

Extracellular vesicle-mediated delivery of survival motor neuron protein: A potential therapeutic strategy for Spinal Muscular Atrophy

Charlotte René

Thesis submitted to the University of Ottawa in partial fulfillment of the requirements for the degree of

Doctorate of Philosophy in Microbiology and Immunology

Department of Biochemistry, Microbiology and Immunology

Faculty of Medicine

University of Ottawa

Abstract

Spinal Muscular Atrophy (SMA) is a genetic neuromuscular disorder caused by homozygous mutation or deletion of the survival motor neuron 1 (*SMN1*) gene, leading to a low quantity of SMN protein in cells. This depletion of SMN protein preferentially leads to death of motor neurons and, consequently, muscle atrophy, in addition to defects in many other peripheral tissues. Although several disease-modifying therapies for SMA exist, there is no cure, and new therapies need to be developed. SMN protein is naturally loaded into extracellular vesicles (EVs), which are sub-micron-sized, membrane-bound particles released from all cell types and found in all bodily fluids. The innate ability of EVs to deliver cargo to recipient cells has caused these vesicles to gain interest as therapeutic delivery vehicles. In this thesis, I explore EVs as a delivery vehicle of SMN protein. The work presented herein demonstrates that adenovirus-mediated overexpression of SMN protein in HepG2 cells leads to the release of EVs loaded with high levels of SMN protein into conditioned medium. Application of this medium to recipient cells in tissue culture led to uptake of the SMN protein, which subsequently transited to the nucleus and co-localized with Gemin2 protein, forming nuclear gem-like structures similar to the native SMN protein. This work demonstrates that SMN protein can be delivered to cells through EVs, however SMN protein-loaded EVs would likely need to be administered on a daily basis to maintain a therapeutic threshold of SMN protein. Thus, I built upon this work by utilizing a gene therapy approach to engineer the liver of mice to release extracellular vesicles enriched with SMN protein, facilitating systemic distribution of the protein throughout the animal. The findings contained within this thesis demonstrate a viable approach to achieve body-wide distribution

of SMN protein, which could be utilized as a potential therapeutic strategy to treat SMA. This approach could be used to deliver numerous therapeutic cargoes, holding promise as a potential therapy for patients presenting with diverse diseases.

Acknowledgements

First, I would like to thank my graduate supervisor Dr. Robin Parks, for going above and beyond with the support and guidance you have provided me for the majority of my adult life (to date). Thank you for answering my never-ending questions about anything and everything, encouraging my curiosity, and for challenging me to refine my skills. I am immensely proud to have been one of your graduate students. I am truly grateful to have had the opportunity to learn and grow under your supervision, and I am excited to carry these invaluable experiences with me as I begin my career.

I would also like to thank the members of my thesis advisory committee, Dr. Dylan Burger, Dr. Rashmi Kothary, and Dr. Jodi Warman-Chardon, for all of the insight you shared and the confidence you helped me build as a researcher.

I am very grateful for the companionship of the past and present members of the Parks lab, as well as many of the other trainees in the Regenerative Medicine Program at the Ottawa Hospital Research Institute. Thank you for making every day as fun as possible. In particular, I would like to thank Kathy Poulin for always having my back, and for the hundreds of morning coffees we spent together.

I would like to thank my grandmother Anna and my mother Julie for demonstrating how to persevere and be resilient. I am sincerely appreciative of the love, support, encouragement, and understanding given to me by my friends and family, and especially by my boyfriend Patrick. Thank you all for the unwavering support over the years, none of this would have been possible without you.

Table of Contents

Abstract.....	ii
Acknowledgements.....	iv
Table of Contents.....	v
List of Abbreviations.....	vii
List of Figures.....	x
List of Tables.....	xii
Chapter 1: General Introduction.....	1
1.1 Spinal Muscular Atrophy.....	2
1.1.1 Genetics.....	2
1.1.2 SMN Protein.....	3
1.1.3 Clinical Manifestation.....	7
1.1.3.1 Classification of SMA.....	7
1.1.3.2 SMA as a Multisystem Disease.....	9
1.1.4 Therapeutics for SMA.....	16
1.1.4.1 Nusinersen.....	16
1.1.4.2 Risdiplam.....	19
1.1.4.3 Onasemnogene abeparvovec.....	21
1.1.4.4 Combination Therapy.....	25
1.2 Extracellular Vesicles.....	26
1.2.1 Subtypes.....	27
1.2.2 Cargo Loading.....	31
1.2.3 Internalization of EVs.....	32
1.2.4 Liver-derived EVs.....	34
1.3 Rationale, Hypothesis, and Objectives.....	36
Chapter 2: Extracellular vesicles efficiently deliver survival motor neuron protein to cells in culture.....	37
2.1 Abstract.....	39
2.2 Introduction.....	40
2.3 Results.....	43
2.3.1 Development and characterization of a donor cell line for Production of EVs loaded with SMN protein.....	43
2.3.2 Generation of a recipient cell line containing HA-tagged Gemin2.....	49
2.3.3 Enhancing the quantity of 3F-SMN protein in EVs through Ad-vector mediated overexpression in the donor cell line.....	53
2.3.4 EVs can deliver SMN protein to recipient cells.....	57
2.4 Discussion.....	67
2.5 Materials and Methods.....	72

2.6 References.....	81
2.7 Acknowledgements.....	92
Chapter 3: Use of gene therapy to engineer the liver to release extracellular vesicles that distribute a therapeutic protein throughout the body of mice.....	94
3.1 Abstract.....	96
3.2 Introduction.....	97
3.3 Results	
3.3.1 EVs efficiently deliver SMN protein to recipient SMA patient fibroblasts.....	100
3.3.2 Characterization of an Ad vector expressing Cre-activatable bioluminescent SMN fusion protein.....	104
3.3.3 Systemic administration of AdfloxNlucSMNfluc leads to high levels of NlucSMN fusion protein in the liver of Alb-Cre mice.....	108
3.3.4 NlucSMN protein is released from the liver within EVs.....	112
3.3.5 NlucSMN protein is present in peripheral tissues of AdfloxNlucSMNFluc-treated Alb-Cre mice.....	114
3.4 Discussion.....	122
3.5 Materials and Methods.....	128
3.6 References.....	138
3.7 Acknowledgements.....	147
Chapter 4: General Discussion and Conclusions.....	148
4.1 SMN protein-loaded EVs.....	149
4.2 <i>in situ</i> production and systemic distribution of liver-derived protein.....	154
4.3 Conclusions.....	161
General Introduction and Discussion References.....	162
Appendix 1: Authorizations.....	190
Appendix 2: Supplemental Data.....	193
Appendix 3: Published Reviews.....	202
Appendix 3.1: Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles.....	203
Appendix 3.2: Expanding the Availability of Onasemnogene Aboaparvovec to Older Patients: The Evolving Treatment Landscape for Spinal Muscular Atrophy.....	223
Appendix 3.3: Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency.....	236
Appendix 4: Curriculum Vitae.....	251

List of Abbreviations

3D: three-dimensional

3F-SMN: Triple FLAG-tagged SMN protein

AAV: adeno-associated virus

AAV9: adeno-associated virus serotype 9

ACVS: Animal Care and Veterinary Services

Ad: adenovirus

Alb-Cre: B6.Cg-Speer6-ps1^{Tg(Alb-Cre)}32MGN/J transgenic mice

ALIX: ALG-2-interacting protein X

ALS: amyotrophic lateral sclerosis

ARDS: acute respiratory distress syndrome

ARMMs: ARRDC1-mediated microvesicles

ARRDC1: Arrestin domain-containing protein 1

ASO: antisense oligonucleotide

ATCC: American Type Culture Collection

BBB: blood-brain barrier

CBA: chicken beta-actin

CM: conditioned medium

CMV: cytomegalovirus

CNS: central nervous system

Co-IP: co-immunoprecipitation

CRISPR: Clustered Interspaced Short Palindromic Repeats

CSF: cerebrospinal fluid

CTD: carboxy terminal domain

DMD: Duchenne muscular dystrophy

DMEM: Dulbecco's Modified Eagle Medium

DRB: 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole

Dtx: diphtheria toxin

E1: adenovirus early region 1

E3: adenovirus early region 3

EMCV: encephalomyocarditis virus

EMG: electromyography

ENS: enteric nervous system

EWS: ewing's sarcoma

EVs: extracellular vesicles

FBS: fetal bovine serum

Fluc: firefly luciferase

FOXM1: Forkhead Box M1

Fus: fused in sarcoma

GDNF: glial cell-derived neurotrophic factor

Gems: gemini of Cajal bodies

GFP: green fluorescent protein

GMAP: Global Managed Access Program

h: hours

HA: human influenza hemagglutinin
HDA: helper-dependent adenovirus
hpi: hours post-infection
IP: intraperitoneal
iPSCs: induced pluripotent stem cells
IRES: internal ribosome entry site
ISEV: International Society for Extracellular Vesicles
IT: intrathecal
ISS-N1: intronic splicing silencer N1
IV: intravenous
m: minutes
MADD: MAP kinase-activating death domain protein
miRNA: micro ribonucleic acid
MOI: multiplicity of infection
MRI: magnetic resonance imaging
mRNA: messenger ribonucleic acid
MEM: Minimum Essential Medium
MSC: mesenchymal stem cell
MUAP: motor unit action potentials
MUNE: motor unit number estimation
MVBs: multivesicular bodies
MVs: microvesicles
NHP: non-human primate
Nluc: NanoLuc luciferase
NTA: nanoparticle tracking analysis
OA: onasemnogene abeparvovec

PBS: phosphate-buffered saline
PBST: phosphate-buffered saline containing 0.1% Tween 20
PEG: polyethylene glycol
PVDF: polyvinylidene difluoride
RLB: reporter lysis buffer
rRNA: ribosomal ribonucleic acid
s: seconds
scAAV: self-complementary adeno-associated virus
SDS: sodium dodecyl sulphate
SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis
Se: selenium
siRNA: small interfering ribonucleic acid
snRNAs: small nuclear ribonucleic acids
snoRNPs: small nucleolar ribonuclear proteins
snRNPs: small nuclear ribonuclear proteins
SMA: Spinal Muscular Atrophy
SMN: survival motor neuron
SMN1: survival motor neuron 1
SMN2: survival motor neuron 2
SOD1: superoxide dismutase
SV40: simian virus 40
TA: tibialis anterior

TAT: transactivator of transcription

TFF: tangential flow filtration

TGS1: trimethylguanosine synthase 1

TSG101: tumor susceptibility gene
101

TTC: tetanus toxin fragment C

UTR: untranslated region

vp: viral particles

ZPR1: zinc-finger protein

List of Figures

Chapter 1: General Introduction

Figure 1.1 The <i>SMN1</i> and <i>SMN2</i> genes, mRNA transcripts, and protein products.....	4
Figure 1.2 Primary tissue pathology and peripheral tissue defects documented in patients with SMA, and mouse and cell culture models of SMA.....	11
Figure 1.3 Release and internalization of EVs.....	28

Chapter 2: Extracellular vesicles efficiently deliver survival motor neuron protein to cells in culture

Figure 2.1 The presence of a 3xFLAG-tag on SMN protein does not affect protein localization within the cell or ability to interact with normal cellular protein partners.....	45
Figure 2.2 EVs released by HepG2:3F-SMN cells contain 3F-SMN protein.....	48
Figure 2.3 Generation and characterization of an A549 cell line expressing an HA-tagged Gemin2 protein.....	51
Figure 2.4 Adenoviral-mediated overexpression of 3F-SMN protein in HepG2 cells leads to the release of EVs containing high levels of the 3F-SMN protein.....	55
Figure 2.5 Application of conditioned medium containing EVs loaded with 3F-SMN protein onto A549:HA-Gemin2 cells results in FLAG-positive nuclear foci.....	58
Figure 2.6 EV-delivered 3F-SMN protein is internalized by recipient A549:HA-Gemin2 cells and localizes in gem-like structures.....	61
Figure 2.7 3F-SMN protein co-localizes with HA-Gemin2 in nuclear gem-like structures in A549:HA-Gemin2 cells.....	63
Figure 2.8 EV-mediated delivery of mCherry-SMN and 3F-SMN protein to A549 cells and fibroblasts derived from an SMA patient.....	66

Chapter 3: Use of gene therapy to engineer the liver to release extracellular vesicles that distribute a therapeutic protein throughout the body of mice

Figure 3.1 EV-delivered 3F-SMN protein is internalized by recipient fibroblasts and localizes in nuclear gem-like structures.....	102
Figure 3.2 Cre-mediated induction of <i>NlucSMN</i> expression from AdfloxNlucSMN in tissue culture.....	106
Figure 3.3 Administration of AdfloxNlucSMNFluc to Alb-Cre mice leads	

to robust expression of NlucSMN protein in the liver.....	110
Figure 3.4 NlucSMN protein is contained in the EV fraction of blood plasma samples from AdfloxBNlucSMNFluc-treated Alb-Cre mice.....	113
Figure 3.5 Liver-derived production of NlucSMN protein leads to high levels of protein in peripheral tissues.....	115
Figure 3.6 Liver-derived production of NlucSMN protein leads to high levels of the protein in muscle and brain.....	117
Figure 3.7 Visualization of liver-derived SMN protein in the TA muscle of mice using immunofluorescence microscopy.....	119

List of Tables

Chapter 1: General Introduction

Table 1.1 Approved disease-modifying therapies for treatment of SMA.....17

Table 1.2 Diameter and mechanism of biogenesis of extracellular vesicles and particles.....30

Chapter 1: General Introduction

Spinal muscular atrophy (SMA) is a devastating neuromuscular disorder that can be fatal without treatment for patients with severe disease. SMA pathogenesis is caused by low cellular levels of survival motor neuron (SMN) protein. There are several therapies available for treatment of SMA that each focus on increasing cellular levels of SMN protein, however, they are not without limitations. The Parks lab previously showed that SMN protein is naturally contained within extracellular vesicles (EVs). EVs are membrane-bound particles released from virtually all cell types that have gained attention as therapeutic delivery vehicles. Thus, this thesis explores the potential of EVs as delivery vehicles of SMN protein, offering a novel therapeutic strategy for treatment of SMA.

1.1 Spinal Muscular Atrophy

SMA is a genetic neuromuscular disorder characterized by a loss of the alpha motor neurons of the spinal cord and progressive muscle weakness and atrophy¹. Muscles of the trunk and proximal limbs are most affected in SMA and can include those involved in breathing¹. In severe forms of the disease, death occurs due to respiratory distress^{1,2}. The disorder was initially described at the end of the 19th century by Werdnig³ and Hoffmann⁴, and the causative gene was identified 100 years later⁵. SMA has a ~1 in 54 pan-ethnic carrier frequency⁶ and a prevalence of ~1 in 15 000 live births⁷. Several therapies are available for treatment of SMA⁸⁻¹⁰, however there is no cure for the disease.

1.1.1 Genetics

The vast majority of patients (~95%) who have SMA are diagnosed with 5q-dependent SMA (referred to as “SMA” in this text), an autosomal recessive disorder

caused by a homozygous mutation or deletion of the survival motor neuron 1 (*SMN1*) gene¹¹ (Figure 1.1). The *SMN1* gene contains nine exons – 1, 2a, 2b, 3, 4, 5, 6, 7, and 8, the last of which remains untranslated^{5,12}. Transcription of *SMN1* produces *SMN1* messenger ribonucleic acid (mRNA), which is then translated to full-length SMN protein, and thus cellular levels of this protein are depleted in SMA¹¹.

Humans have two paralogous genes present on chromosome 5q13, *SMN1* and *SMN2*, that have a key difference: a C-to-T base-transition at position 840 in *SMN2* relative to *SMN1*^{13,14}. This silent point mutation corresponds to position 6 of exon 7, consequently leading to the majority of *SMN2* mRNA transcripts to skip exon 7, and thus most of the protein produced by *SMN2* is truncated and less stable than full-length SMN protein^{15,16}. Importantly, *SMN2* can produce ~10% of the full-length SMN protein produced by *SMN1*¹⁴, however this level of SMN protein is unable to fully compensate for the loss of *SMN1*. *SMN2* is variably amplified in humans, and higher levels of SMN protein within cells positively correlates with greater copies of *SMN2*^{17,18}. Most likely due to this increased level of SMN protein in cells, more copies of *SMN2* commonly leads to less severe disease for a patient with SMA^{18,19}.

1.1.2 SMN Protein

Full-length SMN protein is 294 amino acids in length, and is present in both the nucleus and cytoplasm of cells (Figure 1.1)^{20,21}. In the nucleus, SMN protein is present in structures called gemini of Cajal bodies (gems). These structures are located adjacent to other nuclear structures called Cajal bodies, which contain a high concentration of spliceosomal small nuclear ribonuclear proteins (snRNPs) and coilin protein²²⁻²⁴. A decrease in cellular levels of SMN protein is associated with a

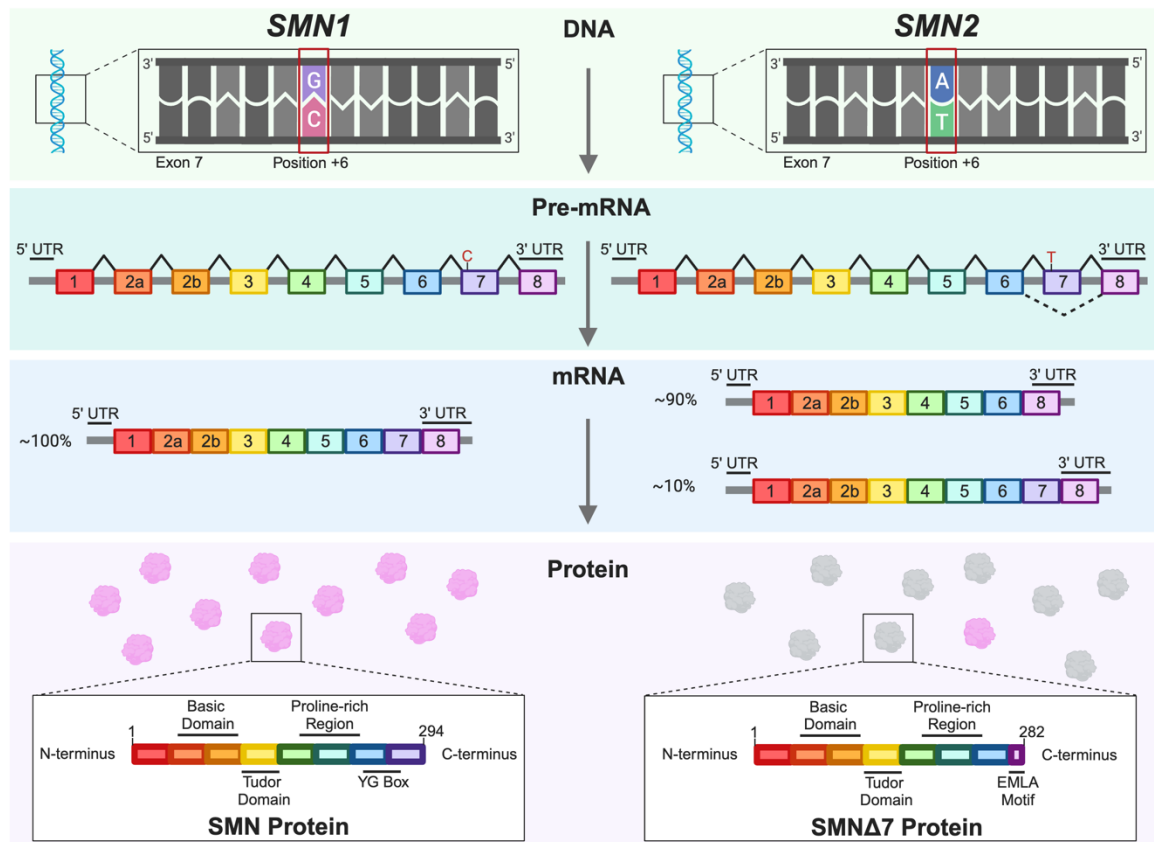


Figure 1.1 The *SMN1* and *SMN2* genes, mRNA transcripts, and protein products. Humans have two highly homologous genes, *SMN1* and *SMN2*. Following transcription of *SMN1*, pre-mRNA is spliced to produce mRNA transcripts containing exons 1, 2a, 2b, 3, 4, 5, 6, 7, and 8. This mRNA, except mRNA from exon 8 which is in the 3' untranslated region (UTR), is translated to produce full-length SMN protein. In contrast, the *SMN2* gene has a critical C-to-T nucleotide transition at position +6 of exon 7. This transition leads to the majority of mRNA transcripts from *SMN2* to be spliced without exon 7, leading to the translation of SMNΔ7 protein, which does not possess the 16 C-terminal amino acids of full-length SMN protein, and has an additional four amino acids (EMLA) translated from exon 8. Figure generated with BioRender. Adapted from Butchbach 2016⁷⁸.

decrease in number of nuclear gems in cells derived from SMA patients^{21,25} and other SMN-depleted cells^{26,27}, and an increase in SMN protein level increases nuclear gem number^{28,29}.

SMN protein self-associates and also associates with Gemin proteins 2-8 and Unrip to form the SMN complex, a multiprotein complex crucial for assembly of the snRNPs essential for pre-mRNA splicing^{14,30-34}. In the cytoplasm, the SMN complex binds to Sm proteins and small nuclear RNAs (snRNAs), then assembles seven Sm proteins in a heptameric ring on the uridine-rich site of snRNAs, forming the building blocks of the snRNPs that make up the spliceosome^{35,36}. The SMN complex is also thought to be involved in small nucleolar RNP (snoRNP) assembly, which are involved in post-transcriptional processing and modification of ribosomal RNA (rRNA) in the nucleolus^{37,38}. SMN protein may also be involved in the biogenesis of telomerase, a RNP complex that elongates telomeres by adding repeat sequences at chromosomal ends³⁹⁻⁴¹. Furthermore, SMN protein has been shown to play a role in numerous cellular functions, such as mRNA transport^{42,43}, translational regulation^{42,44,45}, resolution of R-loops in genetic terminal regions⁴⁶, actin dynamics^{47,48}, endocytosis⁴⁹⁻⁵¹, and mitochondrial function⁵²⁻⁵⁴. Although there are many functions of SMN protein, the precise role of the protein that leads to SMA pathogenesis remains unknown.

SMN protein contains several protein motifs (Figure 1.1). Exons 2a and 2b encode the basic domain, a nucleic-acid binding domain that is the binding site of Gemin2^{55,56}, p53⁵⁷, and COPI⁵⁸ proteins. Downstream of this domain is the Tudor domain, which is encoded by exon 3 and interacts with a large number of proteins

that contain RGG/RG motifs^{59,60}. The Tudor domain interacts with numerous proteins, including coilin⁶¹, histone 3⁶², GAR1³⁷, fused in sarcoma (FUS)⁶³, fibrillarin³⁷, Ewing's sarcoma (EWS)⁶⁴, Sm proteins⁶⁵, and the carboxy terminal domain (CTD) of RNA polymerase II⁴⁶. A proline-rich sequence downstream of the Tudor domain interacts with profilin proteins that are involved in actin dynamics⁶⁶. Close to the C-terminus is the YG box, characterized by a YxxG motif and is encoded by a downstream region of exon 6 and an upstream region of exon 7^{67,68}. This region, along with the 16 most-C-terminal amino acids of SMN protein, facilitates self-oligomerization, which is required for stability and subcellular localization of the protein^{67,69,70}. The C-terminal region of SMN protein interacts with several proteins, including Gemin3⁷¹, zinc-finger protein (ZPR1)⁷², SIN3A⁷³, and trimethylguanosine synthase 1 (TGS1)⁷⁴. Also in the C-terminal region is an amino acid residue that regulates protein stability through phosphorylation⁷⁵, and a QNQKE motif that acts as a nuclear export signal⁷⁶.

The majority of mRNA generated from transcription of *SMN2* lacks exon 7, and is subsequently translated to produce a truncated isoform of SMN protein lacking the region encoded by exon 7, termed SMN Δ 7 protein⁵. This isoform cannot perform all of the functions of full-length SMN protein, and demonstrates impairments in oligomerization⁶⁷ and binding of key protein partners such as Sm proteins⁷⁷. Skipping of exon 7 leads to a EMLA motif encoded by exon 8 to be added to the C-terminus of the protein⁷⁸, which has been shown to act as a degradation signal⁶⁹. Indeed, SMN Δ 7 protein is less stable than full-length SMN protein, with ~50% of the half-life of full-length SMN protein *in vitro*^{16,69}. Both full-length SMN and

SMN Δ 7 proteins are degraded by the ubiquitin-proteasome system, however full-length SMN protein has been shown to be mostly mono- or di-ubiquitinated, whereas SMN Δ 7 protein can be highly ubiquitinated and thus more vulnerable to proteasome degradation⁷⁹. Although 10-15% of healthy people do not have any copies of the *SMN2* gene and thus no SMN Δ 7 protein⁸⁰, the protein is not non-functional. Expression of SMN Δ 7 protein modestly increased survival in mice entirely lacking full-length SMN and Smn proteins, indicating that this protein does have a function⁷⁸. Although the exact function of SMN Δ 7 has yet to be fully elucidated, it is clear that this isoform cannot perform all of the functions of full-length SMN protein.

1.1.3 Clinical Manifestation

1.1.3.1 Classification of SMA

SMA is classified into five types based on age of onset and motor function achieved⁸¹. Skeletal muscle weakness is the common feature of all SMA subtypes. Infants with SMA type 0 represent the youngest of patients, with onset occurring in utero. Life expectancy of patients with SMA type 0 is reduced drastically, with most not surviving past 6 months of age. These infants present with severe hypotonia, weakness, respiratory impairment, areflexia (the absence of deep tendon reflexes), facial septal defects, and joint contractures^{82,83}. SMA type 1, also called Werdnig-Hoffmann disease, is the most common type of SMA, accounting for approximately 50% of SMA diagnoses. Children with SMA type 1 never achieve the ability to sit, and present with severe hypotonia and weakness. The age of onset is between birth and 6 months of age, and most infants succumb to respiratory failure before they reach age two without treatment^{2,84,85}. The onset of SMA type 2 is later, with

symptoms appearing between 7 and 18 months of age. These patients achieve the ability to sit without any assistance, but never acquire the ability to walk due to significant proximal weakness. An individual with SMA type 2 typically survives into adulthood but may have a shortened life span compared to normal⁸¹. SMA type 3, also known as Kugelberg-Welander disease, has an onset after 18 months of age. These individuals are able to sit and walk unassisted early in life, however approximately 50% of people with SMA type 3 who had a disease onset ≤ 12 years of age will lose the ability to walk in their second decade of life⁸⁶. Patients with SMA type 3 typically have a normal lifespan⁸¹. The least severe form of the disease is SMA type 4, which has an adult onset, and presents with weakness and some impairment of daily activities⁸⁷.

In recent years, people living with SMA have been described as “non-sitters,” “sitters,” and “walkers” based on their motor status⁸⁸. The shift from describing patients as SMA type 0-4 to these new classifications is due to changing disease trajectory with the availability of disease-modifying therapies in the last decade⁸⁹. For example, two patients who would have been diagnosed with SMA type 1 may have very different needs based on how well they respond to treatment. A “non-sitter” is someone who has never sat independently, and mainly includes children that fit the SMA type 1 or 2 diagnostic criteria⁸⁸. These patients have muscle weakness in their upper bodies, limbs, and faces, and show no reflexes. “Non-sitters” likely have difficulty breathing and swallowing and may require ventilation. In this group, contractures and scoliosis are common. A “sitter” is a person who can sit independently for at least 10 seconds (s)⁸⁸. People living with SMA type 2 or 3, as

well as patients with SMA type 1 who received treatment early, are commonly members of this group. “Sitters” typically have weak muscles in their upper arms and legs and generally show no reflexes⁸⁸. These patients may develop contractures and scoliosis and may have difficulty breathing. A “walker” is described as someone who can walk unassisted for at least 10 minutes (m) and can represent a patient who would have been diagnosed with SMA type 3 or 4, or patients with SMA types 1 or 2 who were treated pre-symptomatically⁸⁸. These people may experience some difficulty swallowing and breathing, have weak reflexes, and muscle weakness in their upper limbs.

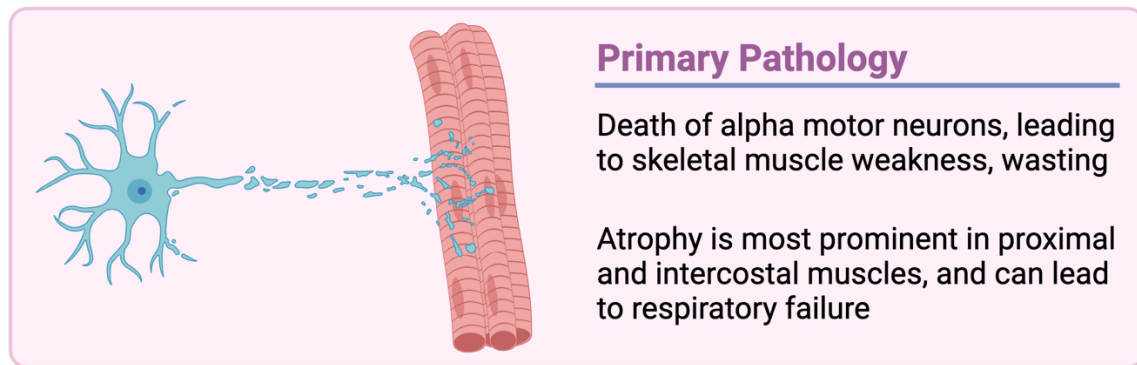
1.1.3.2 SMA as a Multisystem Disease

SMA is characterized by the loss of the alpha motor neurons, leading to skeletal muscle atrophy and weakness⁹⁰. Alpha motor neurons, also called lower motor neurons, have cell bodies located within the ventral horn of the spinal cord and the brain stem, with axons extending outside the central nervous system (CNS) to innervate extrafusal muscle fibres to initiate contraction⁹¹. The proximal and intercostal muscles are most affected in SMA, whereas the diaphragm and distal muscles are less severely affected^{1,90,92}. In severe forms of the disease, intercostal muscle weakness leads to poor clearance of respiratory secretions, decreased lung capacity, nocturnal hypoventilation, and respiratory failure⁹³. Histopathology of tissue collected from patients with SMA reveals atrophic fibres with areas of hypertrophic fibres in the skeletal muscle and a severe loss of motor neurons in the spinal cord⁹⁴⁻⁹⁶. This is coupled with electromyography (EMG) demonstrating changes of fibrillation potentials, indicative of active denervation, and enlarged

motor unit action potentials (MUAPs), in addition to low motor unit number estimation (MUNE) scores, indicating alpha motor neuron loss^{94,97-99}. Additionally, repetitive nerve stimulation studies have demonstrated neuromuscular junction dysfunction in patients with SMA, which can cause muscle weakness and fatigue¹⁰⁰⁻¹⁰². Furthermore, there are many peripheral tissues defects that have been noted in preclinical models with low SMN protein levels, and in clinical studies (Figure 1.2).

Defects in the brain have been noted in severe models and cases of SMA. A study of three infants with SMA type 0 documented progressive, supratentorial brain atrophy characterized by severe white matter reduction and hippocampal atrophy visible by magnetic resonance imaging (MRI)¹⁰³. Brains of a severe mouse model of SMA that have very low levels of SMN protein were significantly reduced in size and weight compared to healthy littermates at a symptomatic stage¹⁰⁴. Similar to observations in humans, hippocampus size and cell density within the hippocampus of affected mice were significantly decreased compared to healthy littermates. A study that examined the brains of patients with SMA types 1, 2, and 3 found that 43% of these patients had macrostructural brain abnormalities compared to 10% of healthy controls¹⁰⁵. The most common structural abnormalities were supratentorial ventriculomegaly and widening of arachnoid spaces, and patients with SMA type 1 had the highest rate of these abnormalities and the largest lateral ventricle volume. A similar study reported white and grey matter abnormalities in patients with SMA type 2 or 3 compared to healthy controls¹⁰⁶.

Cardiac defects have been noted in several mouse models of SMA. Severe mouse models of SMA have exhibited arrhythmia, bradycardia, dilated cardiomyo-



Peripheral Tissue Pathology

Lung

Emphysema^M, ruptured alveolar septa^M, enlarged alveolar space^M

Heart

Septal defects^H, dilated cardiomyopathy^M, decreased contractility^M, bradycardia^M, interstitial fibrosis^M, arrhythmia^M

Liver

Dyslipidemia^{H,M}, steatosis^{H,M}

Pancreas

Pancreatic cell imbalance^{H,M}, hyperglycemia^{H,M}, gene dysregulation^C

Intestines

Constipation^H, delayed gastric emptying^{H,M}, shorter bowel and small villi^M

Testes

Cryptorchidism^H, infertility^{H,M}, degenerated seminiferous tubules^M

Vasculature

Digital necrosis^H, vascular thrombosis^H, tissue hypovascularity^H, ear and tail necrosis^M

Brain

Structural brain abnormalities^H, reduced hippocampus size^{H,M}

Thymus

Abnormal cytokine profile^M, reduced size^M

Skeletal Muscle

Defects in myoblast fusion and myotube morphology^C

Spleen

Congested red pulp^H, accessory spleens^H, reduced red and/or white pulp^M, reduced size^M

Kidney

Medullary calcification and interstitial fibrosis^H, glomerular and tubular abnormalities^H, reduced size and nephron number^M

H: Humans
M: Mice
C: Human cell culture

Figure 1.2 Primary tissue pathology and peripheral tissue defects documented in patients with SMA, and mouse and cell culture models of SMA. SMA is characterized by the death of alpha motor neurons, which leads to skeletal muscle weakness and atrophy. Defects in tissues of the lungs, heart, liver, pancreas, intestines, testes, vasculature, spleen, skeletal muscle, thymus, and brain have been documented in patients with SMA and models of the disease. Figure generated with BioRender. Adapted from Yeo and Darras (2020)⁹⁹.

pathy, decreased cardiac contractility, interstitial fibrosis, and an overall decrease in cardiac function¹⁰⁷⁻¹¹⁰. In a study of 65 infants with SMA, three of four (75%) patients with 1 copy of *SMN2* had atrial or ventricular septal defects, whereas no major cardiac defects were noted in infants with 2 or 3 copies of *SMN2*¹¹¹.

The liver has also been shown to be impacted in SMA. Patients diagnosed with SMA are susceptible to developing dyslipidemia and fatty liver (steatosis)^{112,113}. In some cases, steatosis has been shown to persist in patients with SMA types 1, 2, or 3 who are taking therapeutics for SMA¹¹⁴. Similarly, mouse models of SMA have also exhibited dyslipidemia and steatosis^{112,115}. Hepatocyte-like cells derived from SMA patient induced pluripotent stem cells (iPSCs) also showed increased lipid accumulation, and dysregulation of genes implicated in several processes, including those involved in metabolic function, lipid metabolism, gluconeogenesis, drug metabolism, and fatty acid oxidation¹¹⁴.

Respiratory complications are common in patients with SMA due to weakness of respiratory muscles¹¹⁶, however lung tissue may also be impacted by low levels of SMN protein. Lung tissue of a severe mouse model of SMA exhibited emphysema, ruptured alveolar septa, and enlarged alveolar spaces compared to control¹¹⁷.

Lymphoid organs have also been demonstrated to be affected in SMA. In a severe mouse model of SMA, the spleen is significantly smaller, is missing a capsule, lacks white pulp, and has significantly fewer B lymphocytes than healthy littermates¹¹⁸. In this study, low levels of B lymphocytes in the spleen were accompanied by low circulating levels of B lymphocytes. In a similar study, the

spleens of a less severe SMA mouse model were also smaller than control and exhibited little white pulp and a mislocalization of immune cells¹¹⁹. In these mice, the thymus was overall smaller than control and had a much smaller cortex relative to medulla. Both the thymus and spleen had abnormal cytokine profiles in this model. Another study found that red pulp macrophages in the spleen, which are involved in iron metabolism and phagocytosis of pathogens in the blood, were significantly reduced in mouse models of SMA¹²⁰. Several abnormalities have been noted in spleens of patients with SMA type 1, including accessory spleens and congested red pulp¹¹⁸.

Structure and function of the kidney may also be impacted in SMA. In patients with SMA type 1, findings of medullary calcifications along collecting ducts and medullary interstitial fibrosis were reported, along with glomerular and tubular changes in some patients¹²¹. Granular casts and amorphous crystals present in the urine of these patients are indicative of kidney injury and suggests these patients had impaired kidney function. Children with SMA type 1 have also been identified as having an increased risk of kidney stones (nephrolithiasis)¹²². In a severe mouse model of SMA, kidneys were smaller, with significantly reduced nephron number and renal vasculature, and exhibited dysregulation of nephrin and collagen IV¹²³. Levels of glial cell-derived neurotrophic factor (GDNF), required for nephron development, were significantly reduced in affected mice compared to control.

Glucose metabolism and pancreatic defects have been documented in a mouse model of SMA. Compared to wild type mice, these mice have an increased number of glucagon-producing α -cells and decreased number of insulin-producing

β -cells, and consequently display fasting hyperglycemia, hyperglucagonemia, and glucose resistance¹²⁴. Interestingly, heterozygous littermates of this SMA mouse model that do not present with neuromuscular pathology display an increased number of β -cells, hyperinsulinemia, and weight gain compared to wild type mice following a high fat diet, indicating that even mildly reduced levels of Smn protein can have an impact on the pancreas¹²⁵. In patients with SMA type 1, abnormal islet composition has been reported, where islets were overall disorganized and/or had an increased number of α -cells¹²⁴. Greater prevalence of hyperglycemia and insulin resistance have been reported in both pediatric and adult patients with SMA compared to the general population¹²⁶⁻¹²⁸

In mouse models of SMA, gastrointestinal and enteric nervous system (ENS) dysfunction has been reported. These animals exhibited constipation, delayed gastric emptying, slow intestinal transit, and reduced colonic motility, which was likely caused by observed disrupted ENS signalling to smooth muscle of the colon¹²⁹. Another study of an SMA mouse model demonstrated shorter bowel and small intestine villi lengths compared to control, in addition to decreased vascular density and increased enteric neuron number in the small intestine¹³⁰. Impaired feces excretion and reduced fibre size of the colon smooth muscle cells have also been identified in a mouse model of SMA¹³¹. Patients with SMA commonly experience a variety of gastrointestinal complications, including delayed gastric emptying, constipation, abdominal distention, and gastroesophageal reflux^{132,133}.

In SMA, atrophy of skeletal muscle is known to be caused by denervation following the death of alpha motor neurons that have low cellular levels of SMN

protein⁹⁴⁻⁹⁶, however there may be additional roles for SMN protein in skeletal muscle function. In tissue culture, depletion of SMN protein has caused defects in myoblast fusion and myotube morphology^{134,135}. In a mouse model where Smn protein was depleted exclusively in the skeletal muscle, these mice had damaged myofibers and poor motor function, but no decrease in spinal cord motor neuron numbers compared to control mice¹³⁶. Muscle weakness present before overt motor neuron loss muscle denervation has been noted in mouse models of SMA¹³⁷. These data suggest an intrinsic dysfunction in skeletal muscle caused by low levels of SMN protein.

Case studies of vascular defects in SMA patients have been reported. In one such report, two patients with SMA type 1 developed digital necrosis¹³⁸. Another study similarly reported digital necrosis in two infants with SMA type 1, one of which also had vascular thrombosis¹³⁹. Significantly lower retinal branching vessel complexity and generalized microvasculopathy has been reported in children with SMA type 2 or 3 compared to healthy children, however this had no impact on visual acuity¹⁴⁰. Hypovascularity has been documented in muscle biopsies from SMA patients and spinal cord and muscle tissue from a mouse model of SMA¹⁴¹. Ear and tail necrosis are common phenotypes in mouse models of SMA, which persist following SMN protein restoration in some cases¹⁴²⁻¹⁴⁶.

Male reproductive organ development and fertility are also impacted in SMA. Men with adult-onset SMA are far more likely to receive a diagnosis of testicular hypofunction or infertility than the male general population¹⁴⁷. Undescended testes (cryptorchidism) have been reported in patients with SMA type 1 or 2 at a far greater

incidence than the general male population^{148,149}. Impaired testis development, low sperm count, reduced fertility, and degenerated seminiferous tubules have been reported in a mild mouse model of SMA¹⁵⁰.

Taken together, these studies clearly show that low levels of SMN protein are associated with defects in numerous peripheral tissues, and it is possible more tissue and cell types will be demonstrated to be impacted by low SMN protein levels in the future. Thus, restoring SMN protein levels in cells other than motor neurons is likely required for optimal therapeutic efficacy.

1.1.4 Therapeutics for SMA

Several therapeutics have been developed and approved for treatment of SMA, each focusing on increasing cellular levels of SMN protein¹⁵¹⁻¹⁵³ (Table 1.1). The availability of these therapies has changed the natural history of SMA, and some patients have far surpassed their projected lifespan^{154,155}. Although the three available therapeutics have the same goal of increasing cellular SMN protein concentration, each has a different mechanism of action and thus unique strengths and weaknesses.

1.1.4.1 Nusinersen

Nusinersen, marketed as Spinraza, was the first drug approved by the FDA for treatment of SMA⁹. Nusinersen is an antisense oligonucleotide (ASO) that is administered intrathecally (IT) through a series of four loading doses over the course of two months, followed by maintenance doses every four months^{156,157}. Nusinersen is a modified 2'-O-methoxyethyl ASO on a phosphate backbone that targets the intronic splicing silencer N1 (ISS-N1), an inhibitory element that is present down-

Table 1.1 Approved disease-modifying therapies for treatment of SMA.

Name	Formulation	Mechanism of Action	Dose	Delivery Method
Nusinersen (Spinraza)	Antisense oligonucleotide (ASO)	Targets intronic splicing silencer N1 to promote incorporation of exon 7 in <i>SMN2</i> mRNA	12 mg in 5 ml volume	Intrathecal infusion of 4 loading doses over 2 months, followed by maintenance dose every 4 months
Risdiplam (Evysdi)	Small molecule	Stabilizes 5' splice site on <i>SMN2</i> mRNA to promote inclusion of exon 7	0.15 mg-0.25 mg/kg up to 5 mg maximum	Daily oral administration
Onasemnogene AAV9 (Zolgensma)	Adeno-associated virus serotype 9 (AAV9) gene therapy vector	Delivers functional <i>SMN1</i> cDNA	1.1×10^{14} vp/kg in 2.0 vg/ml solution	Single intravenous infusion

stream of the 5' splice site in exon 7 of *SMN2*^{156,158}. Inhibition of ISS-N1 promotes the inclusion of exon 7 during *SMN2* pre-mRNA splicing, ultimately increasing production of full-length SMN transcript and protein¹⁵⁶⁻¹⁵⁹. IT administration of the drug is required as the ASO formulation cannot cross the blood-brain barrier (BBB)¹⁴². In preclinical studies, survival and motor function were increased in several mouse models of SMA^{142,159-161}. At present, all patients receive the same dose of nusinersen (12 mg of the ASO in a 5 ml volume), regardless of their severity of disease, age, weight, or height¹⁶².

In the ENDEAR¹⁵⁷ (NCT023192074) and CHERISH¹⁶³ (NCT02292537) Phase 3 clinical trials, the safety and efficacy of 12 mg doses of nusinersen were examined in symptomatic children with SMA aged ≤ 7 months and 2 – 12 years old, respectively. Following 13 months of treatment in the ENDEAR trial, 51% of infants treated with nusinersen demonstrated clinically meaningful improvements in motor function, compared with 0% in the control group¹⁵⁷. In this study, infants who had a shorter disease duration were more likely to have improvements in motor function. Similarly, 57% of children who received nusinersen in the CHERISH trial achieved a clinically meaningful change in motor function compared to 27% in the control group¹⁶³. The efficacy of nusinersen was most clear in the Phase 2 NURTURE clinical trial, in which treatment was initiated at ≤ 6 weeks of age, prior to the onset of symptoms¹⁶⁴. All children achieved sitting without support, and 88% achieved walking independently. This study further emphasized the need for inclusion of SMA on newborn screening panels. Importantly, nusinersen was determined to be well

tolerated in these trials, with most of the adverse events determined to be likely related to lumbar puncture, which included vomiting and headache^{157,163,164}.

Although nusinersen is well tolerated overall, use of ASOs like nusinersen comes with some associated risks, such as renal toxicity, thrombocytopenia and coagulation abnormalities¹⁶². Recently, ASOs delivered into the cerebrospinal fluid (CSF) have been linked to neurotoxicity in mice caused by changes in intercellular levels of calcium¹⁶⁵. In adult patients with SMA, treatment with nusinersen has demonstrated stabilization of disease progression and even clinically meaningful improvements in motor function in some patients^{166,167}. However, a meta-analysis of patients with SMA type 2 or 3 has reported ~10% of patients experience a decline in clinical status following treatment with nusinersen¹⁶⁸. Although nusinersen is not an effective treatment for all patients, the drug is overall considered to be reasonably safe, reasonably effective, and is available for a wide age group, with patients between 3 days and 80 years old having received the drug¹⁶⁹. However, nusinersen is designed to increase levels of full-length SMN transcript and protein in the CNS, and thus cannot correct defects caused by low cellular levels of SMN protein in many tissues.

1.1.4.2 Risdiplam

Risdiplam, marketed as Evrysdi, is a small molecule splicing modifier available to treat patients of all ages with SMA^{10,170}. Similar to nusinersen, the drug acts on *SMN2* to increase production of full-length SMN protein, however the small molecule formulation allows risdiplam to cross the BBB and increase SMN protein levels in both the CNS and peripheral tissues^{153,171}. Risdiplam promotes the inclusion of exon

7 in *SMN2* mRNA transcripts through stabilization of the U1 snRNP on the 5' splice site of exon 7 by binding directly to the intermolecular RNA helix, thus increasing cellular levels of full length SMN protein¹⁷². In a mouse model of SMA, intraperitoneal (IP) injection of risdiplam daily at doses ranging 0.1 – 3 mg/kg led to a dose-dependent increase in SMN protein levels in brain and muscle tissue, and a dose-dependent increase in survival and body weight¹⁷¹.

Risdiplam is taken by patients as an oral solution once daily, where the dose is based on patient age and weight at 0.15 mg – 0.25 mg/kg up to 20 kg, at which point 5 mg is the maximum recommended dose for all patients, independent of weight or age¹⁷³. Following 24 months of treatment with risdiplam in infants with symptomatic SMA in the Phase 2/3 FIREFISH clinical trial (NCT02913482), 83% of infants were alive without the need for permanent ventilation, and 44% were able to sit independently¹⁷⁴. The safety and efficacy of risdiplam was also examined in people with SMA types 2 or 3 aged 2-25 years old in the Phase 2/3 SUNFISH clinical trial (NCT02908685). Following 24 months of treatment, 58% of patients showed stabilization of motor function and 32% showed a clinically meaningful improvement following 24 months of treatment¹⁷⁵. In this study, adverse events included pyrexia, diarrhea, and rash. Overall, the treatment was determined to be safe and well tolerated. Similar to nusinersen, the efficacy of risdiplam is most clear when administered to pre-symptomatic infants ≤ 6 weeks old, as investigated in the Phase 3 RAINBOWFISH (NCT03779334) clinical trial. In the interim analysis, all infants treated with risdiplam were alive at 12 months without permanent ventilation and maintained their swallowing and feeding abilities¹⁷⁶. Furthermore, most infants

gained new motor milestones within the normal development window. No treatment-related serious adverse events were identified, although 92% of participants did experience adverse events, none lead to discontinuation of treatment.

The mechanism of action of risdiplam makes other genes vulnerable to off-target alternative splicing, unlike nusinersen, which is designed to only target *SMN2* pre-mRNA. In animal models, risdiplam has been associated with alternative splicing of off-target genes Forkhead Box M1 (*FOXM1*) and MAP kinase-activating death domain protein (*MADD*), which are associated with the cell cycle and apoptosis, respectively¹⁷¹. *FOXM1* is thought to play a key role during spermatogenesis, as it is highly expressed within spermatocytes during prophase in meiosis I¹⁷⁷. Thus, alternative splicing of *FOXM1* could potentially interfere with the production of mature sperm¹⁷⁸. In rats who received doses of risdiplam up to almost 9 mg/kg/day for two to 26 weeks, several animals exhibited an increase in degenerated spermatocytes and seminiferous tubule degeneration, which advanced to atrophy or necrosis in some cases¹⁷⁹. These adverse effects were reversible upon discontinuation of treatment in some but not all animals. The same study examined the effect of risdiplam in cynomolgus macaques, where 0.8 mg/kg/day for 39 weeks, or 6 or 10 mg/kg/day for up to 14 days lead to seminiferous tubule degeneration in all treated animals, which was reversible in most cases. Together, these studies demonstrate that risdiplam is overall well-tolerated and can provide stabilization or improvements in motor function in a subset of patients, however there are some adverse effects related to treatment.

1.1.4.3 Onasemnogene abeparvovec

Onasemnogene abeparvovec (OA), marketed as Zolgensma, is another disease-modifying therapy for SMA, although this drug does not act on *SMN2* like nusinersen and risdiplam. OA is a self-complementary adeno-associated virus serotype 9 (scAAV9) gene therapy vector containing *SMN1* cDNA under control of a hybrid cytomegalovirus (CMV) enhancer-chicken beta-actin promoter (CBA)¹⁵². The CBA promoter driving transgene expression is able to provide ubiquitous expression, allowing SMN protein levels to increase in the CNS and peripheral tissues following IV administration^{180,181}. Preclinical studies of an AAV9 vector encoding a CBA-driven *GFP* demonstrated that the vector is able to transduce motor neurons following intravenous (IV) administration in neonatal mice, although astrocytes were predominantly transduced in adult mice¹⁸². The critical preclinical study that demonstrated the efficacy of scAAV9-CBA-SMN has since been retracted^{143,183}, however many other studies have illustrated rescue in survival and motor function, in addition to other pathologies, provided by treatment^{181,184,185}.

Initially, the safety and efficacy of OA was examined in the phase 1 START trial (NCT02122952), which treated symptomatic infants <8 months old with SMA type 1¹⁸⁶. Infants received either a low- (6.7×10^{13} viral genomes (vg)/kg) or a high-dose (1.1×10^{14} vg/kg) of OA by IV infusion. All 15 infants in this study were alive without the need for mechanical ventilation at 20 months of age, a stark improvement from the 8% survival rate observed in the historical cohort. Increase in survival was accompanied by an overall increase in motor function scores compared to baseline in the high-dose cohort. The infants in the high-dose group were alive without the need for permanent ventilation and maintained previously achieved

motor milestones at up to 7.5 years post-dosing, demonstrating the longevity of OA^{154,155}.

