it, cancelled many of their ongoing clinical trials, including a Phase III trial [220, 223]. However, in November 2005, Shanghai Sunway Biotech (Shanghai, China) announced that it had received regulatory approval from the Chinese government for use of an oncolytic Ad deleted of E1B-55kD, in combination with cisplatin, for treatment of nasopharyngeal carcinomas [223], making this the second Ad-based gene therapeutic that is commercially available (see below for a discussion of the first Ad-based gene therapy commercial product).

1.8 Human Clinical Trials Utilizing Ad-based Vectors. At present, Ads are being used in about 22% of all gene therapy clinical trials, with the vast majority of these trials directed towards treatment of cancer [1]. Some of the shortcomings of E1-deleted Ad vectors, mainly short-term transgene expression and induction of an immune response, are not of concern in most cancer trials, since the therapeutic is usually only required short term (*i.e.* once the tumor is gone, the virus is no longer needed). As shown in Table 1, clinical trials with Ad vectors follow a trend found throughout the field of gene therapy: many Phase I trials are conducted, few of which progress to Phase II or III. Failure to proceed to a Phase II or III trial is not usually a result of safety or toxicity issues but, rather, a lack of evidence for efficacy in the Phase I study (although technically Phase I trials are not designed to examine efficacy, almost all clinical researchers look for some measure of efficacy to provide support for Phase II trials). This is a common problem in the gene therapy community, where

promising results are obtained in in-bred mouse models of the disease, but similar results are not observed in the more diverse human population.

1.8.1 Ad-mediated clinical trials for cancer. Novel gene therapeutics utilizing Ad-based vectors have advanced to twenty-four Phase II/III or III clinical trials and two trials for post-marketing surveillance in phase IV (both Ad-p53). Of these twenty-four clinical trials utilizing Ad, seventeen are directed towards cancer. Eleven of these seventeen trials explore the use of Ad encoding p53 to treat a variety of cancers, including ovarian, nasopharyngeal, non-small cell lung carcinoma, head and neck, hepatocellular, glioblastoma multiforme, prostate, and cervical cancer. The two Phase IV studies will assess Ad-p53 for thyroid cancer. Involved in detecting DNA damage, p53 controls cell fate by deciding on whether the damage is repairable, or whether the cell should undergo apoptosis [214]. Understandably, approximately half of tumors contain a mutated p53 which allows the cells to accumulate DNA damage while circumventing activation of the p53 checkpoint during G2 of the cell cycle. Numerous studies have shown that reintroducing a functional copy of p53 into p53-defective cancer cells leads to reestablishment of appropriate damage surveillance and, frequently, death of the cell [224]. When examining all human clinical gene therapy trials, including all vector systems, approximately 80 out of the 2210 total trials involve the delivery of p53 for treatment of a variety of cancer types (reviewed in [225]), underscoring the perceived importance of this gene. Nine of the eleven phase II/III or III clinical trials for Ad-p53 involve evaluation in combination with chemotherapy and/or radiation therapy. The available data from early clinical trials indicates that the virus is well tolerated, although some relatively minor adverse events were reported, including transient fever, pain at the injection site, and fatigue. The phase I and II clinical trials with Ad-p53 have used a variety

of doses and administration routes, resulting in variable rates of tumor regression or stabilization of disease, ultimately prolonging survival in some patients [226]. Taken together, the data from these early trials were sufficiently compelling to warrant progression to Phase III study.

In October 2003, the Shenzhen SiBono GeneTech biotech company received government approval to produce and distribute their Ad-p53 virus (trademarked as Gendicine) for treatment of head and neck squamous cell carcinoma [227]. Gendicine was the world's first commercially available gene therapy product. In the 16 months following its official launch in April 2004, 2600 patients were treated with Gendicine, and this number was expected to increase to 50,000 by 2006 [228]. The exact number of patients that have been treated with Gendicine is not readily available. Although currently approved only for head and neck squamous cell carcinoma, the product is in late stage testing for a variety of other cancer indications [227]. Given the large number of patients that will receive Gendicine, the gene therapy community should look forward to very solid, statistical significant data regarding efficacy and safety of Ad and the Ad-p53 product. As of 2009, data on 2500 Gendicine treated patients had been published [229].

Of the remaining six cancer-directed trials, three utilize traditional E1-deleted vectors and three involve conditionally replicating vectors. Two of the E1-deleted vectors are designed to specifically target the tumor vasculature, either to inhibit angiogenesis or kill endothelial cells. Endostatin is a 20 kDa C-terminal fragment of collagen XVIII, and has been shown to have potent anti-angiogenic activity [230]. Ad-mediated delivery of endostatin has shown efficacy in preclinical and early clinical trials in a variety of cancers [231, 232]. The second vector that targets vasculature expresses a chimeric Fas and human

TNF receptor 1 protein (Fas signaling is activated only upon TNF- α binding to the receptor component of the chimeric protein), under regulation in the vector by the pre-proendothelin-1 promoter [233]. This vector is being evaluated in combination with bevacizumab for treatment of glioblastoma. Two additional trials deliver classic cell suicide genes, either the herpes simplex virus thymidine kinase gene (HSV TK) in a replication-defective vector or a replication competent Ad vector expressing a fusion protein of yeast cytosine deaminase (yCD) and HSV TK. Both of these trials are for prostate cancer, and both are in combination with conventional radiation therapy. HSV TK acts through converting the prodrug ganciclovir into a nucleotide analogue that causes DNA synthesis chain termination and replication arrest. yCD converts 5-fluorcytidine into 5-fluorouracil, which is an irreversible inhibitor of thymidylate synthase, thus impairing production of dTMP and ultimately significantly impacting the levels of dTTP within the cell. Both vectors showed sufficient efficacy in Phase II study to warrant advancement to Phase III [234-236].

The final two cancer-directed phase III trials utilize oncolytic vectors. The first is rendered tumor-selective due to placement of E1A expression under regulation by E2F-1 promoter. E2F-1 expression is misregulated in cancer cells that are defective in the retinoblastoma pathway [237], thus making this virus selective for replication in these specific cell types. The virus, designated CG0070, also expresses GM-CSF (granulocyte-macrophage colony-stimulating factor), which can aid in establishing anti-tumor immunity [238]. The other oncolytic vector in Phase III trial is deficient in E1B, similar to ONYX-015 [220].

1.8.2 Ad-mediated clinical trials for vascular disease. The remaining seven Ad-mediated clinical trials were directed towards vascular disease, six trials using Ad to deliver fibroblast

growth factor-4 (FGF-4) and one exploring the efficacy of vascular endothelial growth factor (VEGF). FGF-4 is involved in the growth and migration of many cell types in the developing vessel wall, and Ad-mediated delivery of FGF-4 showed efficacy in various animal models of ischemia and early human clinical trials to treat myocardial and critical limb ischemia [239-241]. Most patients in these trials experienced only relatively mild adverse side-effects, including minor fever and asymptomatic elevation in liver enzymes in a few patients [240, 241]. However, fourteen severe adverse events occurred, including accelerated toe pain, myalgia, and peripheral edema, and several patients required toe or limb amputation as a result of advancement of disease. Interestingly, two Phase IIb/III trials utilizing the Ad-FGF-4 vector uncovered a gender-specific bias in efficacy. Patients (n=532) were treated using two doses of Ad5-FGF-4, 1×10^9 or 1×10^{10} viral particles per patient, but the trial was halted when a preliminary analysis of the data indicated that the primary end point, an increase in exercise treadmill time (ETT) from baseline at 12 weeks, would not reach significance [242]. Subsequent additional analysis showed that although in male treated patients the placebo effect was large and not different from Ad5-FGF-4 treated patients, the placebo effect in women was negligible and the treatment effect was significantly greater than placebo. At least one additional Phase III trial has been proposed to test Ad5-FGF-4 as a therapeutic for angina pectoris, which will include examining potential gender-specific effects [243].