Following the success of the START trial, the Phase 3 STR1VE-US (NCT03306277) and STR1VE-EU (NCT03461289) trials examined the efficacy of the 1.1×10^{14} vg/kg dose of OA in children less than 6 months of age diagnosed with SMA type 1^{187,188}. In these trials, over 90% of infants survived free of permanent ventilation at 14 months, compared to 26% in the natural history cohort, and approximately half achieved independent sitting at 18 months, a milestone that was not achieved in the natural history cohort. Similar to observations in clinical trials of other therapeutics for SMA, early initiation of treatment with OA was demonstrated to be beneficial in the Phase 3 SPR1NT trial (NCT03505099), which treated pre-symptomatic infants less than 6 weeks old^{189,190}. At 14 months post-dosing, all infants survived free of permanent ventilation. Importantly, all children were able to sit independently within the normal development window, and all children with 3 copies of *SMN2* were able to walk within the normal development window. However, 90% of patients treated with OA have elevated serum aminotransferases and/or bilirubin, which can be indicative of liver injury, but can be managed with corticosteroids¹⁹¹.

Overall, a favourable risk-benefit profile was demonstrated in these ground-breaking studies¹⁸⁷⁻¹⁹⁰. However, these trials examined the safety of OA in infants weighing <8.5 kg. Findings from the Global Managed Access Program (GMAP) indicated that the safety profile of treatment with OA in patients with SMA weighing ≥8.5 were consistent with previous safety data¹⁹². In contrast, a recently published

case study of two children diagnosed with SMA type 1 that weighed 20 kg at the time of OA administration highlighted the need to consider the risk-benefit profile of AAV gene therapy in heavier patients¹⁹³. These children were both previously treated with nusinersen, however chose to switch to a different treatment modality following difficulties related to IT administration. Both patients required treatment with steroids to control elevated transaminases, and initially exhibited improvements in motor function, which declined to baseline level for one patient and below baseline level for the other, although it is unclear why this change occurred.

Indeed, there are safety concerns with OA administration in older patients, as the approved therapeutic dose has been calculated based on weight, and consequently larger patients need greater absolute doses of vector. Following IV infusion, much of the vector accumulates in the liver and peripheral tissues. Unfortunately, the high dose of AAV required for CNS transduction can cause serious adverse events, including acute liver failure in large animal models¹⁹⁴ and in human patients¹⁹¹, and thrombotic microangiopathy in human patients¹⁹⁵. Use of high dose AAV also raises other safety concerns, as AAV can insert in the genome of the cell, which has been associated with hepatocellular carcinoma in humans¹⁹⁶ and mice¹⁹⁷⁻¹⁹⁹, although not in all studies²⁰⁰⁻²⁰³. In short, although AAV-mediated gene therapy can be very effective, it does not come without risk.

To reach a wider patient population, IT administration of OA has been explored in the Phase 1 STRONG trial (NCT03381729) as a method to provide a more direct transduction of the CNS, potentially resulting in transduction of motor neurons with lower absolute doses of vector, as reviewed in René and Parks

2023²⁰⁴. Overall, the investigators of the STRONG trial concluded that IT OA was safe and well-tolerated in this study and demonstrated efficacy for children 2 to 5 years old²⁰⁵. Unfortunately, OA has yet to be explored in an adult population, which represents 50-60% of people living with SMA²⁰⁶. Taken together, these studies demonstrate the efficacy of OA as a therapeutic for SMA, although there are concerns related to safety of the drug and the unmet need to treat a wide age range of patients with SMA.

1.1.4.4 Combination Therapy

As not all patients show a full response to therapy, the safety and efficacy of combination therapy are being explored in clinical trials. One such study is the RESPOND trial (NCT04488133), which is investigating the use of 12 mg doses of nusinersen in children ≤ 36 months old who previously were treated with OA. Similarly, the JEWELFISH clinical trial (NCT03032172) is investigating the safety and efficacy of risdiplam in patients with SMA who previously were treated with another disease-modifying therapy, including OA. Following 24 months of treatment with risdiplam, all children previously treated with OA experienced at least one adverse event, and no serious adverse events related to risdiplam²⁰⁷. All of these patients had an increase in SMN protein levels in the blood and an increase in motor function was reported at month 24 compared to baseline. Overall, combination therapy may be a better therapeutic option for patients with SMA who have a suboptimal response to monotherapy.

Indeed, many patients have suboptimal clinical responses to the currently available disease-modifying therapies. Nusinersen and risdiplam both act on the

SMN2 gene, and a recent *in vitro* study has demonstrated that some patients respond better to one drug compared to the other, as assessed by the amount of full-length SMN transcript produced²⁰⁸. The same study reported that the amount of full-length SMN protein that is produced by cells following treatment with either nusinersen or risdiplam is independent of how many copies of *SMN2* are present. Thus, *SMN2* may not be a predictable target for SMA therapeutics. Furthermore, AAV9 vectors, including OA, remain episomal following internalization by the cell, and therefore SMN protein produced by OA will be diluted out of dividing cells over time²⁰³. Consequently, OA may not be able to correct pathology caused by low levels of SMN protein in peripheral tissues over long periods of time. Thus, delivering SMN protein to recipient cells, as opposed to using gene replacement or gene-targeting strategies, may be an ideal therapeutic approach to treat SMA. A potential method to deliver SMN protein is through the use of extracellular vesicles (EVs), membrane-bound particles that naturally contain SMN protein^{209,210}. EVs are released and taken up by practically all cell types²¹¹, and thus could be used to replenish SMN protein levels in a wide variety of tissues in patients with SMA.

1.2 Extracellular Vesicles

EVs are small, membrane-bound particles released from virtually all cell types²¹¹. Initially, EVs were thought to be involved only in the elimination of cellular waste²¹², but these particles perform a wide variety of functions²¹³. EVs transport proteins, lipids, and nucleic acids between cells as a method of communication²¹⁴. The composition of biomolecules within EVs and the physiological function of the

vesicles are determined by the type and physiological state of the cell from which the EVs are secreted²¹¹.

EVs have gained attention as a therapeutic delivery platform as the particles have many features that make them attractive as therapeutic vehicles in addition to their innate ability to shuttle cargo between cells. EVs can deliver cargo to recipient cells situated at a distance from where the EVs originate and can transit the BBB²¹⁵⁻²¹⁸. The lipid bilayer membrane of EVs protect the encapsulated cargo from enzymatic degradation, further solidifying their utility as therapeutic delivery vehicles^{219,220}. Also, it has been demonstrated in many studies that EVs administered to an allogeneic recipient induce minimal immunogenicity *in vitro* or *in vivo*²²¹⁻²²³. Some EVs can target specific cell types for uptake through proteins presented on the EV surface and a complementary “receptor” on the surface of the desired cell²¹⁷, and it is possible to engineer EVs to target a specific cell type^{224,225}.

1.2.1 Subtypes

The term EV refers to a heterogeneous group of particles that are released from cells, encompassing many different subtypes that are classified on the basis of biogenesis and size. The most extensively researched subtypes are exosomes, microvesicles (MVs), and apoptotic bodies (Figure 1.3).

Exosomes are formed through invagination of the membrane of the early endosome, giving rise to multivesicular bodies (MVBs), and are released by the cell after the MVB fuses with the plasma membrane of the cell^{213,226,227}. Exosomes have a diameter of approximately 40 – 150 nm. As opposed to originating within the endosomal network like exosomes, MVs are generated by budding at the plasma

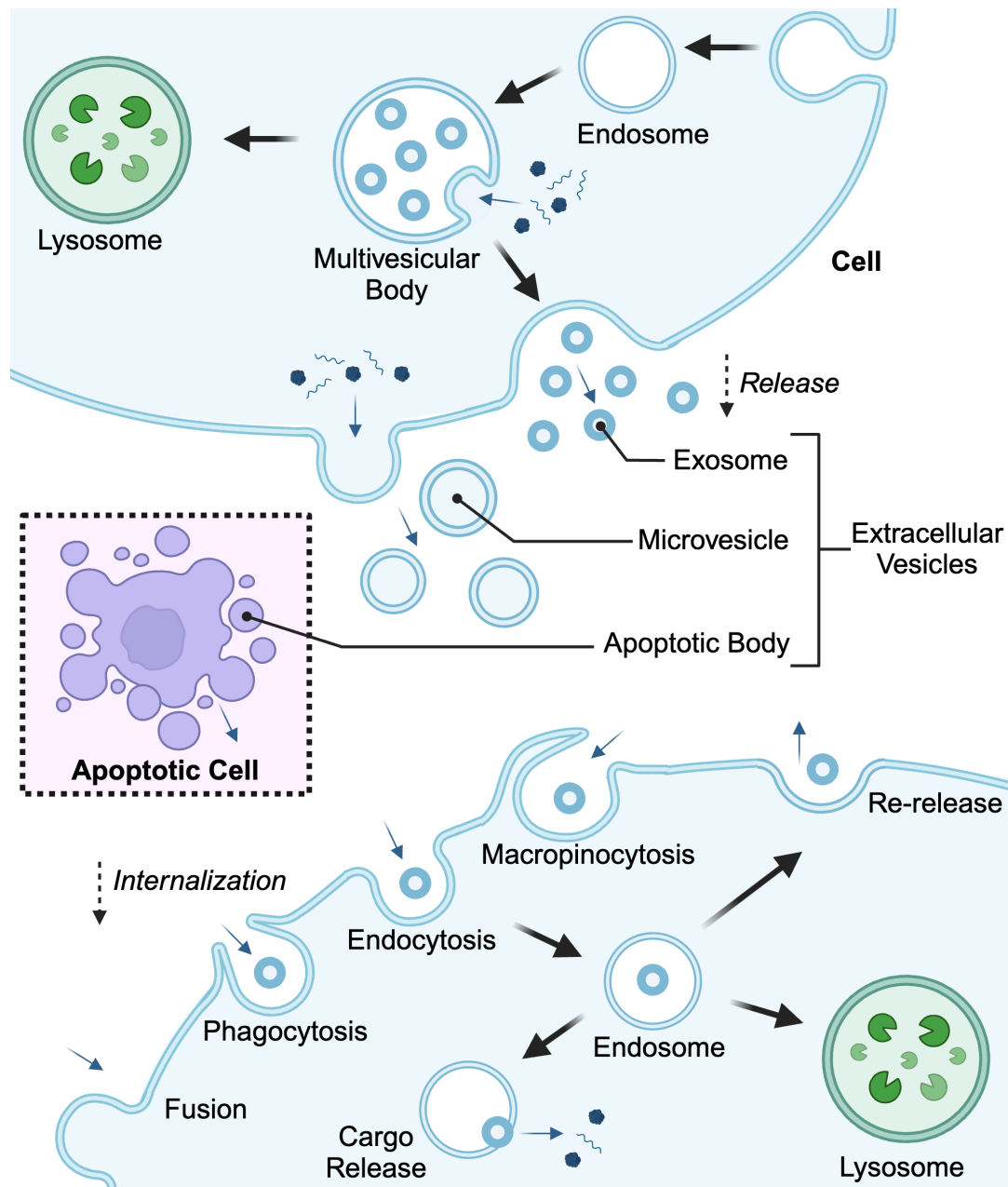


Figure 1.3 Release and internalization of EVs. Exosomes are formed through invagination of the endosome, and are released when the MVB fuses with the cell membrane, or degraded upon fusion of the MVB with the lysosome. MVs are released following budding of the plasma membrane of the cell. Apoptotic bodies are released following blebbing of apoptotic cells. These EVs can be internalized by recipient cells by fusing with the cell membrane, phagocytosis, macropinocytosis, or endocytosis. An EV contained within the endosome must fuse with the membrane of the endosome to release vesicular cargo into the cytoplasm. Alternatively, the endosome can fuse with the lysosome, degrading the EV, or with the cell membrane, re-releasing the EV. Figure is not to scale. Figure generated with BioRender.

membrane of the cell, and are approximately 40 – 1000 nm in diameter^{211,227,228}. The biogenesis of exosomes and MVs are explained in greater detail in René and Parks 2024²²⁹. Apoptotic bodies are generated through blebbing from the plasma membrane of dying cells, and can be 1000 – 5000 nm in diameter, however, vesicles released by apoptotic cells can be as small as 100 nm^{227,230}.

In addition to these three main subtypes, there are several other EV subtypes that have been identified (Table 1.2). Large oncosomes are even larger than apoptotic bodies at ~1000 – 10,000 nm in diameter^{231,232}. Large oncosomes are released from large protrusions of the plasma membrane of non-apoptotic cancer cells, and may play a role in tumor progression^{231,232}. Exophers are another large type of EV, with a diameter of ~4000 nm²³³. These vesicles bud off of the plasma membrane like microvesicles, and at least part of their function is to remove dysfunctional organelles and damaged, dysfunctional, and/or aggregated proteins²³³⁻²³⁵. Migrating cells generate another subclass of EVs called migrasomes on the ends of long, tubular strands called retraction fibres that trail behind motile cells²³⁶. Migrasomes are ~500 – 3000 nm in diameter, filled with vesicles ~50 – 100 nm in diameter, and are rich in cytokines, chemokines, and growth factors^{236,237}. A much smaller subclass of EVs has also been identified called Arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs). These vesicles are 30 – 80 nm in diameter and are generated by budding at the plasma membrane through interaction between ARRDC1 and tumor susceptibility gene 101 (TSG101)^{228,238}.

In addition to these identified subtypes of EVs, a few types of non-membrane

Table 1.2 Diameter and mechanism of biogenesis of extracellular vesicles and particles.

Subtype	Mechanism of Biogenesis	Approximate Diameter
Exosomes	Within the endosomal network	40-150 nm
Microvesicles (MVs)	Budding at cell plasma membrane	40-1000 nm
Apoptotic bodies	Blebbing of apoptotic cell plasma membrane	100-5000 nm
Large oncosomes	Released from protrusions of cancer cell membrane	1000-10,000 nm
Exophers	Budding at cell plasma membrane	4000 nm
Migrasomes	At retraction fibres of migrating cells	500-3000 nm
Arrestin domain-containing protein 1 (ARRDC1)-mediated microvesicles (ARMMs)	Budding at the plasma membrane mediated by interaction between ARRDC1 and TSG101 proteins	30-80 nm
Exomeres	Unknown	≤50 nm
Supermeres	Unknown	≤35 nm

bound extracellular particles have been described. One such type are exomeres, which are very small particles, with a diameter ≤ 50 nm^{239,240}. The process by which these particles are formed has yet to be elucidated. Supermeres, another type of extracellular particle, are even smaller, with a diameter ≤ 35 nm^{241,242}. Like the exomeres, the biogenesis of these particles remains unclear.

Clearly, there are many subtypes of EVs, and these subtypes can have overlapping particle diameter and common cargo content, making it difficult to differentiate between particle types²⁴³. In the absence of observing the biogenesis of the particle in question (i.e. whether the vesicle arises from fusion of the MVB with the plasma membrane or is formed through budding at the cell membrane), it is more accurate to refer to EVs as either small (≤ 200 nm) or large (> 200 nm) EVs.

1.2.2 Cargo Loading

EVs naturally contain a wide variety of proteins, lipids, and nucleic acids, and the composition of these biomolecules can depend on the physiological state and type of cell releasing the EVs, and the subtype of EVs being released²⁴³. The cargo innately carried by EVs may be desirable for some therapeutic contexts, such as in the case of mesenchymal stem cell (MSC)-derived EVs for treatment of COVID-19-induced acute respiratory distress syndrome (ARDS). MSCs have been explored in clinical trial as a potential therapeutic for COVID-19 symptoms as these cells can inhibit production of pro-inflammatory cytokines that can lead to a cytokine storm through modulation of the immune response²⁴⁴⁻²⁴⁷. As MSC-derived EVs retain many of the beneficial properties of MSCs due to their cargo content^{248,249}, these EVs have demonstrated benefit as a treatment for COVID-19-induced ARDS^{250,251}.

In addition to utilizing cargo naturally contained within EVs for therapeutic benefit, it is also possible to load EVs with cargo of interest using either exogenous²⁵²⁻²⁵⁴ or endogenous^{210,255} cargo loading methods. In exogenous cargo loading, cargo is loaded after EVs are harvested from a biofluid. There are many different methods that can be used to exogenously load cargo into EVs, which include electroporation, sonication, freeze-thaw cycles, extrusion, and the use of pore-forming agents^{254,256-258}. Many of these methods rely on transient disruption of the EV membrane to load the cargo, which can result in permanent damage of the membrane. Alternatively, in endogenous cargo loading, cargo is incorporated into EVs during their biogenesis^{210,255}. One common way is to overexpress a protein of interest within cells, which release EVs loaded with this protein. For example, the Parks lab has demonstrated plasmid- or adenovirus (Ad)-mediated overexpression of *SMN1* in cells lead to the release of EVs loaded with elevated concentrations of SMN protein²¹⁰. In addition to conventional overexpression, several strategies have been developed to increase the efficiency of endogenous cargo loading, subsequently increasing the concentration of cargo of interest within EVs. These methods include fusing target cargo to proteins that are abundant in EVs^{259,260} and utilization of cargo loading motifs^{261,262}.

1.2.3 Internalization of EVs

Once an EV has been released by the parental cell into the extracellular space, the EV is able to shuttle vesicular cargo to a recipient cell that may be located in close proximity or at a distance from the parental cell²¹⁸. There are several different ways that EVs can be internalized by recipient cells, which can vary

depending on cell type²⁶³. The most common of internalization is endocytosis²⁶³⁻²⁶⁵, and other methods include macropinocytosis^{264,266}, phagocytosis²⁶⁷, and membrane fusion²⁶⁸ (Figure 1.3). Once EVs are internalized by endocytosis, the vesicles are contained within the endosome and must “escape” this endosome by fusing with the endosomal membrane, releasing cargo content into the cytoplasm for functional cargo delivery^{220,269,270}. Alternatively, the internalized EVs can stay encapsulated within the endosome as it matures into the MVB and are subsequently re-released as exosomes into the extracellular space²²⁰, or the endosome can fuse with a lysosome, resulting in degradation of the EVs and their contents²⁷¹⁻²⁷³.

EVs are released and taken up by virtually all cell types, however the type of cells that preferentially take up EVs can vary based on the cell type from which the EVs are released^{218,259,274}. Furthermore, biodistribution of EVs can depend on whether the vesicles were administered by IV, intranasal, IP, or subcutaneous administration, or if the vesicles were produced *in situ* and taken up by recipient cells within the same animal^{218,275}. For example, a comparative study of IV and intranasal administration of EVs in non-human primates (NHPs) reported greater accumulation of IV administered EVs in the liver, spleen, lungs, colon, and CSF compared to intranasal administration, and similar levels of EV accumulation in the brain, kidney, and heart²¹⁵.

To administer EVs generated in tissue culture, large amounts of EVs first need to be produced and then purified. There are many methods utilized for purification of EVs, including differential ultracentrifugation, size-exclusion chromatography, filter concentration, polyethylene glycol (PEG) precipitation,

density gradients, and tangential flow filtration (TFF)²⁴³. Each of these methods has a different degree of recovery and specificity, and unique strengths and weaknesses²⁷⁶⁻²⁸⁰. The method of EV purification chosen for a given study in part depends on the intended application, and the resulting product must be characterized to determine the purity, quality and composition of the particles. The International Society for Extracellular Vesicles (ISEV) has released a series of consensus papers^{243,281} that describe preferred methods to evaluate EV samples.

1.2.4 Liver-derived EVs

EVs are released by many cell types into the bloodstream²⁸²⁻²⁸⁷, including by the cells of the liver^{218,288,289}. In fact, it has been reported that ~15% of circulating EVs originate from liver cells^{218,274}. The liver is composed of many cell types, the most abundant being hepatocytes that make up 70-80% of the mass of the organ²⁹⁰. EVs released by hepatocytes can easily cross fenestrations in the sinusoidal endothelium of the liver, carrying cargo into the blood stream that enter circulation²⁹¹. Hepatocytes have numerous functions that include synthesis of many proteins such as serum albumin²⁹² and transferrin²⁹³, production of bile salts²⁹⁴, and metabolism of drugs and toxins²⁹⁵. Other cells that make up the liver include resident macrophages called Kupffer cells, hepatic stellate cells, and liver sinusoidal endothelial cells²⁹⁰. The liver releases proteins that signal changes in lipid metabolism, peripheral insulin action, and glycemic control through paracrine and endocrine signaling²⁹⁶⁻³⁰⁰. Secreted proteins possess an N-terminal signaling peptide, causing the proteins to be released into the extracellular environment, where they transport signals to target cells³⁰¹. However, 80-90% of proteins

released by the liver do not possess this secretory peptide, and thus are not released by classic secretion but by an alternative mechanism²⁹⁶⁻²⁹⁸. One such alternative is through the release of these proteins within EVs.

Recently, a study demonstrated the ability of EVs released by the liver to act on peripheral tissues through endocrine signaling²⁹⁸. Interestingly, IP administration of liver-derived EVs increased glucose effectiveness in the skeletal muscle and insulin secretion in the pancreas of mice, lowering blood glucose level. Another study demonstrated that EVs isolated from hepatocytes of early-stage obese mice were taken up by skeletal muscle and adipose tissues and promoted insulin sensitivity when administered IV in recipient mice³⁰². This study indicated that microRNA (miRNA) miR-3075 delivered by the EVs was responsible for the observed effects. Conversely, it has been reported miRNAs are rarely delivered to their target cells by EVs, suggesting other cargo such as proteins make up the bulk of what is delivered by the vesicles³⁰³. In addition, liver-derived EVs have also been shown to interact with brain endothelial and neuronal cells in tissue culture³⁰⁴. EVs secreted by hepatocellular carcinoma HepG2 cells contain selenoprotein P, and these EVs delivered selenium (Se) to neuronal and brain endothelial cells in an *in vitro* BBB model³⁰⁴. Notably, delivery of supplemental Se can prevent neurodegeneration, synaptic dysfunction, and morphological changes caused by Se deficiency^{305,306}. Furthermore, selenoprotein P delivered by HepG2 EVs protected N2a cells from amyloid β -induced cell death³⁰⁴, which is central to Alzheimer's disease neuropathology³⁰⁷. Together, these studies demonstrate the utility of EVs released from cells of the liver to deliver cargo of interest to a wide variety of cell

types. Overall, EVs clearly have many attributes that make the vesicles attractive therapeutic delivery vehicles.

1.3 Rationale, Hypothesis, and Objectives

As overexpression of a gene of interest in cells can lead to production of EVs loaded with the desired protein cargo, transduction of hepatocytes with a gene therapy vector could be used to load a protein of interest into EVs that are released from the liver into circulation. Many viral^{308,309} and non-viral^{310,311} vectors give rise to high levels of protein synthesis in hepatocytes following systemic administration, and thus could be utilized to generate liver-derived EVs loaded with protein cargo of interest. Following systemic administration, 90% of Ad vector localize to the liver^{312,313}, and these vectors can give rise to transgene expression for up to 7 years in NHPs³¹⁴ and for the lifetime of mice³⁰⁸, and thus Ad is a suitable vector to engineer the liver to produce a therapeutic protein. I hypothesize that an Ad-based system will lead to the release of liver-derived EVs loaded with SMN protein that are distributed throughout the body, and these EVs will deliver SMN protein to recipient cells. My objectives are to:

1. Examine the ability of EVs loaded with SMN protein to deliver this SMN protein to recipient cells in tissue culture.
2. Generate and evaluate an Ad-based system for endogenous release of liver-derived EVs loaded with SMN protein in mice.

Chapter 2: Extracellular vesicles efficiently deliver survival motor neuron protein to cells in culture

Statement of contributions:

C.A.R. conducted cell culture, immunoblot, co-immunoprecipitation, nuclear gem and foci quantification, immunofluorescence staining, microscopy, extracellular vesicle isolation and characterization, viral vector propagation and characterization, viral transduction experiments, media transfer experiments, statistical analysis, and figure generation.

R.J.P. conducted molecular cloning of plasmid constructs, and edited figures.

C.A.R. and R.J.P. analysed data

C.A.R. wrote the initial draft of the manuscript

C.A.R. and R.J.P. edited the manuscript.

Extracellular vesicles efficiently deliver survival motor neuron protein to cells in culture

Charlotte A. René¹⁻³ and Robin J. Parks^{1-4*}

¹Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada, K1H 8L6

²Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada, K1H 8L1

³Centre for Neuromuscular Disease, University of Ottawa, Ottawa, Ontario, Canada, K1Y 4E9

⁴Department of Medicine, The Ottawa Hospital and University of Ottawa, Ottawa, Ontario, Canada, K1H 8L6

*Corresponding Author

Robin J. Parks

Regenerative Medicine Program

501 Smyth Road

Ottawa Hospital Research Institute

Ottawa, Ontario, Canada K1H 8L6

e-mail: rparks@ohri.ca

2.1 Abstract

Spinal Muscular Atrophy (SMA) is a genetic neuromuscular disorder caused by homozygous mutation or deletion of the survival motor neuron 1 (*SMN1*) gene, leading to a low quantity of SMN protein in cells. This depletion of SMN protein preferentially leads to death of motor neurons and, consequently, muscle atrophy, in addition to defects in many other peripheral tissues. SMN protein is naturally loaded into extracellular vesicles (EVs), which are sub-micron-sized, membrane-bound particles released from all cell types. The innate ability of EVs to deliver cargo to recipient cells has caused these vesicles to gain interest as therapeutic delivery vehicles. In this study, we show that adenovirus-mediated overexpression of SMN protein in HepG2 cells leads to the release of EVs loaded with high levels of SMN protein into conditioned medium. Application of this medium to recipient cells in tissue culture led to uptake of the SMN protein, which subsequently transited to the nucleus and co-localized with Gemin2 protein, forming nuclear gem-like structures similar to the native SMN protein. Overall, this work demonstrates that SMN protein can be delivered to cells through EVs, which holds promise as a potential therapy for patients with SMA.

2.2 Introduction

Spinal muscular atrophy (SMA) is a neuromuscular disorder caused by homozygous mutation or deletion of the survival motor neuron 1 (*SMN1*) gene¹, resulting in inadequate levels of survival motor neuron (SMN) protein within cells². Depletion of SMN protein causes degeneration of alpha motor neurons in the spinal cord, leading to progressive muscle weakness and atrophy³. For the most common and severe form of SMA, Type I, patients typically succumb to respiratory failure around 8 months of age if untreated⁴⁻⁶. Although SMA is primarily considered a neuromuscular disease, low levels of SMN protein also has detrimental effects on many other tissues and organs, including the heart^{7,8}, liver^{9,10}, thymus¹¹, pancreas^{12,13}, gastrointestinal and enteric nervous system¹⁴, and spleen^{11,15}. Several therapies are available for treatment of SMA, each focusing on increasing SMN protein levels¹⁶⁻¹⁸. Although the use of these therapeutics can improve both the length and quality of life, patients can show varying response to treatment^{16,17,19,20}. Furthermore, each treatment comes with advantages and risks²¹⁻²⁴, suggesting that there is still a need to investigate alternative approaches to treatment.

SMN protein is present in both the nucleus and cytoplasm of cells^{25,26}. In the nucleus, SMN protein localizes to structures called gemini of Cajal bodies (gems). SMN protein associates with Gemin proteins 2-8 and Unrip to form the SMN complex, a multiprotein complex crucial for assembly of small nuclear ribonucleoproteins (snRNPs) which are essential for pre-messenger ribonucleic acid (mRNA) splicing²⁷⁻³³. The SMN complex is also believed to play a role in the

assembly of small nucleolar ribonucleoproteins (snoRNPs), which are involved in post-transcriptional processing and modification of ribosomal RNA in the nucleolus^{34,35}. There is also some evidence that the SMN complex is associated with telomerase, a complex that adds repeat sequences at chromosomal ends, thus elongating telomeres³⁶⁻³⁸. SMN protein has been implicated in numerous other cellular functions, including translational regulation³⁹⁻⁴¹, active transport of mRNA^{42,43}, actin dynamics^{44,45}, and resolution of R-loops in genetic terminal regions⁴⁶. Although many functions of SMN protein have been described, the precise cellular role of SMN protein that results in pathogenesis of SMA remains unclear.

Interestingly, SMN protein is naturally loaded into extracellular vesicles (EVs)⁴⁷⁻⁵². EVs are sub-micron-sized, membrane-bound particles released from all cell types into bodily fluids, or culture media in tissue culture⁵³. There are several types of EVs that are classified based on mechanism of biogenesis and diameter, the most extensively characterized being exosomes and microparticles⁵³. EVs are packed with proteins, nucleic acids, and lipids, and can deliver these biomolecules to recipient cells that take up the EVs, which can be located near or distant from where the vesicles originate⁵³⁻⁵⁵. EVs possess many desirable attributes that can be exploited to deliver therapeutic cargo, such as a lipid bilayer that protects contents from degradation in the bloodstream⁵⁶, the ability to transit the blood-brain barrier (BBB)⁵⁷⁻⁵⁹, and the capacity to be bioengineered to increase loading of desirable therapeutic cargo⁶⁰. EVs have been used successfully to deliver a variety of therapeutic proteins in many different studies. For example, delivery of catalase-

loaded EVs led to rapid uptake of the protein by neuronal cells in culture, and neuroprotection in a mouse model of Parkinson's disease⁶¹. Similarly, neprilysin-loaded EVs provided neuroprotection and improved behavioural function in a mouse model of Alzheimer's disease⁶².

In this study, we show that overexpression of SMN protein in tissue culture leads to the release of EVs containing high quantities of the SMN protein. Importantly, these EVs were able to transfer the protein to recipient cells, where the exogenous SMN protein localized to nuclear gem-like structures and interacted with normal cellular protein partners. Our work suggests that EVs loaded with SMN protein may represent a new therapeutic class to treat patients with SMA.

2.3 Results

2.3.1 Development and characterization of a donor cell line for production of EVs loaded with SMN protein.

For our studies of EV-mediated delivery of SMN protein to recipient cells, we needed to distinguish between endogenous and exogenous SMN protein. We, and many others, have shown that the presence of an N-terminal FLAG tag does not appear to affect protein localization and ability to interact with natural protein partners⁶³⁻⁶⁵. We also previously showed that two common methods can be used to generate cell lines expressing a FLAG-tagged version of SMN protein that is subsequently efficiently loaded into EVs: plasmid-based expression and selection of a stable cell line or adenovirus (Ad)-mediated overexpression of the protein⁴⁷. We initially explored the first option, generation of a stable cell line, as this represents a simpler “one component” production system (i.e. only the EV-producer cell line), compared to the “two-component” Ad system that requires a vector and an EV-producer cell line. Furthermore, Ad-mediated overexpression of a protein typically results in supraphysiological levels of the protein^{47,63,66}, which would impede our ability to determine whether the protein was acting as normal in the producer cell line.

We generated a stable cell line expressing a triple FLAG-tagged version of the SMN (3F-SMN) protein using the HepG2 cell line, originally derived from a human male hepatocellular carcinoma⁶⁷. We chose HepG2 cells, since we previously showed that this cell line naturally releases EVs with high SMN protein content⁴⁷. In the stable cell line, SMN expression is controlled by the human

cytomegalovirus (CMV) immediate early enhancer/promoter, with the *SMN1* cDNA located upstream of an internal ribosome entry site (IRES) derived from the encephalomyocarditis virus (EMCV)⁶⁸, hygromycin resistance gene, and simian virus 40 (SV40) polyadenylation sequence. The pooled population of hygromycin resistant cells, designated HepG2:3F-SMN, were first analyzed by immunoblot. Both the parental HepG2 and HepG2:3F-SMN cell lines showed a protein of ~38 kDa, representing the endogenous SMN protein (Figure 2.1A). The signal for this SMN protein in the HepG2:3F-SMN cell line was ~75% of that observed in the parental HepG2 cells. HepG2:3F-SMN also contained an SMN-positive band of ~41 kDa, representing the 3F-SMN protein. Densitometry analysis showed that the total level of SMN protein (*i.e.* the ~38 kDa SMN and ~41 kDa 3F-SMN) in HepG2:3F-SMN cells was approximately 1.5-fold higher than the level of endogenous SMN protein in the parental HepG2 cells. As anticipated, there was no FLAG signal present in parental HepG2 cells, but a positive signal of ~41 kDa in the HepG2:3F-SMN cell line. We also observed a minor band of ~35 kDa in both the SMN and FLAG immunoblots, which may represent a proteolytically cleaved form of SMN protein. A co-immunoprecipitation (co-IP) experiment was performed to confirm that the N-terminal triple FLAG tag did not interfere with the ability of the protein to self-associate or interact with normal cellular partners, such as Gemin2. As shown in Figure 2.1B, pulldown of the tagged SMN protein with antibody to FLAG resulted in co-IP of both endogenous SMN and Gemin2 proteins. Finally, we examined localization of the tagged SMN protein within the cells by immunofluorescence. In parental HepG2 cells, SMN protein was located in both the cytoplasm and nucleus,

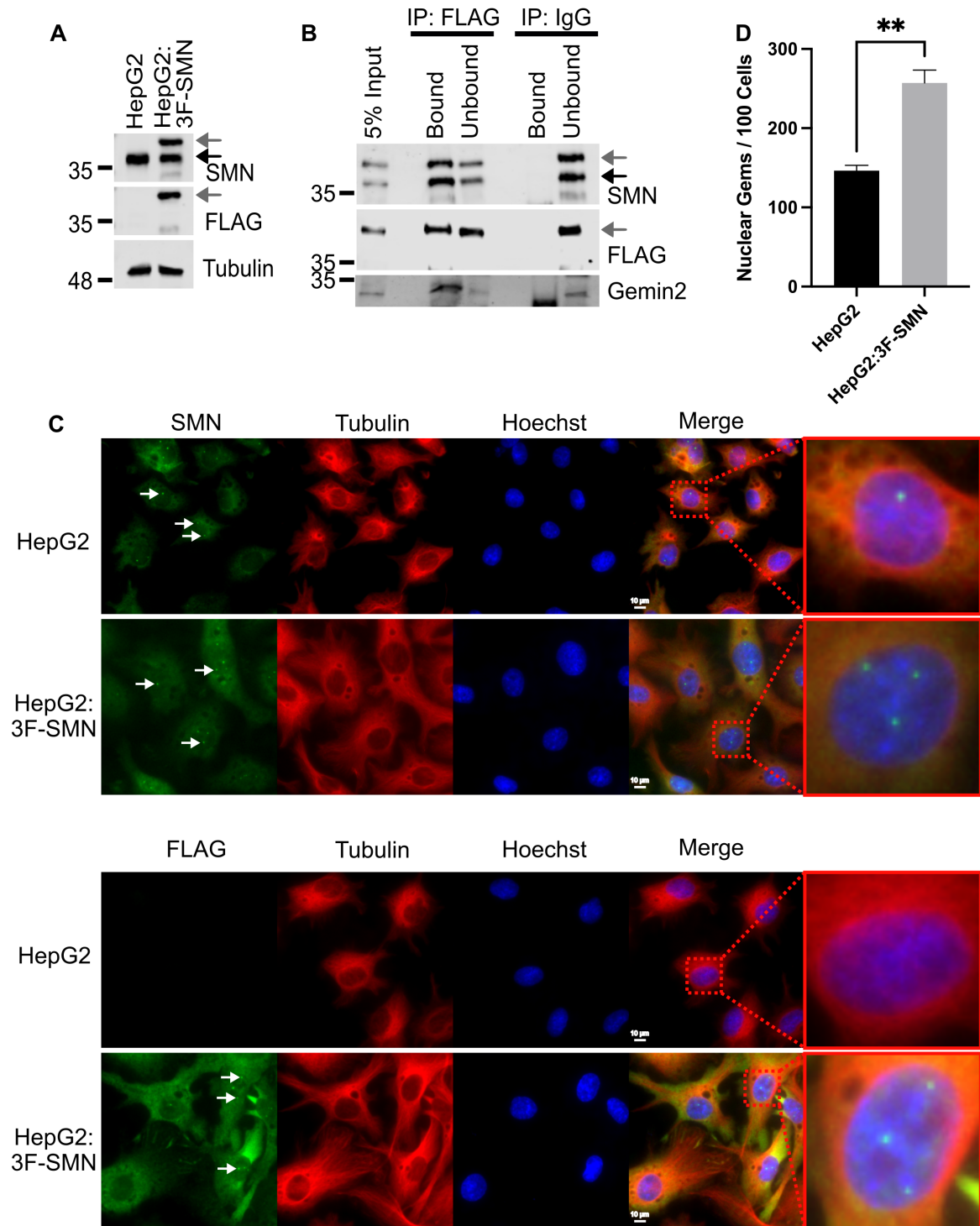


Figure 2.1 The presence of a 3xFLAG-tag on SMN protein does not affect protein localization within the cell or ability to interact with normal cellular protein partners. Panel A: A HepG2 cell line stably expressing a 3xFLAG-tagged SMN protein was assessed for protein expression by immunoblot. Grey arrows indicate the 3F-SMN protein and the black arrow indicates endogenous SMN

protein. Panel B: Co-immunoprecipitation (Co-IP) was performed using anti-FLAG or isotype control (IgG). Input, bound, and unbound fractions were assessed by immunoblot. Grey arrows indicate the 3F-SMN protein and the black arrow indicates endogenous SMN protein. Panel C: Epifluorescence microscopy images of HepG2:3F-SMN cells stained with antibodies to tubulin and SMN, or tubulin and FLAG. Arrows indicate representative nuclear gems. Scale bars represent 10 μ m. Panel D: Quantified nuclear gems per 100 cells in epifluorescence microscopy images of HepG2 and HepG2:3F-SMN stained with anti-tubulin and anti-SMN. Data was tested for significance using an unpaired t-test, where two asterisk (**) indicates $p < 0.01$. Data represents 3 independent experiments.

with nuclear-localized SMN protein localized in gem-like foci (Figure 2.1C). For HepG2:3F-SMN, the 3F-SMN protein was also located in both the cytoplasm and nucleus and, again, within gem-like structures in the nucleus. The average integrated SMN signal intensity in HepG2:3F-SMN cells was ~1.2-fold above that of HepG2 cells, supporting the increase in total SMN protein level shown in Figure 2.1A. Interestingly, the average number of gems per 100 cells $\pm$ SD was 146 ± 11.5 and 257 ± 28.2 in the HepG2 and HepG2:3F-SMN cell lines, respectively (Figure 2.1D), consistent with the observation that gem number roughly correlated with level of SMN expression within the cell²⁶. Overall, these data demonstrate that HepG2:3F-SMN cells were effectively engineered to overexpress SMN protein that localizes normally and interacts with normal cellular partners in the producer cell line.

We next characterized EVs released from the HepG2:3F-SMN cell line, and specifically the incorporation of the 3F-SMN protein into EVs. HepG2 and HepG2:3F-SMN cells were plated at equal density, and equal volumes of conditioned medium (CM) were collected from these cells after 72 h. EVs were isolated by ultracentrifugation from the CM and resuspended in equal volumes of PBS. We first characterized the size and number of EVs isolated from the HepG2 and HepG2:3F-SMN cell lines by nanoparticle tracking analysis (NTA) (Figure 2.2A). The majority of particles isolated from both cell lines were ≤ 200 nm in diameter, and there appeared to be no significant difference between the concentration or distribution of particle sizes isolated from HepG2 and HepG2:3F-SMN cells. The peak particle diameter for EVs isolated by ultracentrifugation from HepG2 and

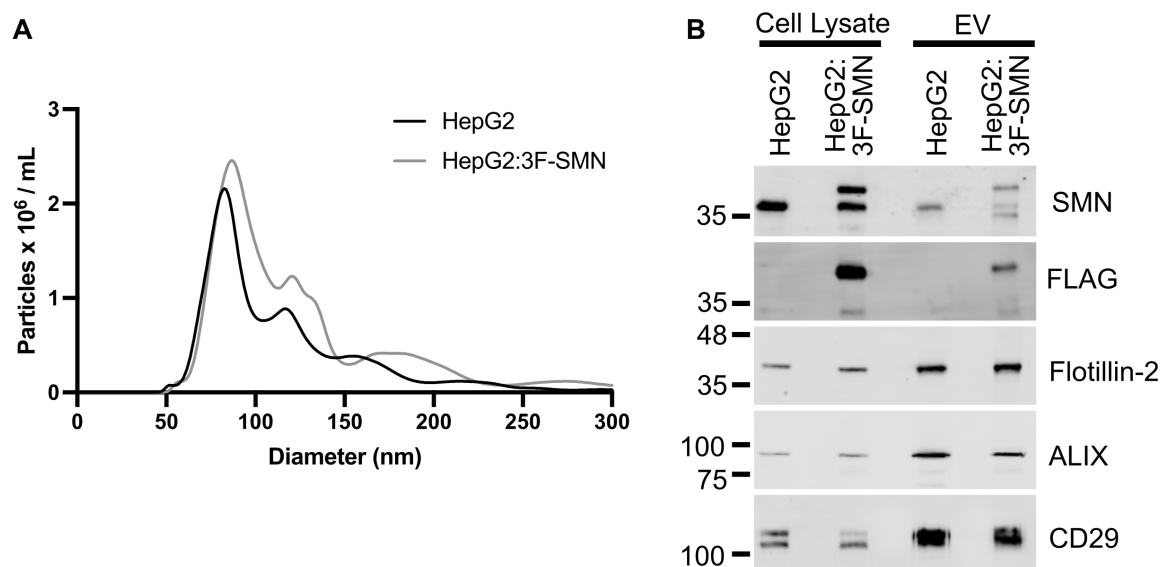


Figure 2.2 EVs released by HepG2:3F-SMN cells contain 3F-SMN protein. Panel A: Conditioned medium (CM) was collected from HepG2 and HepG2:3F-SMN cells 72 h post-plating. Cellular debris and apoptotic bodies were removed by low-speed centrifugation, and EVs were isolated by ultracentrifugation. Particle diameter was assessed by nanoparticle tracking analysis. Panel B: Protein content of isolated EVs was examined by immunoblot with the indicated antibodies, with total cellular lysates from the two cell lines included as controls.

HepG2:3F-SMN were 87 nm and 91 nm, respectively. We next analyzed the protein content of the EVs by immunoblot, and we included lysates from the cells as control (Figure 2.2B). EVs isolated from both cell types had similar Flotillin-2, ALIX (ALG-2-interacting protein X), and CD29 (integrin beta-1) protein signals, common markers of EVs⁵³. As anticipated, EVs released by parental HepG2 cells had a single SMN protein species of ~38 kDa representing the native protein. For EVs isolated from HepG2:3F-SMN cells, we observed a signal corresponding to the native SMN protein, but also SMN-positive signals at approximately 41 and 35 kDa. The 41 kDa species represents the 3xFLAG-tagged version of SMN protein, as a band of similar size was detected on immunoblots probed with antibody to FLAG, and the 35 kDa species may represent a proteolytically cleaved product. The overall SMN protein signal within EVs isolated from HepG2:3F-SMN cells was approximately 2.5-fold higher than that observed in EVs isolated from parental HepG2 cells, consistent with the level of expression of SMN protein within the two cell lines. Taken together, these data show that the presence of the 3xFLAG tag on the SMN protein does not appear to adversely affect localization within the cell, interaction with normal protein partners, or the ability of the protein to be incorporated into EVs.

2.3.2 Generation of a recipient cell line containing HA-tagged Gemin2

SMN protein naturally co-localizes and interacts with Gemin2 protein within the nucleus of cells²⁹, an observation that we ultimately hoped to use as a read-out for successful delivery of SMN protein from our EVs. However, Gemin2 protein is also naturally contained in EVs⁴⁸, precluding our ability to distinguish between

association of the delivered 3F-SMN protein with Gemin2 protein that was co-delivered in the EVs and native protein within the recipient cell. We therefore generated a recipient cell line stably expressing a human influenza hemagglutinin (HA)-tagged Gemin2 protein. We used A549 cells to establish the cell line, as they are a flat, robust cell line ideal for immunofluorescence studies. For this cell line, expression is once again controlled by the human CMV immediate early enhancer/promoter, with the N-terminal tagged *HA-Gemin2* cDNA located upstream of an EMCV-derived IRES, hygromycin resistance gene, and SV40 polyadenylation sequence. The pooled population of hygromycin resistant cells, designated A549:HA-Gemin2, were subsequently analyzed by immunoblot (Figure 2.3). In this cell line, the HA-tagged Gemin2 protein was expressed at approximately the same level as endogenous protein (Figure 2.3A), and was capable of interacting with SMN protein as assessed by co-IP (Figure 2.3B). Immunofluorescence microscopy showed that the HA-tagged Gemin2 protein was present in both the cytoplasm and nucleus, similar to endogenous protein, and localized in the nucleus to gem-like structures (Figure 2.3C). Thus, the presence of the HA tag on Gemin2 protein does not appear to affect protein localization or normal protein interactions. Interestingly, immunofluorescence microscopy revealed that A549:HA-Gemin2 cells possessed less SMN-containing nuclear gems than parental A549 cells (Figure 2.3D, 2.3E). Indeed, the A549 cell line possessed 134 ± 22.5 SMN-positive nuclear gems per 100 cells, whereas A549:HA-Gemin2 cell line possessed 38 ± 9.8 SMN-positive gems per 100 cells $\pm$ SD. Nuclear gems are comprised of SMN protein along with several members of the Gemin protein family, amongst other proteins^{69,70}. Previous work

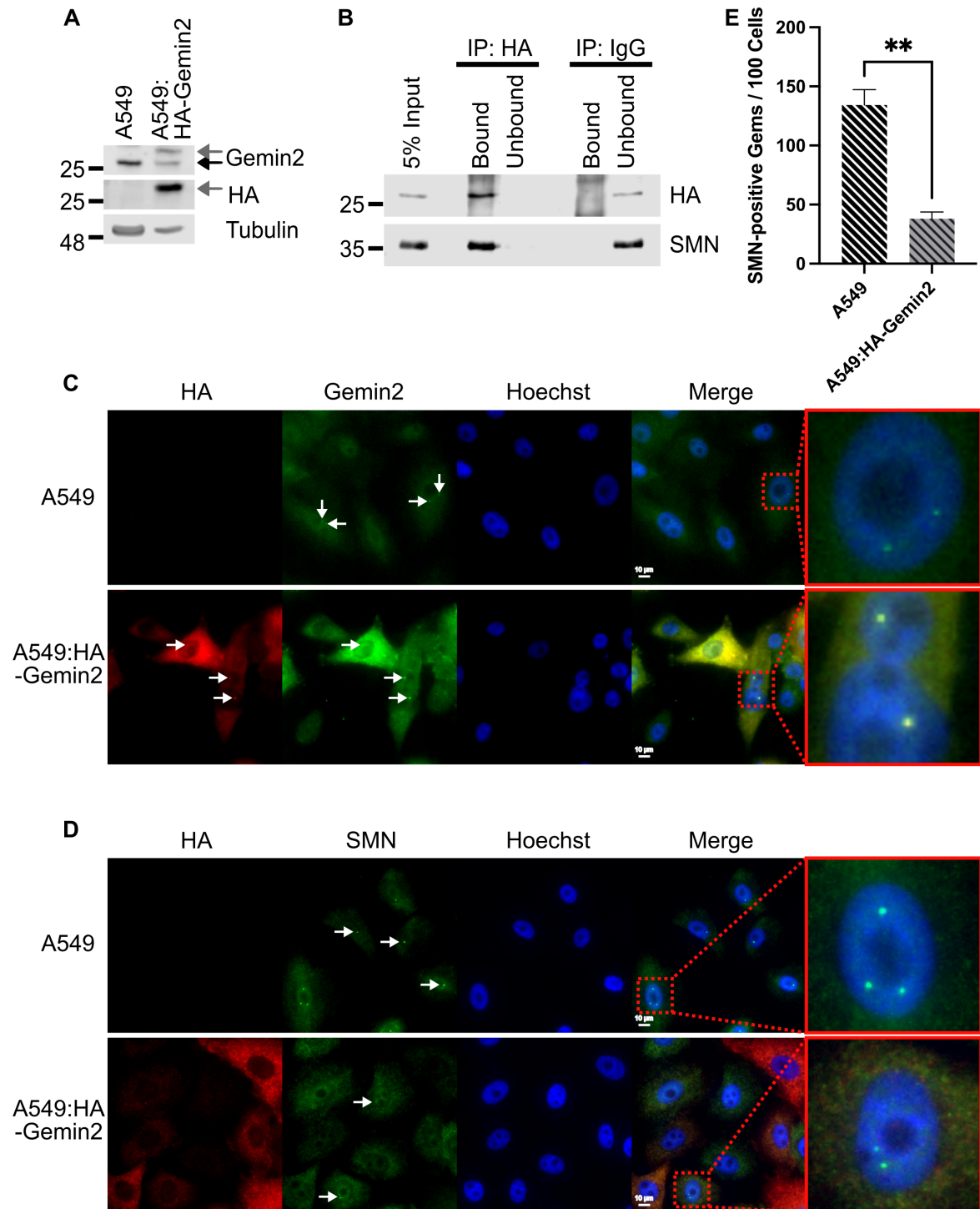


Figure 2.3 Generation and characterization of an A549 cell line expressing an HA-tagged Gemin2 protein. Panel A: An A549 cell line stably expressing HA-tagged Gemin2 protein was assessed by immunoblot with the indicated antibodies. Panel B: Co-immunoprecipitation (Co-IP) was performed using anti-HA or isotype control (IgG). Input, bound, and unbound fractions were assessed by immunoblot. Panel C: Epifluorescence microscopy images of A549:HA-Gemin2 cells stained with

anti-HA and anti-Gemin2. Arrows indicate nuclear gems. Panel D: Epifluorescence microscopy images of A549:HA-Gemin2 cells stained with anti-HA and anti-SMN, with arrows indicating nuclear gem-like structures. Scale bars represent 10 μ m. Panel E: Quantified SMN-positive nuclear gems per 100 cells in epifluorescence microscopy images as processed in panel D. Data was tested for significance using an unpaired t-test, where two asterisk (**) indicates $p < 0.01$. Data represents 3 independent experiments.

has shown that knockdown of SMN protein or any of the Gemin proteins reduces the number of gems observed within the nucleus of the cell⁶⁹. Conversely, overexpression of Gemin4, which is naturally found predominantly in the nucleus of cells, causes a relocalization of the endogenous SMN protein to almost exclusive nuclear localization, and a concomitant increase in the number of nuclear gems⁷¹. In A549:HA-Gemin2 cells, HA-Gemin2 was found in both the cytoplasm and the nucleus, which correlated with a reduction in the number of SMN protein-positive gems in the nucleus of these cells, suggesting that the endogenous SMN protein was partly relocalized by the overexpressed Gemin2 to the cytoplasm in these cells. Nevertheless, these data suggest that A549:HA-Gemin2 is a suitable cell line to evaluate EV-mediated delivery of 3F-SMN protein.

2.3.3 Enhancing the quantity of 3F-SMN protein in EVs through Ad-vector mediated overexpression in the donor cell line.

We initially examined whether application of CM (*i.e.* clarified medium from cells, without concentration of the EVs) from HepG2:3F-SMN could result in detectable uptake of the protein on A549:HA-Gemin2 cells, but were unable to detect FLAG-positive signal by immunofluorescence microscopy (data not shown). Subsequent work showed that if we concentrated the EVs from CM approximately 200-fold through ultracentrifugation, we could detect significant FLAG-positive signal on the recipient cells. However, this FLAG-positive signal appeared to be primarily large aggregates that were likely on the cell surface, which precluded us from confidently detecting internalization and correct localization of the protein (data

not shown).