In summary, a number of Ad-based vectors have advanced to clinical trial and shown efficacy in human patients. It is hoped that these vectors will provide sufficient efficacy in the Phase III studies to promote their advancement to widespread availability to provide new treatment options for some of these devastating conditions.

1.9 Ad-OTC. With the wide-scale testing and availability of Ad-based vectors for vascular disease or cancer, the future of Ad in gene therapy appears very bright, at least for some applications. However, several years ago, Ad clinical and basic researchers faced serious concerns regarding the use of Ad in gene therapy, and even broader criticisms regarding the validity of the "gene therapy" approach to treating disease. These legitimate questions arose as a result of the first death of a gene therapy clinical trial participant that was directly attributed to delivery of the gene therapy vector. The Phase I trial involved escalating dose delivery of an E1/E4-deleted Ad vector encoding OTC for treatment of OTC deficiency (OTCD) [244]. Patients with OTCD develop severe hyperammonemia and excessive elevation of ammonia in the brain, which can cause encephalopathy, coma, and brain damage. The OTC gene is located on the X chromosome and males born with no OTC activity usually die within a few weeks of birth. Patients with partial OTC activity are susceptible to hyperammonemic crisis throughout their life, and dietary restriction is only partially effective at managing OTCD. Patients entered into the trial received between 1.4×10^{11} and 3.8×10^{13} total virus particles of the Ad-OTC vector. The second last patient entered in the trial, an asymptomatic female, received a dose of 3.6×10^{13} of Ad-OTC, which was accompanied by only a transient rise in fever and liver enzymes [245]. The final patient received a similar dose of vector, 3.8×10^{13} virus particles. However, shortly after vector administration, the patient experienced a severe adverse event, with the following report logged with the Recombinant DNA Advisory Committee (RAC) and the National Institutes of Health (NIH)[246]:

"Patient death due to adult respiratory distress syndrome, multiple organ failure, and disseminated intravascular coagulation. Within 12 hours of receiving the intrahepatic

adenoviral vector administration, patient experienced fever, nausea, and back pain. The following morning after vector administration, patient experienced elevated ammonia levels and jaundice. During days 2-4 after vector injection, patient experienced disseminated intravascular coagulation, adult respiratory distress syndrome, and kidney and liver failure. Patient died four days after vector administration."

After an intensive review of clinical and postmortem findings, it was determined that the patient death was most likely a result of a systemic, Ad-vector induced shock syndrome, culminating in the events described above [247]. There has also been significant discussion regarding possible clinical procedural issues and conflict of interest [248, 249]. Immediately following this death, the NIH ordered the suspension of several technically-similar gene therapy clinical trials (several more were suspended voluntarily), and the establishment of a RAC Working Group on Ad vector Safety and Toxicity (AdSAT). The report from AdSAT has been released [250], and included several recommendations such as improved methods for standardizing vector dose and potency, improved collection and dissemination of data on vector safety and toxicity, and increased clarity of the risks and benefits on the informed consent documents used in gene therapy trials. With respect to Ad vector use in gene therapy, AdSAT concluded that "human gene transfer experiments using Ad-based vectors should continue - with caution [250]." Since the release of this report, Ad-mediated gene transfer has continued, and we now have two commercially available gene therapy products based on an Ad platform.

1.10 Concluding Remarks. The death of a participant in a gene therapy trial was, perhaps, not unexpected. In 1995, an NIH-sponsored panel was asked to evaluate the rationale and potential of gene therapy, and their findings were published as the Orkin-Motulsky Report

[251]. They concluded that, "Somatic gene therapy is a logical and natural progression in the application of fundamental biomedical science to medicine and offers extraordinary potential, in the long-term, for the management and correction of human disease." However, it was clearly recognized that all gene therapy transfer vectors have shortcomings, and that we have an inadequate understanding of the biological interactions between the host and the vector. Ad serves as a prime example of a vector system that has evolved over time in an attempt to reduce or eliminate identified deficiencies in order to improve vector function and safety. Since Ad was first proposed as a gene therapy transfer vehicle, there has been a significant amount of research aimed at characterizing the many interactions between the virus, the infected cell, and the host organism. Our knowledge is far from complete; however, it is hoped that the continued efforts of virologists and gene therapy researchers will improve the safety and efficacy of this gene transfer vehicle for gene therapy applications, and provide new hope for patients afflicted with these devastating, currently incurable diseases.

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Table 1. Gene therapy clinical trials using adenovirus vectors (1989-2015).^a

Disease	Clinical Phase						Total
	I	I/II	II	II/III	III	IV	
Cancer							
immune modulator	75	14	19				108
tumor suppressor	33	10	24	5	6	2	80
cell suicide gene	44	9	6		2		61
oncolytic	29	12	5	1	1		48
vaccine	17	4	4				25
other	2	15	4		2		23
Vascular	20	7	8	4	3		42
Infectious Disease	30	7	3				40
Monogenic	15	2					17
Other	11	1	2				14
Total	276	81	75	10	14	2	458

^aData in this table was compiled from the Gene Therapy Clinical Trial database maintained by Wiley and Sons (<http://www.abedia.com/wiley/>). An effort was made to exclude trials which had incomplete data on the current status of their approval, or which were abandoned. This table provides a rough outline of human clinical trials utilizing Ad-based vectors, but should not be considered definitive or complete.

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247. Raper, S.E., et al., *Fatal systemic inflammatory response syndrome in a ornithine transcarbamylase deficient patient following adenoviral gene transfer*. Mol Genet Metab, 2003. **80**(1-2): p. 148-58.
248. Yarborough, M. and R.R. Sharp, *Public trust and research a decade later: what have we learned since Jesse Gelsinger's death?* Mol Genet Metab, 2009. **97**(1): p. 4-5.
249. Wilson, J.M., *Lessons learned from the gene therapy trial for ornithine transcarbamylase deficiency*. Mol Genet Metab, 2009. **96**(4): p. 151-7.
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253. Saha, B., C.M. Wong, and R.J. Parks, *The adenovirus genome contributes to the structural stability of the virion*. Viruses, 2014. **6**(9): p. 3563-83.

Curriculum Vitae

Leslie Nash

Education

Visiting Ph.D. student. Supervisors: Dr. M. Wood, Dr. I. Mager, Dr. M. Bowerman	Completed
Oxford University	2017/02-2017/06
Ph.D. Biochemistry. Supervisor: Dr. R.J. Parks	<i>In progress</i>
University of Ottawa/ Ottawa Hospital Research Institute	2015/01-2019/01
M.Sc. Health Sciences. Supervisor: Dr. W.E. Ward.	Degree Received
Brock University	2012/09-2014/09
B.Sc. (Honors) Biotechnology. Supervisor: Dr. P.M. Zelisko	Degree Received
Brock University	2008/09-2014/04

Recognitions

International

2017 EMBO Short Term Fellowship

National

2017 Michael Smith Foreign Study Supplement – CIHR
2016-2019 Frederick Banting and Charles Best Canada Graduate Scholarships Doctoral Award (CGS-D), Canadian Institute Health Research (CIHR)
2013-2014 Alexander Graham Bell, National Science and Engineering Research Council
2013-2014 NSERC (CGS-M)
3 Minute Thesis Competition: Canadian finalist,
2nd place Ontario,
1st place Brock University