We previously showed that we could significantly increase the quantity of SMN protein within EVs through the use of Ad-mediated overexpression⁴⁷. This approach would allow us to increase the quantity of 3F-SMN protein in each EV, and avoid the complications associated with EV concentration noted above. HepG2 cells were transduced with a non-replicating Ad vector containing a gene for expression of 3F-SMN protein (Ad3F-SMN) at varying multiplicities of infection (MOI). Seventy-two hours post-infection (hpi), cell lysates and CM were collected. As shown in Figure 2.4A, infection of HepG2 cells with Ad3F-SMN resulted in a dose-dependent increase in quantity of 3F-SMN protein within the cells, which amounted to a 15- to 25-fold increase in total SMN protein within the cells (Figure 2.4B). A similar relationship was noted for SMN protein content in EVs isolated from CM from the infected cells, with a >20-fold increase in SMN protein content at the highest MOI examined (Figure 2.4C). Thus, HepG2 cells expressing 3F-SMN protein through Ad vector transduction release EVs loaded with ~10-fold more SMN protein than EVs isolated from the HepG2:3F-SMN cell line. We also characterized the size and concentration of EVs isolated from HepG2 cells transduced with Ad3F-SMN by NTA (Figure 2.4D). The populations of EVs released from HepG2 cells transduced at different MOIs appear to have similar concentrations and size distributions, primarily ≤ 200 nm, and are not significantly different from EVs released from mock-infected cells. All samples had a similar median particle diameter of ~100 nm, regardless of MOI. The NTA data demonstrates that Ad transduction has no overt effect on release of EVs from HepG2 cells at the MOIs used, and the population of particles are

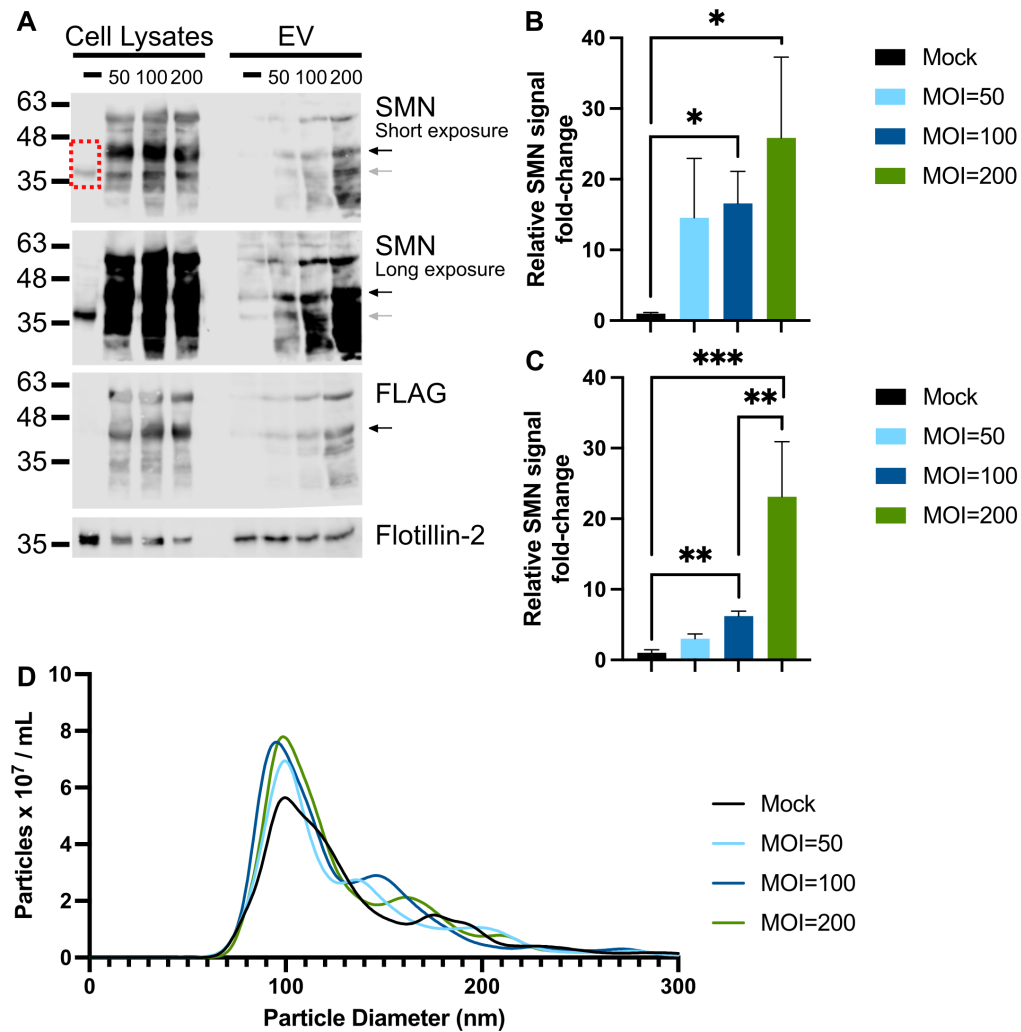


Figure 2.4 Adenoviral-mediated overexpression of 3F-SMN protein in HepG2 cells leads to the release of EVs containing high levels of the 3F-SMN protein. Panel A: HepG2 cells were infected with Ad3F-SMN at an MOI=50, 100, 200, or mock infected (PBS). Seventy-two hpi, cell lysates were collected and EVs were isolated using polyethylene glycol (PEG). Protein content of the EVs was examined by immunoblot. Endogenous SMN protein is indicated by a grey arrow and 3F-SMN protein is indicated by a black arrow. The red square indicates the region used for quantification of SMN protein signal for panel B and C. Panel B: SMN protein signal in cell lysates were quantified, and the fold change in SMN protein signal relative to endogenous SMN protein signal is shown. Panel C: SMN protein signal in EVs was quantified and presented as the fold change in SMN protein signal relative to endogenous SMN protein signal. Panel D: Particle size and diameter of isolated EVs was assessed by nanoparticle tracking analysis. In panels B and C, significance was calculated after transforming data by log transformation, then tested for significance using one-way ANOVA followed by Tukey's multiple

comparisons test. An asterisk (*) indicates $p < 0.05$, two asterisk (**) indicates $p < 0.01$, and three asterisk (***) indicates $p < 0.001$. Data represents 3 independent experiments.

consistent with small EVs. Collectively, this data shows that Ad-mediated overexpression of SMN protein can be utilized as a technique to significantly increase the concentration of SMN protein contained in EVs. For the remainder of this study, “3F-SMN CM” indicates CM collected from HepG2 cells transduced with Ad3F-SMN at an MOI=200.

2.3.4 EVs can deliver SMN protein to recipient cells.

We next examined whether CM from Ad3F-SMN-transduced cells could be used to transfer 3F-SMN protein to recipient cells. HepG2 cells were transduced with Ad3F-SMN at an MOI=200 or mock infected with PBS and, 72 h later, CM was collected for transfer onto A549:HA-Gemin2 cells. Of note, for all media transfer experiments, the CM was subjected to low-speed centrifugation to remove cell debris and large EV (e.g. apoptotic bodies) prior to application to the recipient cells, but was not concentrated or further processed. Although the residual Ad3F-SMN inoculum was washed from the HepG2 cells prior to the 72 h incubation, small amounts of Ad vector could be present in the medium, which may lead to *de novo* synthesis of 3F-SMN protein in the A549:HA-Gemin2 cell line, confounding our analysis. We used two strategies to alleviate this concern. First, transcription from an infecting Ad genome typically does not initiate before ~2 h after application of the virus^{72,73}, so we limited our CM incubation to 2 h. Second, we performed our incubation of CM with the A549:HA-Gemin2 cells in the presence or absence of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), an inhibitor of RNA polymerase transcription elongation⁷⁴. We previously showed that DRB effectively prevents

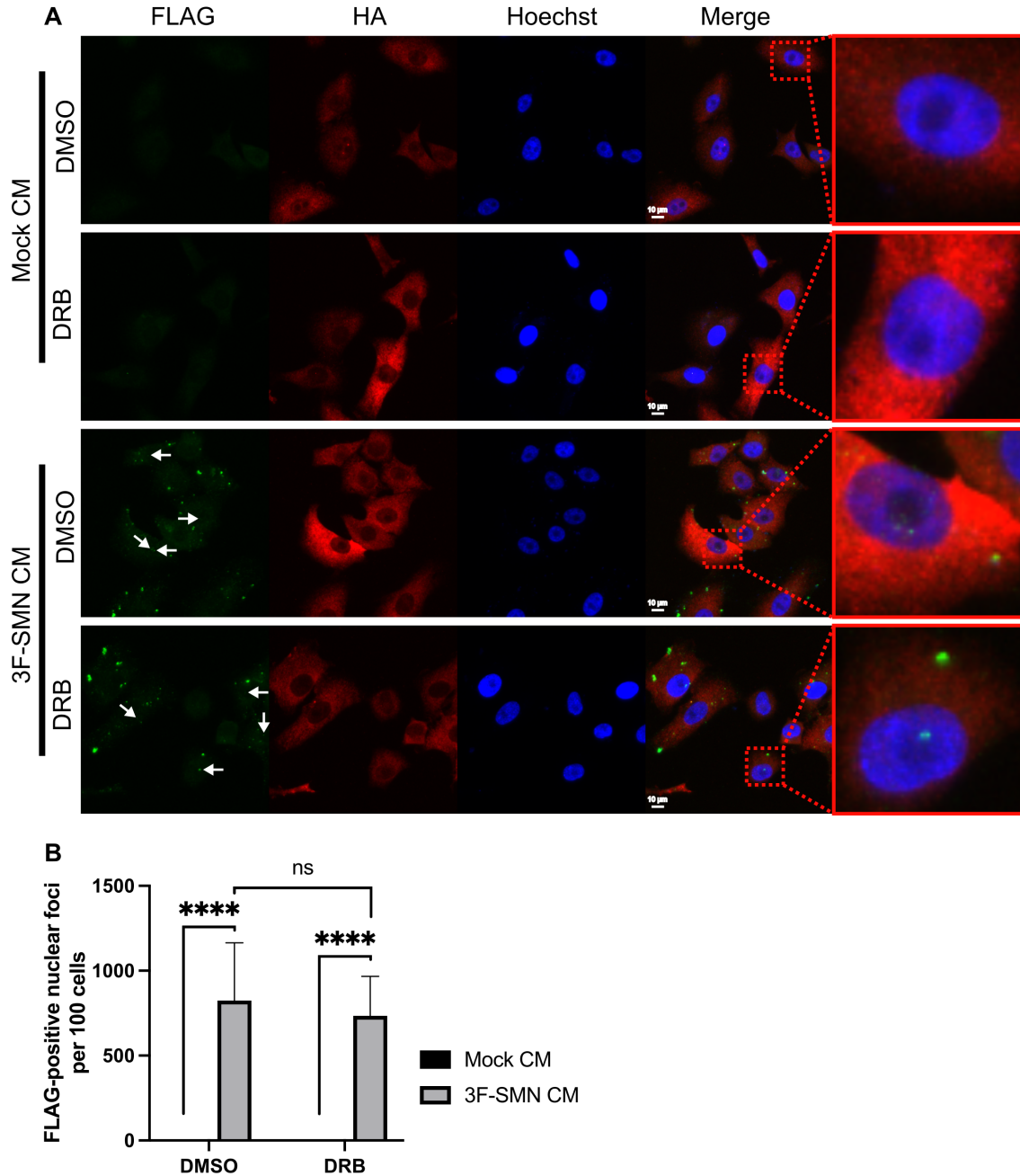


Figure 2.5 Application of conditioned medium containing EVs loaded with 3F-SMN protein onto A549:HA-Gemin2 cells results in FLAG-positive nuclear foci.

Panel A: Conditioned medium (CM) from HepG2 cells infected with Ad3F-SMN or mock-infected was applied to A549:HA-Gemin2 cells in the presence or absence of 100 μ M 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB). Two h post-treatment, the cells were washed and processed for immunofluorescent staining with anti-FLAG and anti-HA, and images were captured by epifluorescence microscopy. Arrows indicate representative nuclear gem-like foci. Scale bars represent 10 μ m. Panel B: FLAG-positive nuclear foci were quantified and

expressed per 100 cells. Significance was calculated after transforming data by log transformation using two-way ANOVA followed by Tukey's multiple comparisons test. Four asterisk (****) indicates $p < 0.0001$. Data represents 3 independent experiments.

transcription from the Ad genome^{75,76}, and thus would prevent gene expression from any vector inadvertently transferred in the CM. Control CM (from mock-infected HepG2) or 3F-SMN CM was applied to A549:HA-Gemin2 cells for 2 h, in the presence or absence of DRB, and then analyzed for the presence of 3F-SMN protein by epifluorescence microscopy. As expected, no FLAG signal was detected in the mock treatment groups (Figure 2.5A). In cells treated with 3F-SMN CM, a similar FLAG signal was visible in both DRB- and vehicle-treated groups. Indeed, in DRB- and vehicle-treated groups, ~730 and ~820 FLAG-positive nuclear foci per 100 cells, respectively, were detected, which may represent a combination of 3F-SMN protein in nuclear gems and also other smaller foci. Thus, at least a portion of the delivered 3F-SMN protein was localizing to the nucleus. We also observed concentrated FLAG signal within the cytoplasm of treated cells. Overall, this data suggests that EVs can be used to deliver SMN protein to recipient A549:HA-Gemin2 cells in tissue culture.

To more precisely evaluate the localization of the EV-delivered 3F-SMN protein in the A549:HA-Gemin2 cells, we performed confocal microscopy and examined orthogonal projections generated from three-dimensional (3D) reconstructions. As in our previous experiment, CM was applied for 2 h in the presence or absence of DRB to inhibit *de novo* gene expression from any residual Ad vector that may have been present. Once again, no FLAG signal was observed in cells treated with mock CM (Figure 2.6A). For both vehicle- and DRB-treated cells, application of 3F-SMN CM resulted in the presence of FLAG signal in both the cytoplasm and nucleus. An analysis of orthogonal projections of the images

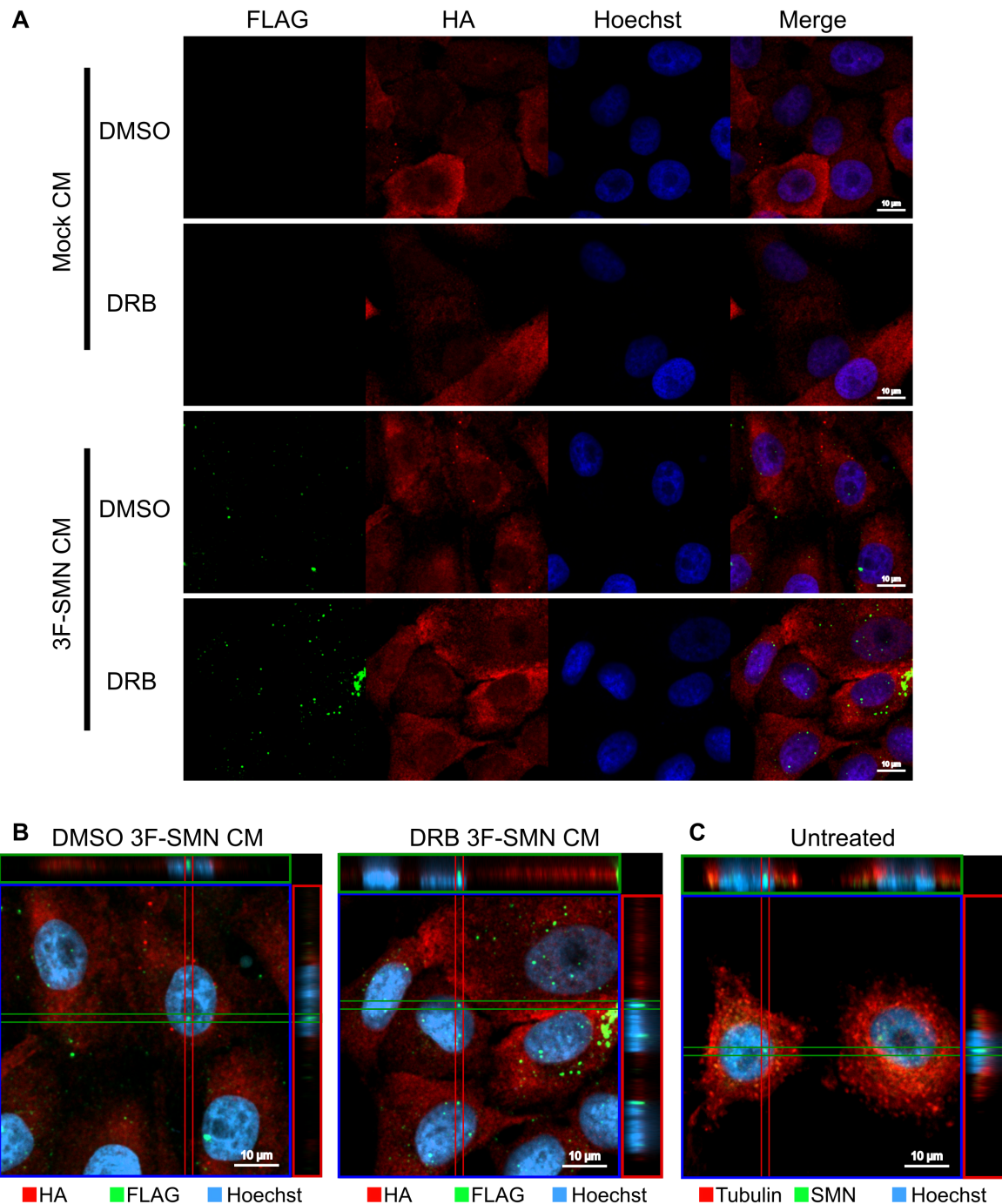


Figure 2.6 EV-delivered 3F-SMN protein is internalized by recipient A549:HA-Gemin2 cells and localizes in gem-like structures. Panel A: Confocal immunofluorescence images of A549:HA-Gemin2 cells that were treated for 2 h with mock or 3F-SMN CM, and processed with antibodies to HA and FLAG. Panel B: Images in panel A were examined through orthogonal projections generated from z-stacks. Panel C: Orthogonal projections generated of z-stacks of parental A549 cells stained with antibodies to tubulin and SMN. Scale bars represent 10 μ m.

presented in Figure 2.6A clearly showed that treatment with 3F-SMN CM resulted in the presence of 3F-SMN protein in specific foci in the nucleus that resemble nuclear gems (Figure 2.6B). Indeed, the nuclear structures observed in 3F-SMN treated cells appear similar in size and morphology to native SMN-containing gems in untreated cells (Figure 2.6C). Interestingly, in both treated and untreated cells, the SMN-positive nuclear gems appear more like cylindrical structures rather than spheres, that are oriented perpendicular to the plane of the coverslip on which the cells were grown.

We also examined whether the 3F-SMN protein present in nuclear gem-like structures co-localized with HA-Gemin2 protein (Figure 2.7A). As expected, no FLAG signal was present in A549:HA-Gemin2 cells treated with CM from mock-infected HepG2 cells. In cells treated with 3F-SMN CM, there was a clear overlap between FLAG and HA signals indicating these proteins co-localized in the nucleus. Indeed, in orthogonal projections of 3F-SMN treated cells, there were distinct merged regions of yellow signals in the nucleus, clearly illustrating co-localization of the HA-Gemin2 and 3F-SMN proteins (Figure 2.7B, 2.7C). We also examined whether EVs could be used to deliver SMN protein tagged with mCherry. CM was prepared as in our previous experiments: HepG2 cells were transduced with AdmCherry-SMN at an MOI=200 for 1 h, CM was collected 72 h post-infection, and centrifuged to remove large debris. Once again, the EV were not concentrated from the CM before application to the recipient cells. The mCherry-SMN CM was applied to recipient parental A549 cells (*i.e.* not overexpressing the HA-tagged Gemin2 protein) for 2 h, and delivery of mCherry-SMN protein was analyzed by confocal

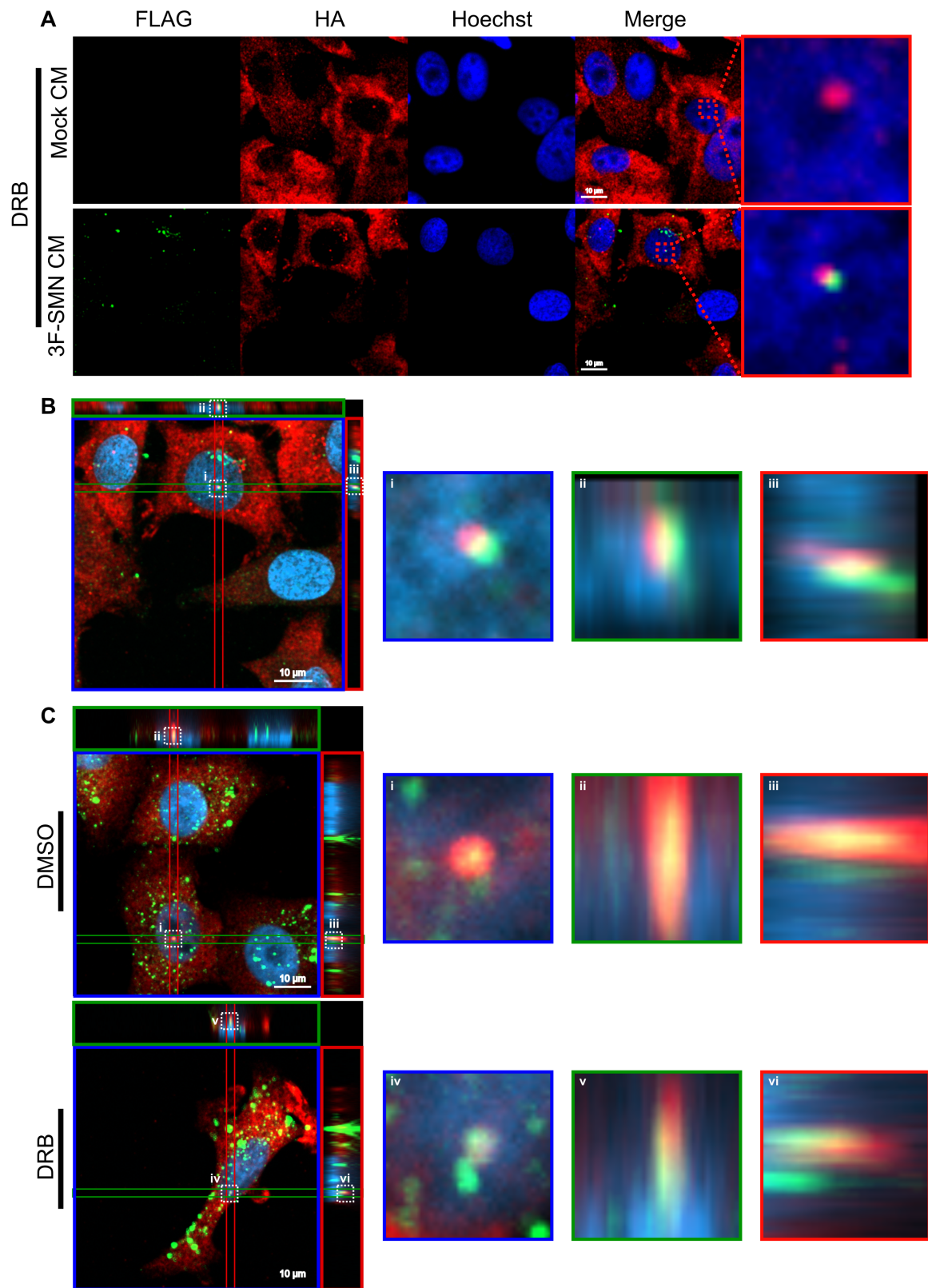


Figure 2.7 3F-SMN protein co-localizes with HA-Gemin2 in nuclear gem-like structures in A549:HA-Gemin2 cells. Panel A: Confocal immunofluorescence

images of A549:HA-Gemin2 cells that were treated for 2 h with mock or 3F-SMN CM, and processed with antibodies to HA and FLAG. Panel B: The image in panel A (lower panel) was further examined through orthogonal projections generated from z-stacks. Panel C: Additional images and orthogonal projections from A549:HA-Gemin2 cells treated with 3F-SMN CM showing colocalization of 3F-SMN and HA-Gemin2 proteins. Scale bars represent 10 μ m.

microscopy. As in our previous experiments, mCherry-SMN protein was present in the cytoplasm and nucleus of treated A549 cells (Figure 2.8A). In the nucleus, mCherry-SMN protein localized in nuclear gem-like structures that appeared similar in size and morphology to those observed in Figures 2.6 and 2.7. Taken together these data suggest that EVs can efficiently deliver SMN protein to A549 cancer cells in culture.

Finally, we examined whether EVs could efficiently deliver SMN protein to fibroblasts derived from an SMA patient. Fibroblasts were treated for 2 h with mock or 3F-SMN CM, and delivery of 3F-SMN protein was assessed by confocal microscopy. As expected, no FLAG signal was present in the cells treated with mock CM (Figure 2.8B). In cells treated with 3F-SMN CM, there was a strong FLAG signal present in both the cytoplasm and nucleus of these cells. Within the nucleus, FLAG-positive foci resembling nuclear gems were present. Thus, EVs can be used to deliver SMN protein to SMN-deficient cells.

Overall, these data show that EVs can effectively deliver SMN protein to recipient cells in tissue culture, including cells with low levels of SMN protein, resulting in accumulation of the protein in both the cytoplasm and gem-like structures within the nucleus of the treated cells.

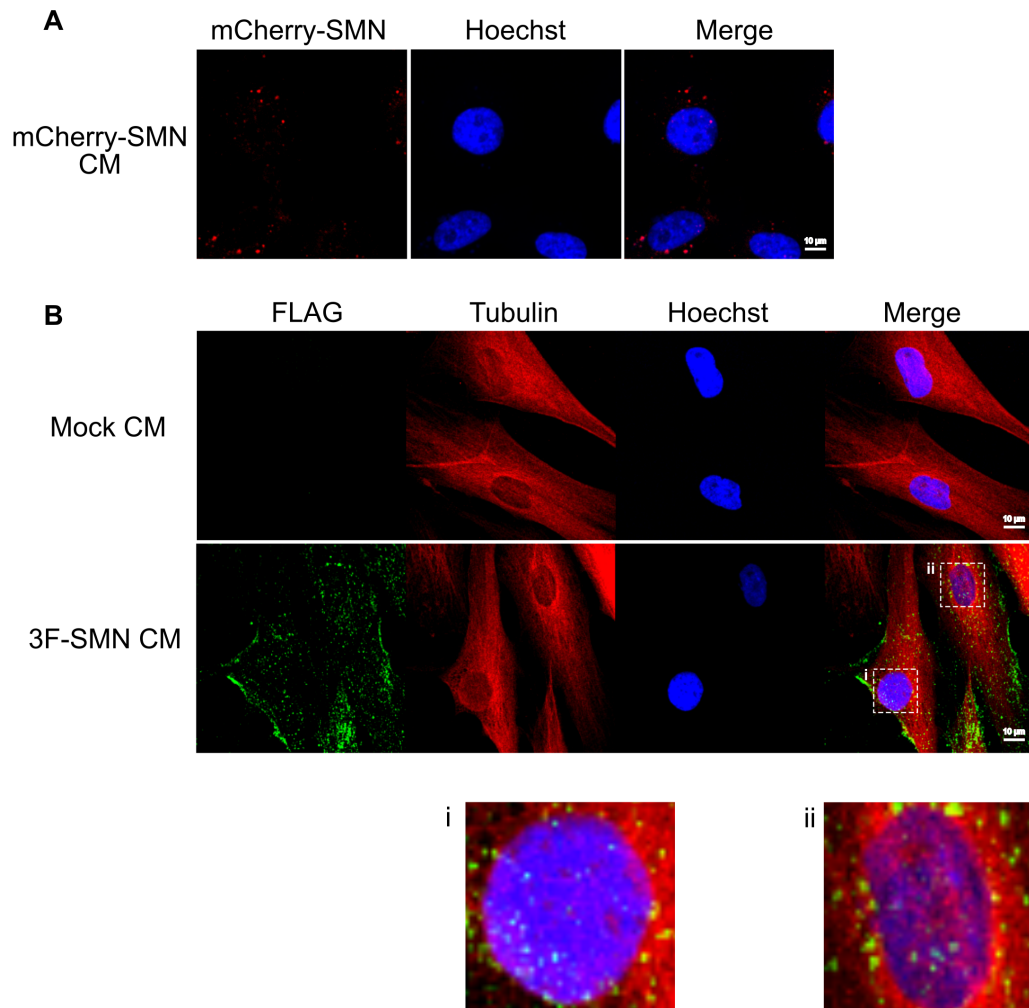


Figure 2.8 EV-mediated delivery of mCherry-SMN and 3F-SMN protein to A549 cells and fibroblasts derived from a patient with SMA. Panel A: CM from HepG2 cells infected with AdmCherry-SMN was applied to A549 cells. Two h post-treatment, the cells were washed, fixed, and stained with Hoechst to visualize nuclei. Images were captured by confocal microscopy. Panel B: CM from HepG2 cells infected with Ad3F-SMN or mock-infected was applied to fibroblasts derived from a patient with SMA. Two h post-treatment, the cells were washed and processed for immunofluorescence staining with anti-FLAG and anti-tubulin, and images were captured by confocal microscopy. Scale bars represent 10 μ m.

2.4 Discussion

SMA is a devastating disease characterized by degeneration of the alpha motor neurons of the spinal cord leading to atrophy of skeletal muscle, although primary defects also occur in many other tissues and organs^{3,77,78}. Severe forms of the disease are lethal at a very early age if left untreated. SMA is caused by inadequate levels of SMN protein within cells^{1,2}. All three of the currently approved therapeutics for treatment of SMA focus on increasing cellular levels of SMN protein. Nusinersen is an anti-sense oligonucleotide (ASO) that acts on *SMN2* pre-mRNA to promote inclusion of exon 7, ultimately leading to greater production of full-length protein^{16,79,80}. However, patients can show a variable response to treatment, with up to 50% of treated patients failing to respond in clinical trial, for reasons that are unknown¹⁹. Furthermore, ASOs, including nusinersen, come with risks, such as coagulation abnormalities, renal toxicity, and thrombocytopenia²¹, and intrathecal administration also comes with risks, including meningitis⁸¹ and post-lumbar puncture syndrome⁸². Risdiplam is another drug available for treatment of SMA that increases production of full-length SMN protein also by modulating splicing of *SMN2* pre-mRNA²². Daily oral administration of risdiplam led to modest improvements in motor function in clinical trial, but lower than that observed with nusinersen^{79,83}. In animal models, risdiplam has been associated with retinal toxicity and off-target splicing of other genes²². Onasemnogene abeparvovec is an adeno-associated virus (AAV) gene therapy drug that increases cellular levels of SMN protein through delivery of a functional copy of the *SMN1* cDNA^{84,85}. Many of the children treated in clinical trial gained new motor milestones and survived without the need for

permanent ventilation, which rarely occurs in untreated patients⁸⁴⁻⁸⁸. However, many safety concerns have been raised due to the high absolute dose of vector delivered to patients, up to 2.3×10^{15} vector genomes⁸⁹, and the severe adverse events that have occurred in some treated patients or preclinical models, including thrombotic microangiopathy²⁴, necrotizing enterocolitis⁹⁰, and acute liver failure^{23,91,92}. As there are several issues with the available therapies for treatment of SMA, there is still a need for development of alternative therapeutics.

A number of different approaches have been investigated to deliver SMN protein to cells. A recombinant SMN protein fused to the catalytic and transmembrane domains of diphtheria toxin (Dtx) and tetanus toxin fragment C (TTC) was investigated as a method to target the SMN protein specifically to neurons⁹³. Unfortunately, in tissue culture, the SMN-Dtx-TTC fusion protein remained at the surface of primary neurons, and was unable to transit to the nucleus. A fusion protein of SMN and the cell-penetrating peptide transactivator of transcription (TAT) of human immunodeficiency virus was also unable to be internalized by rat cortical neurons or SH-SY5Y cells, a neuroblastoma cell line⁹⁴. We have now shown that an Ad-based vector can be used to greatly enhance the quantity of SMN protein within EVs released from cells, and that such EVs can very effectively deliver the protein to recipient cells. Previous studies have shown that many EV-delivered proteins aggregate in the cytoplasm, or fail to escape the endosome and consequently these EVs and their contents are degraded in the lysosome⁹⁵. Importantly, in our study, the delivered 3F-SMN protein localized to gem-like structures in the nucleus and co-localized with cellular Gemin2 protein.

Thus, we have developed a new and effective method to deliver SMN protein to cells in tissue culture.

EV-mediated delivery of SMN protein could be useful as a therapeutic approach to supply SMN protein to patients with SMA. EVs can naturally cross the BBB to efficiently deliver cargo to recipient cells in the CNS^{57,58,96}. *In vivo*, systemically administered EVs can deliver cargo to cells of various tissues including the brain, heart, kidney, lung, and skeletal muscle^{58,97}. Thus, SMN protein-loaded EVs would likely be able to replenish SMN protein levels in a wide variety of cell types in patients with SMA. However, due to the ~6-hour half-life of SMN protein^{98,99}, daily injection of SMN-loaded EVs would likely be required to ensure the treatment remained effective. There are a number of methods that could be used to prepare the large batches of EVs that would be required for such a therapy, such as tangential flow filtration (TFF) that can effectively purify EVs from large volumes of biofluid^{53,100,101}. EVs have been shown to be safe in clinical trials for treatment of COVID-19-induced pneumonia and respiratory failure¹⁰²⁻¹⁰⁴, cancer^{105,106}, and wound healing¹⁰⁷. EVs have several other advantages as therapeutic platforms, such as their relatively stable shelf life^{108,109} and low immunogenicity when delivered to allogenic recipients¹¹⁰.

EVs loaded with SMN protein could also be useful as a transient or top-up therapy. Typically, there is a delay between initial diagnosis and initiation of treatment for patients with SMA, which is usually ~1-2 months for administration of onasemnogene abeparvovec, during which the individual may continue to decline due to disease progression^{2,111,112}. SMN-loaded EVs could be used to supply SMN

protein during this lag-time. EVs can cross the placental barrier¹¹³, suggesting administration of SMN-loaded EVs could be a potential therapeutic strategy to deliver supplemental SMN protein to a fetus identified as at-risk of severe SMA, to slow SMA pathology until alternative therapy can be administered post-birth. Supplemental SMN protein delivered in EVs could also benefit patients receiving nusinersen and/or risdiplam. Patients receiving these drugs can show varying responses, with some patients not gaining or maintaining motor milestones^{19,79,83,114,115}. Supplemental SMN protein therapy could augment the therapeutic benefit of these drugs in these patients. In short, SMN-loaded EVs could provide an additional supply of SMN protein, potentially providing therapeutic benefit to a spectrum of patients with SMA.

All current therapeutics for SMA increase SMN protein levels in peripheral tissues, including liver and muscle¹¹⁶⁻¹¹⁸. For example, systemic administration of onasemnogene abeparvovec in human patients with SMA led to ~300 vector genomes per hepatocyte¹¹⁷, and high levels of SMN mRNA transcripts relative to GAPDH in liver tissue, which would almost certainly be accompanied by significant increases in SMN protein expression in the liver. The liver readily releases EVs⁹⁷ that can be found in blood^{119,120}. It is intriguing to speculate that at least some of the therapeutic efficacy of these three approved treatments may be due to the production of SMN protein at sites other than the CNS, such as the liver, resulting in release of EVs containing the SMN protein, which can then be taken up by virtually all tissues in the body. Full-length SMN mRNA transcripts have been detected within EVs isolated from the serum of patients with SMA treated with nusinersen, but not

within EVs isolated from cerebral spinal fluid (CSF)¹²¹. These EVs could deliver the SMN mRNA to cell throughout the body, resulting in an increase in full-length SMN protein synthesis in these cells in nusinersen-treated patients with SMA. Since EVs readily cross the BBB⁵⁸, our work suggests a new approach in which therapeutics do not necessarily need to be targeted directly to the CNS. Peripheral tissues, such as liver or muscle, could be harnessed as factories to produce therapeutic EVs that circulate and impact tissues throughout the body, including the CNS. This approach could be used for any disease to achieve wide-spread delivery of a therapeutic protein throughout the body.

Conclusion

We have shown that EVs can deliver SMN protein to recipient cells in tissue culture. This delivered SMN protein localized to the nucleus and interacted with normal cellular protein partners, indicating the delivered protein was able to perform in a manner similar to endogenous SMN protein. Our findings highlight a novel approach for delivery of SMN protein to cells, which could be harnessed to develop a new therapeutic strategy for treatment of SMA.

2.5 Materials and Methods

Cell culture. A459 cells¹²² were obtained from the American Type Culture Collection (ATCC) (CCL-185, ATCC, Manassas, VA) and were grown in Minimum Essential Medium (MEM) (M2279, Sigma, Oakville, ON) supplemented with 10% (v/v) fetal bovine serum (FBS) (35-077-CV, Corning, Corning, NY), 2 mM GlutaMAX (35050061, Gibco, Grand Island, NY), and 1X antimycotic-antibiotic (15240062, Gibco). A549:HA-Gemin2 cells stably expressing an N-terminal HA-tagged Gemin2 protein were generated as follows. pRP3268 contains the cytomegalovirus (CMV) immediate early enhancer/promoter driving expression of a HA-tagged human *GEMIN2* cDNA linked to a gene for hygromycin resistance through an internal ribosome entry site (IRES). A549 cells were transfected with pRP3268 using lipofectamine 2000 (Invitrogen, Carlsbad, CA), and pooled resistant cells were selected at 200 µg/mL hygromycin (Invitrogen). HepG2 cells⁶⁷ (HB-8065, ATCC), were grown in Dulbecco's Modified Eagle Medium (D5671, DMEM, Sigma) supplemented with 10% FBS (Gibco), 2 mM GlutaMAX (Gibco), and 1X antimycotic-antibiotic (Gibco). HepG2:3F-SMN cells stably expressing an N-terminal triple FLAG-tagged SMN protein were generated as follows. pLN103 contains the CMV immediate early enhancer/promoter driving expression of an N-terminal triple FLAG-tagged human *SMN1* cDNA linked to a gene for hygromycin resistance through an IRES. HepG2 cells were transfected with pLN103 with Lipofectamine 2000 (Invitrogen), and pooled resistant cells were selected at 400 µg/mL hygromycin (Invitrogen). The human fibroblast cell line GM00232 was obtained from Coriell Institute for Medical Research (Camden, NJ). GM00232 was originally collected from

a 7-month old male diagnosed with SMA type 1 with a homozygous deletion of exons 7 and 8 of *SMN1* and 2 copies of *SMN2*. Cells were maintained in MEM (Sigma) supplemented with 15% (v/v) FBS (Gibco), 2 mM GlutaMAX (Gibco), and 1X antimycotic-antibiotic (Gibco). All cells were incubated at 37°C, 5% CO₂ in a humidified chamber.

Viral infection. Ad3F-SMN is an adenovirus vector lacking early region 1 (E1) and E3 and possessing an expression cassette containing the CMV immediate early enhancer/promoter driving expression of a triple FLAG-tagged *SMN1* cDNA. AdmCherry-SMN is an adenovirus vector lacking E1 and E3 and possessing an expression cassette containing the CMV immediate early enhancer/promoter driving expression of an mCherry-tagged *SMN1* cDNA. Ad3F-SMN and AdmCherry-SMN were grown, purified, and titered on 293 cells as previously described¹²³.

HepG2 cells were infected with Ad3F-SMN or AdmCherry-SMN at the indicated multiplicity of infection (MOI) or mock infected with an equivalent volume of phosphate buffered saline (PBS) (D8537, Sigma) for 1 h at 37°C, with rocking to redistribute the virus every 15 min. Cells were then washed three times with PBS to remove unattached virus. Cell lysates and conditioned medium (CM) were collected 72 hpi.

Isolation of extracellular vesicles (EVs). EVs were isolated from CM to concentrate the particles for immunoblot analysis. Ten 150 mm dishes of HepG2 or HepG2:3F-SMN cells seeded at a density of 5x10⁶ cells per dish were used for EV

isolation. Seventy-two hours post-plating, CM was collected and EVs isolated by differential ultra-centrifugation, adapted from Théry et al¹²⁴. The CM was centrifuged at 2,500xg for 15 min to pellet apoptotic bodies and cellular debris. The resulting supernatant was collected and subjected to ultra-centrifugation for 90 min at 120,000xg at 4°C in a SW32 Ti rotor (Beckman Coulter, Mississauga, ON). The resulting pellets were resuspended in PBS (Sigma), pooled, and ultra-centrifuged at 120,000xg in a SW41 Ti rotor (Beckman Coulter) for 90 min at 4°C. The resulting pellet was resuspended in 300µL of PBS and stored at -20°C.

EVs were isolated from HepG2 cells infected with Ad3F-SMN using a polyethylene glycol (PEG)-based approach. HepG2 cells were plated in one 100 mm dish per treatment condition (4.4×10^6 cells per dish) and, next day, infected with Ad3F-SMN as described above. Seventy-two hpi, CM was collected and centrifuged at 2,500xg for 15 min, the resulting supernatant was collected and mixed 1:1 with 16% PEG (w/v) prepared in PBS (Sigma) and incubated at 4°C overnight. Following incubation, the CM/PEG solution was centrifuged at 1,500xg for 30 min to pellet the EVs. The supernatant was discarded and the pellet was washed with 500 µL of PBS and centrifuged at 1,500xg for 5 min. The supernatant was discarded and the resulting pellet was washed with 500 µL PBS, then resuspended in 300 µL of PBS and stored at -20°C.

Concentration and diameter of the EVs was determined by nanoparticle tracking analysis (NTA) using a NanoSight NS300 (Malvern Panalytical, Westborough, MA). NanoSight NTA 3.4 software was used to capture videos and analyze isolated particles. Samples were diluted in 0.1 µm filtered PBS immediately

before analysis. For each sample, 2 independent analyses were conducted using a syringe pump, each capturing 60 s of video 3 times. Videos were captured using the following settings: syringe pump speed 50, focus 129, screen gain 2.2, camera level 14. Particles were analyzed using the following settings: screen gain 10, detection threshold 6.

CM Treatments. For all media transfer experiments, the CM was subjected to low-speed centrifugation to remove cell debris and large EVs (e.g. apoptotic bodies) prior to application to the recipient cells, but was not concentrated or further processed. HepG2 cells were plated in 100 mm dishes (4.4×10^6 cells per dish) and, next day, infected with Ad3F-SMN or AdmCherry-SMN as described above at an MOI=200. Seventy-two hpi, CM was collected and centrifuged at 2,500xg for 15 min to remove apoptotic bodies and cellular debris. CM was either used immediately or frozen at -80°C until time of use. For frozen CM, the CM was thawed in a 37°C water bath and centrifuged at 2,500xg for 15 min to pellet residual debris prior to application to the recipient cells.

For recipient cells that were to undergo immunofluorescent staining, cells were seeded at a density of 3×10^4 cells per well in 8-well chambered slides (80841, Ibidi, Fitchburg, WI). The next day, medium was removed and the cells were pre-treated with 100 μM of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) resuspended in dimethyl sulfoxide (DMSO) or vehicle control, both at a final DMSO concentration of 0.001% v/v for 1 h at 37°C . Two hundred μL of CM per well was supplemented with 100 μM of DRB or vehicle, both at a final DMSO concentration

of 0.001% v/v, and the cells were treated for 2 h at 37°C. The cells were washed 3 times with PBS and prepared for immunofluorescence staining as detailed below. Parental A549 cells and fibroblasts derived from a patient with SMA underwent the same process without the addition of DRB.

Co-immunoprecipitation. Cells in 150 mm dishes were trypsinized and counted, and 5×10^6 cells were lysed in 500 μ L of RIPA buffer (New England Biolabs). Lysates were cleared by centrifugation at 21,130xg for 10 min at 4°C, and the supernatant transferred to a fresh tube. The cleared lysate was aliquoted into 4 tubes (100 μ L/tube), and the volume adjusted to 400 μ L with RIPA buffer (New England Biolabs). The remaining portion of cleared lysate was retained to use as the input sample during immunoblot analysis. Four μ g of anti-FLAG (F1804, Sigma), mouse IgG isotype control (5415, Cell Signalling, Danvers, MA), anti-HA (ab236632, Abcam), or rabbit IgG isotype control (2729, Cell Signalling) was added to the diluted lysate and mixed by rotation at 4°C overnight. Forty μ L of Dynabeads protein G (10004D, Invitrogen) or Dynabeads protein A (10001D, Invitrogen) were washed in RIPA buffer, then added to each tube of lysate/antibody solution and incubated at 4°C for 90 min. The bound and unbound fractions were separated using a magnetic tube rack. The bound fraction was washed 3 times with RIPA buffer and resuspended in 40 μ L 2x Laemmli buffer (62.5 mM Tris-HCl pH 6.8, 25% w/v glycerol, 5% v/v β -mercaptoethanol, 2% w/v sodium dodecyl sulphate (SDS), 0.01% w/v bromophenol blue).

Immunoblot. Cells and EVs were either lysed in passive lysis buffer (Promega) at the time of collection then mixed with an equal volume of 2x Laemmli buffer or lysed directly in 2x Laemmli buffer, and stored at -20°C . Cells cultured in 35 mm, 100 mm, and 150 mm dishes were collected in 200 μL , 500 μL , and 1000 μL final volume of buffer, respectively. Samples were boiled for 10 min prior to protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA). Membranes were blocked with Intercept (PBS) Blocking Buffer (927-70001, LI-COR Biosciences, Nebraska) for 1 h at RT and probed with the following primary antibodies diluted in blocking buffer supplemented with 0.2% Tween 20: SMN (1:5000 1 h at room temperature (RT) for cell lysates, 1:1000 overnight at 4°C for EVs; BD Transduction, Mississauga, ON), FLAG (1:5000 1 h at RT for cell lysates, 1:1000 overnight at 4°C for EVs; F1804, Sigma), Tubulin (1:10000 1 h at RT; ab59670, Abcam, Cambridge, UK or CP06, Millipore), Flotillin-2 (1:1000 overnight at 4°C ; C42A3, Cell Signaling Technologies, Danvers, MA), Gemin2 (1:5000 1 h at RT; ab7084, Abcam), ALIX (1:1000 overnight at 4°C ; ab186429, Abcam), CD29/Integrin beta-1 (1:1000 overnight at 4°C ; 04-1109, Millipore), HA (1:5000 overnight at 4°C ; ab236632, Abcam). Membranes were washed three times in PBS containing 0.1% Tween 20 (PBST), and incubated with the following fluorescently-labelled secondary antibodies in blocking buffer supplemented with 0.2% Tween 20 and 0.01% SDS: IRDye goat anti-mouse 680 (926-68070, LI-COR), IRDye goat anti-mouse 800 (925-32210), IRDye goat anti-rabbit 680 (925-68071, LI-COR), IRDye goat anti-rabbit 800 (926-32211, LI-COR).

Membranes were developed using Odyssey CLx and visualized using LI-COR Image Studio 4.0 software.

Immunofluorescence staining and microscopy. Cells were fixed in 4% paraformaldehyde in PBS for 10 min at RT, washed 3 times with PBS, and permeabilized with 0.25% Triton X-100 (Sigma) in PBS for 10 min at RT. The cells were blocked in 10% donkey serum (S30, Millipore) in PBS for 30 min at RT, and then incubated in 10% donkey serum (Millipore) overnight at 4°C with the following antibodies: SMN (1:250; BD Transduction), FLAG (1:250; F1804, Sigma), Tubulin (1:500; ab59670, Abcam), Gemin2 (1:250; ab7084, Abcam), HA (1:250, ab236632, Abcam). The cells were washed three times for 5 min with 1% donkey serum (Millipore) in PBS, then incubated with the following secondary antibodies in 10% donkey serum in PBS (Millipore) for 1 h at RT; Alexa Fluor donkey anti-rabbit 488 (1:1000; A21206, Invitrogen, Eugene, OR), Alexa Fluor donkey anti-rabbit 594 (1:1000; A21207, Invitrogen), Alexa Fluor donkey anti-mouse 488 (1:1000; A21202, Invitrogen) or Alexa Fluor donkey anti-mouse 594 (1:1000; A21203, Invitrogen). Following incubation with secondary antibody, the cells were stained with Hoechst 33342 at a concentration of 1 µg/mL (H3570, Life Technologies, Eugene, OR) in 1% donkey serum in PBS (Millipore) for 5 min at RT to visualize nuclei. The cells were washed three times for 5 min each with 1% donkey serum in PBS. Epifluorescence microscopy images were captured with a Zeiss Axio Imager M2 upright microscope, and processed using FIJI software¹²⁵. Confocal images were captured with a Zeiss LSM 900 inverted confocal microscope, and processed using FIJI. Orthogonal

projections were generated from 4-9 z-stacks captured every 1.0 μm using ZEN blue version 3.2 (Carl Zeiss Microscopy, Oberkochen, Germany). Fluorescence intensity in the images was quantified using CellProfiler¹²⁶.

Nuclear gem quantification was conducted as follows: For the data included in figures 2.1 and 2.3, the number of nuclear gems per nucleus was counted in three independent fields of view each containing 50-80 cells, and converted to the number of nuclear gems per 100 nuclei for each field of view. Statistical significance was assessed using each independent fields of view as a separate replicate. For the data included in figure 2.5, three independent media transfer experiments were conducted. For each experiment, three images per treatment group were captured. The average number of gems per nucleus was quantified in each image, and the average of the three images, each containing 60-100 nuclei, was calculated to gather each independent replicate. The number of nuclear gems per nucleus was then converted to the number of nuclear gems per 100 nuclei. Statistical significance was assessed using each independent experiment as a separate replicate.

Statistical analysis. All statistical analyses were performed using Prism version 9.1.0, and the test performed is indicated in the figure legend. Parametric tests were chosen to evaluate statistical significance as the data is not ranked or ordinal, and is normally distributed. Data is presented as the mean $\pm$ standard deviation. Data in figures 2.4 and 2.5 was transformed by log transformation to analyze the data in different treatment groups on a similar scale. In all tests, one asterisk (*) indicates $p < 0.05$, two asterisk (**) indicates $p < 0.01$, three asterisk (***) indicates $p < 0.001$, and

four asterisk (****) indicates $p < 0.0001$.