Provincial

2015, 2016 Ontario Graduate Scholarship, University of Ottawa

Local

2017 Mobility Bursary, University of Ottawa
2015-2016 Destination 20/20 Scholarship, University of Ottawa
2015-2019 Excellence Scholarship, University of Ottawa
2015-2019 Entrance Scholarship, University of Ottawa
2015 Spirit of Brock Award, Brock University
2014 President's Surgite Award, Brock University
2013-2014 Dean's Excellence Scholarship, Brock University
2013-2014 Ralph D. Morris Graduate Student Award, Brock University
2012-2013 Graduate Student Advocacy Bursary, Brock University
2012-2013 Graduate Student Association Bursary, Brock University
2012-2013 Bluma Appel Graduate Entrance Scholarship for Excellence in Mathematics and Science, Brock University
2012-2013 Tomlinson Entrance Award for Excellence in Mathematics and Science, Brock University
2012-2013 Entrance Scholarship, Brock University
2011-2012 Distinguished Graduating Student Award – Biotechnology, Brock University
2011-2012 Chemical Society Merit Award – Biotechnology, Brock University
2010-2012 Harrison-Thompson Award, Brock University

Conference Awards

2018	Outstanding poster presentation award, American Society for Gene and Cell Therapy Conference
2018	1 st place: PhD oral presentation, BMI Day, University of Ottawa
2016-2017	3 rd place: PhD poster presentation, Ottawa Hospital Research Institute
2015-2016	2 nd place: IMPACT award, Identification of Marketable Products, Applications and Commercializable Technologies. Ottawa Hospital Research Institute
2015-2016	2 nd place: PhD oral presentation, BMI Day, University of Ottawa
2014-2015	Nominated for Emerging Leader in Nutrition, Experimental Biology, San Diego
2011-2012	1 st place: oral presentation, Western New York American Chemical Society (ACS) Undergraduate Research Symposium
2011-2012	2 nd place: oral presentation, Southern Ontario Undergraduate Student Chemistry Conference, Guelph
2012-2014	Travel funding awards = 5

Employment

2014-2015	Research assistant: Rooibos tea flavonoids increase mineral content in human osteoblast-like cells, Brock University
2014-2015	Research assistant: Leave the pack behind, Brock University
2014	Marker/grader: SCIE 1P51, Science and Society II, Brock University
2013-2015	Ridley College tutor: Chemistry, Biology and General Science
2013-2014	Course coordinator and teaching assistant: PEKN 2P09, Human Physiology, Brock University
2013-2014	Bridging Our Worlds (Aboriginal Science Camp): Lab demonstrator
2013-2014	Teaching assistant: SCIE 1P50, Science and society, Brock University
2013-2015	Teaching assistant: ECON 1P92, macro-economics, Brock University
2013	Lab demonstrator & teaching assistant: CHEM 1P00, Introduction to chemistry, Brock University
2012, 2014	Lab demonstrator: CHEM 2P63, Biophysical chemistry, Brock University
2011-2014	Scientifically Yours (Science Camp for Women), Residence Coordinator, Brock University
2011-2012	Research assistant: Towards the directed evolution of trypsin; Immobilization of proteases for synthesis of SiO ₂ , Brock University

Activities

Academic

2018-2019	The Science to Business Network – Ottawa Chapter: Bridging academia and industry
2013-2015	Dean's hiring committee for the Faculty of Applied Health Sciences, Brock University
2013-2014	Vice President of Communications: Graduate Student Association Brock University
2013-2014	Freedom of expression and dialogue: Senate ad hoc committee Brock University
2013-2014	Grad plus: graduate professional development committee, Brock University

2013-2014	Mapping New Knowledge: Brock University conference committee Brock University
2012-2014	Mentorship award adjudication committee, Brock University
2012-2014	Student-supervisor handbook committee: development of new policies for students and supervisors to improve relationships and prevent conflicts Brock University
2012-2013	Program representative: Graduate Student Association, Brock University
2012-2013	President's Surgite award adjudication committee, Brock University

Non-academic

2015-2018	Walk for Muscular Dystrophy
2015-2016	Walk for ALS
2014	Congress 2014: Research Matters volunteer
2014	Relay for Life Captain for Brock University Graduate Studies team
2013	Coordinator for the Graduate Student Association -Wellspring Niagara charity gala, raised over \$5000 in silent auction donations
2012-2015	Welland Humane Society (WHS) & WHS Cat Adoption Centre
2012-2013	Multiple Sclerosis Society: Walk for a cure, raised \$300
2012	Shinerama: Cystic Fibrosis Canada, raised \$750
2011-2014	Brock University biotechnology representative for the Ontario University Fair
2009-2012	Student Welcome Awareness Team, Brock University

Contributions

Invited Lectures

2014	Ph.D. seminar series (AHSC 7P00): From tea to 3MT and everything in between, Brock University
2014	Human physiology (PEKN 2P09): Bone anatomy and metabolism, Brock University

Conference Presentations: Oral

1. **Nash L.A.** and R.J. Parks (2018). Alterations in exosomes derived from models of spinal muscular atrophy. *Biochemistry Microbiology and Immunology Seminar Day*. Ottawa, ON. (***This presentation was awarded 1st place for best PhD oral presentation***)
2. **Nash L.A.**, E.R. McFall, Perozzo, A. M., Turner, M., Poulin, K. L., Repentigny, Y.-D., H. McMillan, J. Warman Chardon, R. Kothary and, R. J. Parks (2018). Harnessing exosome-based approaches for development of biomarkers and novel therapeutics in spinal muscular atrophy. *Department of Medicine Research Day*. Ottawa Hospital Research Institute, Ottawa, ON. (***This abstract was selected for an oral presentation by a committee***)
3. **Nash L.A.** and R.J. Parks (2016). Exosomal Survival Motor Neuron Protein, a Biomarker and Therapeutic for Spinal Muscular Atrophy. *Biochemistry Microbiology and Immunology Seminar Day*. Ottawa, ON.
4. **Nash L.A.**, E.R. McFall, H. McMillan, J. Warman Chardon, R. Kothary and, R. J. Parks (2015). SMN in Exosomes: A Potential Biomarker for SMA. *Ottawa Hospital Research Institute's Research Day*. Ottawa, ON. (***This abstract was selected for an oral presentation by a committee***)
5. **Nash L. A.** and R.J. Parks (2015). A Novel Approach to Gene Therapy for Spinal Muscular Atrophy. *Biochemistry Microbiology and Immunology Seminar Day*. Ottawa, ON. (***This presentation was awarded 2nd place for best PhD oral presentation***)

6. **Nash L. A.** and W.E Ward (2014). *Aspalathus linearis* tea flavonoids to promote mineralization *in vitro*. *Ontario Exercise Physiology*. Barrie, ON.
7. **Nash L.A.** and W.E. Ward (2014). Simplici·Tea: Investigating tea as a dietary strategy for better bone health. 3 Minute Thesis Competition. St. Catharines, ON. (**This presentation was awarded 1st place at the institutional level, 2nd place in provincial finals, Canadian finalist**)
8. **Nash L.A.** and W.E. Ward (2014). Tea Flavonoids Increase Hydroxyapatite Production in Osteoblast-like Cells. *Experimental Biology*. FASEB J. 259.7. San Diego, CA. (**published, peer reviewed abstract**)
9. **Nash L.A.** and W.E. Ward (2013). Investigation of rooibos tea flavonoids at stimulating mineralization in osteoblast-like cells. *Annual Chemistry and Biochemistry Graduate Research Conference*. Montreal, QC.
10. **Nash L.A.** and W.E. Ward (2013). Effect of Bioactives from Black Tea on Bone Health. *Mapping New Knowledge Graduate Conference*. St. Catharines, ON.
11. **Nash L.A.**, H. Gordon and P.M. Zelisko (2012). Protease Mediated Hydrolysis of an Si-O Bond for Future Biosilica Production via Directed Evolution. *Annual Chemistry and Biochemistry Graduate Research Conference*. Montreal, QC.
12. **Nash L.A.** and P.M. Zelisko (2012). Directed Evolution of Trypsin for the Enzyme Mediated Sol-Gel Processing of TEOS, *40th Southern Ontario Undergraduate Student Chemistry Conference (SOU SCC)*, Guelph, ON. (**This presentation was awarded second prize in the Biological Chemistry Division**)
13. **Nash L.A.** and P.M. Zelisko (2012). Enzyme Hydrolysis of Tetraethoxysilane: The Directed Evolution of Trypsin for the Mediated Sol-Gel Processing of TEOS, *Western New York American Chemical Society (ACS) Undergraduate Research Symposium*, Buffalo, NY. (**This presentation was awarded first prize for best oral presentation**).
14. **Nash L.A.** and P.M. Zelisko (2012). Enzyme-Mediated Hydrolysis of Tetraethoxysilane: A Step Towards the Directed Evolution of Trypsin, *44th Silicon Symposium*, St. Catharines, ON.