2.6 References

- 1 Lefebvre, S. *et al.* Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* **80**, 155-165, doi:10.1016/0092-8674(95)90460-3 (1995).
- 2 Lefebvre, S. *et al.* Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat Genet* **16**, 265-269, doi:10.1038/ng0797-265 (1997).
- 3 Kolb, S. J. & Kissel, J. T. Spinal Muscular Atrophy. *Neurol Clin* **33**, 831-846, doi:10.1016/j.ncl.2015.07.004 (2015).
- 4 Kolb, S. J. *et al.* Natural history of infantile-onset spinal muscular atrophy. *Ann Neurol* **82**, 883-891, doi:10.1002/ana.25101 (2017).
- 5 Finkel, R. S. *et al.* Observational study of spinal muscular atrophy type I and implications for clinical trials. *Neurology* **83**, 810-817, doi:10.1212/WNL.0000000000000741 (2014).
- 6 Burns, J. K., Kothary, R. & Parks, R. J. Opening the window: The case for carrier and perinatal screening for spinal muscular atrophy. *Neuromuscul Disord* **26**, 551-559, doi:10.1016/j.nmd.2016.06.459 (2016).
- 7 Bevan, A. K. *et al.* Early heart failure in the SMN Δ 7 model of spinal muscular atrophy and correction by postnatal scAAV9-SMN delivery. *Hum Mol Genet* **19**, 3895-3905, doi:10.1093/hmg/ddq300 (2010).
- 8 Shababi, M. *et al.* Partial restoration of cardio-vascular defects in a rescued severe model of spinal muscular atrophy. *J Mol Cell Cardiol* **52**, 1074-1082, doi:10.1016/j.yjmcc.2012.01.005 (2012).
- 9 Szunyogova, E. *et al.* Survival Motor Neuron (SMN) protein is required for normal mouse liver development. *Sci Rep* **6**, 34635, doi:10.1038/srep34635 (2016).
- 10 Deguise, M. O. *et al.* Abnormal fatty acid metabolism is a core component of spinal muscular atrophy. *Ann Clin Transl Neurol* **6**, 1519-1532, doi:10.1002/acn3.50855 (2019).
- 11 Deguise, M. O. *et al.* Immune dysregulation may contribute to disease pathogenesis in spinal muscular atrophy mice. *Hum Mol Genet* **26**, 801-819, doi:10.1093/hmg/ddw434 (2017).
- 12 Bowerman, M. *et al.* Glucose metabolism and pancreatic defects in spinal muscular atrophy. *Ann Neurol* **72**, 256-268, doi:10.1002/ana.23582 (2012).
- 13 Bowerman, M. *et al.* Defects in pancreatic development and glucose metabolism in SMN-depleted mice independent of canonical spinal muscular atrophy neuromuscular pathology. *Hum Mol Genet* **23**, 3432-3444, doi:10.1093/hmg/ddu052 (2014).

- 14 Gombash, S. E. *et al.* SMN deficiency disrupts gastrointestinal and enteric nervous system function in mice. *Hum Mol Genet* **24**, 5665, doi:10.1093/hmg/ddv292 (2015).
- 15 Thomson, A. K. *et al.* Survival of motor neurone protein is required for normal postnatal development of the spleen. *J Anat* **230**, 337-346, doi:10.1111/joa.12546 (2017).
- 16 Chiriboga, C. A. *et al.* Results from a phase 1 study of nusinersen (ISIS-SMN(Rx)) in children with spinal muscular atrophy. *Neurology* **86**, 890-897, doi:10.1212/WNL.0000000000002445 (2016).
- 17 Baranello, G. *et al.* Risdiplam in Type 1 Spinal Muscular Atrophy. *N Engl J Med* **384**, 915-923, doi:10.1056/NEJMoa2009965 (2021).
- 18 Mendell, J. R. *et al.* Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N Engl J Med* **377**, 1713-1722, doi:10.1056/NEJMoa1706198
10.1056/NEJMoa1706198. (2017).
- 19 Mercuri, E. *et al.* Nusinersen versus Sham Control in Later-Onset Spinal Muscular Atrophy. *N Engl J Med* **378**, 625-635, doi:10.1056/NEJMoa1710504 (2018).
- 20 René, C. A. & Parks, R. J. Expanding the Availability of Onasemnogene Abeparvovec to Older Patients: The Evolving Treatment Landscape for Spinal Muscular Atrophy. *Pharmaceutics* **15**, doi:10.3390/pharmaceutics15061764 (2023).
- 21 Biogen. *Spinraza prescribing information*, <https://www.spinraza.com/content/dam/commercial/spinraza/caregiver/en_us/pdf/spinraza-prescribing-information.pdf> (2020).
- 22 Ratni, H. *et al.* Discovery of Risdiplam, a Selective Survival of Motor Neuron-2 (SMN2) Gene Splicing Modifier for the Treatment of Spinal Muscular Atrophy (SMA). *J Med Chem* **61**, 6501-6517, doi:10.1021/acs.jmedchem.8b00741 (2018).
- 23 Chand, D. *et al.* Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy. *J Hepatol* **74**, 560-566, doi:10.1016/j.jhep.2020.11.001 (2021).
- 24 Yazaki, K., Sakuma, S., Hikita, N., Fujimaru, R. & Hamazaki, T. Child Neurology: Pathologically Confirmed Thrombotic Microangiopathy Caused by Onasemnogene Abeparvovec Treatment for SMA. *Neurology* **98**, 808-813, doi:10.1212/WNL.0000000000200676 (2022).
- 25 Pagliardini, S. *et al.* Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet* **9**, 47-56, doi:10.1093/hmg/9.1.47 (2000).

- 26 Coover, D. D. *et al.* The survival motor neuron protein in spinal muscular atrophy. *Hum Mol Genet* **6**, 1205-1214, doi:10.1093/hmg/6.8.1205 (1997).
- 27 Li, D. K., Tisdale, S., Lotti, F. & Pellizzoni, L. SMN control of RNP assembly: from post-transcriptional gene regulation to motor neuron disease. *Semin Cell Dev Biol* **32**, 22-29, doi:10.1016/j.semcdb.2014.04.026 (2014).
- 28 Lorson, C. L., Hahnen, E., Androphy, E. J. & Wirth, B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc Natl Acad Sci U S A* **96**, 6307-6311, doi:10.1073/pnas.96.11.6307 (1999).
- 29 Liu, Q., Fischer, U., Wang, F. & Dreyfuss, G. The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell* **90**, 1013-1021, doi:10.1016/s0092-8674(00)80367-0 (1997).
- 30 Fischer, U., Liu, Q. & Dreyfuss, G. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell* **90**, 1023-1029, doi:10.1016/s0092-8674(00)80368-2 (1997).
- 31 Battle, D. J., Kasim, M., Wang, J. & Dreyfuss, G. SMN-independent subunits of the SMN complex. Identification of a small nuclear ribonucleoprotein assembly intermediate. *J Biol Chem* **282**, 27953-27959, doi:10.1074/jbc.M702317200 (2007).
- 32 Carissimi, C., Saieva, L., Gabanella, F. & Pellizzoni, L. Gemin8 is required for the architecture and function of the survival motor neuron complex. *J Biol Chem* **281**, 37009-37016, doi:10.1074/jbc.M607505200 (2006).
- 33 Goulet, B. B., Kothary, R. & Parks, R. J. At the "junction" of spinal muscular atrophy pathogenesis: the role of neuromuscular junction dysfunction in SMA disease progression. *Curr Mol Med* **13**, 1160-1174, doi:10.2174/15665240113139990044 (2013).
- 34 Pellizzoni, L., Baccon, J., Charroux, B. & Dreyfuss, G. The survival of motor neurons (SMN) protein interacts with the snoRNP proteins fibrillarin and GAR1. *Curr Biol* **11**, 1079-1088, doi:10.1016/s0960-9822(01)00316-5 (2001).
- 35 Renvoisé, B. *et al.* The loss of the snoRNP chaperone Nopp140 from Cajal bodies of patient fibroblasts correlates with the severity of spinal muscular atrophy. *Hum Mol Genet* **18**, 1181-1189, doi:10.1093/hmg/ddp009 (2009).
- 36 Bachand, F., Boisvert, F. M., Côté, J., Richard, S. & Autexier, C. The product of the survival of motor neuron (SMN) gene is a human telomerase-associated protein. *Mol Biol Cell* **13**, 3192-3202, doi:10.1091/mbc.e02-04-0216 (2002).

- 37 Poole, A. R. & Hebert, M. D. SMN and coilin negatively regulate dyskerin association with telomerase RNA. *Biol Open* **5**, 726-735, doi:10.1242/bio.018804 (2016).
- 38 Blackburn, E. H. & Collins, K. Telomerase: an RNP enzyme synthesizes DNA. *Cold Spring Harb Perspect Biol* **3**, doi:10.1101/cshperspect.a003558 (2011).
- 39 Sanchez, G. *et al.* A novel function for the survival motoneuron protein as a translational regulator. *Hum Mol Genet* **22**, 668-684, doi:10.1093/hmg/dds474 (2013).
- 40 Chaytow, H., Huang, Y. T., Gillingwater, T. H. & Faller, K. M. E. The role of survival motor neuron protein (SMN) in protein homeostasis. *Cellular and molecular life sciences : CMLS* **75**, 3877-3894, doi:10.1007/s00018-018-2849-1 (2018).
- 41 Lauria, F. *et al.* SMN-primed ribosomes modulate the translation of transcripts related to spinal muscular atrophy. *Nature cell biology* **22**, 1239-1251, doi:10.1038/s41556-020-00577-7 (2020).
- 42 Rossoll, W. *et al.* Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *J Cell Biol* **163**, 801-812, doi:10.1083/jcb.200304128 (2003).
- 43 Ting, C. H. *et al.* The spinal muscular atrophy disease protein SMN is linked to the Golgi network. *PLoS One* **7**, e51826, doi:10.1371/journal.pone.0051826 (2012).
- 44 Bowerman, M. *et al.* SMN, profilin IIa and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis. *Mol Cell Neurosci* **42**, 66-74, doi:10.1016/j.mcn.2009.05.009 (2009).
- 45 Bowerman, M., Beauvais, A., Anderson, C. L. & Kothary, R. Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model. *Hum Mol Genet* **19**, 1468-1478, doi:10.1093/hmg/ddq021 (2010).
- 46 Zhao, D. Y. *et al.* SMN and symmetric arginine dimethylation of RNA polymerase II C-terminal domain control termination. *Nature* **529**, 48-53, doi:10.1038/nature16469 (2016).
- 47 Nash, L. A. *et al.* Survival Motor Neuron Protein is Released from Cells in Exosomes: A Potential Biomarker for Spinal Muscular Atrophy. *Sci Rep* **7**, 13859, doi:10.1038/s41598-017-14313-z (2017).
- 48 Hurwitz, S. N. *et al.* Proteomic profiling of NCI-60 extracellular vesicles uncovers common protein cargo and cancer type-specific biomarkers. *Oncotarget* **7**, 86999-87015, doi:10.18632/oncotarget.13569 (2016).
- 49 Skogberg, G. *et al.* Characterization of human thymic exosomes. *PLoS One* **8**, e67554, doi:10.1371/journal.pone.0067554 (2013).

- 50 Xu, X. *et al.* Quantitative proteomic analysis of exosomes from umbilical cord mesenchymal stem cells and rat bone marrow stem cells. *Proteomics* **23**, e2200204, doi:10.1002/pmic.202200204 (2023).
- 51 Benedikter, B. J. *et al.* Ultrafiltration combined with size exclusion chromatography efficiently isolates extracellular vesicles from cell culture media for compositional and functional studies. *Sci Rep* **7**, 15297, doi:10.1038/s41598-017-15717-7 (2017).
- 52 Liang, B. *et al.* Characterization and proteomic analysis of ovarian cancer-derived exosomes. *J Proteomics* **80**, 171-182, doi:10.1016/j.jprot.2012.12.029 (2013).
- 53 Welsh, J. A. *et al.* Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J Extracell Vesicles* **13**, e12404, doi:10.1002/jev2.12404 (2024).
- 54 Mathivanan, S., Ji, H. & Simpson, R. J. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics* **73**, 1907-1920, doi:10.1016/j.jprot.2010.06.006 (2010).
- 55 Wiklander, O. P. *et al.* Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. *J Extracell Vesicles* **4**, 26316, doi:10.3402/jev.v4.26316 (2015).
- 56 Wiklander, O. P. B., Brennan, M., Lötvall, J., Breakefield, X. O. & El Andaloussi, S. Advances in therapeutic applications of extracellular vesicles. *Sci Transl Med* **11**, doi:10.1126/scitranslmed.aav8521 (2019).
- 57 Morad, G. *et al.* Tumor-Derived Extracellular Vesicles Breach the Intact Blood-Brain Barrier. *ACS Nano* **13**, 13853-13865, doi:10.1021/acsnano.9b04397 (2019).
- 58 Driedonks, T. *et al.* Pharmacokinetics and biodistribution of extracellular vesicles administered intravenously and intranasally to. *J Extracell Biol* **1**, doi:10.1002/jex2.59 (2022).
- 59 René, C. A. & Parks, R. J. Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles. *Pharmaceutics* **13**, doi:10.3390/pharmaceutics13040492 (2021).
- 60 René, C. A. & Parks, R. J. Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency. *Mol Ther Methods Clin Dev* **32**, 101259, doi:10.1016/j.omtm.2024.101259 (2024).
- 61 Haney, M. J. *et al.* Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release* **207**, 18-30, doi:10.1016/j.jconrel.2015.03.033 (2015).
- 62 Izadpanah, M. *et al.* Extracellular Vesicles as a Neprilysin Delivery System Memory Improvement in Alzheimer's Disease. *Iran J Pharm Res* **19**, 45-60, doi:10.22037/ijpr.2020.112062.13508 (2020).

- 63 Goulet, B. B., McFall, E. R., Wong, C. M., Kothary, R. & Parks, R. J. Supraphysiological expression of survival motor neuron protein from an adenovirus vector does not adversely affect cell function. *Biochem Cell Biol* **91**, 252-264, doi:10.1139/bcb-2012-0094 (2013).
- 64 Aoto, K., Takabayashi, S., Mutoh, H. & Saitsu, H. Generation of Flag/DYKDDDDK Epitope Tag Knock-In Mice Using. *Int J Mol Sci* **23**, doi:10.3390/ijms231911915 (2022).
- 65 Babaylova, E. S. *et al.* mRNA regions where 80S ribosomes pause during translation elongation in vivo interact with protein uS19, a component of the decoding site. *Nucleic Acids Res* **48**, 912-923, doi:10.1093/nar/gkz1145 (2020).
- 66 DiDonato, C. J., Parks, R. J. & Kothary, R. Development of a gene therapy strategy for the restoration of survival motor neuron protein expression: implications for spinal muscular atrophy therapy. *Hum Gene Ther* **14**, 179-188, doi:10.1089/104303403321070874 (2003).
- 67 Knowles, B. B., Howe, C. C. & Aden, D. P. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science* **209**, 497-499, doi:10.1126/science.6248960 (1980).
- 68 Bochkov, Y. A. & Palmenberg, A. C. Translational efficiency of EMCV IRES in bicistronic vectors is dependent upon IRES sequence and gene location. *Biotechniques* **41**, 283-284, 286, 288 passim, doi:10.2144/000112243 (2006).
- 69 Feng, W. *et al.* Gemin3 modulate the expression and activity of the SMN complex. *Hum Mol Genet* **14**, 1605-1611, doi:10.1093/hmg/ddi168 (2005).
- 70 Carissimi, C. *et al.* Gemin8 is a novel component of the survival motor neuron complex and functions in small nuclear ribonucleoprotein assembly. *J Biol Chem* **281**, 8126-8134, doi:10.1074/jbc.M512243200 (2006).
- 71 Meier, I. D., Walker, M. P. & Matera, A. G. Gemin4 is an essential gene in mice, and its overexpression in human cells causes relocalization of the SMN complex to the nucleoplasm. *Biol Open* **7**, doi:10.1242/bio.032409 (2018).
- 72 Ross, P. J. *On the role of chromatin in the regulation of adenovirus vector transgene expression* Doctorate of Philosophy thesis, University of Ottawa, (2009).
- 73 Glenn, G. M. & Ricciardi, R. P. Detailed kinetics of adenovirus type-5 steady-state transcripts during early infection. *Virus Res* **9**, 73-91, doi:10.1016/0168-1702(88)90051-2 (1988).
- 74 Yamaguchi, Y., Wada, T. & Handa, H. Interplay between positive and negative elongation factors: drawing a new view of DRB. *Genes Cells* **3**, 9-15, doi:10.1046/j.1365-2443.1998.00162.x (1998).

- 75 Giberson, A. N. *et al.* Human adenoviral DNA association with nucleosomes containing histone variant H3.3 during the early phase of infection is not dependent on viral transcription or replication. *Biochem Cell Biol* **96**, 797-807, doi:10.1139/bcb-2018-0117 (2018).
- 76 Ross, P. J. *et al.* Assembly of helper-dependent adenovirus DNA into chromatin promotes efficient gene expression. *J Virol* **85**, 3950-3958, doi:10.1128/JVI.01787-10 (2011).
- 77 Yeo, C. J. J. & Darras, B. T. Overturning the Paradigm of Spinal Muscular Atrophy as Just a Motor Neuron Disease. *Pediatr Neurol* **109**, 12-19, doi:10.1016/j.pediatrneurol.2020.01.003 (2020).
- 78 Nash, L. A., Burns, J. K., Chardon, J. W., Kothary, R. & Parks, R. J. Spinal Muscular Atrophy: More than a Disease of Motor Neurons? *Curr Mol Med* **16**, 779-792, doi:10.2174/1566524016666161128113338 (2016).
- 79 Finkel, R. S. *et al.* Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. *N Engl J Med* **377**, 1723-1732, doi:10.1056/NEJMoa1702752 (2017).
- 80 Passini, M. A. *et al.* Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci Transl Med* **3**, 72ra18, doi:10.1126/scitranslmed.3001777 (2011).
- 81 Günther, R. *et al.* Long-term efficacy and safety of nusinersen in adults with 5q spinal muscular atrophy: a prospective European multinational observational study. *Lancet Reg Health Eur* **39**, 100862, doi:10.1016/j.lanepe.2024.100862 (2024).
- 82 Cordts, I. *et al.* Intrathecal nusinersen administration in adult spinal muscular atrophy patients with complex spinal anatomy. *Ther Adv Neurol Disord* **13**, 1756286419887616, doi:10.1177/1756286419887616 (2020).
- 83 Mercuri, E. *et al.* Risdiplam in types 2 and 3 spinal muscular atrophy: A randomised, placebo-controlled, dose-finding trial followed by 24 months of treatment. *Eur J Neurol* **00**, 1-12, doi:10.1111/ene.15499 (2022).
- 84 Day, J. W. *et al.* Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy in patients with two copies of SMN2 (STR1VE): an open-label, single-arm, multicentre, phase 3 trial. *Lancet Neurol* **20**, 284-293, doi:10.1016/s1474-4422(21)00001-6 10.1016/S1474-4422(21)00001-6. Epub 2021 Mar 17. (2021).
- 85 Mendell, J. R. *et al.* Five-Year Extension Results of the Phase 1 START Trial of Onasemnogene Abeparvovec in Spinal Muscular Atrophy. *JAMA Neurol* **78**, 834-841, doi:10.1001/jamaneurol.2021.1272 (2021).
- 86 Mercuri, E. *et al.* Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy type 1 (STR1VE-EU): an open-label,

- single-arm, multicentre, phase 3 trial. *Lancet Neurol* **20**, 832-841, doi:10.1016/S1474-4422(21)00251-9 (2021).
- 87 Strauss, K. A. *et al.* Onasemnogene abeparvovec for presymptomatic infants with two copies of SMN2 at risk for spinal muscular atrophy type 1: the Phase III SPR1NT trial. *Nat Med* **28**, 1381-1389, doi:10.1038/s41591-022-01866-4 (2022).
- 88 Strauss, K. A. *et al.* Onasemnogene abeparvovec for presymptomatic infants with three copies of SMN2 at risk for spinal muscular atrophy: the Phase III SPR1NT trial. *Nat Med* **28**, 1390-1397, doi:10.1038/s41591-022-01867-3 (2022).
- 89 Novartis. *Novartis to initiate SMART Phase 3b global study of Zolgensma in children up to 21 kg, building on real-world experience*, <<https://www.novartis.com/news/media-releases/novartis-initiate-smart-phase-3b-global-study-zolgensma-children-21-kg-building-real-world-experience>> (2021).
- 90 Gaillard, J., Gu, A. R. & Neil Knierbein, E. E. Necrotizing Enterocolitis following Onasemnogene Abeparvovec for Spinal Muscular Atrophy: A Case Series. *J Pediatr* **260**, 113493, doi:10.1016/j.jpeds.2023.113493 (2023).
- 91 Silverman, E. *Novartis reports two children died from acute liver failure after treatment with Zolgensma gene therapy*, <<https://www.statnews.com/pharmalot/2022/08/11/novartis-zolgensma-liver-failure-gene-therapy-death/>> (2022).
- 92 Novartis. *Zolgensma acute liver failure update*, <<https://www.novartis.com/news/zolgensma-acute-liver-failure-update>> (2022).
- 93 Francis, J. W. *et al.* A survival motor neuron:tetanus toxin fragment C fusion protein for the targeted delivery of SMN protein to neurons. *Brain Res* **995**, 84-96, doi:10.1016/j.brainres.2003.09.063 (2004).
- 94 Anderton, R. S., Meloni, B. P., Mastaglia, F. L. & Boulos, S. Investigation of a recombinant SMN protein delivery system to treat spinal muscular atrophy. *Translational Neuroscience* **5**, 8-16 (2014).
- 95 Pham, T. T. *et al.* Endosomal escape of nucleic acids from extracellular vesicles mediates functional therapeutic delivery. *Pharmacol Res* **188**, 106665, doi:10.1016/j.phrs.2023.106665 (2023).
- 96 Chen, C. C. *et al.* Elucidation of Exosome Migration across the Blood-Brain Barrier Model In Vitro. *Cell Mol Bioeng* **9**, 509-529, doi:10.1007/s12195-016-0458-3 (2016).
- 97 Miotto, P. M. *et al.* Liver-derived extracellular vesicles improve whole-body glycaemic control via inter-organ communication. *Nat Metab* **6**, 254-272, doi:10.1038/s42255-023-00971-z (2024).

- 98 Burnett, B. G. *et al.* Regulation of SMN protein stability. *Mol Cell Biol* **29**, 1107-1115, doi:10.1128/MCB.01262-08 (2009).
- 99 Han, K. J. *et al.* Ubiquitin-specific protease 9x deubiquitinates and stabilizes the spinal muscular atrophy protein-survival motor neuron. *J Biol Chem* **287**, 43741-43752, doi:10.1074/jbc.M112.372318 (2012).
- 100 Lorenzini, B. *et al.* Producing vesicle-free cell culture additive for human cells extracellular vesicles manufacturing. *J Control Release* **355**, 501-514, doi:10.1016/j.jconrel.2023.01.073 (2023).
- 101 Andriolo, G. *et al.* Methodologies for Scalable Production of High-Quality Purified Small Extracellular Vesicles from Conditioned Medium. *Methods Mol Biol* **2668**, 69-98, doi:10.1007/978-1-0716-3203-1_7 (2023).
- 102 Lightner, A. L. *et al.* Bone Marrow Mesenchymal Stem Cell-Derived Extracellular Vesicle Infusion for the Treatment of Respiratory Failure From COVID-19: A Randomized, Placebo-Controlled Dosing Clinical Trial. *Chest*, doi:10.1016/j.chest.2023.06.024 (2023).
- 103 Chu, M. *et al.* Nebulization Therapy with Umbilical Cord Mesenchymal Stem Cell-Derived Exosomes for COVID-19 Pneumonia. *Stem Cell Rev Rep* **18**, 2152-2163, doi:10.1007/s12015-022-10398-w (2022).
- 104 Zhu, Y. G. *et al.* Nebulized exosomes derived from allogenic adipose tissue mesenchymal stromal cells in patients with severe COVID-19: a pilot study. *Stem Cell Res Ther* **13**, 220, doi:10.1186/s13287-022-02900-5 (2022).
- 105 Guo, M. *et al.* Autologous tumor cell-derived microparticle-based targeted chemotherapy in lung cancer patients with malignant pleural effusion. *Sci Transl Med* **11**, doi:10.1126/scitranslmed.aat5690 (2019).
- 106 Kim, M. S. *et al.* Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine* **12**, 655-664, doi:10.1016/j.nano.2015.10.012 (2016).
- 107 Johnson, J. *et al.* First-in-human clinical trial of allogeneic, platelet-derived extracellular vesicles as a potential therapeutic for delayed wound healing. *J Extracell Vesicles* **12**, e12332, doi:10.1002/jev2.12332 (2023).
- 108 Wu, J. Y., Li, Y. J., Hu, X. B., Huang, S. & Xiang, D. X. Preservation of small extracellular vesicles for functional analysis and therapeutic applications: a comparative evaluation of storage conditions. *Drug Deliv* **28**, 162-170, doi:10.1080/10717544.2020.1869866 (2021).
- 109 Görgens, A. *et al.* Identification of storage conditions stabilizing extracellular vesicles preparations. *J Extracell Vesicles* **11**, e12238, doi:10.1002/jev2.12238 (2022).
- 110 Zhu, X. *et al.* Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles

- derived from HEK293T cells. *J Extracell Vesicles* **6**, 1324730, doi:10.1080/20013078.2017.1324730 (2017).
- 111 Servais, L. *et al.* Real-World Outcomes in Patients with Spinal Muscular Atrophy Treated with Onasemnogene Abeparvovec Monotherapy: Findings from the RESTORE Registry. *J Neuromuscul Dis* **11**, 425-442, doi:10.3233/JND-230122 (2024).
- 112 Gallotta, I. *et al.* Neuron-specific knock-down of SMN1 causes neuron degeneration and death through an apoptotic mechanism. *Hum Mol Genet* **25**, 2564-2577, doi:10.1093/hmg/ddw119 (2016).
- 113 Sheller-Miller, S., Choi, K., Choi, C. & Menon, R. Cyclic-recombinase-reporter mouse model to determine exosome communication and function during pregnancy. *Am J Obstet Gynecol* **221**, 502.e501-502.e512, doi:10.1016/j.ajog.2019.06.010 (2019).
- 114 Hagenacker, T. *et al.* Nusinersen in adults with 5q spinal muscular atrophy: a non-interventional, multicentre, observational cohort study. *Lancet Neurol* **19**, 317-325, doi:10.1016/S1474-4422(20)30037-5 (2020).
- 115 Darras, B. T. *et al.* Risdiplam-Treated Infants with Type 1 Spinal Muscular Atrophy versus Historical Controls. *N Engl J Med* **385**, 427-435, doi:10.1056/NEJMoa2102047 (2021).
- 116 Poirier, A. *et al.* Risdiplam distributes and increases SMN protein in both the central nervous system and peripheral organs. *Pharmacol Res Perspect* **6**, e00447, doi:10.1002/prp2.447 (2018).
- 117 Thomsen, G. *et al.* Biodistribution of onasemnogene abeparvovec DNA, mRNA and SMN protein in human tissue. *Nat Med* **27**, 1701-1711, doi:10.1038/s41591-021-01483-7 (2021).
- 118 Finkel, R. S. *et al.* Treatment of infantile-onset spinal muscular atrophy with nusinersen: a phase 2, open-label, dose-escalation study. *Lancet* **388**, 3017-3026, doi:10.1016/S0140-6736(16)31408-8 (2016).
- 119 Povero, D. *et al.* Circulating extracellular vesicles with specific proteome and liver microRNAs are potential biomarkers for liver injury in experimental fatty liver disease. *PLoS One* **9**, e113651, doi:10.1371/journal.pone.0113651 (2014).
- 120 Achour, B. *et al.* Liquid Biopsy Enables Quantification of the Abundance and Interindividual Variability of Hepatic Enzymes and Transporters. *Clin Pharmacol Ther* **109**, 222-232, doi:10.1002/cpt.2102 (2021).
- 121 Trifunov, S. *et al.* Full-Length SMN Transcript in Extracellular Vesicles as Biomarker in Individuals with Spinal Muscular Atrophy Type 2 Treated with Nusinersen. *J Neuromuscul Dis* **10**, 653-665, doi:10.3233/JND-230012 (2023).

- 122 Giard, D. J. *et al.* In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst* **51**, 1417-1423, doi:10.1093/jnci/51.5.1417 (1973).
- 123 Ross, P. J. & Parks, R. J. Construction and characterization of adenovirus vectors. *Cold Spring Harb Protoc* **2009**, pdb.prot5011, doi:10.1101/pdb.prot5011 (2009).
- 124 Théry, C., Amigorena, S., Raposo, G. & Clayton, A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. *Curr Protoc Cell Biol* **Chapter 3**, Unit 3.22, doi:10.1002/0471143030.cb0322s30 (2006).
- 125 Schindelin, J. *et al.* Fiji: an open-source platform for biological-image analysis. *Nat Methods* **9**, 676-682, doi:10.1038/nmeth.2019 (2012).
- 126 Stirling, D. R. *et al.* CellProfiler 4: improvements in speed, utility and usability. *BMC Bioinformatics* **22**, 433, doi:10.1186/s12859-021-04344-9 (2021).

2.7 Acknowledgements

Data Availability: The data that support the findings of this study are available on request.

Acknowledgements: The authors are grateful to Kathy Poulin for excellent technical assistance, and to Drs. Rashmi Kothary (Ottawa Hospital Research Institute) and Dylan Burger (Ottawa Hospital Research Institute) for valuable discussion. pLN107, the plasmid used to generate Ad3F-SMN, was constructed by Leslie Nash as a graduate student in the Parks Lab. This work was supported by a collaborative grant from the Canadian Institutes of Health Research (CIHR, PJT-178120) to R.J.P. and Drs. Rashmi Kothary (OHRI), Panayiotis O. Vacratsis (University of Windsor) and Jodi Warman-Chardon (The Ottawa Hospital), and additional research funds to R.J.P. from CIHR (NHG-107767, MOP-136898, PYY-175428) and Cure SMA/Cure SMA Canada. C.A.R. was supported by a Queen Elizabeth II Graduate Scholarship in Science and Technology from the Ontario Provincial Government (Canada).

Author Contributions: Conceptualization: C.A.R., R.J.P.; Formal Analysis: C.A.R., R.J.P.; Funding acquisition: C.A.R., R.J.P.; Investigation: C.A.R.; Methodology: C.A.R., R.J.P.; Project administration: R.J.P.; Supervision: R.J.P.; Visualization: C.A.R., R.J.P.; Writing – original draft: C.A.R.; Writing – review & editing: C.A.R., R.J.P.

All authors have read and agreed to the published version of the manuscript.

Competing Interests: The authors declare no competing interests.

Keywords: Extracellular vesicles, neuromuscular disease, nuclear gems, Spinal Muscular Atrophy, therapeutic delivery, therapeutic protein

Chapter 3: Use of gene therapy to engineer the liver to release extracellular vesicles that distribute a therapeutic protein throughout the body of mice

Statement of contributions:

C.A.R. conducted cell culture, immunoblot, nuclear gem quantification, immunofluorescence staining of cells in culture, immunofluorescence staining of tissue in Figure 3.7 and Supplemental Figure S6, microscopy, extracellular vesicle isolation and characterization, extracellular vesicle depletion, viral vector propagation and characterization of AdfloxNlucSMN, AdNlucSMN, AdfloxNlucSMNFluc, AdEmpty, and AdmCherry-SMN, cell culture viral transduction experiments, media transfer experiments, tissue homogenization, protein quantification, luciferase assay, statistical analysis, figure generation, and assisted with tissue collection from mice.

Tissue sectioning, fixation, and staining presented in Figure 3.3E was performed by the University of Ottawa Louise Pelletier Histology Core Facility.

K.L.P. conducted the immunoblot in Figure 3.3B and Supplemental Figure S5A, and viral vector propagation and characterization of AdCre and AdGFP.

J.P. performed tail vein injections on mice and assisted with tissue collection.

R.J.P. conducted molecular cloning of plasmid constructs, and edited figures.

C.A.R. and R.J.P. analysed data, and wrote and edited the manuscript.

K.L.P., J.P., J.C.B., D.B., P.O.V., J.W-C., and R.K edited the manuscript.

**Use of gene therapy to engineer the liver to release extracellular vesicles
that distribute a therapeutic protein throughout the body of mice**

Charlotte A. René^{a-c}, Kathy L. Poulin^a, Julia Petryk^d, John C. Bell^{b,d,e},
Dylan Burger^{e-h}, Panayiotis O. Vacratsisⁱ, Jodi Warman-Chardon^{a,c,e,h,j},
Rashmi Kothary^{a-c,e,h}, and Robin J. Parks^{a-c,e*}

^aRegenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada, K1H 8L6

^bDepartment of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, Ontario, Canada, K1H 8L1

^cCentre for Neuromuscular Disease, University of Ottawa, Ottawa, Ontario, Canada, K1Y 4E9

^dCancer Therapeutics Program, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada, K1H 8L6

^eDepartment of Medicine, The Ottawa Hospital and University of Ottawa, Ottawa, Ontario, Canada, K1H 8L6

^fInflammation and Chronic Disease Program, Kidney Research Centre, Ottawa Hospital Research Institute, Ottawa, Ontario, Canada, K1H 8L6

^gSchool of Pharmaceutical Sciences, University of Ottawa, Ottawa, Ontario, Canada, K1H 8M5

^hDepartment of Cellular and Molecular Medicine, University of Ottawa, Ottawa, Ontario, Canada, K1H 8L1

ⁱDepartment of Chemistry and Biochemistry, University of Windsor, Windsor, Ontario, Canada, N9B 3P4

^jDepartment of Genetics, Children's Hospital of Eastern Ontario, Ottawa, Ontario, Canada, K1H 8L1

Short title: Liver-derived extracellular vesicles distribute protein body-wide

Keywords: extracellular vesicles, spinal muscular atrophy, adenovirus, liver

*Corresponding Author

Robin J. Parks

Room C4415 - Regenerative Medicine Program

501 Smyth Road

Ottawa Hospital Research Institute

Ottawa, Ontario, Canada K1H 8L6

e-mail: rparks@ohri.ca

3.1 Abstract

A key challenge in gene therapy is achieving efficient delivery of a therapeutic to the desired target tissue or, for some diseases, body-wide. In this study, we used a gene therapy approach to engineer the liver of mice to release extracellular vesicles enriched with a therapeutic protein, the survival of motor neuron (SMN) protein, facilitating systemic distribution of the protein throughout the animal, including critical regions such as the brain and spinal cord. Our findings demonstrate a viable approach to achieve body-wide distribution of a therapeutic protein, which holds promise as a potential therapy for patients presenting with diverse diseases.

3.2 Introduction

A central challenge in gene therapy of genetic and acquired disorders is the ability to efficiently deliver the therapeutic to the desired target tissue. For diseases affecting multiple cell types and tissues, this may necessitate delivery of the therapeutic body-wide. For example, for treatment of spinal muscular atrophy (SMA), a genetic neuromuscular disorder, therapies to date have focused primarily on restoring the levels of survival motor neuron (SMN) protein in alpha motor neurons of the spinal cord, widely considered a key tissue affected in individuals with the disorder¹. Recently approved disease-modifying therapies for the treatment of SMA have significantly improved the quality of life and survival of many individuals affected by the disorder²⁻⁵. However, low SMN protein concentration actually impacts many different tissue types, including brain^{6,7}, gastrointestinal and enteric nervous system⁸, heart^{9,10}, liver^{11,12}, lung¹³, kidney^{14,15}, pancreas^{16,17}, spleen^{18,19}, skeletal muscle²⁰⁻²², and thymus¹⁸, and thus SMA appears to be a body-wide disease. Indeed, application of the approved therapies has led to an evolution of the SMA phenotype, with now a greater appreciation of some of these non-central nervous system (CNS) contributors to the disease in individuals with SMA that are now living longer²³⁻²⁶. Thus, truly effective treatment for SMA may require restoring SMN protein levels in all tissues in the body.

Numerous studies have shown that extracellular vesicles (EVs) can deliver protein to all tissues of the body, and also have an innate ability to cross the blood-brain barrier (BBB) and deliver cargo to the CNS²⁷⁻³². Thus, EVs have gained significant attention as therapeutic delivery vehicles. EVs are small, membrane

bound particles released from all cell types and found in all bodily fluids³³. There are several subtypes of EVs, with most studies focusing on small EVs, which are on average less than 200 nm in diameter and primarily comprised of exosomes and microvesicles³⁴. Although originally thought to be involved only in the elimination of cellular waste, EVs actually perform an array of physiological functions in the body³⁵. EVs are involved in intercellular communication, transferring proteins, lipids, and nucleic acids to recipient cells, which may be located close to or distant from the site of EV origin³⁶. The specific biomolecules contained in the EVs are dictated by the type and physiological state of the originating cell³³.

As mentioned, SMA is a genetic neuromuscular disorder characterized by atrophy of skeletal muscle due to degeneration of the alpha motor neurons of the spinal cord^{1,37,38}. The disorder is caused by homozygous mutation or deletion of the survival of motor neuron 1 (*SMN1*) gene³⁹, resulting in inadequate levels of survival motor neuron (SMN) protein⁴⁰. In the most severe form of the disease, SMA Type I, patients succumb to respiratory failure before they reach two years of age if untreated⁴¹⁻⁴³. SMN protein is found in both the cytoplasm of the cell and in small nuclear bodies called gems (gemini of Cajal bodies)^{44,45}. The SMN protein is involved in many processes, including the assembly of spliceosomal small nuclear ribonuclear proteins (snRNPs) essential for pre-mRNA splicing⁴⁶⁻⁵¹, translational regulation⁵², active transport^{53,54}, actin dynamics^{55,56}, and resolution of R-loops in genetic terminal regions⁵⁷. The precise cellular role of SMN protein that results in pathogenesis of SMA remains unknown. Previously, we demonstrated that SMN protein is naturally released from cells in EVs, and that the quantity of SMN protein

in the EVs reflects the state of the cell⁵⁸. For example, a fibroblast cell line derived from a patient with SMA released EVs with less SMN protein compared to cells derived from healthy controls^{58,59}. Furthermore, cells engineered to overexpress the SMN protein, either stably or through adenovirus (Ad) vector-mediated expression of the *SMN1* gene, release EVs containing a greatly enhanced quantity of SMN protein^{58,60}. Importantly, we showed that EVs loaded with SMN protein can efficiently deliver the protein to recipient cells in culture, and this delivered protein can transit to the nucleus where it co-localizes with normal protein partners⁶⁰. Thus, EVs loaded with SMN protein may represent a novel therapy for SMA. However, SMN protein has a natural half-life of only ~6 h^{61,62}, suggesting that a therapy based solely on delivery of therapeutic EVs would need to be readministered at least on a daily basis to maintain therapeutic levels of the SMN protein in the recipient cells. In this study, we show that the liver of a mouse can be engineered to release EVs containing enhanced levels of the SMN protein which subsequently distribute throughout the body of the animal. Thus, we have developed a novel approach to achieve body-wide distribution of a therapeutic protein for treatment of SMA and, potentially, other disorders, that unlike current SMA-directed therapies, can restore protein to all affected tissues in the body, including the CNS.

3.3 Results

3.3.1 EVs efficiently deliver SMN protein to recipient SMA patient fibroblasts.

Previously, we demonstrated that infection of HepG2 hepatocellular carcinoma cells with a replication-defective Ad vector expressing the SMN protein led to the release of EVs containing high levels of the SMN protein⁶⁰. Furthermore, these EVs could efficiently deliver the SMN protein to recipient human A549 adenocarcinoma cells in culture, and the delivered protein co-localized with endogenous protein partners in the nucleus of the recipient cells. Cells with reduced levels of SMN protein show aberrant endocytosis⁶³⁻⁶⁵, a common method of EV internalization⁶⁶, and intracellular trafficking^{67,68}, which could affect the ability of the cell to take up EVs. We first confirmed our observation that EVs could also effectively deliver SMN protein to fibroblast cells derived from a patient with SMA, using confocal microscopy and orthogonal projection analysis. As in our previous work, we utilized conditioned medium (CM) from HepG2 cells that had been transduced with a replication-defective Ad vector encoding a triple-FLAG-tagged SMN (3F-SMN) protein as a source of EVs⁶⁰. We confirmed loading of the 3F-SMN protein into EVs released from the transduced HepG2 cells (Supplemental Figure S2). CM from mock- or Ad3F-SMN-transduced HepG2 cells was applied to the recipient SMN-deficient fibroblasts in the absence or presence of 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), an inhibitor of RNA polymerase transcription elongation, to block *de novo* gene expression from any residual Ad3F-SMN remaining in the CM, and the association of 3F-SMN protein was examined 2 h later. As shown in Figure 3.1A, no FLAG signal was observed in cells treated with CM

from mock-transduced cells whereas significant signal was observed in cells treated with CM containing EVs loaded with 3F-SMN protein. A similar FLAG signal was observed in the presence or absence of DRB, suggesting no significant affect from any Ad3F-SMN that may have been present in the CM. Importantly, ultracentrifugation-mediated depletion of EVs from the 3F-SMN CM resulted in a significant reduction in transfer of the 3F-SMN protein to the SMN-deficient cells (Supplemental Figure S3), suggesting that the FLAG signal we observed was due to protein delivery mediated by EVs. We also examined orthogonal projections generated from three-dimensional (3D) reconstructions from the images captured by confocal microscopy. In cells, SMN protein is routinely found in both the cytoplasm and in small nuclear bodies called gems^{44,45}. In both vehicle and DRB treatment groups, FLAG-positive foci were present throughout the cytoplasm and nucleus of the cells treated with CM containing EVs loaded with 3F-SMN protein (Figure 3.1B). Specifically, within the nucleus, FLAG-positive nuclear bodies, reminiscent of gem-like structures, were present, which were more clearly illustrated in the ZX (Figure 3.1B, panels ii and v) and ZY (Figure 1B, panels iii and vi) cross sections. These nuclear bodies are similar to those observed in A549 lung adenocarcinoma cells treated with 3F-SMN protein loaded EVs, which we showed localized with endogenous Gemin2 protein⁶⁰, suggesting these structures are indeed nuclear gems. Together, these data indicate that cells deficient in SMN protein are able to readily take up EV-delivered 3F-SMN protein, and that the protein can transit to the nucleus to form gem-like structures. Thus, EVs loaded with SMN protein may represent a novel therapy to restore SMN protein levels for treatment

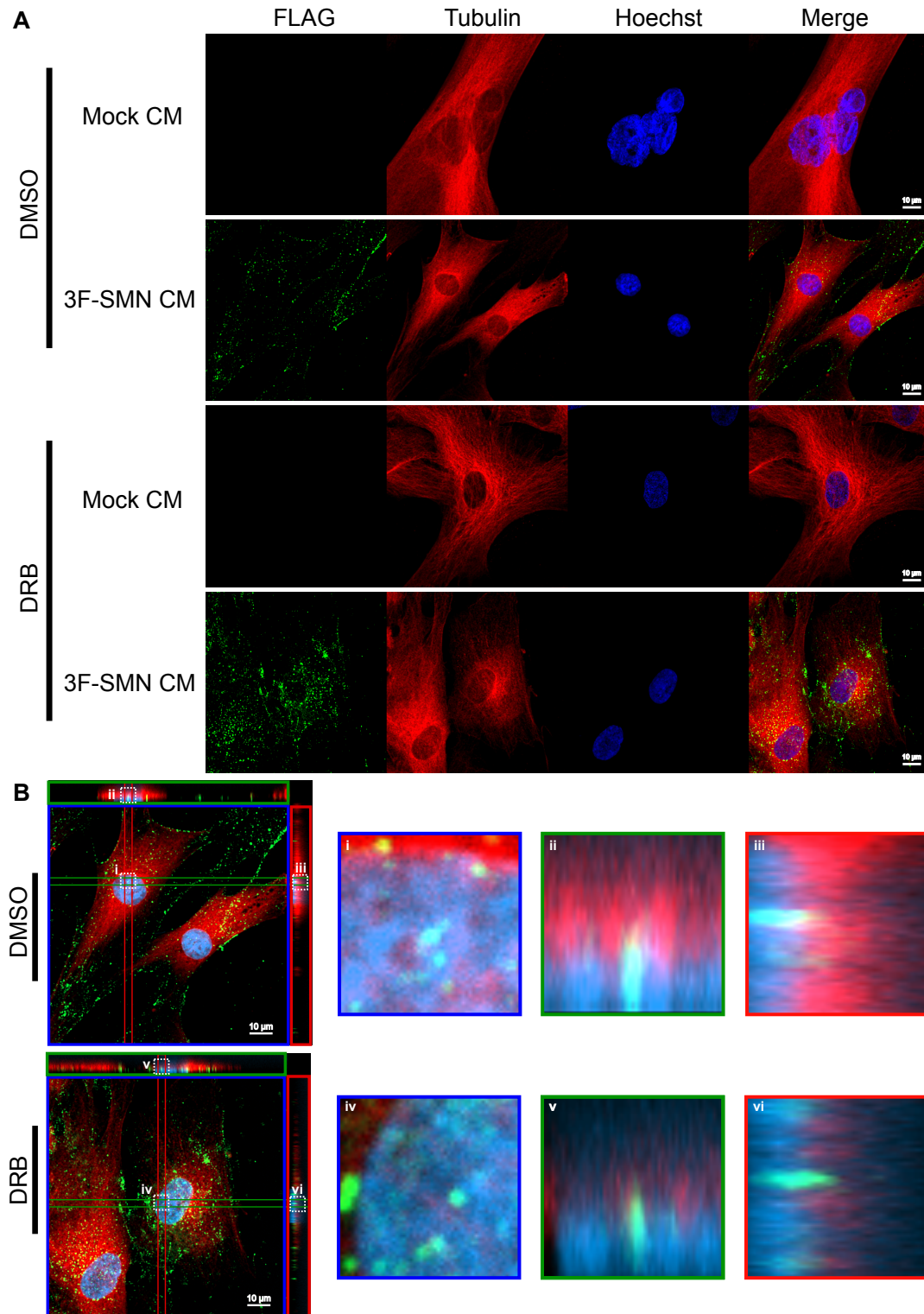


Figure 3.1 EV-delivered 3F-SMN protein is internalized by recipient fibroblasts and localizes in nuclear gem-like structures. Panel A: CM containing EVs loaded with the 3F-SMN protein or mock CM was applied to fibroblasts derived from an individual with SMA in the presence or absence of 100 μ M DRB, an inhibitor of RNA

polymerase transcription elongation, to block de novo gene expression from residual Ad3F-SMN that may be contained in the CM. Two hours later, the treated cells were processed for immunofluorescent staining with antibodies to FLAG and tubulin, and images were captured by confocal microscopy. Scale bars represent 10 μm . Panel B: Orthogonal projections generated of z-stacks of treated fibroblasts from Panel A. Scale bars represent 10 μm .

of SMA.

3.3.2 Characterization of an Ad vector expressing a Cre-activatable bioluminescent SMN fusion protein.

Several groups have shown that repeated administration of EVs can effectively treat diseases over prolonged periods⁶⁹⁻⁷¹. Similarly, EVs loaded with SMN protein present a potential novel treatment modality for patients with SMA. Several approaches are available for the production and purification of large quantities of clinical-grade EVs^{34,72,73}. However, given the approximately 6 h half-life of SMN protein⁶², it is likely that daily administration of therapeutic EVs might be required to ensure proper maintenance of SMN protein levels. To circumvent the need for repeated administration of therapeutic EVs, we explored a novel approach to utilize the liver as a “factory” for the production and release of EVs loaded with SMN protein. Several studies have shown that gene therapy can be used to significantly increase the levels of a protein in the liver of a mouse⁷⁴⁻⁷⁷. For Ad vectors, systemic administration results in greater than 90% of the vector localizing to the liver^{78,79}, and a single vector administration can lead to transgene expression in the liver for the lifetime of a mouse⁸⁰, and for at least 7 years in non-human primates⁸¹. Studies have also shown that the liver contributes approximately 15% of the circulating EVs found in blood^{82,83}. Thus, the liver may be an ideal organ for the production of SMN protein-loaded EVs for systemic delivery of the protein throughout the body.

To evaluate whether liver-derived SMN protein can be detected in peripheral

tissues, we wished to ensure that Ad-mediated transgene expression was confined to the liver. We utilized transgenic mice that express Cre recombinase protein from the liver-specific albumin promoter⁸⁴, and thus only express Cre protein in hepatocytes. We then designed an Ad vector that would only “turn-on” transgene expression in the presence of Cre recombinase (Figure 3.2A). This vector, designated AdfloxNlucSMN, possesses a *loxP*-flanked green fluorescent protein (*GFP*) cDNA in which the start codon for the gene is located upstream of the 5' *loxP* site, and also contains a downstream *NlucSMN* gene that lacks a start codon (ATG). Cre-mediated recombination results in removal of the *GFP* cDNA and enables in-frame translation of the NlucSMN protein. Consequently, systemic administration of AdfloxNlucSMN is expected to predominantly target the liver, resulting in production of the NlucSMN fusion protein exclusively in hepatocytes. Thus, any NlucSMN protein present in peripheral tissues would originate from the liver. We also generated a control vector that provides constitutive expression of *NlucSMN*, designated AdNlucSMN (Figure 3.2A).