Conference Presentations: Poster

1. **Nash L.A.**, McFall, E.R., Perozzo, A.M., Turner, M., Poulin, K.L., De Repentigny, Y., Burns, J.K., McMillan, H.J., Chardon, J.W., Burger, D., Kothary, R., Parks, R.J. (2018). Harnessing exosome-Based Approaches for Development of Biomarkers and Novel Therapeutics in Spinal Muscular Atrophy. *American Society for Cell and Gene Therapy*. Chicago, IL, USA. (**This poster was awarded for outstanding presentation**).
2. **Nash L.A.**, McFall, E.R., Perozzo, A.M., Turner, M., Poulin, K.L., De Repentigny, Y., Burns, J.K., McMillan, H.J., Chardon, J.W., Burger, D., Kothary, R., Parks, R.J. (2018). Utilization of exosomes as novel prognostic biomarkers and therapeutic vehicles for spinal muscular atrophy. *Gordon Conference: Extracellular Vesicles*. Newry, ME, USA.
3. **Nash L.A.**, McFall, E.R., Perozzo, A.M., Turner, M., Poulin, K.L., De Repentigny, Y., Burns, J.K., McMillan, H.J., Chardon, J.W., Burger, D., Kothary, R., Parks, R.J. (2017). Exosomes, a novel biomarker for Spinal Muscular Atrophy *Ottawa Hospital Research Institute Conference*. Ottawa, ON.
4. **Nash L.A.**, McFall, E.R., Perozzo, A.M., Turner, M., Poulin, K.L., De Repentigny, Y., Burns, J.K., McMillan, H.J., Chardon, J.W., Burger, D., Kothary, R., Parks, R.J. (2017). Alterations of exosomes in derived from models of spinal muscular atrophy. *International Conference on Neuromuscular Disease and Biology*. Ottawa, ON.
5. **Nash L.A.**, E.R. McFall, D. Burger, R. Kothary, and R.J. Parks (2016). Exploiting Exosomes as Biomarkers and Therapy for Spinal Muscular Atrophy. *Ottawa Hospital Research Institute Conference*. Ottawa, ON. (**Awarded third prize in the PhD category**)
6. **Nash L.A.**, E.R. McFall, D. Burger, R. Kothary, and R.J. Parks (2016). Exploiting Exosomes as Biomarkers and Therapy for Spinal Muscular Atrophy. *Keystone*

Symposia: Exosomes/Microvesicles: Novel Mechanisms of Cell-Cell Communication. Keystone, Colorado.