We first evaluated the ability of AdfloxNlucSMN to provide Cre-mediated induction of NlucSMN expression in tissue culture. A549 cells, a lung adenocarcinoma cell line commonly used for tissue culture studies of Ad, were transduced with AdfloxNlucSMN or AdNlucSMN along with AdGFP (negative control) or AdCre. Protein from transduced cells was isolated 24 hours post infection (hpi) and expression of the NlucSMN fusion protein was assessed by immunoblot (Figure 3.2B) and Nluc activity assay (Figure 3.2C). As shown in Figure 3.2B, co-transduction of A549 cells with AdGFP and AdfloxNlucSMN did not result in

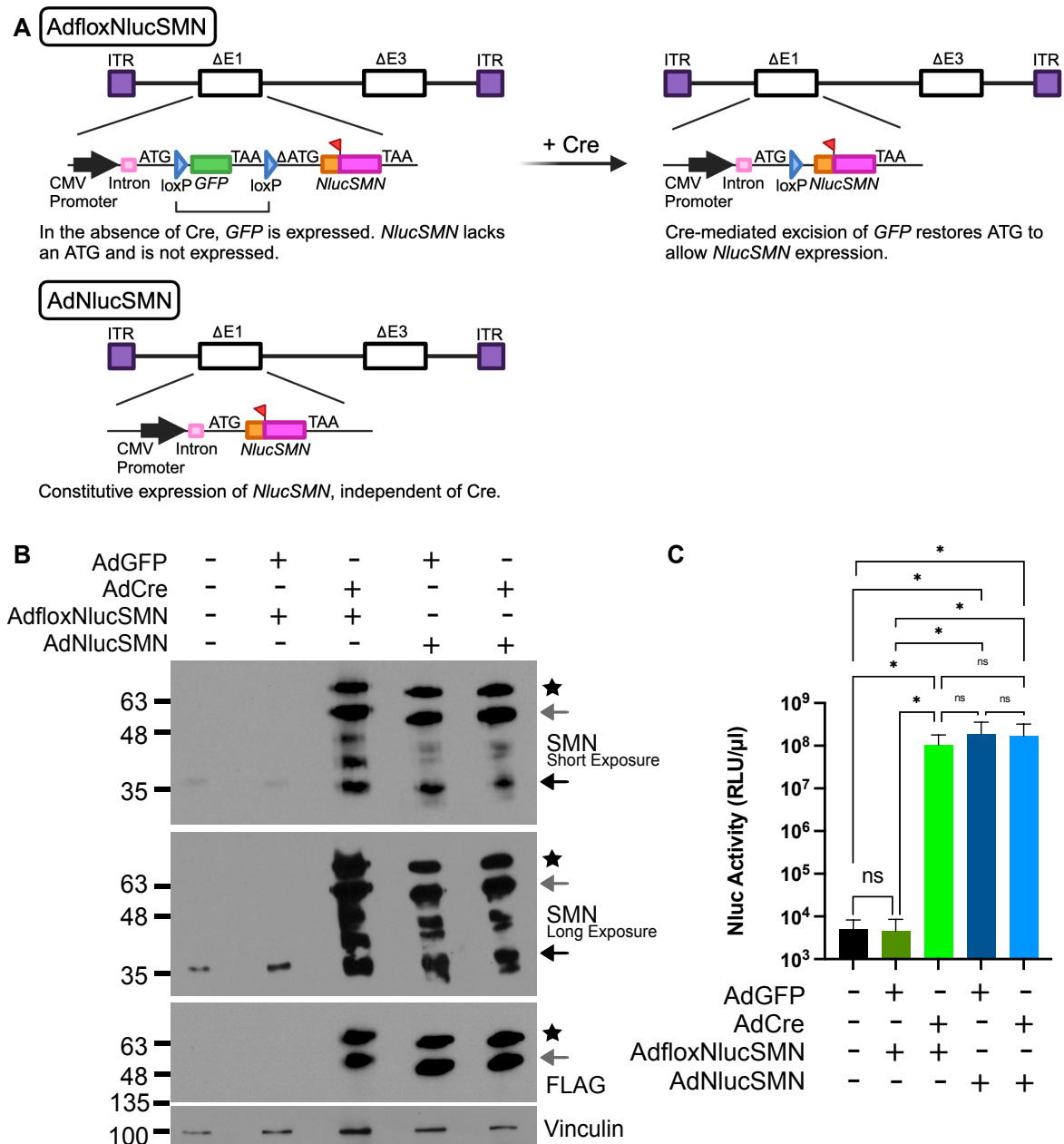


Figure 3.2 Cre-mediated induction of *NlucSMN* expression from AdfloxNlucSMN in tissue culture. Panel A: Schematic representation of AdfloxNlucSMN and AdNlucSMN. AdfloxNlucSMN is an early region 1 (E1)/E3 deleted human adenovirus serotype 5 (HAdV-5)-based vector possessing a *loxP*-flanked green fluorescent protein (GFP) cDNA in which the start codon for the gene is located upstream of the 5' *loxP* site, and a downstream *NlucSMN* gene lacking a start codon (Δ ATG). Cre-mediated recombination results in removal of the GFP gene and places the start codon in-frame with the *NlucSMN* gene, thereby permitting expression of the protein. Transgene expression is driven by the human CMV immediate early enhancer/promoter. AdNlucSMN is a E1/E3 deleted HAdV-5-based vector in which a CMV enhancer/promoter drives constitutive expression of the

NlucSMN fusion protein (*i.e.* NlucSMN is expressed in both the absence and presence of Cre). Panel B: A549 cells were transduced at a multiplicity of infection (MOI)=10 with AdfloxNlucSMN or AdNlucSMN, in combination with an E1/E3 deleted HAdV-5-based vector expressing Cre (AdCre) or GFP (AdGFP), also at an MOI=10, or mock transduced. Cells were lysed 24 hpi and expression of NlucSMN was assessed by immunoblot. Grey arrows indicate NlucSMN fusion protein, black arrows indicate the endogenous SMN protein and a star marks an aberrant SMN protein. Panel C: Nluc activity as assessed by luciferase assay. Data is plotted on a logarithmic scale, while statistical significance was determined on log-transformed data using one-way ANOVA followed by Tukey's multiple comparisons test. One asterisk (*) indicates $p < 0.05$. Data represents 3 independent experiments, where cells were transduced in duplicate, and luminescence of each sample was read in duplicate. Schematic in Panel A generated with BioRender.

expression of NlucSMN protein, whereas co-transduction of AdCre and AdfloxNlucSMN yielded robust expression of the NlucSMN protein. Indeed, the quantity of NlucSMN protein present in these cells was similar to the level of protein produced through constitutive expression from AdNlucSMN. Quantitative analysis of Nluc activity present in crude protein lysates from cells transduced with AdfloxNlucSMN and AdCre showed similar expression as AdNlucSMN, which was a 4-log induction of expression relative to the uninduced state (Figure 3.2C). Overall, this data shows that the Cre-activatable vector AdfloxNlucSMN provides minimal background expression of NlucSMN and high Cre-mediated induction.

3.3.3 Systemic administration of AdfloxNlucSMN leads to high-levels of NlucSMN fusion protein in the liver in Alb-Cre mice.

We next examined activation of expression from AdfloxNlucSMN in the Alb-Cre mice compared to the C57BL/6 parental strain that lacks Cre. For this study, we used a derivative of AdfloxNlucSMN that also encoded a CMV-driven firefly luciferase (*Fluc*) gene in the E3 region of the vector, designated AdfloxNlucSMNFluc (Figure 3.3A). In this vector, NlucSMN is only expressed in the presence of Cre (*i.e.* in the liver of Alb-Cre mice), whereas Fluc would be constitutively expressed in all tissues that the vector transduces. Thus, Fluc expression can be used to monitor vector distribution, as well as normalize Nluc activity (Supplemental Figure S4).

Five-week-old Alb-Cre and C57BL/6 mice were administered PBS or AdfloxNlucSMNFluc at a dose of 5×10^{12} viral particles (vp)/kg by tail vein injection. Seventy-two hpi, tissues were collected, snap frozen in liquid nitrogen and stored at

-80°C until the time of analysis. Plasma was collected at the time of sacrifice and also stored at -80°C until the time of analysis. We first performed immunoblot analysis on liver lysates from the treated animals to analyze protein expression from our vector. As shown in Figure 3.3B, induction of expression of the NlucSMN fusion protein was observed in the liver of vector-treated Alb-Cre mice, with no detectable NlucSMN protein in liver lysates from vector-treated C57BL/6 mice. In the AdfloxNlucSMNFluc-treated Alb-Cre mice, we observed several protein bands on immunoblots probed with SMN or FLAG antibody, ranging in size from 20 to 65 kDa. The NlucSMN fusion protein is predicted to be ~48 kDa, and a predominant band of this size was noted on both SMN and FLAG immunoblots. We also observed prominent bands of 38 kDa on the SMN immunoblot and a doublet of ~20 kDa on the FLAG blot, which are consistent with the molecular weight of SMN and Nluc proteins, respectively. Thus, some proteolytic degradation of the fusion protein may have occurred near the junction of the two proteins. The ~65 kDa band likely represents an aberrant splice product that we have observed previously when using Ad vectors encoding SMN protein⁸⁵, and may represent splicing from the SMN gene to downstream Ad sequences^{85,86}. To estimate the level of overexpression of SMN protein in the liver of treated mice, we compared the SMN protein signal in PBS-treated Alb-Cre mice to the combined SMN and NlucSMN signal in AdfloxNlucSMNFluc-treated Alb-Cre mice by immunoblot, which showed a level of overexpression of approximately 7.5-fold (Supplemental Figure S5A, S5B).

We also analyzed Fluc and Nluc activity in liver tissue lysates. As expected, C57BL/6 and Alb-Cre mice treated with AdfloxNlucSMNFluc showed a similar level

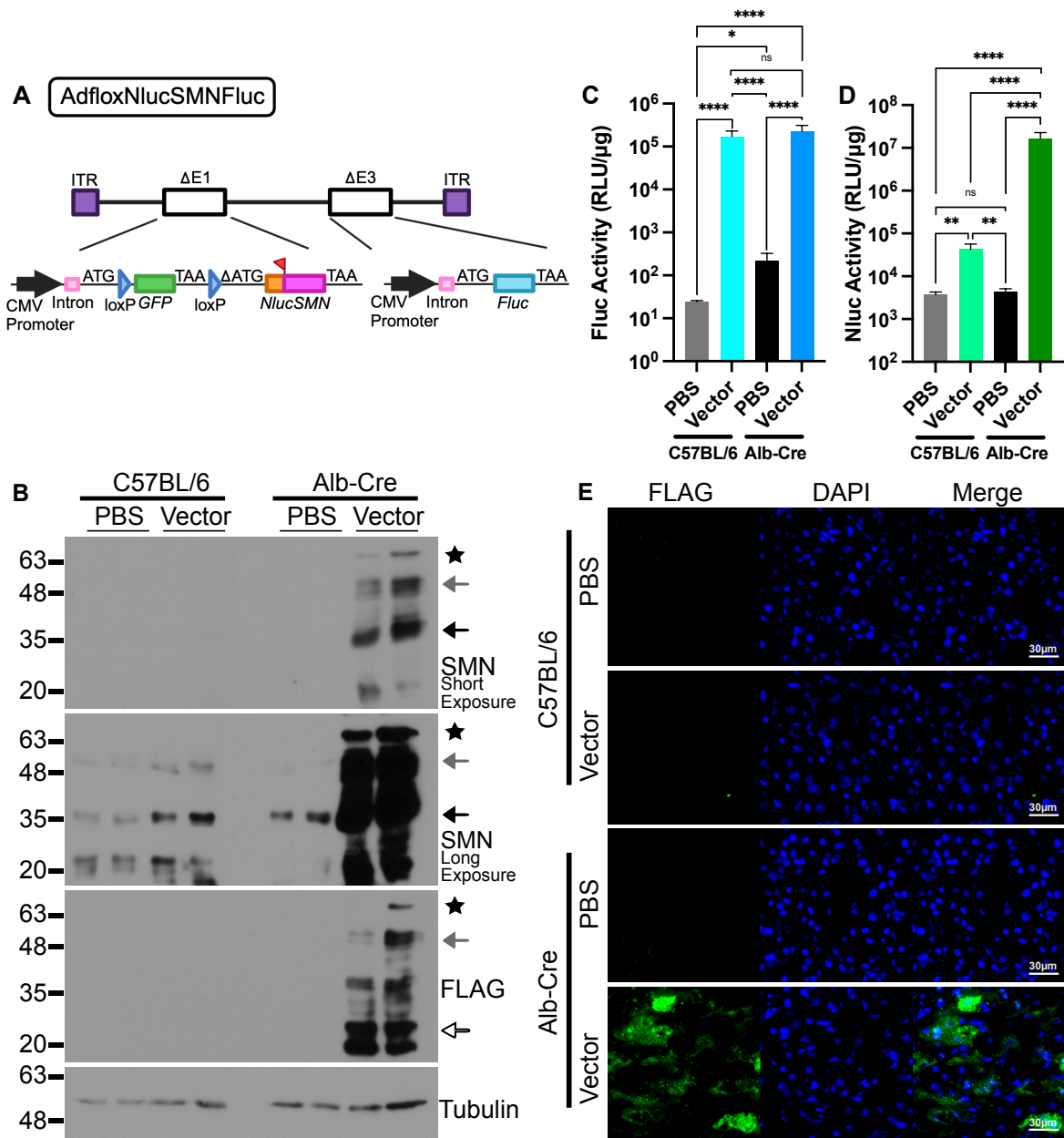


Figure 3.3 Administration of AdfloNlucSMNFluc to Alb-Cre mice leads to robust expression of NlucSMN protein in the liver. Panel A: Schematic representation of AdfloNlucSMNFluc. AdfloNlucSMNFluc is an early region 1 (E1)/E3 deleted HAdV-5-based vector that contains a CMV driven expression cassette comprised of a *loxP*-flanked *GFP* cDNA in which the start codon for the gene is located upstream of the 5' *loxP* site, and a downstream *NlucSMN* gene lacking a start codon (Δ ATG), and also contains a CMV-*Fluc* expression cassette within the E3 region. Panel B: Five week old female C57BL/6 and Alb-Cre mice were injected by tail vein with 5×10^{12} vp/kg AdfloNlucSMNFluc. Blood and tissues were harvested 72 hpi. The levels of NlucSMN fusion protein were assessed in liver tissue by immunoblot. Grey arrows indicate NlucSMN fusion protein, black arrows indicate

endogenous SMN protein, white arrows indicate Nluc protein, and the black star indicates an aberrant protein product. Samples from two different mice from each treatment group is shown. Panel C: Fluc activity in liver tissue was assessed by luciferase assay. Panel D: Nluc activity in liver tissue was assessed by luciferase assay. Data in panels C and D is plotted on a logarithmic scale, but was assessed by one-way ANOVA for statistical significance using log-transformed data followed by Tukey's multiple comparisons test. One asterisk (*) indicates $p < 0.05$, two asterisk (**) indicates $p < 0.01$, and four asterisk (****) indicates $p < 0.0001$. Data represents 3 mice per treatment group. Panel E: Liver tissue sections were stained with anti-FLAG antibody and imaged by epifluorescence microscopy. Scale bars represent 30 μm . Of note, the methanol/acetone fix used in processing the tissues inactivates GFP, thus inhibiting fluorescence emission from the GFP in tissues from animals treated with AdfloxNlucSMNFluc. Schematic in Panel A generated with BioRender.

of Fluc activity in liver (Figure 3.3C), indicating that there was no difference in liver transduction efficiency in the two different strains of mice. Although C57BL/6 mice treated with AdfloxNlucSMNFluc did show an ~10-fold increase in Nluc activity in the liver compared to PBS-treated mice, suggesting low level of spurious expression from the vector, Alb-Cre mice showed an over 100-fold increase above this background level (Figure 3.3D). Thus, we observed robust induction of expression of the NlucSMN fusion protein in the liver of vector-treated Alb-Cre mice. Sections of liver from the various treated mice showed an induction of NlucSMN protein levels in tissue from the AdfloxNlucSMNFluc-treated Alb-Cre mice, with no detectable protein in the other treatment groups (Figure 3.3E, Supplemental Figure S5). Thus, Cre-mediated induction of expression from AdfloxNlucSMNFluc in Alb-Cre mice provides an excellent system to evaluate EV-mediated distribution of protein originating from liver.

3.3.4 NlucSMN fusion protein is released from the liver within EVs.

Following our determination that systemic administration of AdfloxNlucSMNFluc leads to robust NlucSMN fusion protein expression in the liver of Alb-Cre mice, we investigated whether the liver-derived protein was released into circulation in EVs. Plasma samples from C57BL/6 or Alb-Cre mice treated with PBS or AdfloxNlucSMNFluc were evaluated for Fluc and Nluc activity. Fluc activity in whole plasma samples was below the threshold of detection in the vector-treated animals and was not significantly different from that observed in PBS-treated mice (Figure 3.4A). In contrast, Nluc activity in plasma of vector-treated Alb-Cre mice was

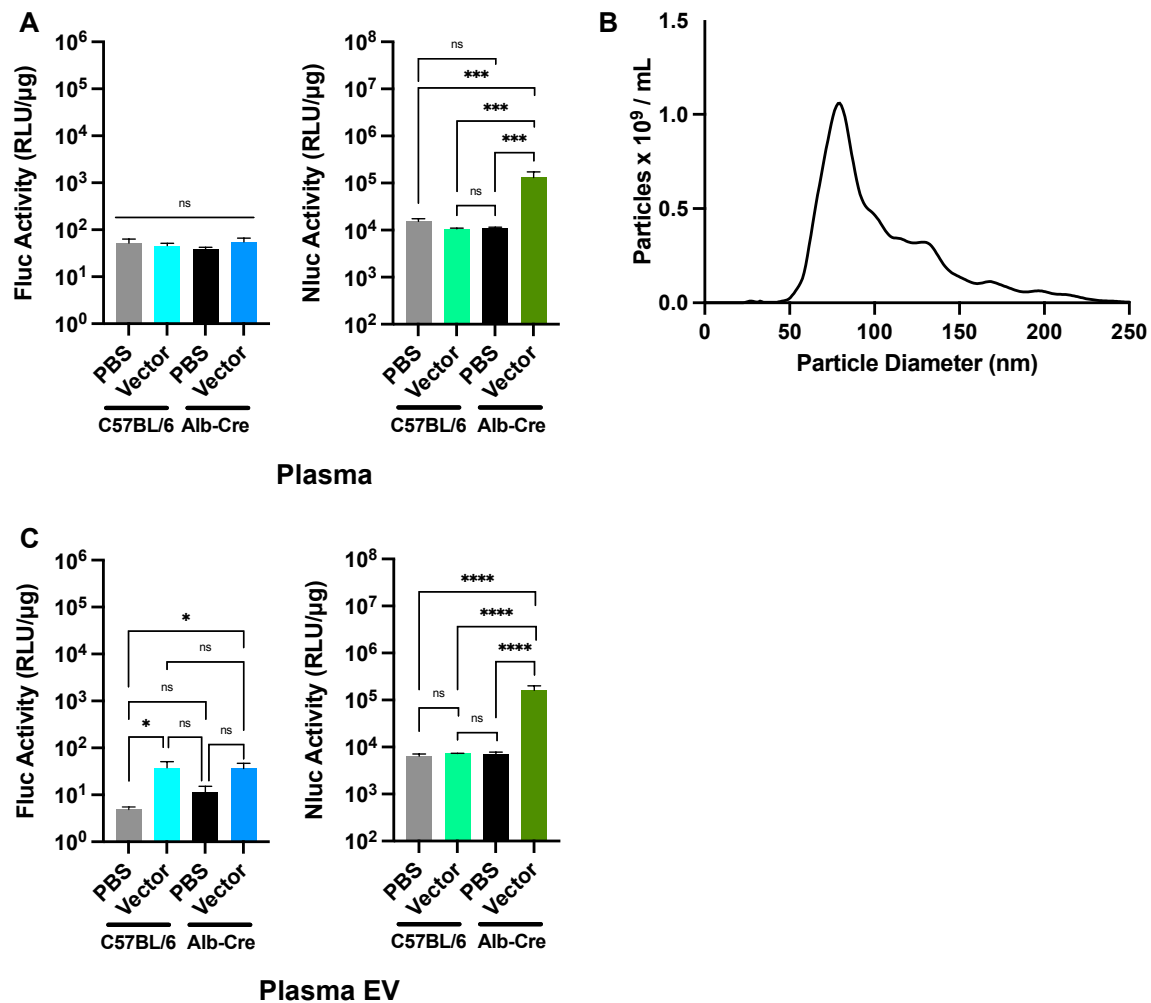


Figure 3.4 NlucSMN protein is contained in the EV fraction of blood plasma samples from AdfloNlucSMNFluc-treated Alb-Cre mice. Mice were treated with AdfloNlucSMNFluc as described in Figure 4, and plasma samples isolated 72 h post-treatment. Panel A: Fluc and Nluc activity in plasma was assessed by luciferase assay. Panel B: EVs isolated from plasma of control, untreated mice using Exoquick reagent were assessed for particle diameter and concentration by nanoparticle tracking analysis. Of note, this plasma was not from the same animals shown in panel A, but is provided to illustrate the typical EV profile obtained from this isolation method. Panel C: Fluc and Nluc activity in EVs isolated from plasma was assessed by luciferase assay. Data is plotted on a logarithmic scale, but assessed by one-way ANOVA for statistical significance using log-transformed data followed by Tukey's multiple comparisons test. One asterisk (*) indicates $p < 0.05$, two asterisk (**) indicates $p < 0.01$, three asterisk (***) indicates $p < 0.001$, and four asterisk (****) indicates $p < 0.0001$. Data represents 3 mice per treatment group.

~10-fold higher than background activity observed in PBS controls or vector-treated C57BL/6 mice, suggesting that NlucSMN protein from the liver was released into circulation. We further evaluated Fluc and Nluc activity in EVs isolated from the plasma from the various treatment groups using Exoquick reagent to isolate the EVs. As shown in Figure 3.4B, the particles isolated from plasma had a size distribution consistent with small EVs. Within the isolated particles, we observed a similar level of enrichment of Fluc activity in EVs isolated from plasma derived from both strains of mice treated with vector (Figure 3.4C). We also observed significant Nluc activity in the EVs isolated from plasma collected from AdfloxNlucSMNFluc-treated Alb-Cre mice. Given that *NlucSMN* expression is only activated in the liver of Alb-Cre mice, these data indicate that the NlucSMN protein produced in the liver is packaged into EVs which can then be found circulating in the blood of treated mice.

3.3.5 Liver-derived SMN protein is present in peripheral tissues of vector-treated mice.

As EVs loaded with NlucSMN protein had entered circulation in vector-treated Alb-Cre mice, we next examined if NlucSMN protein was distributed to peripheral tissues of the mouse. Tibialis anterior (TA) muscle, kidney, spinal cord, brain, heart, and lung tissue lysates were analysed by dual luciferase assay to examine the level of luciferase activity (Figures 3.5A, 3.5B). In both C57BL/6 and Alb-Cre mice, increased Fluc activity was found in all tissues of vector-treated mice compared to mice administered PBS (Figure 3.5A). This Fluc activity would be a

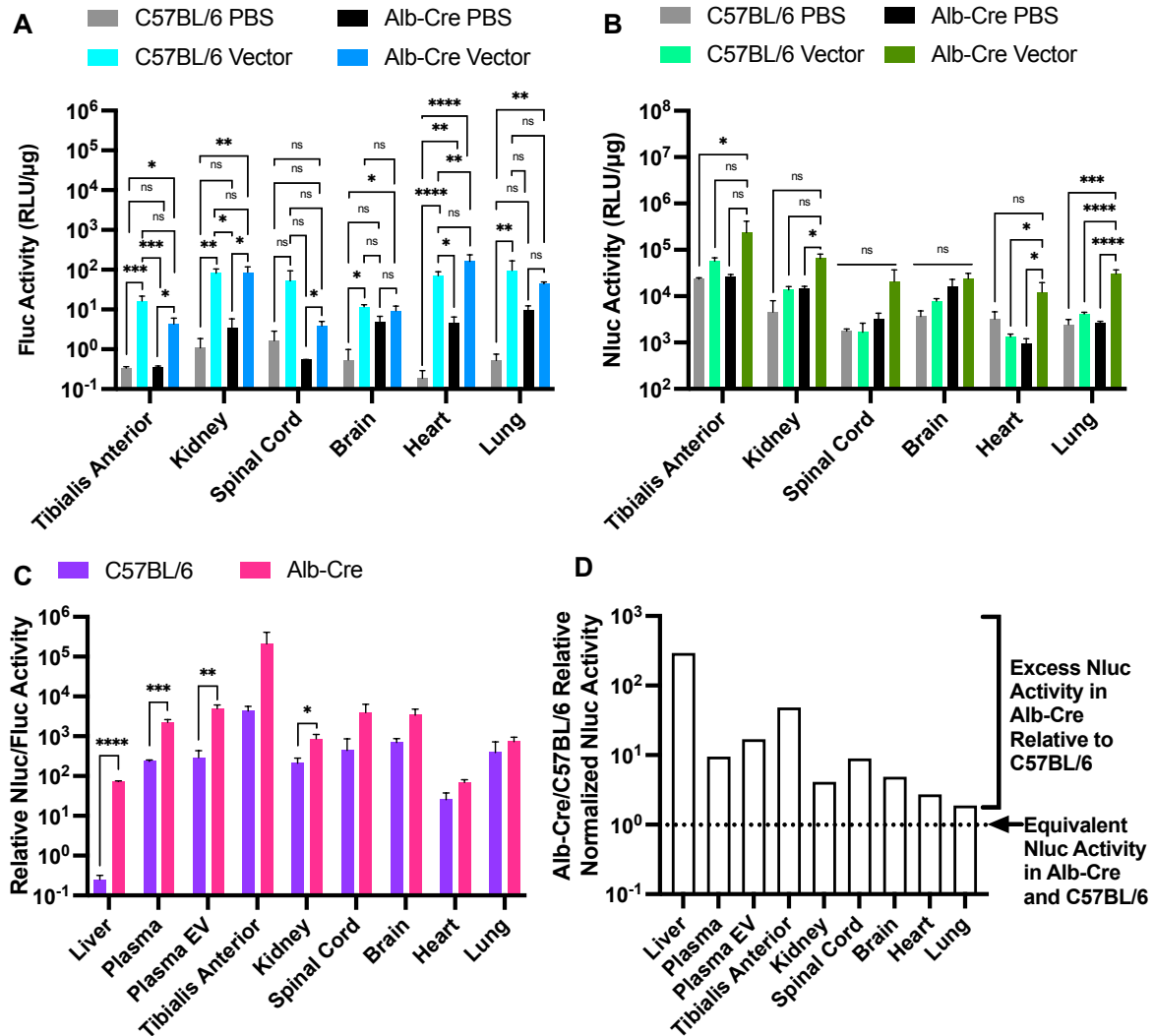


Figure 3.5 Liver-derived production of NlucSMN protein leads to high levels of the protein in peripheral tissues. Mice were treated with AdfloxNlucSMNFluc, and tissue samples were isolated 72 h post-treatment. Panel A: Fluc activity in TA muscle, kidney, spinal cord, brain, heart, and lung tissue was analyzed by luciferase assay. Panel B: Nluc activity in the tissues was analyzed by luciferase assay. Panel C: Nluc activity normalized to Fluc activity for the various tissues. Panel D: The ratio of normalized Nluc activity for Alb-Cre mice to C57BL/6 mice is shown. Equal Nluc expression in both animals would yield a value of 10^0 (*i.e.* 1), while enhanced Nluc activity in the tissues would yield a value above this line. Data in panels A and B is plotted on a logarithmic scale, but assessed by one-way ANOVA for statistical significance using log-transformed data followed by Tukey's multiple comparisons test. Data in panel C was tested for statistical significance by unpaired t-test. One asterisk (*) indicates $p < 0.05$, two asterisk (**) indicates $p < 0.01$, three asterisk (***) indicates $p < 0.001$, and four asterisk (****) indicates $p < 0.0001$. Data represents 3 mice per treatment group.

combination of EV-mediated transit of Fluc protein produced in the liver into these tissues and also any primary transduction of vector directly into these tissues. As mentioned, unlike adeno-associated virus (AAV), Ad does not efficiently extravasate, and thus exhibits only extremely low transduction of tissues other than liver following systemic administration in mice^{79,87,88}. C57BL/6 mice treated with AdfloxNlucSMNFluc showed only background levels of Nluc activity in all tissues (Figure 3.5B). In contrast, we observed enhanced Nluc activity in many tissues examined in the AdfloxNlucSMNFluc-treated Alb-Cre mice. Normalizing Nluc activity to the Fluc activity in the various tissues showed an elevated level of Nluc in four of the nine tissues examined in the Alb-Cre mice relative to the C57BL/6 (Figure 3.5C). Indeed, calculation of the fold increase in normalized Nluc activity in Alb-Cre relative to C57BL/6 illustrated that all tissues showed a trend towards greatly enhanced relative expression (Figure 3.5D). Unsurprisingly, the highest relative abundance of Nluc activity was observed in the liver, followed by plasma/plasma EVs, TA muscle, spinal cord and brain. We used immunoblot analysis to directly examine the quantity of NlucSMN protein in TA and brain. As shown in Figure 3.6, we detected the NlucSMN protein in both tissues. For the TA samples, densitometry analysis of the immunoblots showed that the quantity of the 48 kDa NlucSMN protein in the two samples analyzed was ~40 and 90% of their respective endogenous 38 kDa SMN protein signal (Figure 3.6A). As expected, the 48 kDa NlucSMN protein was also detected with anti-FLAG antibody. We also detected proteins of ~20 and 38 kDa with the anti-FLAG antibody in the animals treated with vector, similar to the size of the putative proteolytic cleavage products of the Nluc and SMN fusion protein observed

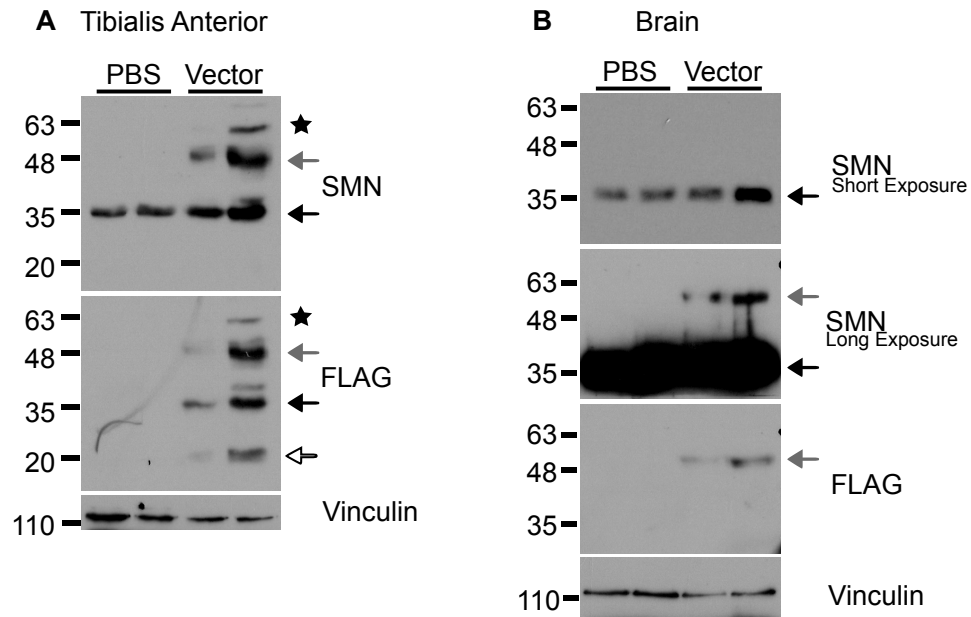


Figure 3.6 Liver-derived production of NlucSMN protein leads to high levels of the protein in muscle and brain. Mice were treated with AdfloxNlucSMNFluc, and tissue samples were isolated 72 h post-treatment. Protein lysates from TA (panel A) and brain (panel B) were assessed by immunoblot with antibodies to SMN and FLAG, with vinculin as a loading control. Grey arrows indicate NlucSMN fusion protein, black arrows represent SMN protein, the white arrows indicate Nluc protein, and the black stars indicate an aberrant protein product. Samples from two mice from each treatment group are shown.

in the liver of treated animals (Figure 3.3B). This ~38 kDa proteolytic SMN protein likely contributed to the apparent 2- to 3-fold higher signal for the 38 kDa SMN protein in the TA muscle of animals treated with vector relative to the animals treated with PBS, when normalized to the vinculin loading control. Thus, overall, a 7.5-fold overexpression of NlucSMN in the liver (Figure 3.3, Supplemental Figure S5) led to a ~40-90% increase in SMN protein in the TA muscle of treated animals. In the brain, the NlucSMN protein was also detected with both the SMN and FLAG antibodies, but was present at a much lower level than the endogenous SMN protein (Figure 3.6B).

As shown in Figures 3.5 and 3.6A, the TA muscle appeared to have the highest level of SMN protein uptake of all tissues examined. We performed immunofluorescence microscopy on histological sections of TA muscle to examine the localization of the liver-derived SMN protein within this tissue. In this experiment, wildtype C57BL/6 mice were administered 5×10^{12} vp/kg by tail vein injection of either an E1/E3-deleted Ad vector contained a CMV-driven *SMN* gene that contained an N-terminal mCherry and FLAG epitope tag, designated AdmCherry-SMN, or with the same dose of control Ad vector lacking a transgene (AdEmpty), or PBS. TA muscle was collected 72 hpi and processed for epifluorescence imaging using antibodies to FLAG or laminin. As anticipated, no FLAG signal was detected in the PBS- or AdEmpty-treated animals (Figure 3.7). In contrast, the FLAG-tagged SMN protein was detected in the TA muscle of AdmCherry-SMN-treated mice, and appeared to co-localize with the basal lamina surrounding the myofibers in the TA muscle tissue. Importantly, examination of tissue sections isolated from varying

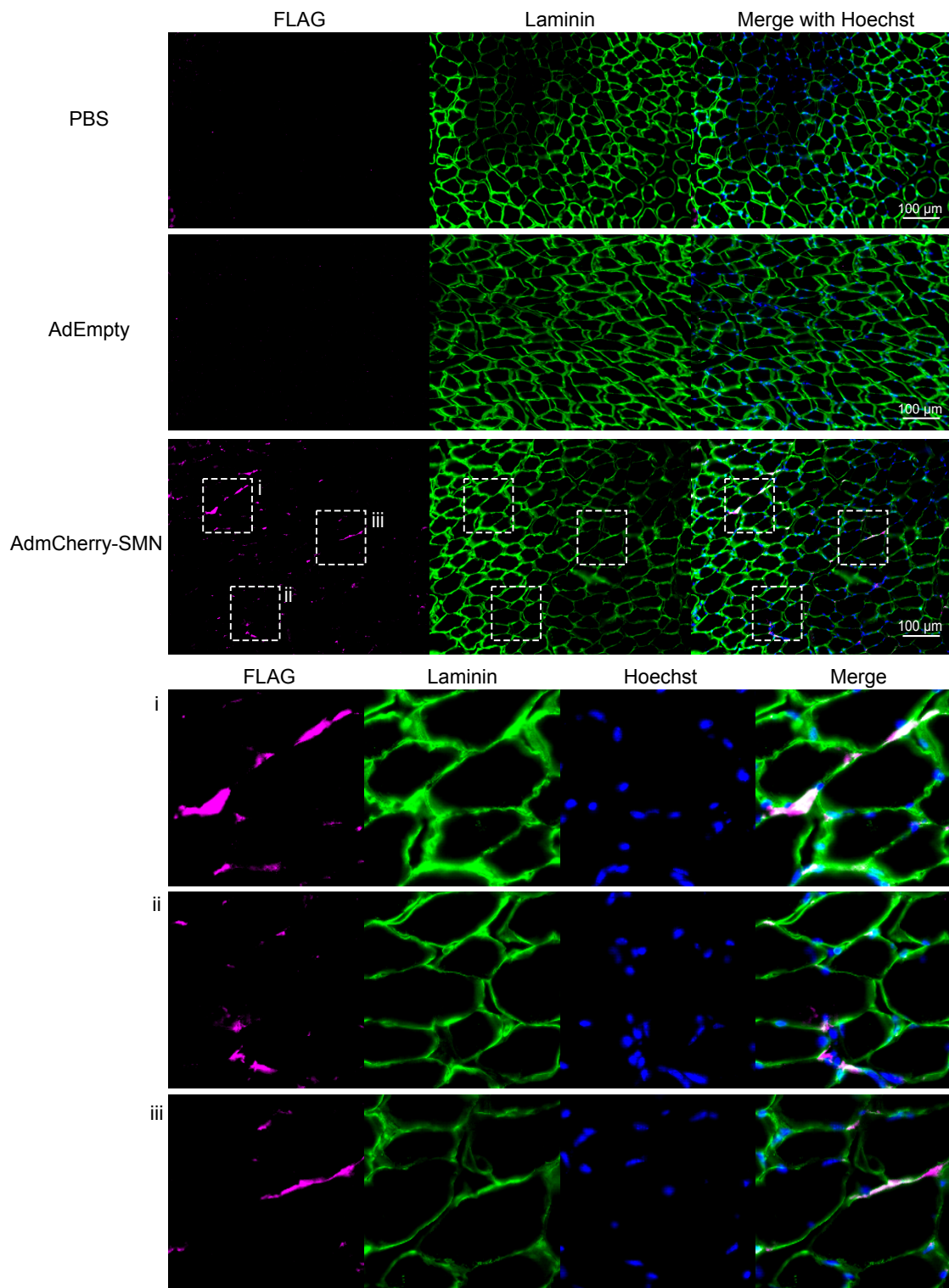


Figure 3.7 Visualization of liver-derived SMN protein in the TA muscle of mice using immunofluorescence microscopy. Mice were treated with 5×10^{12} vp/kg of AdmCherry-SMN, AdEmpty, or PBS, and TA muscles were collected 72 h post-treatment. Muscle tissue sections were stained with anti-FLAG and anti-laminin

antibodies and imaged by epifluorescence microscopy. Scale bars represent 100 μm .

positions within the TA muscle showed that the mCherry-SMN protein had distributed throughout the muscle (Supplemental Figure S6). We were not able to conclusively detect liver-derived SMN protein directly within the myofibers of treated mice, likely reflecting the limit of sensitivity of standard immunofluorescence microscopy. However, numerous studies have shown that systemic delivery of EVs in mice or rats results in cellular uptake⁸⁹⁻⁹¹, suggesting that at least a portion of the liver-derived SMN protein present in TA muscle is likely internalized by the cells. Overall, our data shows that a gene therapy approach can effectively convert the liver into a “factory” for producing EVs containing a therapeutic protein that distributes throughout the body and is taken up by peripheral tissues, including the CNS.

3.4 Discussion

EVs have garnered significant attention as therapeutic delivery vehicles for a number of conditions and disorders⁹²⁻⁹⁵. EVs can deliver cargo to all tissues in the body²⁷ and cross the BBB⁹⁶, exhibit low immunogenicity^{97,98}, and are relatively stable⁹⁹. Previously, we demonstrated that EVs can deliver SMN protein to cells in culture, and that the delivered protein localizes to gem-like structures in the nucleus of recipient cells similar to endogenous protein⁶⁰. We have extended these findings to show that EVs can effectively deliver SMN protein to fibroblast cells derived from an individual with SMA. Cells with low levels of SMN protein show defects in endocytosis^{63-65,68} and intracellular trafficking^{67,68}, both of which are essential for effective EV-mediated delivery of a therapeutic protein. Administration of CM containing 3F-SMN-loaded EVs to the SMA fibroblasts resulted in uptake of the protein (Figure 3.1), indicating that deficits in these pathways do not impede the use of EVs as a therapeutic for this disease. That delivery of the 3F-SMN protein was mediated by EVs was supported by EV depletion studies: ultracentrifuge-based depletion of EVs from CM significantly reduced delivery of the 3F-SMN protein to the recipient cells (Supplemental Figure S3). Using 3D reconstruction of confocal microscopy images, we showed that the delivered protein was present in both the cytoplasm and nucleus of recipient cells. In the nucleus, the 3F-SMN protein localized to nuclear bodies reminiscent of gem-like structures (Figure 3.1). In previous work, we showed that human cancer cells treated with CM containing EVs loaded with 3F-SMN protein also showed similar nuclear bodies, and that the 3F-SMN signal co-localized with Gemin2 protein⁶⁰, suggesting these structures are

indeed nuclear gems and that the delivered protein is in the proper cellular compartmentalization for functional activity. Taken together, these data suggest that SMN protein-loaded EVs may represent a novel therapeutic for treatment of patients with SMA. However, given the naturally short half-life of SMN protein within the cell (~ 6 h^{61,62}), daily re-administration of these EVs would likely be required to maintain a therapeutic benefit. Unfortunately, production of large quantities of pure EVs has remained a major barrier to clinical application in the field of EV-based therapeutics¹⁰⁰.

To overcome the challenge of producing large quantities of pure EVs for therapeutic use, we utilized a gene therapy approach to engineer the liver of mice to release SMN protein into circulation. Hepatocytes naturally release EVs¹⁰¹, and the liver contributes approximately 15% of the total EVs found in blood¹⁰²⁻¹⁰⁴. To determine if the liver could serve as a factory for production of EVs loaded with SMN protein that subsequently distribute throughout the body, we utilized an Ad vector containing a Cre-inducible cassette for expression of *NlucSMN* in combination with Alb-Cre transgenic mice to ensure protein was produced solely in the liver (Figure 3.2, Supplemental Figure S4, S5). Systemic delivery of AdfloxNlucSMN to Alb-Cre mice resulted in an ~ 7.5 -fold overexpression of the NlucSMN protein in the liver, relative to endogenous SMN protein, with no protein detected in vector-treated wildtype C57BL/6 mice (Figure 3.3, Supplemental Figure S5C). NlucSMN protein was subsequently detected in the plasma of treated mice and specifically in the EV fraction of the plasma (Figure 3.4). NlucSMN protein was also detected in all tissues examined by luminescent assay, including the brain and spinal cord (Figure 3.5). In

the TA muscle, NlucSMN protein was also easily detectable by immunoblot and was present at a level equivalent to ~40-90% of the endogenous protein (Figure 3.6A), suggesting effective distribution to this tissue. NlucSMN protein was also detected by immunoblot of protein samples from the brain of treated mice (Figure 3.6B), consistent with previous observations that EV can readily cross the BBB²⁷⁻³². Based on relative Nluc activity observed in the various tissues (Figure 3.5), we estimate that the liver-derived EVs delivered the equivalent of ~10% of the endogenous level of SMN protein to the spinal cords of these mice (Figure 3.5). In the *Smn*^{2B} mouse model of SMA, *Smn*^{2B/-} mice that have ~25% of wildtype levels of the SMN protein in the spinal cord have a significantly reduced lifespan (~28 days) and exhibit all the hallmarks of SMA disease, while *Smn*^{2B/2B} mice that have ~36% of wildtype levels of SMN protein in the spinal cord are phenotypically normal^{105,106}. Thus, our ability to increase the quantity of SMN protein in CNS tissues by 10%, as well as also increase SMN protein levels in all other peripheral tissues, may be expected to be of therapeutic benefit. We used an E1-deleted Ad vector with a Cre-inducible system to ensure NlucSMN protein was produced exclusively in the liver (Figures 3.2, 3.3) in this proof-of-concept study. Mosaicism has been reported in Cre-lox mouse models, in which not all cells in a tissue of interest express Cre^{107,108}, which would effectively prevent maximal induction of *NlucSMN* expression from our vector. Indeed, not all hepatocytes in vector-treated Alb-Cre mice appeared positive for an NlucSMN signal (Figure 3.3E). Constitutive expression of NlucSMN from a gene therapy vector would be expected to yield much higher levels of NlucSMN protein in the liver, and consequently greater quantities of therapeutic protein in peripheral

tissues and the CNS. A combination of helper-dependent Ad, which are deleted of all viral protein coding sequences and associated with very long-term transgene expression relative to traditional E1-deleted Ad vectors^{80,81}, and an appropriate house-keeping promoter, which are less susceptible to epigenetic shut-down¹⁰⁹⁻¹¹¹, could provide high-level expression of the SMN protein for many years. However, engineering of the “donor” tissue could be achieved with any vector system, including AAV, lentivirus, or plasmid, providing a sufficient level of expression is achieved. Compared to AAV, Ad typically gives rise to much higher levels of gene expression per delivered viral genome^{112,113}. Use of a codon-optimized *SMN1* gene or delivery of a gene encoding a phospho-mimetic form of the SMN protein exhibiting higher protein stability^{62,114}, could also enhance the levels of SMN protein in the donor tissue and subsequently in the recipient tissue.

We demonstrated that liver-derived SMN protein distributes throughout skeletal muscle tissue, and was visualized surrounding myofibres in TA muscle (Figure 3.7, Supplemental Figure S6). It is likely that at least a portion of the protein reaching the TA muscle, and other tissues, is internalized into cells. Through the elegant use of reporter mice, many studies have shown that systemic delivery of EVs in mice or rats leads to cellular uptake⁸⁹⁻⁹¹. Using fibroblasts derived from SMA patients, we showed that EV-delivered SMN protein is able to transit to the nucleus and co-localize with normal cellular partners, suggesting the EV-delivered SMN protein is bioavailable and functional⁶⁰. Thus, we would predict that liver-derived SMN protein delivered to peripheral tissues would be similarly functional. We recently showed in the *Smn*^{2B} mouse model of SMA that administration of an AAV

vector encoding *SMN1* under regulation by the liver-specific albumin promoter, resulting in significant transduction of liver and no detectable transgene expression in the CNS, led to improved survival and corrected many disease-associated tissue pathologies, including liver steatosis, muscle fibre cross sectional area, and pancreatic cell imbalance¹². Although many of these effects may be due to restoration of SMN protein levels specifically in the liver, and thus restoration of paracrine signaling from the liver, at least part of this effect could also be due to EV-mediated distribution of SMN protein to peripheral tissues. However, in that study, vector-derived SMN protein was only detected in liver samples and was not detected in spinal cord or TA muscle by immunoblot, suggesting that any EV-mediated transfer of the SMN protein between tissues was low, and below the level of detection. As mentioned, Ad typically provides much higher levels of gene expression per delivered viral genome compared to AAV^{112,113}, which may have affected detection of any EV-transferred protein in that study.

Current SMA therapies approved for treatment of SMA, including nusinersen, risdiplam, and onasemnogene abeparvovec, have all been shown to be present in the liver, and increase SMN protein levels in this tissue^{2,115,116}. We speculate that at least a portion of the therapeutic efficacy of these treatments could be attributable to an increase in SMN protein at sites other than the CNS, such as the liver, resulting in release of SMN-loaded EVs, thus delivering SMN protein to cells body-wide, similar to what we demonstrated in this study. In particular, onasemnogene abeparvovec is administered systemically and results in very high uptake of vector by the liver, up to 400 vector genome copies per hepatocyte¹¹⁶, giving rise to high

levels of SMN mRNA transcripts and almost certainly resulting in substantial quantities of SMN protein in these cells. We expect that these transduced hepatocytes are releasing EVs loaded with SMN protein, which are then able to deliver protein to all tissues in the patient body. Thus, the reliance on direct CNS transduction of the onasemnogene abeparvovec vector may be less critical than previously thought, given the potential systemic benefits of liver EV-derived SMN protein delivery.

Conclusion

In conclusion, we have demonstrated that a gene therapy approach can be used to engineer the liver to release EVs containing a therapeutic protein into circulation, which subsequently distribute to all tissues of the body, including the CNS. Thus, we have developed an approach that may be applicable to many diseases that require body-wide delivery of a therapeutic protein.

3.5 Materials and Methods

Cell culture. GM00232 and AG08498 were obtained from Coriell Institute for Medical Research (Camden, NJ). GM00232 was collected from a 7-month old male who was diagnosed with SMA type 1 with a homozygous deletion of exons 7 and 8 of *SMN1* and 2 copies of *SMN2*. AG08498 was collected from a 1-year old healthy male. Cells were maintained in Minimum Essential Medium (MEM) (M2279, Sigma, Oakville, ON) supplemented with 15% (v/v) fetal bovine serum (FBS) (35-077-CV, Corning, Corning, NY), 2 mM GlutaMAX (35050061, Gibco, Grand Island, NY), and 1X antimycotic-antibiotic (15240062, Gibco). Reduced expression of SMN protein in the GM00232 cell line, relative to the AG08498 control cell line was confirmed (Supplemental Figure S1). A459 cells¹¹⁷ were obtained from the American Type Culture Collection (ATCC) (CCL-185, ATCC, Manassas, VA) and were grown in MEM supplemented with 10% (v/v) FBS, 2 mM GlutaMAX, and 1X antimycotic-antibiotic. HepG2 cells¹¹⁸ (HB-8065, ATCC), were grown in Dulbecco's Modified Eagle Medium (D5671, DMEM, Sigma) supplemented with 10% FBS, 2 mM GlutaMAX, and 1X antimycotic-antibiotic. FBS used in this study was not depleted of EVs, and thus conditioned medium (CM) collected from cells contained both EVs released from the cells and EVs present within FBS. All cells were incubated at 37°C and 5% CO₂ in a humidified chamber.

Viral vectors. Ad3F-SMN is a human adenovirus serotype 5 (HAdV-5)-based vector devoid of early region 1 (E1) and E3, and possessing an expression cassette containing the human cytomegalovirus (CMV) immediate early enhancer/promoter

driving expression of a triple FLAG-tagged *SMN1* cDNA replacing the E1 region, and has been described previously⁶⁰. AdmCherry-SMN is an HAdV-5-based vector lacking the E1 and E3 regions, possessing an expression cassette containing the CMV immediate early enhancer/promoter driving expression of a mCherry- and FLAG-tagged *SMN1* cDNA replacing the E1 region, and has been described previously⁶⁰. AdEmpty (laboratory designation AdRP2014) is an HAdV-5-based vector lacking the E1 and E3 regions with no foreign transgene, and has been previously described¹¹⁹.

AdfloxNlucSMN (laboratory designation AdRP3280) is a HAdV-5 E1/E3 deleted vector containing an expression cassette comprised of the CMV immediate early enhancer/promoter driving expression of a *loxP* flanked green fluorescent protein (GFP). The start codon for the GFP gene is located upstream of the 5' *loxP* site. Downstream of the floxed GFP gene is a gene for expression of a Nanoluciferase-FLAG-SMN (NlucSMN) fusion protein lacking a start codon (ATG). Cre-mediated excision of the GFP gene places the 5' start codon in frame with the NlucSMN gene, thus allowing for expression of the protein. AdfloxNlucSMNFluc (AdRP3281) is similar in structure to AdfloxNlucSMN, but also contains a human CMV immediate early enhancer/promoter driving expression of the firefly luciferase (Fluc) gene replacing the E3 region. AdNlucSMN (AdRP3272) is an E1/E3 deleted HAdV-5-based vector that contains a gene for expression of NlucSMN in the E1 region, under regulation by the human CMV early enhancer/promoter, and provides constitutive expression of the protein. AdGFP is an E1/E3 deleted HAdV-5-based vector that encodes GFP in the E1 region, driven by the human CMV early

enhancer/promoter, and has been described previously¹²⁰. AdCre is an E1/E3 deleted HAdV-5-based vector that possesses the Cre recombinase gene in the E1 region¹²¹, driven by the murine CMV early enhancer/promoter¹²². All Ad vectors were grown, purified, and titered on 293¹²³ or 293N3S¹²⁴ cells as previously described¹²⁵. Cells were transduced with vector at the indicated multiplicity of infection (MOI) or mock transduced with an equivalent volume of phosphate buffered saline (PBS) (D8537, Sigma) for 1 h at 37°C, with rocking to redistribute the virus every 15 min. Cells were then washed three times with PBS to remove unattached virus. Cell lysates and conditioned medium (CM) were collected 72 hpi.

Isolation and analysis of extracellular vesicles (EVs). EVs were isolated from CM for immunoblot analysis as previously described⁶⁰. Briefly, HepG2 cells were transduced with Ad3F-SMN as described above. Seventy-two hpi, CM was collected and centrifuged at 2,500xg for 15 min, then the resulting supernatant was collected and subjected to ultracentrifugation for 90 min at 120,000xg at 4°C in a TLA-100 rotor (Beckman Coulter). The resulting pellets were resuspended in PBS (Sigma), pooled, and subjected to ultracentrifugation at 120,000xg in a TLA-100 rotor for 90 min at 4°C. The resulting pellet was resuspended in 100 µL of PBS and stored at -20°C.

EVs were isolated from 50 µL of mouse plasma using a commercially available kit (Exoquick, EXOQ5A-1, SBI Biosciences, Palo Alto, CA) according to the manufacturer's instructions. The resulting pellet was resuspended in 50 µL of PBS or reporter lysis buffer (E397A, Promega, Madison, WI).

The diameter and concentration of EVs was determined by nanoparticle tracking analysis using a NanoSight NS300 (Malvern Panalytical, Westborough, MA). NanoSight NTA 3.4 software was used to capture videos and analyze isolated particles. Samples were diluted into a working range of the system in 0.1 μ m filtered PBS immediately before analysis. Samples were loaded using a syringe pump at an infusion rate of 50 and 2 independent analyses were conducted for each sample, each capturing 3 videos of 60 s. Capture settings remained consistent across all analyses.