7. **Nash L.A.**, E.R. McFall, D. Burger, R. Kothary, and R.J. Parks (2016). Exosomal Survival Motor Neuron Protein, A Potential Biomarker for Spinal Muscular Atrophy. *SMA Families Research Conference*. Anaheim, California.
8. **Nash L.A.**, E.R. McFall, R. Kothary, R.J. Parks. Exosomes (2016). The Biological Swiss Army Knife for SMA. *Ottawa Hospital Research Institute Conference*. Ottawa, ON. (**Awarded second prize in the IMPACT award**)
9. **Nash L.A.**, E.R. McFall, R. Kothary, R.J. Parks (2015). SMN in Exosomes: A Potential Biomarker for SMA. *International Conference on Neuromuscular Biology, Disease and Biology*. Ottawa, ON.
10. **Nash L.A.** and W.E. Ward (2015). Comparison of Different Tea Types on Osteoblast Activity. *FASEB J.* 924.35. Boston, MA. (**Published and peer-reviewed abstract, abstract nominated for emerging leader in nutrition award**)
11. **Nash L.A.** and W.E. Ward (2014). Flavonoids from Rooibos Tea Promote Cell Death in an Osteosarcoma Cell Line. *Experimental Biology. FASEB J.* 647.10. San Diego, CA, 2014. (**Published and peer-reviewed abstract**)
12. **Nash L.A.** and W.E. Ward (2013). Flavonoid-induced cell death in an osteosarcoma cell line. *Annual Chemistry and Biochemistry Graduate Research Conference*. Montreal, QC.
13. **Nash L.A.**, H. Gordon, P.M. Zelisko (2012). A Green Route to Silica Production. *Annual Chemistry and Biochemistry Graduate Research Conference*. Montreal, QC.
14. **Nash L.A.** and P.M. Zelisko (2012). Biocatalysis of an Alkoxysilane. *Quebec-Ontario Mini Symposium for Bioorganic and Organic Chemistry*. Windsor, ON.
15. **Nash L.A.** and P.M. Zelisko (2012). *Biocatalysis of an Alkoxysilane. Brock University Undergraduate Poster Session*. St. Catharines, ON.

Publications

Peer-Reviewed Articles

1. **Nash, L.A.**, Del Papa, J., Deguise, M.-O., Eshraghi, M., Burger, D., Kothary, R., Parks, R.J. (2018). Exosomes are effective at delivering Survival Motor Neuron protein to recipient cells. *Molecular Therapy*. Submitted.
2. Osorio-Querejeta I., Carregal S., Aierdi A., Mäger I., **Nash L.A.**, Wood M., Egimendia A., Betanzos M., Alberro A., Iparraguirre L., Moles L., Llarena I., Goñi-de-Cerio F., Bijelic G., Ramos P., Muñoz-Culla M., Otaegui D. (2018). MiR-219a-5p enriched exosomes induce OPC differentiation and EAE improvement more efficiently than liposomes and nanoparticles. A comparative study. *Biomaterials*. Submitted.
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9. **Nash L.A.**, Sullivan P. J., Peters S. J. and Ward W. E (2016). Supra physiological Levels of Quercetin Glycosides are Required to Alter Mineralization in Saos2 Cells. *Int. J. Environ. Res. Public Health*. 13(5): 460; doi: 10.3390/ijerph13050460
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1. **Nash L. A.** and Ward W.E. (2015). Comparison of Different Tea Types on Osteoblast Activity. *FASEB J.* 29(1) 924.35
2. **Nash L. A.** and Ward W. E. (2014). Tea flavonoids stimulate mineralization in osteoblast-like cells. *FASEB J.* 28(1) 259.7
3. **Nash L. A.** and Ward W. E. (2014). Flavonoids from rooibos tea promote cell death in an osteosarcoma cell line. *FASEB J.* 28(1) 647.10

Manuscripts in Preparation

1. Hammond, S., Abendroth, F., Burki, U., Bowerman, M., McClorey, G., Meijboom, K., Hazel, G., **Nash, L.A.**, Wood, M. J. (2018). Pre-clinical pharmacokinetics and pharmacodynamics of a glycosylated cell penetrating peptide delivering antisense oligonucleotides to the CNS.

Media Relations

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| 2016 | Interview for Surgite, Brock University Alumni magazine |
| 2014 | Radio interview for 3MT, CFBU 103.7 FM |
| 2014 | Three graduate students win 2014 President's Surgite Awards, Brock News |
| 2014 | Three-minute thesis tests' graduate students' preparation, presentation skills, Brock News |
| 2013 | Graduate students make community outreach a priority, Brock News |