Immunoblot. Cells in 35 mm dishes were lysed directly in 200 μ L of 2x Laemmli buffer (LB, 62.5 mM Tris-HCl pH 6.8, 25% w/v glycerol, 5% v/v β -mercaptoethanol, 2% w/v sodium dodecyl sulphate (SDS), 0.01% w/v bromophenol blue) and stored at -20°C . Isolated EVs in PBS were mixed 1:1 with 5X LB. Mouse tissue lysates were combined with equal volume of 2X LB. All samples were boiled for 10 min prior to protein separation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE). Separated proteins were transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Millipore, Burlington, MA). Membranes were blocked with 5% w/v skim milk powder dissolved in Tris-buffered saline (20 mM Tris-HCl pH 7.6, 135 mM NaCl) containing 0.2% Tween 20 (Thermo Fisher Scientific) (TBST) or Intercept (PBS) Blocking Buffer (927-70001, LI-COR Biosciences, Nebraska) for 1 h at room temperature (RT) and probed with the following primary antibodies diluted in blocking buffer containing 0.2% Tween 20: SMN (1:5000 1 h at RT for cell lysates and tissues, 1:1000 overnight at 4°C for EVs;

BD Transduction, Mississauga, ON), FLAG (1:5000 1 h at RT for cell lysates and tissues, 1:1000 overnight 4°C for EVs; F1804, Sigma), Tubulin (1:10000 1 h at RT; ab59670, Abcam, Cambridge, UK or CP06, Millipore), Flotillin-2 (1:1000 overnight at 4°C; C42A3, Cell Signalling Technologies, Danvers, MA), ALIX (1:1000 overnight at 4°C; ab186429, Abcam), CD29/Integrin beta-1 (1:1000 overnight at 4°C; 04-1109, Millipore), Vinculin (1:10000 1 h at RT; ab129002, Abcam). For membranes to be visualized by chemiluminescence, membranes blocked in 5% skim milk in TBST were washed three times in TBST and incubated with either goat anti-mouse horseradish peroxidase (HRP) conjugate (1706516, BioRad, Hercules, CA) or goat anti-rabbit HRP conjugate (1706515, BioRad). Blots were developed using Immobilon Classico Western HRP Substrate (Millipore) and visualized by standard autoradiography. For membranes to be visualized by infrared fluorescence, membranes blocked in Intercept (PBS) Blocking Buffer were washed three times in PBS containing 0.2% Tween 20 (PBST), and incubated with the following fluorescently-labelled secondary antibodies in blocking buffer containing 0.2% Tween 20 and 0.01% SDS: IRDye goat anti-mouse 680 (926-68070, LI-COR), IRDye goat anti-mouse 800 (925-32210), IRDye goat anti-rabbit 680 (925-68071, LI-COR), IRDye goat anti-rabbit 800 (926-32211, LI-COR). Membranes were developed by Odyssey CLx and visualized using LI-COR Image Studio 4.0 software.

Isolation of conditioned medium and EV transfer treatments. EV uptake experiments in tissue culture were performed directly with conditioned medium from donor cells, without further concentration of the EVs, as previously described⁶⁰.

Briefly, HepG2 cells were plated in 35 mm dishes (6×10^5 cells per dish) and, next day, transduced with Ad3F-SMN at an MOI=200. CM was collected 72 h later, centrifuged at 2,500xg for 15 min to pellet large cellular debris, and used immediately. For studies using CM depleted of EVs, half of the CM was maintained at 4°C, while the other half was subjected to ultracentrifugation at 120,000xg in a TLA-110 rotor overnight at 4°C to pellet the EVs. After centrifugation, the supernatant was retained and used immediately.

For CM treatments, GM00232 were seeded at a density of 3×10^4 cells per well in 8-well chambered slides (80841, Ibidi, Fitchburg, WI). Next day, the medium was removed and the cells were pre-treated with 100 μ M 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB) or vehicle control, both at a final DMSO concentration of 0.001% v/v for 1 h at 37°C. CM (200 μ L per well) was supplemented with DRB or vehicle, both to a final DMSO concentration of 0.001% v/v, and the cells were treated for 2 h at 37°C. DRB was used as an inhibitor of transcription elongation¹²⁶, to prevent *de novo* gene expression from residual HAdV vector that may be retained in the CM. The cells were washed 3 times with PBS and prepared for immunofluorescent staining as detailed below.

Immunofluorescence staining and microscopy. Cells were seeded at a density of 3×10^4 cells per well in 8-well chambered slides (80841, Ibidi, Fitchburg, WI). Next day, the cells were treated with CM as described above. After the 2 h incubation with CM, the cells were fixed with 4% paraformaldehyde (PFA) in PBS for 10 min at RT, washed 3 times with PBS, and permeabilized with 0.25% Triton X-100 (Sigma) in

PBS for 10 min at RT. The cells were blocked in 10% donkey serum (S30, Millipore) in PBS for 30 min at RT, and then incubated in 10% donkey serum (Millipore) overnight at 4°C with the following antibodies: SMN (1:250; BD Transduction), FLAG (1:250; F1804, Sigma), and Tubulin (1:500; ab59670, Abcam). The cells were washed three times for 5 min with 1% donkey serum (Millipore) in PBS, then incubated with the following secondary antibodies in 10% donkey serum in PBS (Millipore) for 1 h at RT; Alexa Fluor donkey anti-rabbit 594 (1:1000; A21207, Invitrogen, Eugene, OR) and Alexa Fluor donkey anti-mouse 488 (1:1000; A21202, Invitrogen). Following incubation with secondary antibody, the cells were stained with Hoechst 33342 at a concentration of 1 µg/mL (H3570, Life Technologies, Eugene, OR) in 1% donkey serum in PBS (Millipore) for 5 min at RT to visualize nuclei. The cells were washed three times for 5 min with 1% donkey serum in PBS (Millipore). Epifluorescence microscopy images were captured with a Zeiss Axio Imager M2 upright microscope, and processed using FIJI¹²⁷. Confocal images were captured with a Zeiss LSM 900 inverted confocal microscope, and processed using FIJI. Orthogonal projections were generated from 12-16 z-stacks captured 0.5 µm apart using ZEN blue version 3.2 (Carl Zeiss Microscopy, Oberkochen, Germany).

Sectioning and staining of mouse livers were performed at the Louise Pelletier Histology Core Facility at the University of Ottawa. Frozen tissue was sectioned by cryostat into 5-7 µm sections. The slides were dried, fixed with methanol/acetone (1:1) for 10 min and air dried. Of note, methanol/acetone fix is known to inactivate GFP¹²⁸, thus inhibiting fluorescence emission from the GFP in tissues from animals treated with AdfloxNlucSMNFluc. Sections were blocked for 30

min with Rodent Block M (Biocare RBM961H) and incubated with FLAG primary antibody (1:250; F1804, Sigma, Oakville, ON) for 2 h at RT. Sections were washed with 1x TBST and incubated with goat anti-mouse 488 and Phalloidin-594 antibodies for 2 h at RT, followed by incubation for 5 min with Vector TrueView Autofluorescence Quenching reagent (SP-8400, Vector Labs) to decrease autofluorescence. Sections were washed and incubated with 5 µg/mL of DAPI (62248, ThermoScientific). Fluorescence images were captured with a Zeiss Axio Imager M2 upright microscope, and processed using FIJI. Fluorescence intensity in the images was quantified using CellProfiler¹²⁹, and normalized to cell area using the tubulin signal.

Mouse tibialis anterior (TA) muscle tissue was fixed in 4% PFA for 24 h at 4°C immediately upon collection. The tissue was cryopreserved by three sequential incubations in 30% sucrose in PBS at 4°C for 24 h. Following cryopreservation, TA muscle tissue was embedded in Tissue-Tek O.C.T. compound (4583, Sakura Finetek, Torrance, CA) and frozen at -80°C. The tissue was sectioned by cryostat into 6 µm sections. Sections were dried for 20 min at RT, rehydrated with PBS for 10 min at RT, and permeabilized with 0.25% Triton X-100 (Sigma) in PBS for 10 min at RT. Sections were blocked for 1 h at RT with 10% goat serum (16210064, Gibco), washed three times for 10 min with PBS at RT, blocked with ReadyProbes Mouse-on-Mouse IgG Blocking Solution (R37621, Invitrogen) for 1 h at RT, and washed three times for 10 min with PBS at RT. Tissue sections were stained with anti-FLAG (1:250; F1804, Sigma) and anti-laminin (1:500; PA1-16730, Invitrogen) in 10% goat serum in PBS overnight at 4°C. The following day, sections were washed three times with 1% goat serum in PBS for 10 min at RT. Sections were incubated with goat anti-

mouse IgG1 647 (1:500; A-21240, Invitrogen) and goat anti-rabbit IgG 488 (1:1000; A-11008, Invitrogen) in 10% goat serum in PBS for 1 h at RT. Following incubation with secondary antibody, the sections were stained with Hoechst 33342 (Life Technologies) at a concentration of 1 μ g/mL in 1% goat serum in PBS for 5 min at RT to visualize nuclei. Sections were washed three times for 5 min at RT with 1% goat serum in PBS. Fluorescence images were captured with a Zeiss Axio Imager M2 upright microscope, and processed using FIJI.

Animal studies. All animal experiments were approved and performed according to the Animal Care and Veterinary Services (ACVS) guidelines at the University of Ottawa and conducted according to the Canadian Council on Animal Care. Mice were housed in ventilated cages in groups of 3 in a 12h/12h light/dark cycle with access to food and water *ad libitum*. For the data presented in Figures 3.3-6 and Supplemental Figure S5, five-week-old female C57BL/6 (Strain #000664, Jackson Laboratories) and B6.Cg-Speer6-ps1^{Tg(Alb-Cre)}32MGN/J (Alb-Cre)⁸⁴ (Strain#003574, Jackson Laboratories) mice were injected by tail vein with 5×10^{12} vp/kg AdfloxNlucSMNFluc ($\sim 1.25 \times 10^{11}$ vp per mouse) in 100 μ L of PBS or with 100 μ L PBS as a control. Three days post vector administration, mice were anesthetized with isoflurane and blood was collected in K2EDTA tubes (Microvette, 20.1314.100, Sarstedt, Nümbrecht, Germany) by cardiac puncture. Tissues were harvested, snap frozen in liquid nitrogen and stored at -80°C until processing. Tissues were combined with Reporter Lysis Buffer (RLB) (Promega, Madison, WI) and homogenized for 30 sec (PowerGen 125, Fisher Scientific). Whole TA muscle, lung, and kidney were

homogenized in 200, 400, and 500 μL of RLB, respectively. Approximately half of the heart, spinal cord, brain, and liver were homogenized in 200, 200, 600, and 1000 μL of RLB, respectively. Homogenized tissues were then frozen for at least 1 h at -80°C , thawed on ice and centrifuged at $15,000\times g$ for 3 min at 4°C to attain a cleared lysate. For the data presented in Figure 3.7 and Supplemental Figure S6, five-week-old female C57BL/6 (Strain #000664, Jackson Laboratories) were injected by tail vein with 5×10^{12} vp/kg ($\sim 1.25\times 10^{11}$ vp per mouse) of AdmCherry-SMN or AdEmpty in 100 μL of PBS or with 100 μL PBS as a control. Three days post vector administration, TA muscles were collected and fixed in 4% PFA.

Luciferase Assay. Luciferase assays were performed with the Nano-Glo Dual Luciferase Reporter Assay kit (N1630, Promega, Madison, WI, USA), and were conducted according to the manufacturer's protocol. Luciferase activity was determined using a 20/20 luminometer (Turner Biosystems, Sunnyvale, CA, USA), and normalized to protein concentration as determined by Bradford assay (BioRad, Hercules, CA, USA).

Statistical analysis. All statistical analyses were performed using Prism version 9.1.0, and the specific test performed is indicated in the figure legends. In all tests, one asterisk (*) indicates $p<0.05$, two asterisks (**) indicates $p<0.01$, three asterisks (***) indicates $p<0.001$, and four asterisks (****) indicates $p<0.0001$.

3.6 References

1. Kolb SJ, Kissel JT. Spinal Muscular Atrophy. *Neurol Clin.* Nov 2015;33(4):831-846.
2. Finkel RS, Chiriboga CA, Vajsar J, et al. Treatment of infantile-onset spinal muscular atrophy with nusinersen: a phase 2, open-label, dose-escalation study. *Lancet.* Dec 17 2016;388(10063):3017-3026.
3. Al-Zaidy S, Pickard AS, Kotha K, et al. Health outcomes in spinal muscular atrophy type 1 following AVXS-101 gene replacement therapy. *Pediatr Pulmonol.* 02 2019;54(2):179-185.
4. Baranello G, Darras BT, Day JW, et al. Risdiplam in Type 1 Spinal Muscular Atrophy. *N Engl J Med.* 03 2021;384(10):915-923.
5. René CA, Parks RJ. Expanding the Availability of Onasemnogene Apeparvovec to Older Patients: The Evolving Treatment Landscape for Spinal Muscular Atrophy. *Pharmaceutics.* Jun 19 2023;15(6).
6. Wishart TM, Huang JP, Murray LM, et al. SMN deficiency disrupts brain development in a mouse model of severe spinal muscular atrophy. *Hum Mol Genet.* Nov 01 2010;19(21):4216-4228.
7. Mendonça RH, Rocha AJ, Lozano-Arango A, et al. Severe brain involvement in 5q spinal muscular atrophy type 0. *Ann Neurol.* Sep 2019;86(3):458-462.
8. Gombash SE, Cowley CJ, Fitzgerald JA, et al. SMN deficiency disrupts gastrointestinal and enteric nervous system function in mice. *Hum Mol Genet.* Oct 2015;24(19):5665.
9. Bevan AK, Hutchinson KR, Foust KD, et al. Early heart failure in the SMNDelta7 model of spinal muscular atrophy and correction by postnatal scAAV9-SMN delivery. *Hum Mol Genet.* Oct 2010;19(20):3895-3905.
10. Shababi M, Habibi J, Yang HT, Vale SM, Sewell WA, Lorson CL. Cardiac defects contribute to the pathology of spinal muscular atrophy models. *Hum Mol Genet.* Oct 15 2010;19(20):4059-4071.
11. Deguise MO, Baranello G, Mastella C, et al. Abnormal fatty acid metabolism is a core component of spinal muscular atrophy. *Ann Clin Transl Neurol.* 08 2019;6(8):1519-1532.
12. Sutton ER, Beauvais A, Yaworski R, et al. Liver Smn Restoration Rescues the Smn2b/- Mouse Model of Spinal Muscular Atrophy: The Key to Rescue Whole Body Pathology? *eBioMedicine.* 2024.
13. Schreml J, Riessland M, Paterno M, et al. Severe SMA mice show organ impairment that cannot be rescued by therapy with the HDACi JNJ-26481585. *Eur J Hum Genet.* Jun 2013;21(6):643-652.
14. Allardyce H, Kuhn D, Hernandez-Gerez E, et al. Renal pathology in a mouse model of severe Spinal Muscular Atrophy is associated with downregulation of Glial Cell-Line Derived Neurotrophic Factor (GDNF). *Hum Mol Genet.* 08 11 2020;29(14):2365-2378.
15. Nery FC, Siranosian JJ, Rosales I, et al. Impaired kidney structure and function in spinal muscular atrophy. *Neurol Genet.* Oct 2019;5(5):e353.
16. Bowerman M, Michalski JP, Beauvais A, Murray LM, DeRepentigny Y, Kothary R. Defects in pancreatic development and glucose metabolism in

- SMN-depleted mice independent of canonical spinal muscular atrophy neuromuscular pathology. *Hum Mol Genet.* Jul 1 2014;23(13):3432-3444.
17. Bowerman M, Swoboda KJ, Michalski JP, et al. Glucose metabolism and pancreatic defects in spinal muscular atrophy. *Ann Neurol.* Aug 2012;72(2):256-268.
 18. Deguise MO, De Repentigny Y, McFall E, Auclair N, Sad S, Kothary R. Immune dysregulation may contribute to disease pathogenesis in spinal muscular atrophy mice. *Hum Mol Genet.* 02 2017;26(4):801-819.
 19. Thomson AK, Somers E, Powis RA, et al. Survival of motor neurone protein is required for normal postnatal development of the spleen. *J Anat.* 02 2017;230(2):337-346.
 20. Kim JK, Jha NN, Feng Z, et al. Muscle-specific SMN reduction reveals motor neuron-independent disease in spinal muscular atrophy models. *J Clin Invest.* Mar 02 2020;130(3):1271-1287.
 21. Shafey D, Côté PD, Kothary R. Hypomorphic Smn knockdown C2C12 myoblasts reveal intrinsic defects in myoblast fusion and myotube morphology. *Exp Cell Res.* Nov 15 2005;311(1):49-61.
 22. McCormack NM, Villalón E, Viollet C, et al. Survival motor neuron deficiency slows myoblast fusion through reduced myomaker and myomixer expression. *J Cachexia Sarcopenia Muscle.* Aug 2021;12(4):1098-1116.
 23. Tizzano EF, Finkel RS. Spinal muscular atrophy: A changing phenotype beyond the clinical trials. *Neuromuscul Disord.* Oct 2017;27(10):883-889.
 24. Antonaci L, Pera MC, Mercuri E. New therapies for spinal muscular atrophy: where we stand and what is next. *Eur J Pediatr.* Jul 2023;182(7):2935-2942.
 25. Mercuri E, Pera MC, Scoto M, Finkel R, Muntoni F. Spinal muscular atrophy - insights and challenges in the treatment era. *Nat Rev Neurol.* Dec 2020;16(12):706-715.
 26. Ruythooren F, Moens P. Spinal Muscular Atrophy Scoliosis in the Era of Background Therapies-A Review of the Literature. *J Clin Med.* Jun 14 2024;13(12).
 27. Driedonks T, Jiang L, Carlson B, et al. Pharmacokinetics and biodistribution of extracellular vesicles administered intravenously and intranasally to. *J Extracell Biol.* Oct 2022;1(10).
 28. Tolomeo AM, Zuccolotto G, Malvicini R, et al. Biodistribution of Intratracheal, Intranasal, and Intravenous Injections of Human Mesenchymal Stromal Cell-Derived Extracellular Vesicles in a Mouse Model for Drug Delivery Studies. *Pharmaceutics.* Feb 07 2023;15(2).
 29. Lázaro-Ibáñez E, Faruqu FN, Saleh AF, et al. Selection of Fluorescent, Bioluminescent, and Radioactive Tracers to Accurately Reflect Extracellular Vesicle Biodistribution. *ACS Nano.* Feb 23 2021;15(2):3212-3227.
 30. Gangadaran P, Li XJ, Lee HW, et al. A new bioluminescent reporter system to study the biodistribution of systematically injected tumor-derived bioluminescent extracellular vesicles in mice. *Oncotarget.* Dec 15 2017;8(66):109894-109914.

31. René CA, Parks RJ. Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles. *Pharmaceutics*. Apr 03 2021;13(4).
32. René CA, Parks RJ. Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency. *Mol Ther Methods Clin Dev*. Jun 13 2024;32(2):101259.
33. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol*. Feb 2013;200(4):373-383.
34. Welsh JA, Goberdhan DCI, O'Driscoll L, et al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J Extracell Vesicles*. Feb 2024;13(2):e12404.
35. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem*. Jul 1987;262(19):9412-9420.
36. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics*. Sep 2010;73(10):1907-1920.
37. Hoffmann J. Ueber chronische spinale Muskelatrophie im Kindesalter, auf familiärer Basis. *Deutsche Zeitschrift für Nervenheilkunde*. 1893;3(6):427-470.
38. Werdnig G. Zwei frühinfantile hereditäre Fälle von progressiver Muskelatrophie unter dem Bilde der Dystrophie, aber anf neurotischer Grundlage. *Archiv für Psychiatrie und Nervenkrankheiten*. 1891;22(2):437-480.
39. Lefebvre S, Bürglen L, Reboullet S, et al. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*. 1995;80(1):155-165.
40. Lefebvre S, Burlet P, Liu Q, et al. Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat Genet*. Jul 1997;16(3):265-269.
41. Finkel RS, McDermott MP, Kaufmann P, et al. Observational study of spinal muscular atrophy type I and implications for clinical trials. *Neurology*. 2014/07/30 2014;83(9):810-817.
42. Thomas NH, Dubowitz V. The natural history of type I (severe) spinal muscular atrophy. *Neuromuscul Disord*. 1994 Sep-Nov 1994;4(5-6):497-502.
43. Zerres K, Rudnik-Schöneborn S. Natural history in proximal spinal muscular atrophy. Clinical analysis of 445 patients and suggestions for a modification of existing classifications. *Arch Neurol*. May 1995;52(5):518-523.
44. Pagliardini S, Giavazzi A, Setola V, et al. Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet*. Jan 2000;9(1):47-56.
45. Coovert DD, Le TT, McAndrew PE, et al. The survival motor neuron protein in spinal muscular atrophy. *Hum Mol Genet*. Aug 1997;6(8):1205-1214.
46. Li DK, Tisdale S, Lotti F, Pellizzoni L. SMN control of RNP assembly: from post-transcriptional gene regulation to motor neuron disease. *Semin Cell Dev Biol*. Aug 2014;32:22-29.

47. Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc Natl Acad Sci U S A*. May 1999;96(11):6307-6311.
48. Liu Q, Fischer U, Wang F, Dreyfuss G. The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell*. 1997;90(6):1013-1021.
49. Fischer U, Liu Q, Dreyfuss G. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell*. 1997;90(6):1023-1029.
50. Battle DJ, Kasim M, Wang J, Dreyfuss G. SMN-independent subunits of the SMN complex. Identification of a small nuclear ribonucleoprotein assembly intermediate. *J Biol Chem*. Sep 2007;282(38):27953-27959.
51. Carissimi C, Saieva L, Gabanella F, Pellizzoni L. Gemin8 is required for the architecture and function of the survival motor neuron complex. *J Biol Chem*. Dec 2006;281(48):37009-37016.
52. Sanchez G, Dury AY, Murray LM, et al. A novel function for the survival motoneuron protein as a translational regulator. *Hum Mol Genet*. Feb 2013;22(4):668-684.
53. Rossoll W, Jablonka S, Andreassi C, et al. Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *J Cell Biol*. Nov 2003;163(4):801-812.
54. Ting CH, Wen HL, Liu HC, Hsieh-Li HM, Li H, Lin-Chao S. The spinal muscular atrophy disease protein SMN is linked to the Golgi network. *PloS one*. 2012;7(12):e51826.
55. Bowerman M, Anderson CL, Beauvais A, Boyl PP, Witke W, Kothary R. SMN, profilin IIa and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis. *Mol Cell Neurosci*. Sep 2009;42(1):66-74.
56. Bowerman M, Beauvais A, Anderson CL, Kothary R. Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model. *Hum Mol Genet*. Apr 2010;19(8):1468-1478.
57. Zhao DY, Gish G, Braunschweig U, et al. SMN and symmetric arginine dimethylation of RNA polymerase II C-terminal domain control termination. *Nature*. Jan 7 2016;529(7584):48-53.
58. Nash LA, McFall ER, Perozzo AM, et al. Survival Motor Neuron Protein is Released from Cells in Exosomes: A Potential Biomarker for Spinal Muscular Atrophy. *Sci Rep*. 10 2017;7(1):13859.
59. Roberto J, Poulin KL, Parks RJ, Vacratsis PO. Label-free quantitative proteomic analysis of extracellular vesicles released from fibroblasts derived from patients with spinal muscular atrophy. *Proteomics*. 07 2021;21(13-14):e2000301.
60. René CA, Parks RJ. Extracellular vesicles efficiently deliver survival motor neuron protein to cells in culture. *Sci Rep*. Feb 15 2025;15(1):5674.
61. Vitte J, Fassier C, Tiziano FD, et al. Refined characterization of the expression and stability of the SMN gene products. *Am J Pathol*. Oct 2007;171(4):1269-1280.

62. Burnett BG, Munoz E, Tandon A, Kwon DY, Sumner CJ, Fischbeck KH. Regulation of SMN protein stability. *Mol Cell Biol.* Mar 2009;29(5):1107-1115.
63. Riessland M, Kaczmarek A, Schneider S, et al. Neurocalcin Delta Suppression Protects against Spinal Muscular Atrophy in Humans and across Species by Restoring Impaired Endocytosis. *Am J Hum Genet.* Feb 02 2017;100(2):297-315.
64. Hosseinibarkooie S, Peters M, Torres-Benito L, et al. The Power of Human Protective Modifiers: PLS3 and CORO1C Unravel Impaired Endocytosis in Spinal Muscular Atrophy and Rescue SMA Phenotype. *Am J Hum Genet.* Sep 01 2016;99(3):647-665.
65. Walsh MB, Janzen E, Wingrove E, et al. Genetic modifiers ameliorate endocytic and neuromuscular defects in a model of spinal muscular atrophy. *BMC Biol.* Sep 16 2020;18(1):127.
66. Costa Verdera H, Gitz-Francois JJ, Schiffelers RM, Vader P. Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis. *J Control Release.* Nov 2017;266:100-108.
67. Nishimura AL, Mitne-Neto M, Silva HC, et al. A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am J Hum Genet.* Nov 2004;75(5):822-831.
68. Dimitriadi M, Derdowski A, Kalloo G, et al. Decreased function of survival motor neuron protein impairs endocytic pathways. *Proc Natl Acad Sci U S A.* Jul 26 2016;113(30):E4377-4386.
69. Kamerkar S, LeBleu VS, Sugimoto H, et al. Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature.* Jun 22 2017;546(7659):498-503.
70. Didiot MC, Hall LM, Coles AH, et al. Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing. *Mol Ther.* 10 2016;24(10):1836-1847.
71. Haney MJ, Klyachko NL, Zhao Y, et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release.* Jun 2015;207:18-30.
72. Andriolo G, Provasi E, Brambilla A, et al. Methodologies for Scalable Production of High-Quality Purified Small Extracellular Vesicles from Conditioned Medium. *Methods Mol Biol.* 2023;2668:69-98.
73. Benedikter BJ, Bouwman FG, Vajen T, et al. Ultrafiltration combined with size exclusion chromatography efficiently isolates extracellular vesicles from cell culture media for compositional and functional studies. *Sci Rep.* Nov 10 2017;7(1):15297.
74. Huard J, Lochmüller H, Acsadi G, Jani A, Massie B, Karpati G. The route of administration is a major determinant of the transduction efficiency of rat tissues by adenoviral recombinants. *Gene Ther.* Mar 1995;2(2):107-115.
75. Schiedner G, Morral N, Parks RJ, et al. Genomic DNA transfer with a high-capacity adenovirus vector results in improved in vivo gene expression and decreased toxicity. *Nat Genet.* 1998;18(2):180-183.
76. Zabaleta N, Unzu C, Weber ND, Gonzalez-Aseguinolaza G. Gene therapy for liver diseases - progress and challenges. *Nat Rev Gastroenterol Hepatol.* May 2023;20(5):288-305.

77. Moscoso CG, Steer CJ. Liver targeted gene therapy: Insights into emerging therapies. *Drug Discov Today Technol.* Dec 2019;34:9-19.
78. Vrancken Peeters MJ, Perkins AL, Kay MA. Method for multiple portal vein infusions in mice: quantitation of adenovirus-mediated hepatic gene transfer. *BioTechniques.* Feb 1996;20(2):278-285.
79. Worgall S, Wolff G, Falck-Pedersen E, Crystal RG. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum Gene Ther.* Jan 01 1997;8(1):37-44.
80. Kim IH, Józkowicz A, Piedra PA, Oka K, Chan L. Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci U S A.* Nov 2001;98(23):13282-13287.
81. Brunetti-Pierri N, Ng T, Iannitti D, et al. Transgene expression up to 7 years in nonhuman primates following hepatic transduction with helper-dependent adenoviral vectors. *Hum Gene Ther.* Aug 2013;24(8):761-765.
82. Royo F, Moreno L, Mleczko J, et al. Hepatocyte-secreted extracellular vesicles modify blood metabolome and endothelial function by an arginase-dependent mechanism. *Sci Rep.* 02 17 2017;7:42798.
83. Newman LA, Useckaite Z, Johnson J, Sorich MJ, Hopkins AM, Rowland A. Selective Isolation of Liver-Derived Extracellular Vesicles Redefines Performance of miRNA Biomarkers for Non-Alcoholic Fatty Liver Disease. *Biomedicines.* Jan 17 2022;10(1).
84. Postic C, Shiota M, Niswender KD, et al. Dual roles for glucokinase in glucose homeostasis as determined by liver and pancreatic beta cell-specific gene knock-outs using Cre recombinase. *J Biol Chem.* Jan 01 1999;274(1):305-315.
85. Goulet BB, McFall ER, Wong CM, Kothary R, Parks RJ. Supraphysiological expression of survival motor neuron protein from an adenovirus vector does not adversely affect cell function. *Biochem Cell Biol.* Aug 2013;91(4):252-264.
86. Nakai M, Komiya K, Murata M, et al. Expression of pIX gene induced by transgene promoter: possible cause of host immune response in first-generation adenoviral vectors. *Hum Gene Ther.* Oct 2007;18(10):925-936.
87. Wang Y, Krushel LA, Edelman GM. Targeted DNA recombination in vivo using an adenovirus carrying the cre recombinase gene. *Proc Natl Acad Sci U S A.* Apr 30 1996;93(9):3932-3936.
88. Johnson M, Huyn S, Burton J, Sato M, Wu L. Differential biodistribution of adenoviral vector in vivo as monitored by bioluminescence imaging and quantitative polymerase chain reaction. *Hum Gene Ther.* Dec 2006;17(12):1262-1269.
89. Zomer A, Maynard C, Verweij FJ, et al. In Vivo imaging reveals extracellular vesicle-mediated phenocopying of metastatic behavior. *Cell.* May 21 2015;161(5):1046-1057.
90. de Jong OG, Murphy DE, Mäger I, et al. A CRISPR-Cas9-based reporter system for single-cell detection of extracellular vesicle-mediated functional transfer of RNA. *Nat Commun.* Feb 28 2020;11(1):1113.

91. Reshke R, Taylor JA, Savard A, et al. Reduction of the therapeutic dose of silencing RNA by packaging it in extracellular vesicles via a pre-microRNA backbone. *Nat Biomed Eng*. Jan 2020;4(1):52-68.
92. Kojima R, Bojar D, Rizzi G, et al. Designer exosomes produced by implanted cells intracerebrally deliver therapeutic cargo for Parkinson's disease treatment. *Nat Commun*. Apr 03 2018;9(1):1305.
93. Lightner AL, Sengupta V, Qian S, et al. Bone Marrow Mesenchymal Stem Cell-Derived Extracellular Vesicle Infusion for the Treatment of Respiratory Failure From COVID-19: A Randomized, Placebo-Controlled Dosing Clinical Trial. *Chest*. Jun 23 2023.
94. Izadpanah M, Dargahi L, Ai J, et al. Extracellular Vesicles as a Neprilysin Delivery System Memory Improvement in Alzheimer's Disease. *Iran J Pharm Res*. 2020;19(2):45-60.
95. Yang T, Martin P, Fogarty B, et al. Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in Danio rerio. *Pharm Res*. Jun 2015;32(6):2003-2014.
96. Morad G, Carman CV, Hagedorn EJ, et al. Tumor-Derived Extracellular Vesicles Breach the Intact Blood-Brain Barrier. *ACS Nano*. 12 2019;13(12):13853-13865.
97. Zhu X, Badawi M, Pomeroy S, et al. Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *J Extracell Vesicles*. 2017;6(1):1324730.
98. Saleh AF, Lázaro-Ibáñez E, Forsgard MA, et al. Extracellular vesicles induce minimal hepatotoxicity and immunogenicity. *Nanoscale*. Apr 2019;11(14):6990-7001.
99. Görgens A, Corso G, Hagey DW, et al. Identification of storage conditions stabilizing extracellular vesicles preparations. *J Extracell Vesicles*. Jun 2022;11(6):e12238.
100. Li G, Chen T, Dahlman J, et al. Current challenges and future directions for engineering extracellular vesicles for heart, lung, blood and sleep diseases. *J Extracell Vesicles*. Feb 2023;12(2):e12305.
101. Miotto PM, Yang CH, Keenan SN, et al. Liver-derived extracellular vesicles improve whole-body glycaemic control via inter-organ communication. *Nat Metab*. Feb 2024;6(2):254-272.
102. Povero D, Eguchi A, Li H, et al. Circulating extracellular vesicles with specific proteome and liver microRNAs are potential biomarkers for liver injury in experimental fatty liver disease. *PLoS One*. 2014;9(12):e113651.
103. Achour B, Al-Majdoub ZM, Grybos-Gajniak A, et al. Liquid Biopsy Enables Quantification of the Abundance and Interindividual Variability of Hepatic Enzymes and Transporters. *Clin Pharmacol Ther*. Jan 2021;109(1):222-232.
104. Fordjour FK, Abuelreich S, Hong X, et al. Exomap1 mouse: a transgenic model for. *bioRxiv*. May 29 2023.
105. Bowerman M, Murray LM, Beauvais A, Pinheiro B, Kothary R. A critical smn threshold in mice dictates onset of an intermediate spinal muscular atrophy

- phenotype associated with a distinct neuromuscular junction pathology. *Neuromuscul Disord*. Mar 2012;22(3):263-276.
106. Eshraghi M, McFall E, Gibeault S, Kothary R. Effect of genetic background on the phenotype of the *Smn2B^{-/-}* mouse model of spinal muscular atrophy. *Hum Mol Genet*. Oct 15 2016;25(20):4494-4506.
 107. Leneuve P, Colnot S, Hamard G, et al. Cre-mediated germline mosaicism: a new transgenic mouse for the selective removal of residual markers from tri-ox conditional alleles. *Nucleic Acids Res*. Mar 01 2003;31(5):e21.
 108. Weisend CM, Kundert JA, Suvorova ES, Prigge JR, Schmidt EE. Cre activity in fetal albCre mouse hepatocytes: Utility for developmental studies. *Genesis*. Dec 2009;47(12):789-792.
 109. Löser P, Jennings GS, Strauss M, Sandig V. Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: involvement of NFkappaB. *J Virol*. Jan 1998;72(1):180-190.
 110. Chen HH, Mack LM, Choi SY, Ontell M, Kochanek S, Clemens PR. DNA from both high-capacity and first-generation adenoviral vectors remains intact in skeletal muscle. *Hum Gene Ther*. Feb 10 1999;10(3):365-373.
 111. Brooks AR, Harkins RN, Wang P, Qian HS, Liu P, Rubanyi GM. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med*. Apr 2004;6(4):395-404.
 112. Martino AT, Nayak S, Hoffman BE, et al. Tolerance induction to cytoplasmic beta-galactosidase by hepatic AAV gene transfer: implications for antigen presentation and immunotoxicity. *PLoS One*. Aug 04 2009;4(8):e6376.
 113. McCaffrey AP, Fawcett P, Nakai H, et al. The host response to adenovirus, helper-dependent adenovirus, and adeno-associated virus in mouse liver. *Mol Ther*. May 2008;16(5):931-941.
 114. Xie Q, Chen X, Ma H, et al. Improved gene therapy for spinal muscular atrophy in mice using codon-optimized hSMN1 transgene and hSMN1 gene-derived promoter. *EMBO Mol Med*. Apr 2024;16(4):945-965.
 115. Poirier A, Weetall M, Heinig K, et al. Risdiplam distributes and increases SMN protein in both the central nervous system and peripheral organs. *Pharmacol Res Perspect*. 12 2018;6(6):e00447.
 116. Thomsen G, Burghes AHM, Hsieh C, et al. Biodistribution of onasemnogene abeparvovec DNA, mRNA and SMN protein in human tissue. *Nat Med*. Oct 2021;27(10):1701-1711.
 117. Giard DJ, Aaronson SA, Todaro GJ, et al. In vitro cultivation of human tumors: establishment of cell lines derived from a series of solid tumors. *J Natl Cancer Inst*. Nov 1973;51(5):1417-1423.
 118. Knowles BB, Howe CC, Aden DP. Human hepatocellular carcinoma cell lines secrete the major plasma proteins and hepatitis B surface antigen. *Science*. Jul 25 1980;209(4455):497-499.
 119. Poulin KL, McFall ER, Chan G, et al. Fusion of Large Polypeptides to Human Adenovirus Type 5 Capsid Protein IX Can Compromise Virion Stability and DNA Packaging Capacity. *J Virol*. Aug 17 2020;94(17).

120. Hubberstey AV, Pavliv M, Parks RJ. Cancer therapy utilizing an adenoviral vector expressing only E1A. *Cancer Gene Ther.* Apr 2002;9(4):321-329.
121. Anton M, Graham FL. Site-specific recombination mediated by an adenovirus vector expressing the Cre recombinase protein: a molecular switch for control of gene expression. *J Virol.* Aug 1995;69(8):4600-4606.
122. Addison CL, Hitt M, Kunsken D, Graham FL. Comparison of the human versus murine cytomegalovirus immediate early gene promoters for transgene expression by adenoviral vectors. *J Gen Virol.* Jul 1997;78 (Pt 7):1653-1661.
123. Graham FL, Smiley J, Russell WC, Nairn R. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J Gen Virol.* Jul 1977;36(1):59-74.
124. Graham FL. Growth of 293 cells in suspension culture. *J Gen Virol.* Mar 1987;68 (Pt 3):937-940.
125. Ross PJ, Parks RJ. Construction and characterization of adenovirus vectors. *Cold Spring Harb Protoc.* May 2009;2009(5):pdb.prot5011.
126. Chodosh LA, Fire A, Samuels M, Sharp PA. 5,6-Dichloro-1-beta-D-ribofuranosylbenzimidazole inhibits transcription elongation by RNA polymerase II in vitro. *J Biol Chem.* Feb 05 1989;264(4):2250-2257.
127. Schindelin J, Arganda-Carreras I, Frise E, et al. Fiji: an open-source platform for biological-image analysis. *Nat Methods.* Jun 28 2012;9(7):676-682.
128. Scandella V, Paolicelli RC, Knobloch M. A novel protocol to detect green fluorescent protein in unfixed, snap-frozen tissue. *Sci Rep.* Sep 04 2020;10(1):14642.
129. Stirling DR, Swain-Bowden MJ, Lucas AM, Carpenter AE, Cimini BA, Goodman A. CellProfiler 4: improvements in speed, utility and usability. *BMC Bioinformatics.* Sep 10 2021;22(1):433.

3.7 Acknowledgements

Data Availability: The data that support the findings of this study are available on request.

Acknowledgements: The authors are grateful to the participants in this study. This work was supported by a collaborative grant from the Canadian Institutes of Health Research (CIHR, PJT-178120) to R.J.P., R.K., P.O.V. and J.W.-C. R.J.P. was also supported by funds from CIHR (NHG-107767, MOP-136898, PYY-175428) and Cure SMA/Cure SMA Canada. C.A.R. was supported by a Queen Elizabeth II Graduate Scholarship in Science and Technology from the Ontario Provincial Government (Canada).

Author Contributions: Conceptualization: C.A.R., P.O.V., J.W.-C., R.K., R.J.P.; Formal Analysis: C.A.R., K.L.P., R.J.P.; Funding acquisition: C.A.R., J.C.B., D.B., P.O.V., J.W.-C., R.K., R.J.P.; Investigation: C.A.R., K.L.P., J.P., D.B.; Methodology: C.A.R., K.L.P., J.P., D.B., R.J.P.; Project administration: R.J.P.; Supervision: J.C.B., D.B., P.O.V., J.W.-C., R.J.P.; Visualization: C.A.R., K.L.P., R.J.P.; Writing – original draft: C.A.R.; Writing – review & editing: C.A.R., K.L.P., J.P., J.C.B., D.B., P.O.V., J.W.-C., R.K., R.J.P.

All authors have read and agreed to the published version of the manuscript.

Declarations of Interest: The authors declare no conflicts of interest.

Chapter 4: General Discussion

SMA is a devastating neuromuscular disease caused by low cellular levels of SMN protein following homozygous mutation or deletion of *SMN1*. There are three therapeutics currently available to treat SMA, each focusing on increasing cellular levels of SMN protein through gene replacement or gene targeting strategies. Unfortunately, not all patients with SMA respond to these therapies, and therefore, there is a need for the development of new therapeutic strategies. The Parks lab has previously demonstrated that SMN protein is naturally contained within EVs, and therefore, EVs may be an ideal therapeutic vehicle to deliver SMN protein. Thus, the overall purpose of the work contained within this thesis was to assess the utility of EVs as a delivery vehicle for SMN protein, in cell culture and animal models. The results obtained are discussed below, as well as the implications and future directions of this work.

4.1 SMN protein-loaded EVs

As detailed in chapters 1, 2, and 3 of this thesis, SMA pathology is caused by low levels of SMN protein, and there is a need for the development of new therapies to treat this devastating disease. In line with previous studies, we have demonstrated that SMN protein is present within EVs (Figure 2.2). Thus, we wanted to utilize EVs as delivery vehicles of this crucial SMN protein. We have shown that Ad-mediated delivery of *SMN1* cDNA to cells can be used to increase the amount of SMN protein contained within EVs (Figure 2.4). The Ad-based method used to increase SMN protein levels in cells and subsequently within EVs released by these cells has many advantages over other cargo loading methods. Loading cargo into EVs after their biogenesis commonly relies on disruption of the EV membrane, which

can be inefficient and cause permanent damage to the membrane^{254,256-258}. Incorporating cargo into EVs during the biogenesis of the vesicles allows for the membrane to remain intact. One such method where cargo is loaded during biogenesis is transfection, where DNA or RNA is incorporated into cells, which then release EVs loaded with the desired cargo^{315,316}. However, transfection complexes may impair the ability of EVs to deliver cargo to target cells and also can generate artefacts that confound analysis of EV-mediated cargo delivery^{315,316}. Thus, transfection is also a flawed method to achieve cargo loading. Alternatively, similar to Ad transduction, lentiviral-mediated transduction of a desired gene has been used to endogenously incorporate cargo into EVs during biogenesis. Unfortunately, the enveloped structure and size of lentivirus makes it difficult to differentiate the released EVs and lentivirus in the extracellular space³¹⁷. In contrast, Ad is a non-enveloped virus³¹⁸, and thus, EVs released by cells can be differentiated from any Ad vector that was not internalized by cells and thus persists in the extracellular space. We have demonstrated that Ad transduction can give rise to high levels of cargo loading into EVs (Figure 2.4), and could be used to load other cargos of interest into EVs. Ad vectors devoid of E1/E3, such as those used in this work, have a cloning capacity of ~8 kb³¹⁹ and helper-dependent Ad vectors (HDAd) devoid of viral coding sequences have a cloning capacity up to ~37 kb³²⁰, thus even very large transgenes can be carried by these vectors. Therefore, Ad transduction of parental cells may be an ideal method to load EVs released by these cells with an abundance of different target cargos during biogenesis, as demonstrated herein with SMN protein.

The results presented in this thesis demonstrate that EVs loaded with SMN protein can deliver this SMN protein to cells in tissue culture (Figure 2.5, 2.8). Importantly, this delivered SMN protein localized in nuclear gem-like structures and co-localized with endogenous protein partners (Figures 2.6, 2.7, 2.8). Previously, other groups have sought to deliver supplemental SMN protein to cells in culture. One approach utilized a recombinant SMN protein fused to the catalytic and transmembrane domains of diphtheria toxin (Dtx) and tetanus toxin fragment C (TTC)³²¹. Unfortunately, the SMN-Dtx-TTC fusion protein was unable to transit to the nucleus, instead remaining at the surface of primary neurons in tissue culture. Another approach utilized a fusion protein of SMN and the human immunodeficiency virus transactivator of transcription (TAT) cell-penetrating peptide, however the TAT-SMN fusion protein was unable to be internalized by SH-SY5Y cells, a neuroblastoma cell line, or rat cortical neurons³²². Recently, another study utilized a TAT-SMN fusion protein to treat murine motor neuron-like NSC-34 cells in tissue culture³²³. Following 48 h of treatment with 100 nM of TAT-SMN, mass spectrometry analysis confirmed that the NSC-34 cells internalized the TAT-SMN fusion protein, however it is unclear if the SMN protein was able to escape the endosome or transit to the nucleus. Indeed, previous studies have indicated that TAT fusion proteins enter the cell by an endocytic pathway and thus need to escape the endosome to perform cellular functions³²⁴, and that TAT fusion proteins do not efficiently localize to the nucleus of treated cells in culture^{325,326}. Importantly, the results presented in this thesis demonstrate the ability of EVs to efficiently deliver SMN protein to recipient cells in culture.

Overall, these data demonstrate the ability of EVs to be used as a vehicle to deliver SMN protein to cells in tissue culture. The data presented in this thesis could be strengthened by demonstrating the ability of SMN protein-loaded EVs to deliver SMN protein to motor neurons in culture. Previous studies have demonstrated the ability of EVs to deliver cargo to primary motor neurons isolated from a mouse model of amyotrophic lateral sclerosis (ALS) and wildtype mice^{327,328}. It would be interesting to assess EV-mediated delivery of SMN protein to primary motor neurons isolated from moderate and severe mouse models of SMA, and to iPSC-derived motor neurons from human patients with SMA. Delivery of SMN protein by EVs to motor neurons with low levels of SMN protein would further support the use of EVs as delivery vehicles of therapeutic SMN protein, a potential novel approach to treat SMA.

Indeed, SMN protein-loaded EVs have the potential to be used as a therapy for SMA. Following IV administration, EVs distribute throughout the body and accumulate in various tissues, including the brain, kidney, heart, spleen, and liver²¹⁵, and can deliver cargo to a wide variety of cell types, importantly including motor neurons³²⁷. Thus, EVs could be used as a delivery vehicle to correct defects caused by low SMN protein levels in many cell types. Daily systemic administration of SMN protein-loaded EVs to a mouse model of SMA would provide insight into the therapeutic benefit of these EVs.

Interestingly, EVs can cross the placental barrier³²⁹, and EVs administered into amniotic fluid reach the fetal lung in rodent models of pulmonary hypoplasia, rescuing fetal lung underdevelopment^{330,331}. The ability of EVs to provide therapeutic

benefit as a prenatal therapy suggests that SMN protein-loaded EVs could be used as a vehicle to deliver supplemental SMN protein to a fetus identified as at-risk of severe SMA. Fetal therapy for SMA has gained attention in recent years, and increasing SMN protein levels *in utero* has demonstrated greater therapeutic benefit compared to initiating treatment after birth in a severe mouse model of SMA³³². Delivering SMN protein *in utero* with EVs could slow SMA pathology until alternative therapy can be administered post-birth. Furthermore, severe forms of SMA are associated with structural abnormalities in the brain¹⁰⁵ and heart¹¹¹, and thus delivery of supplemental SMN protein during fetal development could potentially ameliorate these defects. The utility and efficacy of SMN protein-loaded EVs could be evaluated in a mouse model of SMA by administering SMN protein-loaded EVs IV into pregnant dams, into the amniotic fluid, or into the placenta and evaluating the therapeutic benefit in a severe mouse model of SMA.

In addition, EVs loaded with SMN protein may also be useful as an add-on therapy for SMA. Patients who are treated with nusinersen and/or risdiplam can show varying responses to treatment, with some patients not gaining or maintaining motor milestones^{157,163,333-335}. Recently, an *in vitro* study demonstrated that the amount of full-length SMN protein produced by cells of SMA patients following treatment with risdiplam or nusinersen was independent of *SMN2* copy number and which drug was used²⁰⁸. Thus, patients receiving these therapies may benefit from supplemental SMN protein delivered in EVs. Overall, SMN protein-loaded EVs could potentially provide therapeutic benefit to a spectrum of patients with SMA by supplying supplemental SMN protein.

4.2 *in situ* production and systemic distribution of liver-derived protein

The work presented in chapter 2 demonstrated the ability of EVs to deliver SMN protein to recipient cells that localized similarly to endogenous SMN protein and co-localized with endogenous protein partners, highlighting the potential of EVs to be used as a delivery vehicle of therapeutic SMN protein to treat SMA. This work was expanded upon in chapter 3, further demonstrating the ability of EVs to deliver SMN protein to fibroblasts derived from a patient with SMA. The EV-delivered SMN protein localized in the cytoplasm and nucleus in gem-like structures (Figure 3.1, 3.2), demonstrating that although some cells with low levels of SMN protein can exhibit defects in endocytosis^{49-51,336} and intracellular trafficking^{49,337}, these cells were still able to take up SMN protein-loaded EVs. Thus, EVs loaded with SMN protein may represent a novel therapeutic strategy to replenish low cellular levels of SMN protein in patients with SMA. However, due to the ~6 h half-life of SMN protein within the cell^{15,16}, it is likely that SMN protein-loaded EVs would need to be re-administered every day to maintain a therapeutic benefit. Thus, an alternative approach to circumvent the need for continuous re-administration of EVs was desirable.

As hepatocytes naturally release EVs²⁹⁸, and the liver contributes ~15% of the total EVs in circulation^{218,338,339}, I utilized a gene therapy approach to engineer the liver of mice to release SMN protein into circulation, encapsulated within EVs. To determine if the liver could be used as a factory for the production of SMN protein-loaded EVs that distributed throughout the body, a system was generated to give rise to liver-specific production of a bioluminescent SMN fusion protein. An Ad vector

was created, containing a Cre-inducible cassette for expression of *NlucSMN* to be used in combination with Alb-Cre transgenic mice, giving rise to liver-specific protein production (Figure 3.3). Systemic delivery of this vector led to high levels of NlucSMN protein synthesis in the liver of Alb-Cre mice and no detectable NlucSMN protein in wildtype mice (Figure 3.4). The NlucSMN protein was also detected in plasma and EVs isolated from plasma of vector-treated Alb-Cre mice (Figure 3.5), indicating that the protein produced in the liver was entering circulation in EVs. Importantly, NlucSMN was detected in all tissues examined by luminescent assay (Figure 3.6) and in the brain and TA muscle as assessed by immunoblot (Figure 3.7). Thus, NlucSMN protein produced in the liver was able to enter circulation, likely within EVs, and distribute to peripheral tissues, including the brain and spinal cord. Together, these data indicate that the liver can be used as a “factory” of therapeutic protein loaded EVs that distribute throughout the body.

The clear next step for this work is to test the efficacy of this approach as a therapeutic strategy for SMA by using this same system in a mouse model of the disease. It was recently demonstrated by Sutton et al.¹¹⁵ that modestly increasing SMN protein levels exclusively in the liver of a mouse model of SMA increases survival and corrects many disease-associated pathologies, including muscle fibre cross sectional area, liver steatosis, and pancreatic cell imbalance. These effects may be due to improvements in paracrine signalling from the liver following increase in SMN protein levels in the liver. Overall, the data presented in this study demonstrated that increasing SMN protein level in the liver has a therapeutic benefit in a mouse model of SMA. Thus, following systemic administration of AdSMN into a

mouse model of SMA, subsequently increasing SMN protein level in the liver, it is likely that there would be a therapeutic benefit. However, it would be difficult to definitively determine whether rescue in survival and/or disease-associated pathology was due to the effect of increasing SMN protein level in the liver, or due to distribution of SMN protein to peripheral tissues in EVs, or a combination of both. Inhibition of EV release would allow for differentiation between the benefit of increasing SMN protein level in the liver and systemic distribution and uptake of the protein. However, it is possible that inhibition of EV release could have detrimental consequences on the health of the mice, as EVs are mediators of intercellular communication^{214,288}. Alternatively, continuous systemic administration of SMN protein-loaded EVs would provide insight into the therapeutic benefit of these EVs. Producing SMN protein-loaded EVs in cell culture and administering these EVs by daily IV injection in a mouse model of SMA could replicate the release of EVs by the liver. To more closely replicate endogenous release of EVs by the liver, cells producing high levels of SMN protein could be implanted into a mouse model of SMA, releasing EVs loaded with SMN protein into circulation. This approach has been used before, where subcutaneously implanted 293T cells expressing luciferase or catalase released EVs containing this cargo, delivering the cargo to the brain, spleen, and liver of mice³⁴⁰. Use of implanted liver-derived cells releasing EVs loaded with SMN protein would allow for simple differentiation between the effect of restoring SMN protein level in the liver and the systemic distribution of liver-derived SMN-loaded EVs. Thus, there are several methods that could be used to elucidate

whether the therapeutic benefit demonstrated after increasing SMN protein levels in the liver is due to systemic distribution of the SMN protein within liver-derived EVs.

The work presented herein utilized E1/E3 deleted Ad vectors with CMV promoter-driven transgenes, which are prone to epigenetic silencing³⁴¹. High-level expression of SMN protein in the liver for many years could be provided through the use of HDAd, which are associated with very long-term transgene expression relative to traditional E1-deleted Ad vectors^{308,314}, and a house-keeping promoter that is less susceptible to epigenetic silencing³⁴¹⁻³⁴³. However, the liver could be engineered with a variety of other vector systems. One such vector is AAV, however Ad typically gives rise to much higher levels of gene expression per delivered viral genome^{344,345}. This problem could be circumvented through the use of a codon-optimized *SMN1* cDNA or delivery of a cDNA encoding a phospho-mimetic form of the SMN protein exhibiting higher protein stability^{16,185}. These strategies would enhance the level of SMN protein in the liver, and thus the amount of protein delivered to recipient tissues. Recently, base editing of the *SMN2* gene has been explored as a method to permanently increase production of full-length SMN protein^{346,347}. As the C-to-T transition in *SMN2* relative to *SMN1* is responsible for the exclusion of exon 7 in the majority of *SMN2* pre-mRNA transcripts^{13,14}, using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR)-based adenine base editing to change the T at position 840 of *SMN2* to a C can make the gene function more similarly to *SMN1*. This base editing results in the inclusion of exon 7 in the majority of pre-mRNA transcripts, giving rise to production of more full-length SMN protein^{346,347}. At present, many of the strategies employed to carry out

base editing are packaged in dual AAV vectors, as the relatively small packaging capacity of AAV (~4.5 kb) can prevent the entire system from being incorporated in one AAV vector³⁴⁶⁻³⁴⁸. Interestingly, Ad vectors have gained attention as vehicles for prime and base editors due to their large packaging capacity^{319,320,349}. In fact, the capacity of HDAd vectors is so large that it is possible to generate a vector that includes a cassette encoding *SMN1* cDNA and another encoding an adenine base editor to carry out a T-840-C transition in *SMN2*. Thus, these two methods incorporated in a single vector would each increase production of full-length SMN protein in the liver. The *SMN1* cDNA would rapidly give rise to high levels of SMN protein synthesis that would be sustained for many years, and the base editing of *SMN2* would permanently increase the production of full-length SMN protein. Thus, there are numerous strategies that can be used to increase production of SMN protein in the liver, ultimately leading to the release of EVs loaded with this SMN protein that are distributed throughout the body.

Indeed, there are many methods that can be used to increase SMN protein levels in the liver. It is noteworthy that the current disease-modifying therapies available for SMA, nusinersen, risdiplam, and OA, have all been shown to be present in the liver, increasing SMN protein levels in this tissue^{153,180,350}. For example, IV administration of OA in patients with SMA led to very high uptake of the vector by the liver (~300 vector genomes per hepatocyte), which led to very high levels of *SMN* mRNA transcripts relative to GAPDH in liver tissue¹⁸⁰, and these high levels of mRNA would almost certainly be accompanied by a massive increase in level of SMN protein produced in the liver. Therefore, as these therapeutics increase

SMN protein levels in the liver, and the liver releases EVs into circulation, it is probable that SMN protein is being distributed to peripheral tissues in a process mediated by liver-derived EVs. The SMN protein delivered to tissues by EVs could be responsible for at least a portion of the therapeutic effect of these treatments. Thus, the need for direct, high-level transduction of the CNS may not be as high as currently believed. This could be tested empirically by administering AAV-SMN IV to a mouse model of SMA and comparing the therapeutic effect to a treatment group receiving AAV-SMN that cannot be expressed in hepatocytes. Transgene expression can be dramatically reduced in hepatocytes through the inclusion of miR-122 binding sites in the 3' UTR of a transgene, which has been demonstrated in an AAV vector carrying a codon-optimized *SMN* transgene administered systemically in a severe mouse model of SMA¹⁸⁵. It would be interesting to compare the therapeutic efficacy of AAV-SMN with and without miR-122 binding sites to further elucidate the benefit of SMN protein produced in the liver in a mouse model of SMA. In order for this approach to be viable, the reduction of AAV-mediated *SMN* expression in the liver would need to be below the level required for rescue as observed in the Sutton et al. study¹¹⁵. High levels of hepatocyte transduction are an off-target effect of OA due to the natural biodistribution of AAV¹⁹⁴, however it is likely that liver transduction with the vector is beneficial. Overall, it is clear that the therapies currently available to treat SMA increase SMN protein levels in the liver, however exactly how beneficial this effect is in patients remains unknown.

The work contained within this thesis demonstrates the ability of the liver to be engineered to release extracellular vesicles that distribute a therapeutic protein

throughout the body and central nervous system of mice. Here, SMN was used as the therapeutic protein of interest, however this approach could be used with a variety of other therapeutic proteins for treatment of other diseases, such as Duchenne muscular dystrophy (DMD). DMD is an X-linked recessive disorder caused by mutations in the *DMD* gene that prevent production of the muscle isoform of dystrophin³⁵¹. Dystrophin connects the cytoskeleton of the muscle fibre to the extracellular matrix, and without this vital protein, patients experience severe, progressive muscle wasting, followed by death typically between 20 and 40 years of age from cardiac and/or respiratory failure^{351,352}. Therapeutic development for DMD has been complicated by the fact that the *DMD* gene is the largest known gene in the human genome, and thus the complete gene cannot be packaged into AAV vectors^{353,354}. Micro-and mini-dystrophin genes have been explored, which encode truncated forms of dystrophin protein that function similarly to full-length dystrophin³⁵⁵⁻³⁵⁷. As the NlucSMN protein produced in the liver distributed well to the skeletal muscle (Figure 3.5, 3.6), and Ad vectors have a high packaging capacity, Ad-mediated overexpression of dystrophin in the liver to give rise to systemically distributed dystrophin could deliver this protein to skeletal muscle, and may be a fitting therapeutic strategy to treat DMD.

The novel system of *in situ* production and distribution of therapeutic liver-derived proteins presented in this thesis could be used to deliver therapeutic proteins systemically to treat a countless number of diseases. This approach is applicable to any protein that is naturally loaded into EVs, however, there are several methods that can be used to achieve or enhance loading of target protein cargo, as

described in René and Parks²⁷⁵. Not only can therapeutic proteins be distributed systemically in EVs, but it is also possible to systemically distribute small interfering RNA (siRNA) to decrease levels of detrimental proteins in peripheral tissues. EVs can efficiently be loaded with siRNAs through the incorporation of a pre-miR-451 backbone³⁵⁸. Systemically distributed siRNAs encapsulated in liver-derived EVs have the potential to treat disorders such as Huntington's disease³⁵⁹, Parkinson's disease²²⁵, and *superoxide dismutase 1 (SOD1)*-associated ALS³⁵⁸. Overall, *in situ* production of liver-derived EVs loaded with therapeutic cargo that are systemically distributed to peripheral tissues and the CNS has the potential to be harnessed as a therapeutic strategy for innumerable disease states.

4.3 Conclusions

The data presented within this thesis have demonstrated the ability of EVs to deliver SMN protein to cells in culture. This protein was internalized by recipient cells, localizing in the cytoplasm and nucleus in gem-like structures. The EV-delivered SMN protein was able to interact with endogenous protein partners, overall indicating the SMN protein functioned similarly to endogenous SMN protein, supporting the use of EVs as delivery vehicles of therapeutic SMN protein. It was also demonstrated that a gene therapy approach can be used to engineer the liver to release EVs loaded with SMN protein into circulation. These SMN protein-loaded EVs distributed to many peripheral tissues, including those of the CNS. This approach could be used to deliver numerous different therapeutic cargoes, and has the potential to be utilized as a therapeutic approach to treat SMA, as well as many diseases that require systemic delivery of therapeutic cargo.

General Introduction and Discussion References

References

1. Kolb SJ, Kissel JT. Spinal Muscular Atrophy. *Neurol Clin*. Nov 2015;33(4):831-46. doi:10.1016/j.ncl.2015.07.004
2. Finkel RS, McDermott MP, Kaufmann P, et al. Observational study of spinal muscular atrophy type I and implications for clinical trials. *Neurology*. Aug 2014;83(9):810-7. doi:10.1212/WNL.0000000000000741
3. Werdnig G. Zwei frühinfantile hereditäre Fälle von progressiver Muskelatrophie unter dem Bilde der Dystrophie, aber auf neurotischer Grundlage. *Archiv für Psychiatrie und Nervenkrankheiten*. 1891;22(2):437-480. doi:10.1007/BF01776636
4. Hoffmann J. Ueber chronische spinale Muskelatrophie im Kindesalter, auf familiärer Basis. *Deutsche Zeitschrift für Nervenheilkunde*. 1893;3(6):427-470. doi:10.1007/BF01668496
5. Lefebvre S, Bürglen L, Reboullet S, et al. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell*. Jan 1995;80(1):155-65. doi:10.1016/0092-8674(95)90460-3
6. Sugarman EA, Nagan N, Zhu H, et al. Pan-ethnic carrier screening and prenatal diagnosis for spinal muscular atrophy: clinical laboratory analysis of >72,400 specimens. *Eur J Hum Genet*. Jan 2012;20(1):27-32. doi:10.1038/ejhg.2011.134
7. Belter L, Taylor JL, Jorgensen E, et al. Newborn Screening and Birth Prevalence for Spinal Muscular Atrophy in the US. *JAMA Pediatr*. Sep 01 2024;178(9):946-949. doi:10.1001/jamapediatrics.2024.1911
8. AveXis. AveXis receives FDA approval for Zolgensma®, the first and only gene therapy for pediatric patients with spinal muscular atrophy (SMA). Accessed October 11 2022, 2022. <https://www.novartis.com/news/media-releases/avexis-receives-fda-approval-zolgensma-first-and-only-gene-therapy-pediatric-patients-spinal-muscular-atrophy-sma>
9. Ottesen EW. ISS-N1 makes the First FDA-approved Drug for Spinal Muscular Atrophy. *Transl Neurosci*. Jan 2017;8:1-6. doi:10.1515/tnsci-2017-0001
10. Dhillon S. Risdiplam: First Approval. *Drugs*. Nov 2020;80(17):1853-1858. doi:10.1007/s40265-020-01410-z
11. Verhaart IEC, Robertson A, Wilson IJ, et al. Prevalence, incidence and carrier frequency of 5q-linked spinal muscular atrophy - a literature review. *Orphanet J Rare Dis*. Jul 04 2017;12(1):124. doi:10.1186/s13023-017-0671-8
12. Bürglen L, Lefebvre S, Clermont O, et al. Structure and organization of the human survival motor neurone (SMN) gene. *Genomics*. Mar 15 1996;32(3):479-82. doi:10.1006/geno.1996.0147
13. Monani UR, Lorson CL, Parsons DW, et al. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Hum Mol Genet*. Jul 1999;8(7):1177-83. doi:10.1093/hmg/8.7.1177
14. Lorson CL, Hahnen E, Androphy EJ, Wirth B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc Natl Acad Sci U S A*. May 1999;96(11):6307-11. doi:10.1073/pnas.96.11.6307

15. Vitte J, Fassier C, Tiziano FD, et al. Refined characterization of the expression and stability of the SMN gene products. *Am J Pathol.* Oct 2007;171(4):1269-80. doi:10.2353/ajpath.2007.070399
16. Burnett BG, Muñoz E, Tandon A, Kwon DY, Sumner CJ, Fischbeck KH. Regulation of SMN protein stability. *Mol Cell Biol.* Mar 2009;29(5):1107-15. doi:10.1128/MCB.01262-08
17. Wadman RI, Stam M, Jansen MD, et al. A Comparative Study of SMN Protein and mRNA in Blood and Fibroblasts in Patients with Spinal Muscular Atrophy and Healthy Controls. *PLoS One.* 2016;11(11):e0167087. doi:10.1371/journal.pone.0167087
18. Wadman RI, Jansen MD, Stam M, et al. Intragenic and structural variation in the SMN locus and clinical variability in spinal muscular atrophy. *Brain Commun.* 2020;2(2):fcaa075. doi:10.1093/braincomms/fcaa075
19. Feldkötter M, Schwarzer V, Wirth R, Wienker TF, Wirth B. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am J Hum Genet.* Feb 2002;70(2):358-68. doi:10.1086/338627
20. Pagliardini S, Giavazzi A, Setola V, et al. Subcellular localization and axonal transport of the survival motor neuron (SMN) protein in the developing rat spinal cord. *Hum Mol Genet.* Jan 2000;9(1):47-56. doi:10.1093/hmg/9.1.47
21. Coover DD, Le TT, McAndrew PE, et al. The survival motor neuron protein in spinal muscular atrophy. *Hum Mol Genet.* Aug 1997;6(8):1205-14. doi:10.1093/hmg/6.8.1205
22. Liu Q, Dreyfuss G. A novel nuclear structure containing the survival of motor neurons protein. *EMBO J.* Jul 15 1996;15(14):3555-65.
23. Fakan S, Leser G, Martin TE. Ultrastructural distribution of nuclear ribonucleoproteins as visualized by immunocytochemistry on thin sections. *J Cell Biol.* Jan 1984;98(1):358-63. doi:10.1083/jcb.98.1.358
24. Raska I, Andrade LE, Ochs RL, et al. Immunological and ultrastructural studies of the nuclear coiled body with autoimmune antibodies. *Exp Cell Res.* Jul 1991;195(1):27-37. doi:10.1016/0014-4827(91)90496-h
25. Ebert AD, Yu J, Rose FF, et al. Induced pluripotent stem cells from a spinal muscular atrophy patient. *Nature.* Jan 15 2009;457(7227):277-80. doi:10.1038/nature07677
26. Girard C, Neel H, Bertrand E, Bordonné R. Depletion of SMN by RNA interference in HeLa cells induces defects in Cajal body formation. *Nucleic Acids Res.* 2006;34(10):2925-32. doi:10.1093/nar/gkl374
27. Feng W, Gubitz AK, Wan L, et al. Gemins modulate the expression and activity of the SMN complex. *Hum Mol Genet.* Jun 15 2005;14(12):1605-11. doi:10.1093/hmg/ddi168
28. Hua Y, Vickers TA, Baker BF, Bennett CF, Krainer AR. Enhancement of SMN2 exon 7 inclusion by antisense oligonucleotides targeting the exon. *PLoS Biol.* Apr 2007;5(4):e73. doi:10.1371/journal.pbio.0050073
29. DiDonato CJ, Parks RJ, Kothary R. Development of a gene therapy strategy for the restoration of survival motor neuron protein expression: implications for

- spinal muscular atrophy therapy. *Hum Gene Ther.* Jan 20 2003;14(2):179-88. doi:10.1089/104303403321070874
30. Li DK, Tisdale S, Lotti F, Pellizzoni L. SMN control of RNP assembly: from post-transcriptional gene regulation to motor neuron disease. *Semin Cell Dev Biol.* Aug 2014;32:22-9. doi:10.1016/j.semcdb.2014.04.026
 31. Liu Q, Fischer U, Wang F, Dreyfuss G. The spinal muscular atrophy disease gene product, SMN, and its associated protein SIP1 are in a complex with spliceosomal snRNP proteins. *Cell.* Sep 1997;90(6):1013-21. doi:10.1016/s0092-8674(00)80367-0
 32. Fischer U, Liu Q, Dreyfuss G. The SMN-SIP1 complex has an essential role in spliceosomal snRNP biogenesis. *Cell.* Sep 1997;90(6):1023-9. doi:10.1016/s0092-8674(00)80368-2
 33. Battle DJ, Kasim M, Wang J, Dreyfuss G. SMN-independent subunits of the SMN complex. Identification of a small nuclear ribonucleoprotein assembly intermediate. *J Biol Chem.* Sep 2007;282(38):27953-9. doi:10.1074/jbc.M702317200
 34. Carissimi C, Saieva L, Gabanella F, Pellizzoni L. Gemin8 is required for the architecture and function of the survival motor neuron complex. *J Biol Chem.* Dec 2006;281(48):37009-16. doi:10.1074/jbc.M607505200
 35. Pellizzoni L, Yong J, Dreyfuss G. Essential role for the SMN complex in the specificity of snRNP assembly. *Science.* Nov 29 2002;298(5599):1775-9. doi:10.1126/science.1074962
 36. Meister G, Bühler D, Pillai R, Lottspeich F, Fischer U. A multiprotein complex mediates the ATP-dependent assembly of spliceosomal U snRNPs. *Nat Cell Biol.* Nov 2001;3(11):945-9. doi:10.1038/ncb1101-945
 37. Pellizzoni L, Baccon J, Charroux B, Dreyfuss G. The survival of motor neurons (SMN) protein interacts with the snoRNP proteins fibrillarin and GAR1. *Curr Biol.* Jul 2001;11(14):1079-88. doi:10.1016/s0960-9822(01)00316-5
 38. Renvoisé B, Colasse S, Burlet P, Viollet L, Meier UT, Lefebvre S. The loss of the snoRNP chaperone Nopp140 from Cajal bodies of patient fibroblasts correlates with the severity of spinal muscular atrophy. *Hum Mol Genet.* Apr 2009;18(7):1181-9. doi:10.1093/hmg/ddp009
 39. Bachand F, Boisvert FM, Côté J, Richard S, Autexier C. The product of the survival of motor neuron (SMN) gene is a human telomerase-associated protein. *Mol Biol Cell.* Sep 2002;13(9):3192-202. doi:10.1091/mbc.e02-04-0216
 40. Poole AR, Hebert MD. SMN and coilin negatively regulate dyskerin association with telomerase RNA. *Biol Open.* Jun 2016;5(6):726-35. doi:10.1242/bio.018804
 41. Blackburn EH, Collins K. Telomerase: an RNP enzyme synthesizes DNA. *Cold Spring Harb Perspect Biol.* May 2011;3(5)doi:10.1101/cshperspect.a003558
 42. Rossoll W, Jablonka S, Andreassi C, et al. Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. *J Cell Biol.* Nov 2003;163(4):801-12. doi:10.1083/jcb.200304128

43. Fallini C, Zhang H, Su Y, et al. The survival of motor neuron (SMN) protein interacts with the mRNA-binding protein HuD and regulates localization of poly(A) mRNA in primary motor neuron axons. *J Neurosci*. Mar 09 2011;31(10):3914-25. doi:10.1523/JNEUROSCI.3631-10.2011
44. Sanchez G, Dury AY, Murray LM, et al. A novel function for the survival motoneuron protein as a translational regulator. *Hum Mol Genet*. Feb 2013;22(4):668-84. doi:10.1093/hmg/ddu474
45. Kye MJ, Niederst ED, Wertz MH, et al. SMN regulates axonal local translation via miR-183/mTOR pathway. *Hum Mol Genet*. Dec 01 2014;23(23):6318-31. doi:10.1093/hmg/ddu350
46. Zhao DY, Gish G, Braunschweig U, et al. SMN and symmetric arginine dimethylation of RNA polymerase II C-terminal domain control termination. *Nature*. Jan 2016;529(7584):48-53. doi:10.1038/nature16469
47. Bowerman M, Anderson CL, Beauvais A, Boyl PP, Witke W, Kothary R. SMN, profilin IIa and plastin 3: a link between the deregulation of actin dynamics and SMA pathogenesis. *Mol Cell Neurosci*. Sep 2009;42(1):66-74. doi:10.1016/j.mcn.2009.05.009
48. Bowerman M, Beauvais A, Anderson CL, Kothary R. Rho-kinase inactivation prolongs survival of an intermediate SMA mouse model. *Hum Mol Genet*. Apr 2010;19(8):1468-78. doi:10.1093/hmg/ddq021
49. Dimitriadi M, Derdowski A, Kalloo G, et al. Decreased function of survival motor neuron protein impairs endocytic pathways. *Proc Natl Acad Sci U S A*. Jul 26 2016;113(30):E4377-86. doi:10.1073/pnas.1600015113
50. Riessland M, Kaczmarek A, Schneider S, et al. Neurocalcin Delta Suppression Protects against Spinal Muscular Atrophy in Humans and across Species by Restoring Impaired Endocytosis. *Am J Hum Genet*. Feb 02 2017;100(2):297-315. doi:10.1016/j.ajhg.2017.01.005
51. Walsh MB, Janzen E, Wingrove E, et al. Genetic modifiers ameliorate endocytic and neuromuscular defects in a model of spinal muscular atrophy. *BMC Biol*. Sep 16 2020;18(1):127. doi:10.1186/s12915-020-00845-w
52. Xu CC, Denton KR, Wang ZB, Zhang X, Li XJ. Abnormal mitochondrial transport and morphology as early pathological changes in human models of spinal muscular atrophy. *Dis Model Mech*. Jan 2016;9(1):39-49. doi:10.1242/dmm.021766
53. Miller N, Shi H, Zelikovich AS, Ma YC. Motor neuron mitochondrial dysfunction in spinal muscular atrophy. *Hum Mol Genet*. Aug 15 2016;25(16):3395-3406. doi:10.1093/hmg/ddw262
54. Chemello F, Pozzobon M, Tsansizi LI, et al. Dysfunctional mitochondria accumulate in a skeletal muscle knockout model of Smn1, the causal gene of spinal muscular atrophy. *Cell Death Dis*. Feb 27 2023;14(2):162. doi:10.1038/s41419-023-05573-x
55. Zhang R, So BR, Li P, et al. Structure of a key intermediate of the SMN complex reveals Gemin2's crucial function in snRNP assembly. *Cell*. Aug 05 2011;146(3):384-95. doi:10.1016/j.cell.2011.06.043

56. Sarachan KL, Valentine KG, Gupta K, et al. Solution structure of the core SMN-Gemin2 complex. *Biochem J*. Aug 01 2012;445(3):361-70. doi:10.1042/BJ20120241
57. Young PJ, Day PM, Zhou J, Androphy EJ, Morris GE, Lorson CL. A direct interaction between the survival motor neuron protein and p53 and its relationship to spinal muscular atrophy. *J Biol Chem*. Jan 25 2002;277(4):2852-9. doi:10.1074/jbc.M108769200
58. Custer SK, Todd AG, Singh NN, Androphy EJ. Dilycine motifs in exon 2b of SMN protein mediate binding to the COPI vesicle protein α -COP and neurite outgrowth in a cell culture model of spinal muscular atrophy. *Hum Mol Genet*. Oct 15 2013;22(20):4043-52. doi:10.1093/hmg/ddt254
59. Côté J, Richard S. Tudor domains bind symmetrical dimethylated arginines. *J Biol Chem*. Aug 05 2005;280(31):28476-83. doi:10.1074/jbc.M414328200
60. Tripsianes K, Madl T, Machyna M, et al. Structural basis for dimethylarginine recognition by the Tudor domains of human SMN and SPF30 proteins. *Nat Struct Mol Biol*. Nov 20 2011;18(12):1414-20. doi:10.1038/nsmb.2185
61. Hebert MD, Szymczyk PW, Shpargel KB, Matera AG. Coilin forms the bridge between Cajal bodies and SMN, the spinal muscular atrophy protein. *Genes Dev*. Oct 15 2001;15(20):2720-9. doi:10.1101/gad.908401
62. Sabra M, Texier P, El Maalouf J, Lomonte P. The Tudor protein survival motor neuron (SMN) is a chromatin-binding protein that interacts with methylated lysine 79 of histone H3. *J Cell Sci*. Aug 15 2013;126(Pt 16):3664-77. doi:10.1242/jcs.126003
63. Yamazaki T, Chen S, Yu Y, et al. FUS-SMN protein interactions link the motor neuron diseases ALS and SMA. *Cell Rep*. Oct 25 2012;2(4):799-806. doi:10.1016/j.celrep.2012.08.025
64. Young PJ, Francis JW, Lince D, Coon K, Androphy EJ, Lorson CL. The Ewing's sarcoma protein interacts with the Tudor domain of the survival motor neuron protein. *Brain Res Mol Brain Res*. Nov 06 2003;119(1):37-49. doi:10.1016/j.molbrainres.2003.08.011
65. Bühler D, Raker V, Lührmann R, Fischer U. Essential role for the tudor domain of SMN in spliceosomal U snRNP assembly: implications for spinal muscular atrophy. *Hum Mol Genet*. Dec 1999;8(13):2351-7. doi:10.1093/hmg/8.13.2351
66. Giesemann T, Rathke-Hartlieb S, Rothkegel M, et al. A role for polyproline motifs in the spinal muscular atrophy protein SMN. Profilins bind to and colocalize with smn in nuclear gems. *J Biol Chem*. Dec 31 1999;274(53):37908-14. doi:10.1074/jbc.274.53.37908
67. Lorson CL, Strasswimmer J, Yao JM, et al. SMN oligomerization defect correlates with spinal muscular atrophy severity. *Nat Genet*. May 1998;19(1):63-6. doi:10.1038/ng0598-63
68. Martin R, Gupta K, Ninan NS, Perry K, Van Duyne GD. The survival motor neuron protein forms soluble glycine zipper oligomers. *Structure*. Nov 07 2012;20(11):1929-39. doi:10.1016/j.str.2012.08.024
69. Cho S, Dreyfuss G. A degron created by SMN2 exon 7 skipping is a principal contributor to spinal muscular atrophy severity. *Genes Dev*. Mar 01 2010;24(5):438-42. doi:10.1101/gad.1884910

70. Narayanan U, Achsel T, Lührmann R, Matera AG. Coupled in vitro import of U snRNPs and SMN, the spinal muscular atrophy protein. *Mol Cell*. Oct 22 2004;16(2):223-34. doi:10.1016/j.molcel.2004.09.024
71. Charroux B, Pellizzoni L, Parkinson RA, Shevchenko A, Mann M, Dreyfuss G. Gemin3: A novel DEAD box protein that interacts with SMN, the spinal muscular atrophy gene product, and is a component of gems. *J Cell Biol*. Dec 13 1999;147(6):1181-94. doi:10.1083/jcb.147.6.1181
72. Gangwani L, Mikrut M, Theroux S, Sharma M, Davis RJ. Spinal muscular atrophy disrupts the interaction of ZPR1 with the SMN protein. *Nat Cell Biol*. Apr 2001;3(4):376-83. doi:10.1038/35070059
73. Zou J, Barahmand-pour F, Blackburn ML, Matsui Y, Chansky HA, Yang L. Survival motor neuron (SMN) protein interacts with transcription corepressor mSin3A. *J Biol Chem*. Apr 09 2004;279(15):14922-8. doi:10.1074/jbc.M309218200
74. Mouaikel J, Narayanan U, Verheggen C, et al. Interaction between the small-nuclear-RNA cap hypermethylase and the spinal muscular atrophy protein, survival of motor neuron. *EMBO Rep*. Jun 2003;4(6):616-22. doi:10.1038/sj.embor.embor863
75. Rademacher S, Detering NT, Schüning T, et al. A Single Amino Acid Residue Regulates PTEN-Binding and Stability of the Spinal Muscular Atrophy Protein SMN. *Cells*. Nov 03 2020;9(11)doi:10.3390/cells9112405
76. Zhang H, Xing L, Singer RH, Bassell GJ. QNQKE targeting motif for the SMN-Gemin multiprotein complex in neurons. *J Neurosci Res*. Sep 2007;85(12):2657-67. doi:10.1002/jnr.21308
77. Pellizzoni L, Charroux B, Dreyfuss G. SMN mutants of spinal muscular atrophy patients are defective in binding to snRNP proteins. *Proc Natl Acad Sci U S A*. Sep 28 1999;96(20):11167-72. doi:10.1073/pnas.96.20.11167
78. Le TT, Pham LT, Butchbach ME, et al. SMNDelta7, the major product of the centromeric survival motor neuron (SMN2) gene, extends survival in mice with spinal muscular atrophy and associates with full-length SMN. *Hum Mol Genet*. Mar 15 2005;14(6):845-57. doi:10.1093/hmg/ddi078
79. Han KJ, Foster DG, Zhang NY, et al. Ubiquitin-specific protease 9x deubiquitinates and stabilizes the spinal muscular atrophy protein-survival motor neuron. *J Biol Chem*. Dec 2012;287(52):43741-52. doi:10.1074/jbc.M112.372318
80. Prior TW, Snyder PJ, Rink BD, et al. Newborn and carrier screening for spinal muscular atrophy. *Am J Med Genet A*. Jul 2010;152A(7):1608-16. doi:10.1002/ajmg.a.33474
81. Zerres K, Davies KE. 59th ENMC International Workshop: Spinal Muscular Atrophies: recent progress and revised diagnostic criteria 17-19 April 1998, Soestduinen, The Netherlands. *Neuromuscul Disord*. Jun 1999;9(4):272-8. doi:10.1016/s0960-8966(99)00016-4
82. Dubowitz V. Very severe spinal muscular atrophy (SMA type 0): an expanding clinical phenotype. *Eur J Paediatr Neurol*. 1999;3(2):49-51. doi:10.1053/ejpn.1999.0181

83. MacLeod MJ, Taylor JE, Lunt PW, Mathew CG, Robb SA. Prenatal onset spinal muscular atrophy. *Eur J Paediatr Neurol.* 1999;3(2):65-72. doi:10.1053/ejpn.1999.0184
84. Thomas NH, Dubowitz V. The natural history of type I (severe) spinal muscular atrophy. *Neuromuscul Disord.* 1994 Sep-Nov 1994;4(5-6):497-502. doi:10.1016/0960-8966(94)90090-6
85. Zerres K, Rudnik-Schöneborn S. Natural history in proximal spinal muscular atrophy. Clinical analysis of 445 patients and suggestions for a modification of existing classifications. *Arch Neurol.* May 1995;52(5):518-23. doi:10.1001/archneur.1995.00540290108025
86. Wadman RI, Stam M, Gijzen M, et al. Association of motor milestones, SMN2 copy and outcome in spinal muscular atrophy types 0-4. *J Neurol Neurosurg Psychiatry.* Apr 2017;88(4):365-367. doi:10.1136/jnnp-2016-314292
87. Piepers S, van den Berg LH, Brugman F, et al. A natural history study of late onset spinal muscular atrophy types 3b and 4. *J Neurol.* Sep 2008;255(9):1400-4. doi:10.1007/s00415-008-0929-0
88. Kölbel H, Müller-Felber W. Spinale muskelatrophie (SMA), diagnostik und therapie AWMF online 2020. AWMF. Accessed October 28 2024, https://register.awmf.org/assets/guidelines/022-030I_S1_Spinale-Muskelatrophie-SMA-Diagnostik-Therapie_2021-07_1.pdf
89. Tizzano EF, Finkel RS. Spinal muscular atrophy: A changing phenotype beyond the clinical trials. *Neuromuscul Disord.* Oct 2017;27(10):883-889. doi:10.1016/j.nmd.2017.05.011
90. Lunn MR, Wang CH. Spinal muscular atrophy. *Lancet.* Jun 21 2008;371(9630):2120-33. doi:10.1016/S0140-6736(08)60921-6
91. Stifani N. Motor neurons and the generation of spinal motor neuron diversity. *Front Cell Neurosci.* 2014;8:293. doi:10.3389/fncel.2014.00293
92. Sumner CJ. Molecular mechanisms of spinal muscular atrophy. *J Child Neurol.* Aug 2007;22(8):979-89. doi:10.1177/0883073807305787
93. Fauroux B, Griffon L, Amaddeo A, et al. Respiratory management of children with spinal muscular atrophy (SMA). *Arch Pediatr.* Dec 2020;27(7S):7S29-7S34. doi:10.1016/S0929-693X(20)30274-8
94. Buchthal F, Olsen PZ. Electromyography and muscle biopsy in infantile spinal muscular atrophy. *Brain.* 1970;93(1):15-30. doi:10.1093/brain/93.1.15
95. Mastaglia FL, Walton JN. Histological and histochemical changes in skeletal muscle from cases of chronic juvenile and early adult spinal muscular atrophy (the Kugelberg-Welander syndrome). *J Neurol Sci.* Jan 1971;12(1):15-44. doi:10.1016/0022-510x(71)90249-8
96. Madakshira MG, Singla S, Gupta K, Zahan S, Paria P, Sahu JK. Autopsy of a child with Spinal muscular atrophy Type I (Werdnig-Hoffmann disease). *Autops Case Rep.* Apr 23 2020;10(2):e2020157. doi:10.4322/acr.2020.157
97. Bromberg MB, Swoboda KJ. Motor unit number estimation in infants and children with spinal muscular atrophy. *Muscle Nerve.* Mar 2002;25(3):445-7. doi:10.1002/mus.10050
98. Schneider C, Wassermann MK, Grether NB, Fink GR, Wunderlich G, Lehmann HC. Motor unit number estimation in adult patients with spinal muscular

- atrophy treated with nusinersen. *Eur J Neurol*. Sep 2021;28(9):3022-3029. doi:10.1111/ene.15005
99. Yeo, C. J. J. & Darras, B. T. Overturning the Paradigm of Spinal Muscular Atrophy as Just a Motor Neuron Disease. *Pediatr Neurol* **109**, 12-19, doi:10.1016/j.pediatrneurol.2020.01.003 (2020).
 100. Arnold WD, Severyn S, Zhao S, et al. Persistent neuromuscular junction transmission defects in adults with spinal muscular atrophy treated with nusinersen. *BMJ Neurol Open*. 2021;3(2):e000164. doi:10.1136/bmjno-2021-000164
 101. Wadman RI, Vrancken AF, van den Berg LH, van der Pol WL. Dysfunction of the neuromuscular junction in spinal muscular atrophy types 2 and 3. *Neurology*. Nov 13 2012;79(20):2050-5. doi:10.1212/WNL.0b013e3182749eca
 102. Pera MC, Luigetti M, Pane M, et al. 6MWT can identify type 3 SMA patients with neuromuscular junction dysfunction. *Neuromuscul Disord*. Oct 2017;27(10):879-882. doi:10.1016/j.nmd.2017.07.007
 103. Mendonça RH, Rocha AJ, Lozano-Arango A, et al. Severe brain involvement in 5q spinal muscular atrophy type 0. *Ann Neurol*. Sep 2019;86(3):458-462. doi:10.1002/ana.25549
 104. Wishart TM, Huang JP, Murray LM, et al. SMN deficiency disrupts brain development in a mouse model of severe spinal muscular atrophy. *Hum Mol Genet*. Nov 01 2010;19(21):4216-28. doi:10.1093/hmg/ddq340
 105. Groulx-Boivin E, Oliveira-Carneiro A, Carlson H, et al. Macrostructural Brain Abnormalities in Spinal Muscular Atrophy: A Case-Control Study. *Neurol Genet*. Oct 2024;10(5):e200193. doi:10.1212/NXG.0000000000200193
 106. Shen W, Yan Z, Su S, et al. Gray and white matter abnormalities in children with type 2 and 3 SMA: A morphological assessment. *Eur J Pediatr*. Mar 2024;183(3):1381-1388. doi:10.1007/s00431-023-05397-z
 107. Bevan AK, Hutchinson KR, Foust KD, et al. Early heart failure in the SMNDelta7 model of spinal muscular atrophy and correction by postnatal scAAV9-SMN delivery. *Hum Mol Genet*. Oct 2010;19(20):3895-905. doi:10.1093/hmg/ddq300
 108. Shababi M, Habibi J, Yang HT, Vale SM, Sewell WA, Lorson CL. Cardiac defects contribute to the pathology of spinal muscular atrophy models. *Hum Mol Genet*. Oct 15 2010;19(20):4059-71. doi:10.1093/hmg/ddq329
 109. Heier CR, Satta R, Lutz C, DiDonato CJ. Arrhythmia and cardiac defects are a feature of spinal muscular atrophy model mice. *Hum Mol Genet*. Oct 15 2010;19(20):3906-18. doi:10.1093/hmg/ddq330
 110. Maxwell GK, Szunyogova E, Shorrock HK, Gillingwater TH, Parson SH. Developmental and degenerative cardiac defects in the Taiwanese mouse model of severe spinal muscular atrophy. *J Anat*. Jun 2018;232(6):965-978. doi:10.1111/joa.12793
 111. Rudnik-Schöneborn S, Heller R, Berg C, et al. Congenital heart disease is a feature of severe infantile spinal muscular atrophy. *J Med Genet*. Oct 2008;45(10):635-8. doi:10.1136/jmg.2008.057950

112. Deguise MO, Baranello G, Mastella C, et al. Abnormal fatty acid metabolism is a core component of spinal muscular atrophy. *Ann Clin Transl Neurol.* 08 2019;6(8):1519-1532. doi:10.1002/acn3.50855
113. Miletic M, Stevic Z, Vujovic S, et al. Glucose and Lipid Metabolism Disorders in Adults with Spinal Muscular Atrophy Type 3. *Diagnostics (Basel).* Sep 19 2024;14(18)doi:10.3390/diagnostics14182078
114. Leow DM, Ng YK, Wang LC, et al. Hepatocyte-intrinsic SMN deficiency drives metabolic dysfunction and liver steatosis in spinal muscular atrophy. *J Clin Invest.* May 09 2024;134(12)doi:10.1172/JCI173702
115. Sutton ER, Beauvais A, Yaworski R, et al. Liver Smn Restoration Rescues the Smn2b/- Mouse Model of Spinal Muscular Atrophy: The Key to Rescue Whole Body Pathology? *eBioMedicine.* 2024;doi:doi.org/10.2139/ssrn.4828743
116. Wijngaarde CA, Veldhoen ES, van Eijk RPA, et al. Natural history of lung function in spinal muscular atrophy. *Orphanet J Rare Dis.* Apr 10 2020;15(1):88. doi:10.1186/s13023-020-01367-y
117. Schreml J, Riessland M, Paterno M, et al. Severe SMA mice show organ impairment that cannot be rescued by therapy with the HDACi JNJ-26481585. *Eur J Hum Genet.* Jun 2013;21(6):643-52. doi:10.1038/ejhg.2012.222
118. Thomson AK, Somers E, Powis RA, et al. Survival of motor neurone protein is required for normal postnatal development of the spleen. *J Anat.* 02 2017;230(2):337-346. doi:10.1111/joa.12546
119. Deguise MO, De Repentigny Y, McFall E, Auclair N, Sad S, Kothary R. Immune dysregulation may contribute to disease pathogenesis in spinal muscular atrophy mice. *Hum Mol Genet.* 02 2017;26(4):801-819. doi:10.1093/hmg/ddw434
120. Khairallah MT, Astroski J, Custer SK, Androphy EJ, Franklin CL, Lorson CL. SMN deficiency negatively impacts red pulp macrophages and spleen development in mouse models of spinal muscular atrophy. *Hum Mol Genet.* 03 01 2017;26(5):932-941. doi:10.1093/hmg/ddx008
121. Nery FC, Siranosian JJ, Rosales I, et al. Impaired kidney structure and function in spinal muscular atrophy. *Neurol Genet.* Oct 2019;5(5):e353. doi:10.1212/NXG.0000000000000353
122. Gök A, Saygılı SK, Kuruğöçlü S, Saltık S, Canpolat N. Children With Type 1 Spinal Muscular Atrophy Are at Increased Risk for Nephrolithiasis. *Pediatr Neurol.* Jan 2024;150:32-36. doi:10.1016/j.pediatrneurol.2023.10.006
123. Allardyce H, Kuhn D, Hernandez-Gerez E, et al. Renal pathology in a mouse model of severe Spinal Muscular Atrophy is associated with downregulation of Glial Cell-Line Derived Neurotrophic Factor (GDNF). *Hum Mol Genet.* 08 11 2020;29(14):2365-2378. doi:10.1093/hmg/ddaa126
124. Bowerman M, Swoboda KJ, Michalski JP, et al. Glucose metabolism and pancreatic defects in spinal muscular atrophy. *Ann Neurol.* Aug 2012;72(2):256-68. doi:10.1002/ana.23582
125. Bowerman M, Michalski JP, Beauvais A, Murray LM, DeRepentigny Y, Kothary R. Defects in pancreatic development and glucose metabolism in SMN-depleted mice independent of canonical spinal muscular atrophy

- neuromuscular pathology. *Hum Mol Genet.* Jul 2014;23(13):3432-44. doi:10.1093/hmg/ddu052
126. Rakusa M, Koritnik B, Leonardis L, et al. The endocrine manifestations of adults with spinal muscular atrophy. *Muscle Nerve.* Oct 05 2024;doi:10.1002/mus.28275
 127. Davis RH, Miller EA, Zhang RZ, Swoboda KJ. Responses to Fasting and Glucose Loading in a Cohort of Well Children with Spinal Muscular Atrophy Type II. *J Pediatr.* Dec 2015;167(6):1362-8.e1. doi:10.1016/j.jpeds.2015.09.023
 128. Djordjevic SA, Milic-Rasic V, Brankovic V, et al. Glucose and lipid metabolism disorders in children and adolescents with spinal muscular atrophy types 2 and 3. *Neuromuscul Disord.* Apr 2021;31(4):291-299. doi:10.1016/j.nmd.2021.02.002
 129. Gombash SE, Cowley CJ, Fitzgerald JA, et al. SMN deficiency disrupts gastrointestinal and enteric nervous system function in mice. *Hum Mol Genet.* Oct 2015;24(19):5665. doi:10.1093/hmg/ddv292
 130. Sintusek P, Catapano F, Angkathunkayul N, et al. Histopathological Defects in Intestine in Severe Spinal Muscular Atrophy Mice Are Improved by Systemic Antisense Oligonucleotide Treatment. *PLoS One.* 2016;11(5):e0155032. doi:10.1371/journal.pone.0155032
 131. Yang Y, Vassilakos G, Hammers DW, Yang Z, Barton ER, Sweeney HL. Smooth muscle atrophy and colon pathology in SMN deficient mice. *Am J Transl Res.* 2019;11(3):1789-1799.
 132. Wang CH, Finkel RS, Bertini ES, et al. Consensus statement for standard of care in spinal muscular atrophy. *J Child Neurol.* Aug 2007;22(8):1027-49. doi:10.1177/0883073807305788
 133. Messina S, Pane M, De Rose P, et al. Feeding problems and malnutrition in spinal muscular atrophy type II. *Neuromuscul Disord.* May 2008;18(5):389-93. doi:10.1016/j.nmd.2008.02.008
 134. Shafey D, Côté PD, Kothary R. Hypomorphic Smn knockdown C2C12 myoblasts reveal intrinsic defects in myoblast fusion and myotube morphology. *Exp Cell Res.* Nov 15 2005;311(1):49-61. doi:10.1016/j.yexcr.2005.08.019
 135. McCormack NM, Villalón E, Viollet C, et al. Survival motor neuron deficiency slows myoblast fusion through reduced myomaker and myomixer expression. *J Cachexia Sarcopenia Muscle.* Aug 2021;12(4):1098-1116. doi:10.1002/jcsm.12740
 136. Kim JK, Jha NN, Feng Z, et al. Muscle-specific SMN reduction reveals motor neuron-independent disease in spinal muscular atrophy models. *J Clin Invest.* Mar 02 2020;130(3):1271-1287. doi:10.1172/JCI131989
 137. Boyer JG, Murray LM, Scott K, De Repentigny Y, Renaud JM, Kothary R. Early onset muscle weakness and disruption of muscle proteins in mouse models of spinal muscular atrophy. *Skelet Muscle.* Oct 11 2013;3(1):24. doi:10.1186/2044-5040-3-24
 138. Araujo A, Araujo M, Swoboda KJ. Vascular perfusion abnormalities in infants with spinal muscular atrophy. *J Pediatr.* Aug 2009;155(2):292-4. doi:10.1016/j.jpeds.2009.01.071

139. Rudnik-Schöneborn S, Vogelgesang S, Armbrust S, Graul-Neumann L, Fusch C, Zerres K. Digital necroses and vascular thrombosis in severe spinal muscular atrophy. *Muscle Nerve*. Jul 2010;42(1):144-7. doi:10.1002/mus.21654
140. Zhou H, Hong Y, Scoto M, et al. Microvasculopathy in spinal muscular atrophy is driven by a reversible autonomous endothelial cell defect. *J Clin Invest*. Nov 01 2022;132(21)doi:10.1172/JCI153430
141. Somers E, Lees RD, Hoban K, et al. Vascular Defects and Spinal Cord Hypoxia in Spinal Muscular Atrophy. *Ann Neurol*. Feb 2016;79(2):217-30. doi:10.1002/ana.24549
142. Hua Y, Sahashi K, Hung G, et al. Antisense correction of SMN2 splicing in the CNS rescues necrosis in a type III SMA mouse model. *Genes Dev*. Aug 01 2010;24(15):1634-44. doi:10.1101/gad.1941310
143. Foust KD, Wang X, McGovern VL, et al. Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. *Nat Biotechnol*. Mar 2010;28(3):271-4. doi:10.1038/nbt.1610
144. Chehade L, Deguise MO, De Repentigny Y, et al. Suppression of the necroptotic cell death pathways improves survival in. *Front Cell Neurosci*. 2022;16:972029. doi:10.3389/fncel.2022.972029
145. Cobb MS, Rose FF, Rindt H, et al. Development and characterization of an SMN2-based intermediate mouse model of Spinal Muscular Atrophy. *Hum Mol Genet*. May 01 2013;22(9):1843-55. doi:10.1093/hmg/ddt037
146. Hsieh-Li HM, Chang JG, Jong YJ, et al. A mouse model for spinal muscular atrophy. *Nat Genet*. Jan 2000;24(1):66-70. doi:10.1038/71709
147. Lipnick SL, Agniel DM, Aggarwal R, et al. Systemic nature of spinal muscular atrophy revealed by studying insurance claims. *PLoS One*. 2019;14(3):e0213680. doi:10.1371/journal.pone.0213680
148. Brener A, Lebenthal Y, Shtamler A, et al. The endocrine manifestations of spinal muscular atrophy, a real-life observational study. *Neuromuscul Disord*. Apr 2020;30(4):270-276. doi:10.1016/j.nmd.2020.02.011
149. Bach JR. Medical considerations of long-term survival of Werdnig-Hoffmann disease. *Am J Phys Med Rehabil*. May 2007;86(5):349-55. doi:10.1097/PHM.0b013e31804b1d66
150. Ottesen EW, Howell MD, Singh NN, Seo J, Whitley EM, Singh RN. Severe impairment of male reproductive organ development in a low SMN expressing mouse model of spinal muscular atrophy. *Sci Rep*. Feb 02 2016;6:20193. doi:10.1038/srep20193
151. Haché M, Swoboda KJ, Sethna N, et al. Intrathecal Injections in Children With Spinal Muscular Atrophy: Nusinersen Clinical Trial Experience. *J Child Neurol*. 06 2016;31(7):899-906. doi:10.1177/0883073815627882
152. Mendell JR, Al-Zaidy S, Shell R, et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N Engl J Med*. 11 2017;377(18):1713-1722. doi:10.1056/NEJMoa1706198
153. Poirier A, Weetall M, Heinig K, et al. Risdiplam distributes and increases SMN protein in both the central nervous system and peripheral organs. *Pharmacol Res Perspect*. 12 2018;6(6):e00447. doi:10.1002/prp2.447

154. Mendell JR, Al-Zaidy SA, Lehman KJ, et al. Five-Year Extension Results of the Phase 1 START Trial of Onasemnogene Apeparvovec in Spinal Muscular Atrophy. *JAMA Neurol.* 07 01 2021;78(7):834-841. doi:10.1001/jamaneurol.2021.1272
155. Novartis. Novartis shares Zolgensma long-term data demonstrating sustained durability up to 7.5 years post-dosing; 100% achievement of all assessed milestones in children treated prior to SMA symptom onset. Accessed March 25 2023, 2023. <https://www.novartis.com/news/media-releases/novartis-shares-zolgensma-long-term-data-demonstrating-sustained-durability-75-years-post-dosing-100-achievement-all-assessed-milestones-children-treated-prior-sma-symptom-onset>
156. Chiriboga CA, Swoboda KJ, Darras BT, et al. Results from a phase 1 study of nusinersen (ISIS-SMN(Rx)) in children with spinal muscular atrophy. *Neurology.* Mar 08 2016;86(10):890-7. doi:10.1212/WNL.0000000000002445
157. Finkel RS, Mercuri E, Darras BT, et al. Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. *N Engl J Med.* 11 02 2017;377(18):1723-1732. doi:10.1056/NEJMoa1702752
158. Singh NK, Singh NN, Androphy EJ, Singh RN. Splicing of a critical exon of human Survival Motor Neuron is regulated by a unique silencer element located in the last intron. *Mol Cell Biol.* Feb 2006;26(4):1333-46. doi:10.1128/MCB.26.4.1333-1346.2006
159. Passini MA, Bu J, Richards AM, et al. Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci Transl Med.* Mar 02 2011;3(72):72ra18. doi:10.1126/scitranslmed.3001777
160. Porensky PN, Mitropant C, McGovern VL, et al. A single administration of morpholino antisense oligomer rescues spinal muscular atrophy in mouse. *Hum Mol Genet.* Apr 01 2012;21(7):1625-38. doi:10.1093/hmg/ddr600
161. Zhou H, Janghra N, Mitropant C, et al. A novel morpholino oligomer targeting ISS-N1 improves rescue of severe spinal muscular atrophy transgenic mice. *Hum Gene Ther.* Mar 2013;24(3):331-42. doi:10.1089/hum.2012.211
162. Biogen. Spinraza prescribing information. Accessed October 20 2022, 2022. https://www.spinraza.com/content/dam/commercial/spinraza/caregiver/en_us/pdf/spinraza-prescribing-information.pdf
163. Mercuri E, Darras BT, Chiriboga CA, et al. Nusinersen versus Sham Control in Later-Onset Spinal Muscular Atrophy. *N Engl J Med.* 02 15 2018;378(7):625-635. doi:10.1056/NEJMoa1710504
164. De Vivo DC, Bertini E, Swoboda KJ, et al. Nusinersen initiated in infants during the presymptomatic stage of spinal muscular atrophy: Interim efficacy and safety results from the Phase 2 NURTURE study. *Neuromuscul Disord.* 11 2019;29(11):842-856. doi:10.1016/j.nmd.2019.09.007
165. Jia C, Lei Mon SS, Yang Y, et al. Change of intracellular calcium level causes acute neurotoxicity by antisense oligonucleotides via CSF route. *Mol Ther Nucleic Acids.* Mar 14 2023;31:182-196. doi:10.1016/j.omtn.2022.12.010
166. Pechmann A, Behrens M, Dörnbrack K, et al. Improvements in Walking Distance during Nusinersen Treatment - A Prospective 3-year SMARTCARE

- Registry Study. *J Neuromuscul Dis.* 2023;10(1):29-40. doi:10.3233/JND-221600
167. Arslan D, Inan B, Kilinc M, Bekircan-Kurt CE, Erdem-Ozdamar S, Tan E. Nusinersen for adults with spinal muscular atrophy. *Neurol Sci.* Mar 01 2023;doi:10.1007/s10072-023-06698-9
 168. Coratti G, Cutrona C, Pera MC, et al. Motor function in type 2 and 3 SMA patients treated with Nusinersen: a critical review and meta-analysis. *Orphanet J Rare Dis.* Oct 13 2021;16(1):430. doi:10.1186/s13023-021-02065-z
 169. Spinraza. Spinraza. Accessed October 24 2024, <https://www.spinraza.com/>
 170. Wire B. FDA Approves Genentech's Evrysdi (risdiplam) for Use in Babies Under Two Months With Spinal Muscular Atrophy (SMA). Accessed October 11 2022, 2022. <https://www.businesswire.com/news/home/20220530005033/en/FDA-Approves-Genentech>
 171. Ratni H, Ebeling M, Baird J, et al. Discovery of Risdiplam, a Selective Survival of Motor Neuron-2 (SMN2) Gene Splicing Modifier for the Treatment of Spinal Muscular Atrophy (SMA). *J Med Chem.* Aug 2018;61(15):6501-6517. doi:10.1021/acs.jmedchem.8b00741
 172. Campagne S, Boigner S, Rüdisser S, et al. Structural basis of a small molecule targeting RNA for a specific splicing correction. *Nat Chem Biol.* Dec 2019;15(12):1191-1198. doi:10.1038/s41589-019-0384-5
 173. Genentech. Evrysdi prescribing information. Accessed April 3 2023, 2023. https://www.gene.com/download/pdf/evrysdi_prescribing.pdf
 174. Masson R, Mazurkiewicz-Beldzińska M, Rose K, et al. Safety and efficacy of risdiplam in patients with type 1 spinal muscular atrophy (FIREFISH part 2): secondary analyses from an open-label trial. *Lancet Neurol.* Dec 2022;21(12):1110-1119. doi:10.1016/S1474-4422(22)00339-8
 175. Oskoui M, Day JW, Deconinck N, et al. Two-year efficacy and safety of risdiplam in patients with type 2 or non-ambulant type 3 spinal muscular atrophy (SMA). *J Neurol.* May 2023;270(5):2531-2546. doi:10.1007/s00415-023-11560-1
 176. Finkel RS, Farrar MA, Servais L, et al. RAINBOWFISH: Primary efficacy and safety data in risdipla-treated infants with presymptomatic spinal muscular atrophy SMA. 2023:
 177. O'Donnell L, Pratis K, Wagenfeld A, et al. Transcriptional profiling of the hormone-responsive stages of spermatogenesis reveals cell-, stage-, and hormone-specific events. *Endocrinology.* Nov 2009;150(11):5074-84. doi:10.1210/en.2009-0755
 178. Martin-du Pan RC, Campana A. Physiopathology of spermatogenic arrest. *Fertil Steril.* Dec 1993;60(6):937-46. doi:10.1016/s0015-0282(16)56388-2
 179. Mueller L, Barrow P, Jacobsen B, Ebeling M, Weinbauer G. Reproductive findings in male animals exposed to selective survival of motor neuron 2 (SMN2) gene splicing-modifying agents. *Reprod Toxicol.* Jun 2023;118:108360. doi:10.1016/j.reprotox.2023.108360

180. Thomsen G, Burghes AHM, Hsieh C, et al. Biodistribution of onasemnogene abeparvovec DNA, mRNA and SMN protein in human tissue. *Nat Med*. Oct 2021;27(10):1701-1711. doi:10.1038/s41591-021-01483-7
181. Reilly A, Yaworski R, Beauvais A, Schneider BL, Kothary R. Long term peripheral AAV9-SMN gene therapy promotes survival in a mouse model of spinal muscular atrophy. *Hum Mol Genet*. Feb 28 2024;33(6):510-519. doi:10.1093/hmg/ddad202
182. Foust KD, Nurre E, Montgomery CL, Hernandez A, Chan CM, Kaspar BK. Intravascular AAV9 preferentially targets neonatal neurons and adult astrocytes. *Nat Biotechnol*. Jan 2009;27(1):59-65. doi:10.1038/nbt.1515
183. Foust KD, Wang X, McGovern VL, et al. Retraction Note: Rescue of the spinal muscular atrophy phenotype in a mouse model by early postnatal delivery of SMN. *Nat Biotechnol*. Nov 2022;40(11):1692. doi:10.1038/s41587-022-01497-7
184. Reilly A, Deguise MO, Beauvais A, et al. Central and peripheral delivered AAV9-SMN are both efficient but target different pathomechanisms in a mouse model of spinal muscular atrophy. *Gene Ther*. Sep 2022;29(9):544-554. doi:10.1038/s41434-022-00338-1
185. Xie Q, Chen X, Ma H, et al. Improved gene therapy for spinal muscular atrophy in mice using codon-optimized hSMN1 transgene and hSMN1 gene-derived promotor. *EMBO Mol Med*. Apr 2024;16(4):945-965. doi:10.1038/s44321-024-00037-x
186. Al-Zaidy S, Pickard AS, Kotha K, et al. Health outcomes in spinal muscular atrophy type 1 following AVXS-101 gene replacement therapy. *Pediatr Pulmonol*. 02 2019;54(2):179-185. doi:10.1002/ppul.24203
187. Day JW, Finkel RS, Chiriboga CA, et al. Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy in patients with two copies of SMN2 (STR1VE): an open-label, single-arm, multicentre, phase 3 trial. *Lancet Neurol*. 04 2021;20(4):284-293. doi:10.1016/S1474-4422(21)00001-6
188. Mercuri E, Muntoni F, Baranello G, et al. Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy type 1 (STR1VE-EU): an open-label, single-arm, multicentre, phase 3 trial. *Lancet Neurol*. 10 2021;20(10):832-841. doi:10.1016/S1474-4422(21)00251-9
189. Strauss KA, Farrar MA, Muntoni F, et al. Onasemnogene abeparvovec for presymptomatic infants with two copies of SMN2 at risk for spinal muscular atrophy type 1: the Phase III SPR1NT trial. *Nat Med*. 07 2022;28(7):1381-1389. doi:10.1038/s41591-022-01866-4
190. Strauss KA, Farrar MA, Muntoni F, et al. Onasemnogene abeparvovec for presymptomatic infants with three copies of SMN2 at risk for spinal muscular atrophy: the Phase III SPR1NT trial. *Nat Med*. 07 2022;28(7):1390-1397. doi:10.1038/s41591-022-01867-3
191. Chand D, Mohr F, McMillan H, et al. Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy. *J Hepatol*. Mar 2021;74(3):560-566. doi:10.1016/j.jhep.2020.11.001

192. Chand DH, Mitchell S, Sun R, LaMarca N, Reyna SP, Sutter T. Safety of Onasemnogene Abeparvovec for Patients With Spinal Muscular Atrophy 8.5 kg or Heavier in a Global Managed Access Program. *Pediatr Neurol.* Jul 2022;132:27-32. doi:10.1016/j.pediatrneurol.2022.05.001
193. Finnegan R, Manzur A, Munot P, et al. Risk-benefit profile of onasemnogene abeparvovec in older and heavier children with spinal muscular atrophy type 1. *Neuromuscul Disord.* Sep 2024;42:22-26. doi:10.1016/j.nmd.2024.07.004
194. Hinderer C, Katz N, Buza EL, et al. Severe Toxicity in Nonhuman Primates and Piglets Following High-Dose Intravenous Administration of an Adeno-Associated Virus Vector Expressing Human SMN. *Hum Gene Ther.* 03 2018;29(3):285-298. doi:10.1089/hum.2018.015
195. Yazaki K, Sakuma S, Hikita N, Fujimaru R, Hamazaki T. Child Neurology: Pathologically Confirmed Thrombotic Microangiopathy Caused by Onasemnogene Abeparvovec Treatment for SMA. *Neurology.* 05 10 2022;98(19):808-813. doi:10.1212/WNL.0000000000200676
196. Nault JC, Datta S, Imbeaud S, et al. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat Genet.* Oct 2015;47(10):1187-93. doi:10.1038/ng.3389
197. Zhong L, Malani N, Li M, et al. Recombinant adeno-associated virus integration sites in murine liver after ornithine transcarbamylase gene correction. *Hum Gene Ther.* May 2013;24(5):520-5. doi:10.1089/hum.2012.112
198. Donsante A, Vogler C, Muzyczka N, et al. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene Ther.* Sep 2001;8(17):1343-6. doi:10.1038/sj.gt.3301541
199. Dalwadi DA, Torrens L, Abril-Fornaguera J, et al. Liver Injury Increases the Incidence of HCC following AAV Gene Therapy in Mice. *Mol Ther.* Feb 03 2021;29(2):680-690. doi:10.1016/j.ymthe.2020.10.018
200. Bell P, Moscioni AD, McCarter RJ, et al. Analysis of tumors arising in male B6C3F1 mice with and without AAV vector delivery to liver. *Mol Ther.* Jul 2006;14(1):34-44. doi:10.1016/j.ymthe.2006.03.008
201. Bell P, Wang L, Lebherz C, et al. No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. *Mol Ther.* Aug 2005;12(2):299-306. doi:10.1016/j.ymthe.2005.03.020
202. Gil-Farina I, Fronza R, Kaeppel C, et al. Recombinant AAV Integration Is Not Associated With Hepatic Genotoxicity in Nonhuman Primates and Patients. *Mol Ther.* Jun 2016;24(6):1100-1105. doi:10.1038/mt.2016.52
203. Sabatino DE, Bushman FD, Chandler RJ, et al. Evaluating the state of the science for adeno-associated virus integration: An integrated perspective. *Mol Ther.* Aug 03 2022;30(8):2646-2663. doi:10.1016/j.ymthe.2022.06.004
204. René CA, Parks RJ. Expanding the Availability of Onasemnogene Abeparvovec to Older Patients: The Evolving Treatment Landscape for Spinal Muscular Atrophy. *Pharmaceutics.* Jun 19 2023;15(6)doi:10.3390/pharmaceutics15061764
205. Finkel RS, Darras BT, Mendell JR, et al. Intrathecal Onasemnogene Abeparvovec for Sitting, Nonambulatory Patients with Spinal Muscular

- Atrophy: Phase I Ascending-Dose Study (STRONG). *J Neuromuscul Dis.* Mar 09 2023;doi:10.3233/JND-221560
206. CureSMA. *State of SMA: 2022 Report.* 2023. Accessed October 24 2024. https://www.curesma.org/wp-content/uploads/2023/06/9062023_State-of-SMA_vWeb.pdf
 207. Chiriboga CA, Bruno C, Duong T, et al. JEWELFISH: 24-month results from an open-label study in non-treatment-naïve patients with SMA receiving treatment with risdiplam. *J Neurol.* Aug 2024;271(8):4871-4884. doi:10.1007/s00415-024-12318-z
 208. Signoria I, Zwartkruis MM, Geerlofs L, et al. Patient-specific responses to SMN2 splice-modifying treatments in spinal muscular atrophy fibroblasts. *Mol Ther Methods Clin Dev.* Dec 12 2024;32(4):101379. doi:10.1016/j.omtm.2024.101379
 209. Roberto J, Poulin KL, Parks RJ, Vacratsis PO. Label-free quantitative proteomic analysis of extracellular vesicles released from fibroblasts derived from patients with spinal muscular atrophy. *Proteomics.* 07 2021;21(13-14):e2000301. doi:10.1002/pmic.202000301
 210. Nash LA, McFall ER, Perozzo AM, et al. Survival Motor Neuron Protein is Released from Cells in Exosomes: A Potential Biomarker for Spinal Muscular Atrophy. *Sci Rep.* 10 2017;7(1):13859. doi:10.1038/s41598-017-14313-z
 211. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. *J Cell Biol.* Feb 2013;200(4):373-83. doi:10.1083/jcb.201211138
 212. Johnstone RM, Adam M, Hammond JR, Orr L, Turbide C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J Biol Chem.* Jul 1987;262(19):9412-20.
 213. Yáñez-Mó M, Siljander PR, Andreu Z, et al. Biological properties of extracellular vesicles and their physiological functions. *J Extracell Vesicles.* 2015;4:27066. doi:10.3402/jev.v4.27066
 214. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. *J Proteomics.* Sep 2010;73(10):1907-20. doi:10.1016/j.jprot.2010.06.006
 215. Driedonks T, Jiang L, Carlson B, et al. Pharmacokinetics and biodistribution of extracellular vesicles administered intravenously and intranasally to. *J Extracell Biol.* Oct 2022;1(10)doi:10.1002/jex2.59
 216. Tolomeo AM, Zuccolotto G, Malvicini R, et al. Biodistribution of Intratracheal, Intranasal, and Intravenous Injections of Human Mesenchymal Stromal Cell-Derived Extracellular Vesicles in a Mouse Model for Drug Delivery Studies. *Pharmaceutics.* Feb 07 2023;15(2)doi:10.3390/pharmaceutics15020548
 217. Wiklander OP, Nordin JZ, O'Loughlin A, et al. Extracellular vesicle in vivo biodistribution is determined by cell source, route of administration and targeting. *J Extracell Vesicles.* 2015;4:26316. doi:10.3402/jev.v4.26316
 218. Fordjour FK, Abuelreich S, Hong X, et al. Exomap1 mouse: A transgenic model for. *Extracell Vesicle.* Dec 2023;2doi:10.1016/j.vesic.2023.100030
 219. Bonsergent E, Grisard E, Buchrieser J, Schwartz O, Théry C, Lavieu G. Quantitative characterization of extracellular vesicle uptake and content

- delivery within mammalian cells. *Nat Commun.* Mar 25 2021;12(1):1864. doi:10.1038/s41467-021-22126-y
220. O'Brien K, Ughetto S, Mahjoun S, Nair AV, Breakefield XO. Uptake, functionality, and re-release of extracellular vesicle-encapsulated cargo. *Cell Rep.* Apr 12 2022;39(2):110651. doi:10.1016/j.celrep.2022.110651
 221. Saleh AF, Lázaro-Ibáñez E, Forsgard MA, et al. Extracellular vesicles induce minimal hepatotoxicity and immunogenicity. *Nanoscale.* Apr 2019;11(14):6990-7001. doi:10.1039/c8nr08720b
 222. Zhu X, Badawi M, Pomeroy S, et al. Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *J Extracell Vesicles.* 2017;6(1):1324730. doi:10.1080/20013078.2017.1324730
 223. Kordelas L, Rebmann V, Ludwig AK, et al. MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia.* Apr 2014;28(4):970-3. doi:10.1038/leu.2014.41
 224. Alvarez-Erviti L, Seow Y, Yin H, Betts C, Lakhal S, Wood MJ. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat Biotechnol.* Apr 2011;29(4):341-5. doi:10.1038/nbt.1807
 225. Cooper JM, Wiklander PB, Nordin JZ, et al. Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice. *Mov Disord.* Oct 2014;29(12):1476-85. doi:10.1002/mds.25978
 226. Paulaitis M, Agarwal K, Nana-Sinkam P. Dynamic Scaling of Exosome Sizes. *Langmuir.* 08 2018;34(32):9387-9393. doi:10.1021/acs.langmuir.7b04080
 227. György B, Szabó TG, Pásztói M, et al. Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell Mol Life Sci.* Aug 2011;68(16):2667-88. doi:10.1007/s00018-011-0689-3
 228. Nabhan JF, Hu R, Oh RS, Cohen SN, Lu Q. Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMVs) at plasma membrane by recruitment of TSG101 protein. *Proc Natl Acad Sci U S A.* Mar 2012;109(11):4146-51. doi:10.1073/pnas.1200448109
 229. René CA, Parks RJ. Extracellular vesicles efficient deliver survival motor neuron protein to cells in culture. *Under review.* 2024;
 230. Brodeur A, Migneault F, Lanoie M, et al. Apoptotic exosome-like vesicles transfer specific and functional mRNAs to endothelial cells by phosphatidylserine-dependent macropinocytosis. *Cell Death Dis.* Jul 20 2023;14(7):449. doi:10.1038/s41419-023-05991-x
 231. Minciacchi VR, You S, Spinelli C, et al. Large oncosomes contain distinct protein cargo and represent a separate functional class of tumor-derived extracellular vesicles. *Oncotarget.* May 10 2015;6(13):11327-41. doi:10.18632/oncotarget.3598
 232. Morello M, Minciacchi VR, de Candia P, et al. Large oncosomes mediate intercellular transfer of functional microRNA. *Cell Cycle.* Nov 15 2013;12(22):3526-36. doi:10.4161/cc.26539
 233. Melentijevic I, Toth ML, Arnold ML, et al. C. elegans neurons jettison protein aggregates and mitochondria under neurotoxic stress. *Nature.* Feb 16 2017;542(7641):367-371. doi:10.1038/nature21362

234. Wang Y, Arnold ML, Smart AJ, et al. Large vesicle extrusions from. *Elife*. Mar 02 2023;12doi:10.7554/eLife.82227
235. Arnold ML, Cooper J, Androwski R, et al. Intermediate filaments associate with aggresome-like structures in proteostressed *C. elegans* neurons and influence large vesicle extrusions as exophers. *Nat Commun*. Jul 24 2023;14(1):4450. doi:10.1038/s41467-023-39700-1
236. Ma L, Li Y, Peng J, et al. Discovery of the migrasome, an organelle mediating release of cytoplasmic contents during cell migration. *Cell Res*. Jan 2015;25(1):24-38. doi:10.1038/cr.2014.135
237. Jiang D, Jiang Z, Lu D, et al. Migrasomes provide regional cues for organ morphogenesis during zebrafish gastrulation. *Nat Cell Biol*. Aug 2019;21(8):966-977. doi:10.1038/s41556-019-0358-6
238. Wang Q, Yu J, Kadungure T, Beyene J, Zhang H, Lu Q. ARMMs as a versatile platform for intracellular delivery of macromolecules. *Nat Commun*. 03 2018;9(1):960. doi:10.1038/s41467-018-03390-x
239. Zhang H, Freitas D, Kim HS, et al. Identification of distinct nanoparticles and subsets of extracellular vesicles by asymmetric flow field-flow fractionation. *Nat Cell Biol*. Mar 2018;20(3):332-343. doi:10.1038/s41556-018-0040-4
240. Zhang Q, Higginbotham JN, Jeppesen DK, et al. Transfer of Functional Cargo in Exomeres. *Cell Rep*. Apr 16 2019;27(3):940-954.e6. doi:10.1016/j.celrep.2019.01.009
241. Clancy JW, Boomgarden AC, D'Souza-Schorey C. Profiling and promise of supermeres. *Nat Cell Biol*. Dec 2021;23(12):1217-1219. doi:10.1038/s41556-021-00808-5
242. Zhang Q, Jeppesen DK, Higginbotham JN, et al. Supermeres are functional extracellular nanoparticles replete with disease biomarkers and therapeutic targets. *Nat Cell Biol*. Dec 2021;23(12):1240-1254. doi:10.1038/s41556-021-00805-8
243. Welsh JA, Goberdhan DCI, O'Driscoll L, et al. Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J Extracell Vesicles*. Feb 2024;13(2):e12404. doi:10.1002/jev2.12404
244. Meng F, Xu R, Wang S, et al. Human umbilical cord-derived mesenchymal stem cell therapy in patients with COVID-19: a phase 1 clinical trial. *Signal Transduct Target Ther*. Aug 27 2020;5(1):172. doi:10.1038/s41392-020-00286-5
245. Dilogio IH, Aditjaningsih D, Sugiarto A, et al. Umbilical cord mesenchymal stromal cells as critical COVID-19 adjuvant therapy: A randomized controlled trial. *Stem Cells Transl Med*. Sep 2021;10(9):1279-1287. doi:10.1002/sctm.21-0046
246. Wei F, Kong D, Li T, et al. Efficacy and safety of umbilical cord mesenchymal stem cells for the treatment of patients with COVID-19. *Clinics (Sao Paulo)*. 2021;76:e2604. doi:10.6061/clinics/2021/e2604
247. Feng Y, Huang J, Wu J, et al. Safety and feasibility of umbilical cord mesenchymal stem cells in patients with COVID-19 pneumonia: A pilot study. *Cell Prolif*. Dec 2020;53(12):e12947. doi:10.1111/cpr.12947

248. Yu B, Zhang X, Li X. Exosomes derived from mesenchymal stem cells. *Int J Mol Sci*. Mar 07 2014;15(3):4142-57. doi:10.3390/ijms15034142
249. Lee JH, Park J, Lee JW. Therapeutic use of mesenchymal stem cell-derived extracellular vesicles in acute lung injury. *Transfusion*. Feb 2019;59(S1):876-883. doi:10.1111/trf.14838
250. Zarrabi M, Shahrabaf MA, Nouri M, et al. Allogenic mesenchymal stromal cells and their extracellular vesicles in COVID-19 induced ARDS: a randomized controlled trial. *Stem Cell Res Ther*. Jun 26 2023;14(1):169. doi:10.1186/s13287-023-03402-8
251. Lightner AL, Sengupta V, Qian S, et al. Bone Marrow Mesenchymal Stem Cell-Derived Extracellular Vesicle Infusion for the Treatment of Respiratory Failure From COVID-19: A Randomized, Placebo-Controlled Dosing Clinical Trial. *Chest*. Jun 23 2023;doi:10.1016/j.chest.2023.06.024
252. Antimisiaris SG, Mourtas S, Marazioti A. Exosomes and Exosome-Inspired Vesicles for Targeted Drug Delivery. *Pharmaceutics*. Nov 2018;10(4)doi:10.3390/pharmaceutics10040218
253. Sun D, Zhuang X, Xiang X, et al. A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol Ther*. Sep 2010;18(9):1606-14. doi:10.1038/mt.2010.105
254. Haney MJ, Klyachko NL, Zhao Y, et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J Control Release*. Jun 2015;207:18-30. doi:10.1016/j.jconrel.2015.03.033
255. Armstrong JP, Holme MN, Stevens MM. Re-Engineering Extracellular Vesicles as Smart Nanoscale Therapeutics. *ACS Nano*. 01 2017;11(1):69-83. doi:10.1021/acsnano.6b07607
256. Walker S, Busatto S, Pham A, et al. Extracellular vesicle-based drug delivery systems for cancer treatment. *Theranostics*. 2019;9(26):8001-8017. doi:10.7150/thno.37097
257. Fuhrmann G, Serio A, Mazo M, Nair R, Stevens MM. Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. *J Control Release*. May 10 2015;205:35-44. doi:10.1016/j.jconrel.2014.11.029
258. Kim MS, Haney MJ, Zhao Y, et al. Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine*. Apr 2016;12(3):655-664. doi:10.1016/j.nano.2015.10.012
259. McCann JV, Bischoff SR, Zhang Y, et al. Reporter mice for isolating and auditing cell type-specific extracellular vesicles in vivo. *Genesis*. Jul 2020;58(7):e23369. doi:10.1002/dvg.23369
260. Kooijmans SA, Aleza CG, Roffler SR, van Solinge WW, Vader P, Schiffelers RM. Display of GPI-anchored anti-EGFR nanobodies on extracellular vesicles promotes tumour cell targeting. *J Extracell Vesicles*. 2016;5:31053. doi:10.3402/jev.v5.31053
261. Ferreira JV, da Rosa Soares A, Ramalho J, et al. LAMP2A regulates the loading of proteins into exosomes. *Sci Adv*. Mar 25 2022;8(12):eabm1140. doi:10.1126/sciadv.abm1140

262. Sterzenbach U, Putz U, Low LH, Silke J, Tan SS, Howitt J. Engineered Exosomes as Vehicles for Biologically Active Proteins. *Mol Ther*. Jun 07 2017;25(6):1269-1278. doi:10.1016/j.ymthe.2017.03.030
263. Mulcahy LA, Pink RC, Carter DR. Routes and mechanisms of extracellular vesicle uptake. *J Extracell Vesicles*. 2014;3doi:10.3402/jev.v3.24641
264. Costa Verdera H, Gitz-Francois JJ, Schiffelers RM, Vader P. Cellular uptake of extracellular vesicles is mediated by clathrin-independent endocytosis and macropinocytosis. *J Control Release*. Nov 2017;266:100-108. doi:10.1016/j.jconrel.2017.09.019
265. Chen CC, Liu L, Ma F, et al. Elucidation of Exosome Migration across the Blood-Brain Barrier Model In Vitro. *Cell Mol Bioeng*. Dec 2016;9(4):509-529. doi:10.1007/s12195-016-0458-3
266. Li Y, Wang J, Chen S, et al. miR-137 boosts the neuroprotective effect of endothelial progenitor cell-derived exosomes in oxyhemoglobin-treated SH-SY5Y cells partially via COX2/PGE2 pathway. *Stem Cell Res Ther*. Oct 2020;11(1):330. doi:10.1186/s13287-020-01836-y
267. Feng D, Zhao WL, Ye YY, et al. Cellular internalization of exosomes occurs through phagocytosis. *Traffic*. May 2010;11(5):675-87. doi:10.1111/j.1600-0854.2010.01041.x
268. Parolini I, Federici C, Raggi C, et al. Microenvironmental pH is a key factor for exosome traffic in tumor cells. *J Biol Chem*. Dec 04 2009;284(49):34211-22. doi:10.1074/jbc.M109.041152
269. Hung ME, Leonard JN. A platform for actively loading cargo RNA to elucidate limiting steps in EV-mediated delivery. *J Extracell Vesicles*. 2016;5:31027. doi:10.3402/jev.v5.31027
270. Pham TT, Chen H, Nguyen PHD, Jayasinghe MK, Le AH, Le MT. Endosomal escape of nucleic acids from extracellular vesicles mediates functional therapeutic delivery. *Pharmacol Res*. Feb 2023;188:106665. doi:10.1016/j.phrs.2023.106665
271. Karim MA, Samyn DR, Mattie S, Brett CL. Distinct features of multivesicular body-lysosome fusion revealed by a new cell-free content-mixing assay. *Traffic*. Feb 2018;19(2):138-149. doi:10.1111/tra.12543
272. Tjelle TE, Brech A, Juvet LK, Griffiths G, Berg T. Isolation and characterization of early endosomes, late endosomes and terminal lysosomes: their role in protein degradation. *J Cell Sci*. Dec 1996;109 (Pt 12):2905-14. doi:10.1242/jcs.109.12.2905
273. Mullock BM, Bright NA, Fearon CW, Gray SR, Luzio JP. Fusion of lysosomes with late endosomes produces a hybrid organelle of intermediate density and is NSF dependent. *J Cell Biol*. Feb 09 1998;140(3):591-601. doi:10.1083/jcb.140.3.591
274. Li W, Wang J, Yin X, et al. Construction of a mouse model that can be used for tissue-specific EV screening and tracing. *Front Cell Dev Biol*. 2022;10:1015841. doi:10.3389/fcell.2022.1015841
275. René CA, Parks RJ. Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency. *Mol Ther Methods Clin Dev*. Jun 13 2024;32(2):101259. doi:10.1016/j.omtm.2024.101259

276. Pincela Lins PM, Pirlet E, Szymonik M, Bronckaers A, Nelissen I. Manufacture of extracellular vesicles derived from mesenchymal stromal cells. *Trends Biotechnol.* Jul 2023;41(7):965-981. doi:10.1016/j.tibtech.2023.01.003
277. Giancaterino S, Boi C. Alternative biological sources for extracellular vesicles production and purification strategies for process scale-up. *Biotechnol Adv.* 2023;63:108092. doi:10.1016/j.biotechadv.2022.108092
278. Lai JJ, Hill JJ, Huang CY, et al. Unveiling the Complex World of Extracellular Vesicles: Novel Characterization Techniques and Manufacturing Considerations. *Chonnam Med J.* Jan 2024;60(1):1-12. doi:10.4068/cmj.2024.60.1.1
279. Omrani M, Beyrampour-Basmenj H, Jahanban-Esfahlan R, et al. Global trend in exosome isolation and application: an update concept in management of diseases. *Mol Cell Biochem.* Mar 2024;479(3):679-691. doi:10.1007/s11010-023-04756-6
280. Wang W, Sun H, Duan H, et al. Isolation and usage of exosomes in central nervous system diseases. *CNS Neurosci Ther.* Mar 2024;30(3):e14677. doi:10.1111/cns.14677
281. Théry C, Witwer KW, Aikawa E, et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J Extracell Vesicles.* 2018;7(1):1535750. doi:10.1080/20013078.2018.1535750
282. Komatsu S, Kato N, Kitai H, et al. Detecting and exploring kidney-derived extracellular vesicles in plasma. *Clin Exp Nephrol.* Jul 2024;28(7):617-628. doi:10.1007/s10157-024-02464-z
283. Uil M, Hau CM, Ahdi M, et al. Cellular origin and microRNA profiles of circulating extracellular vesicles in different stages of diabetic nephropathy. *Clin Kidney J.* Jan 2021;14(1):358-365. doi:10.1093/ckj/sfz145
284. Dickens AM, Tovar-Y-Romo LB, Yoo SW, et al. Astrocyte-shed extracellular vesicles regulate the peripheral leukocyte response to inflammatory brain lesions. *Sci Signal.* Apr 04 2017;10(473):doi:10.1126/scisignal.aai7696
285. Delgado-Peraza F, Nogueras-Ortiz C, Simonsen AH, et al. Neuron-derived extracellular vesicles in blood reveal effects of exercise in Alzheimer's disease. *Alzheimers Res Ther.* Sep 20 2023;15(1):156. doi:10.1186/s13195-023-01303-9
286. Blandin A, Dugail I, Hilairet G, et al. Lipidomic analysis of adipose-derived extracellular vesicles reveals specific EV lipid sorting informative of the obesity metabolic state. *Cell Rep.* Mar 28 2023;42(3):112169. doi:10.1016/j.celrep.2023.112169
287. Guescini M, Canonico B, Lucertini F, et al. Muscle Releases Alpha-Sarcoglycan Positive Extracellular Vesicles Carrying miRNAs in the Bloodstream. *PLoS One.* 2015;10(5):e0125094. doi:10.1371/journal.pone.0125094
288. Royo F, Moreno L, Mleczko J, et al. Hepatocyte-secreted extracellular vesicles modify blood metabolome and endothelial function by an arginase-dependent mechanism. *Sci Rep.* 02 17 2017;7:42798. doi:10.1038/srep42798

289. Newman LA, Useckaite Z, Johnson J, Sorich MJ, Hopkins AM, Rowland A. Selective Isolation of Liver-Derived Extracellular Vesicles Redefines Performance of miRNA Biomarkers for Non-Alcoholic Fatty Liver Disease. *Biomedicines*. Jan 17 2022;10(1)doi:10.3390/biomedicines10010195
290. Blouin A, Bolender RP, Weibel ER. Distribution of organelles and membranes between hepatocytes and nonhepatocytes in the rat liver parenchyma. A stereological study. *J Cell Biol*. Feb 1977;72(2):441-55. doi:10.1083/jcb.72.2.441
291. Wetmore BA, Brees DJ, Singh R, et al. Quantitative analyses and transcriptomic profiling of circulating messenger RNAs as biomarkers of rat liver injury. *Hepatology*. Jun 2010;51(6):2127-39. doi:10.1002/hep.23574
292. Feldmann G, Penaud-Laurencin J, Crassous J, Benhamou JP. Albumin synthesis by human liver cells: its morphological demonstration. *Gastroenterology*. Dec 1972;63(6):1036-48.
293. Morgan EH, Peters T. Intracellular aspects of transferrin synthesis and secretion in the rat. *J Biol Chem*. Jun 10 1971;246(11):3508-11.
294. Hofmann AF. The continuing importance of bile acids in liver and intestinal disease. *Arch Intern Med*. 1999 Dec 13-27 1999;159(22):2647-58. doi:10.1001/archinte.159.22.2647
295. Grant DM. Detoxification pathways in the liver. *J Inherit Metab Dis*. 1991;14(4):421-30. doi:10.1007/BF01797915
296. Meex RC, Hoy AJ, Morris A, et al. Fetuin B Is a Secreted Hepatocyte Factor Linking Steatosis to Impaired Glucose Metabolism. *Cell Metab*. Dec 01 2015;22(6):1078-89. doi:10.1016/j.cmet.2015.09.023
297. Montgomery MK, Bayliss J, Devereux C, et al. SMOC1 is a glucose-responsive hepatokine and therapeutic target for glycemic control. *Sci Transl Med*. Sep 02 2020;12(559)doi:10.1126/scitranslmed.aaz8048
298. Miotto PM, Yang CH, Keenan SN, et al. Liver-derived extracellular vesicles improve whole-body glycaemic control via inter-organ communication. *Nat Metab*. Feb 2024;6(2):254-272. doi:10.1038/s42255-023-00971-z
299. Montgomery MK, Bayliss J, Nie S, et al. Deep proteomic profiling unveils arylsulfatase A as a non-alcoholic steatohepatitis inducible hepatokine and regulator of glycemic control. *Nat Commun*. Mar 10 2022;13(1):1259. doi:10.1038/s41467-022-28889-2
300. Misu H, Takamura T, Takayama H, et al. A liver-derived secretory protein, selenoprotein P, causes insulin resistance. *Cell Metab*. Nov 03 2010;12(5):483-95. doi:10.1016/j.cmet.2010.09.015
301. Michael DJ, Cai H, Xiong W, Ouyang J, Chow RH. Mechanisms of peptide hormone secretion. *Trends Endocrinol Metab*. Dec 2006;17(10):408-15. doi:10.1016/j.tem.2006.10.011
302. Ji Y, Luo Z, Gao H, et al. Hepatocyte-derived exosomes from early onset obese mice promote insulin sensitivity through miR-3075. *Nat Metab*. Sep 2021;3(9):1163-1174. doi:10.1038/s42255-021-00444-1
303. Albanese M, Chen YA, Hüls C, et al. MicroRNAs are minor constituents of extracellular vesicles that are rarely delivered to target cells. *PLoS Genet*. Dec 2021;17(12):e1009951. doi:10.1371/journal.pgen.1009951

304. Jin Y, Chung YW, Jung MK, et al. Apolipoprotein E-mediated regulation of selenoprotein P transportation via exosomes. *Cell Mol Life Sci*. Jun 2020;77(12):2367-2386. doi:10.1007/s00018-019-03287-y
305. Caito SW, Milatovic D, Hill KE, Aschner M, Burk RF, Valentine WM. Progression of neurodegeneration and morphologic changes in the brains of juvenile mice with selenoprotein P deleted. *Brain Res*. Jun 29 2011;1398:1-12. doi:10.1016/j.brainres.2011.04.046
306. Peters MM, Hill KE, Burk RF, Weeber EJ. Altered hippocampus synaptic function in selenoprotein P deficient mice. *Mol Neurodegener*. Sep 19 2006;1:12. doi:10.1186/1750-1326-1-12
307. Glenner GG, Wong CW. Alzheimer's disease: initial report of the purification and characterization of a novel cerebrovascular amyloid protein. *Biochem Biophys Res Commun*. May 16 1984;120(3):885-90. doi:10.1016/s0006-291x(84)80190-4
308. Kim IH, Józkowicz A, Piedra PA, Oka K, Chan L. Lifetime correction of genetic deficiency in mice with a single injection of helper-dependent adenoviral vector. *Proc Natl Acad Sci U S A*. Nov 2001;98(23):13282-7. doi:10.1073/pnas.241506298
309. Nakai H, Fuess S, Storm TA, Muramatsu S, Nara Y, Kay MA. Unrestricted hepatocyte transduction with adeno-associated virus serotype 8 vectors in mice. *J Virol*. Jan 2005;79(1):214-24. doi:10.1128/JVI.79.1.214-224.2005
310. Zhu Y, Shen R, Vuong I, et al. Multi-step screening of DNA/lipid nanoparticles and co-delivery with siRNA to enhance and prolong gene expression. *Nat Commun*. Jul 25 2022;13(1):4282. doi:10.1038/s41467-022-31993-y
311. Pattipeiluhu R, Arias-Alpizar G, Basha G, et al. Anionic Lipid Nanoparticles Preferentially Deliver mRNA to the Hepatic Reticuloendothelial System. *Adv Mater*. Apr 2022;34(16):e2201095. doi:10.1002/adma.202201095
312. Vrancken Peeters MJ, Perkins AL, Kay MA. Method for multiple portal vein infusions in mice: quantitation of adenovirus-mediated hepatic gene transfer. *Biotechniques*. Feb 1996;20(2):278-85. doi:10.2144/96202rr05
313. Worgall S, Wolff G, Falck-Pedersen E, Crystal RG. Innate immune mechanisms dominate elimination of adenoviral vectors following in vivo administration. *Hum Gene Ther*. Jan 01 1997;8(1):37-44. doi:10.1089/hum.1997.8.1-37
314. Brunetti-Pierri N, Ng T, Iannitti D, et al. Transgene expression up to 7 years in nonhuman primates following hepatic transduction with helper-dependent adenoviral vectors. *Hum Gene Ther*. Aug 2013;24(8):761-5. doi:10.1089/hum.2013.071
315. McConnell RE, Youniss M, Gnanasambandam B, Shah P, Zhang W, Finn JD. Transfection reagent artefact likely accounts for some reports of extracellular vesicle function. *J Extracell Vesicles*. Oct 2022;11(10):e12253. doi:10.1002/jev2.12253
316. McCann J, Sosa-Miranda CD, Guo H, et al. Contaminating transfection complexes can masquerade as small extracellular vesicles and impair their delivery of RNA. *J Extracell Vesicles*. Oct 2022;11(10):e12220. doi:10.1002/jev2.12220

317. Do Minh A, Star AT, Stupak J, et al. Characterization of Extracellular Vesicles Secreted in Lentiviral Producing HEK293SF Cell Cultures. *Viruses*. Apr 29 2021;13(5)doi:10.3390/v13050797
318. Liu H, Jin L, Koh SB, et al. Atomic structure of human adenovirus by cryo-EM reveals interactions among protein networks. *Science*. Aug 27 2010;329(5995):1038-43. doi:10.1126/science.1187433
319. Bett AJ, Prevec L, Graham FL. Packaging capacity and stability of human adenovirus type 5 vectors. *J Virol*. Oct 1993;67(10):5911-21. doi:10.1128/JVI.67.10.5911-5921.1993
320. Parks RJ, Chen L, Anton M, Sankar U, Rudnicki MA, Graham FL. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. *Proc Natl Acad Sci U S A*. Nov 26 1996;93(24):13565-70. doi:10.1073/pnas.93.24.13565
321. Francis JW, Figueiredo D, vanderSpek JC, et al. A survival motor neuron:tetanus toxin fragment C fusion protein for the targeted delivery of SMN protein to neurons. *Brain Res*. Jan 02 2004;995(1):84-96. doi:10.1016/j.brainres.2003.09.063
322. Anderton RS, Meloni BP, Mastaglia FL, Boulos S. Investigation of a recombinant SMN protein delivery system to treat spinal muscular atrophy. *Translational Neuroscience*. 2014;5:8-16.
323. Brambilla L, Valori CF, Guidotti G, et al. Recombinant SMN protein synergizes with spinal muscular atrophy therapy to counteract pathological motor neuron phenotypes. *Transl Neurodegener*. Dec 17 2024;13(1):63. doi:10.1186/s40035-024-00455-4
324. Ferrari A, Pellegrini V, Arcangeli C, Fittipaldi A, Giacca M, Beltram F. Caveolae-mediated internalization of extracellular HIV-1 tat fusion proteins visualized in real time. *Mol Ther*. Aug 2003;8(2):284-94. doi:10.1016/s1525-0016(03)00122-9
325. Caron NJ, Torrente Y, Camirand G, et al. Intracellular delivery of a Tat-eGFP fusion protein into muscle cells. *Mol Ther*. Mar 2001;3(3):310-8. doi:10.1006/mthe.2001.0279
326. Koutsokeras A, Kabouridis PS. Secretion and uptake of TAT-fusion proteins produced by engineered mammalian cells. *Biochim Biophys Acta*. Feb 2009;1790(2):147-53. doi:10.1016/j.bbagen.2008.11.005
327. Gschwendtberger T, Thau-Habermann N, von der Ohe J, Luo T, Hass R, Petri S. Protective effects of EVs/exosomes derived from permanently growing human MSC on primary murine ALS motor neurons. *Neurosci Lett*. Nov 01 2023;816:137493. doi:10.1016/j.neulet.2023.137493
328. Provenzano F, Nyberg S, Giunti D, et al. Micro-RNAs Shuttled by Extracellular Vesicles Secreted from Mesenchymal Stem Cells Dampen Astrocyte Pathological Activation and Support Neuroprotection in In-Vitro Models of ALS. *Cells*. Dec 04 2022;11(23)doi:10.3390/cells11233923
329. Sheller-Miller S, Choi K, Choi C, Menon R. Cyclic-recombinase-reporter mouse model to determine exosome communication and function during pregnancy. *Am J Obstet Gynecol*. Nov 2019;221(5):502.e1-502.e12. doi:10.1016/j.ajog.2019.06.010

330. Antounians L, Catania VD, Montalva L, et al. Fetal lung underdevelopment is rescued by administration of amniotic fluid stem cell extracellular vesicles in rodents. *Sci Transl Med.* Apr 21 2021;13(590)doi:10.1126/scitranslmed.aax5941
331. Antounians L, Figueira RL, Kukreja B, et al. Fetal hypoplastic lungs have multilineage inflammation that is reversed by amniotic fluid stem cell extracellular vesicle treatment. *Sci Adv.* Jul 26 2024;10(30):eadn5405. doi:10.1126/sciadv.adn5405
332. Kong L, Valdivia DO, Simon CM, et al. Impaired prenatal motor axon development necessitates early therapeutic intervention in severe SMA. *Sci Transl Med.* Jan 27 2021;13(578)doi:10.1126/scitranslmed.abb6871
333. Hagenacker T, Wurster CD, Günther R, et al. Nusinersen in adults with 5q spinal muscular atrophy: a non-interventional, multicentre, observational cohort study. *Lancet Neurol.* 04 2020;19(4):317-325. doi:10.1016/S1474-4422(20)30037-5
334. Darras BT, Masson R, Mazurkiewicz-Bęldzińska M, et al. Risdiplam-Treated Infants with Type 1 Spinal Muscular Atrophy versus Historical Controls. *N Engl J Med.* 07 29 2021;385(5):427-435. doi:10.1056/NEJMoa2102047
335. Mercuri E, Baranello G, Boespflug-Tanguy O, et al. Risdiplam in types 2 and 3 spinal muscular atrophy: A randomised, placebo-controlled, dose-finding trial followed by 24 months of treatment. *Eur J Neurol.* Jul 15 2022;00:1-12. doi:10.1111/ene.15499
336. Hosseinibarkooie S, Peters M, Torres-Benito L, et al. The Power of Human Protective Modifiers: PLS3 and CORO1C Unravel Impaired Endocytosis in Spinal Muscular Atrophy and Rescue SMA Phenotype. *Am J Hum Genet.* Sep 01 2016;99(3):647-665. doi:10.1016/j.ajhg.2016.07.014
337. Nishimura AL, Mitne-Neto M, Silva HC, et al. A mutation in the vesicle-trafficking protein VAPB causes late-onset spinal muscular atrophy and amyotrophic lateral sclerosis. *Am J Hum Genet.* Nov 2004;75(5):822-31. doi:10.1086/425287
338. Povero D, Eguchi A, Li H, et al. Circulating extracellular vesicles with specific proteome and liver microRNAs are potential biomarkers for liver injury in experimental fatty liver disease. *PLoS One.* 2014;9(12):e113651. doi:10.1371/journal.pone.0113651
339. Achour B, Al-Majdoub ZM, Grybos-Gajniak A, et al. Liquid Biopsy Enables Quantification of the Abundance and Interindividual Variability of Hepatic Enzymes and Transporters. *Clin Pharmacol Ther.* Jan 2021;109(1):222-232. doi:10.1002/cpt.2102
340. Kojima R, Bojar D, Rizzi G, et al. Designer exosomes produced by implanted cells intracerebrally deliver therapeutic cargo for Parkinson's disease treatment. *Nat Commun.* Apr 03 2018;9(1):1305. doi:10.1038/s41467-018-03733-8
341. Löser P, Jennings GS, Strauss M, Sandig V. Reactivation of the previously silenced cytomegalovirus major immediate-early promoter in the mouse liver: involvement of NFkappaB. *J Virol.* Jan 1998;72(1):180-90. doi:10.1128/JVI.72.1.180-190.1998

342. Chen HH, Mack LM, Choi SY, Ontell M, Kochanek S, Clemens PR. DNA from both high-capacity and first-generation adenoviral vectors remains intact in skeletal muscle. *Hum Gene Ther.* Feb 10 1999;10(3):365-73. doi:10.1089/10430349950018814
343. Brooks AR, Harkins RN, Wang P, Qian HS, Liu P, Rubanyi GM. Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med.* Apr 2004;6(4):395-404. doi:10.1002/jgm.516
344. Martino AT, Nayak S, Hoffman BE, et al. Tolerance induction to cytoplasmic beta-galactosidase by hepatic AAV gene transfer: implications for antigen presentation and immunotoxicity. *PLoS One.* Aug 04 2009;4(8):e6376. doi:10.1371/journal.pone.0006376
345. McCaffrey AP, Fawcett P, Nakai H, et al. The host response to adenovirus, helper-dependent adenovirus, and adeno-associated virus in mouse liver. *Mol Ther.* May 2008;16(5):931-41. doi:10.1038/mt.2008.37
346. Alves CRR, Ha LL, Yaworski R, et al. Base editing as a genetic treatment for spinal muscular atrophy. *bioRxiv.* Jan 21 2023;doi:10.1101/2023.01.20.524978
347. Arbab M, Matuszek Z, Kray KM, et al. Base editing rescue of spinal muscular atrophy in cells and in mice. *Science.* Apr 21 2023;380(6642):eadg6518. doi:10.1126/science.adg6518
348. Dong JY, Fan PD, Frizzell RA. Quantitative analysis of the packaging capacity of recombinant adeno-associated virus. *Hum Gene Ther.* Nov 10 1996;7(17):2101-12. doi:10.1089/hum.1996.7.17-2101
349. Nakanishi T, Maekawa A, Suzuki M, et al. Construction of adenovirus vectors simultaneously expressing four multiplex, double-nicking guide RNAs of CRISPR/Cas9 and in vivo genome editing. *Sci Rep.* Feb 17 2021;11(1):3961. doi:10.1038/s41598-021-83259-0
350. Finkel RS, Chiriboga CA, Vajsar J, et al. Treatment of infantile-onset spinal muscular atrophy with nusinersen: a phase 2, open-label, dose-escalation study. *Lancet.* Dec 17 2016;388(10063):3017-3026. doi:10.1016/S0140-6736(16)31408-8
351. Duan D, Goemans N, Takeda S, Mercuri E, Aartsma-Rus A. Duchenne muscular dystrophy. *Nat Rev Dis Primers.* Feb 18 2021;7(1):13. doi:10.1038/s41572-021-00248-3
352. Mercuri E, Bönnemann CG, Muntoni F. Muscular dystrophies. *Lancet.* Nov 30 2019;394(10213):2025-2038. doi:10.1016/S0140-6736(19)32910-1
353. Grieger JC, Samulski RJ. Packaging capacity of adeno-associated virus serotypes: impact of larger genomes on infectivity and postentry steps. *J Virol.* Aug 2005;79(15):9933-44. doi:10.1128/JVI.79.15.9933-9944.2005
354. Tennyson CN, Klamut HJ, Worton RG. The human dystrophin gene requires 16 hours to be transcribed and is cotranscriptionally spliced. *Nat Genet.* Feb 1995;9(2):184-90. doi:10.1038/ng0295-184
355. Scott JM, Li S, Harper SQ, et al. Viral vectors for gene transfer of micro-, mini-, or full-length dystrophin. *Neuromuscul Disord.* Oct 2002;12 Suppl 1:S23-9. doi:10.1016/s0960-8966(02)00078-0

- 356. Sakamoto M, Yuasa K, Yoshimura M, et al. Micro-dystrophin cDNA ameliorates dystrophic phenotypes when introduced into mdx mice as a transgene. *Biochem Biophys Res Commun.* May 17 2002;293(4):1265-72. doi:10.1016/S0006-291X(02)00362-5
- 357. Rafael JA, Sunada Y, Cole NM, Campbell KP, Faulkner JA, Chamberlain JS. Prevention of dystrophic pathology in mdx mice by a truncated dystrophin isoform. *Hum Mol Genet.* Oct 1994;3(10):1725-33. doi:10.1093/hmg/3.10.1725
- 358. Reshke R, Taylor JA, Savard A, et al. Reduction of the therapeutic dose of silencing RNA by packaging it in extracellular vesicles via a pre-microRNA backbone. *Nat Biomed Eng.* Jan 2020;4(1):52-68. doi:10.1038/s41551-019-0502-4
- 359. Didiot MC, Hall LM, Coles AH, et al. Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing. *Mol Ther.* 10 2016;24(10):1836-1847. doi:10.1038/mt.2016.126

Appendix 1: Authorizations

Chapter 2: Extracellular vesicles efficiently deliver survival motor neuron protein to cells in culture

- This article, of which I am first author, was published in the Springer Nature journal Scientific Reports. This article is licensed under a Creative Commons Attribution-NonCommercial-NoDerivates (CC BY-NC-ND) 4.0 International License, which permits non-commercial use, sharing, distribution and reproduction in any medium or format with appropriate credit to the original authors and the source when accompanied by a link to the Creative Commons license.
- Creative Commons license: <http://creativecommons.org/licenses/by-nc-nd/4.0/>
- The licensed material has not been modified.
- Citation: René CA, Parks RJ. Extracellular vesicles efficiently deliver survival motor neuron protein to cells in culture. Sci Rep. Feb 15 2025;15(1):5674. doi:10.1038/s41598-025-90083-3

Chapter 3: Use of a gene therapy approach to engineer the liver to release extracellular vesicles that distribute a therapeutic protein throughout the body of mice

- This article, of which I am first author, is under review by the Cell Press journal Molecular Therapy Methods and Clinical Development. Authors are permitted to reuse the work as permitted by the Creative Commons CC BY-NC-ND 4.0 license.

Appendix 3.1: Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles

- This article, of which I am first author, was published in the MDPI journal Pharmaceutics. Authors of MDPI articles retain copywrite and the articles are licensed under Creative Commons CC BY-NC-ND 4.0 license.
- Additional information: <https://www.mdpi.com/authors/rights>
- Citation: René CA, Parks RJ. Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles. Pharmaceutics. Apr 03 2021;13(4)doi:10.3390/pharmaceutics13040492

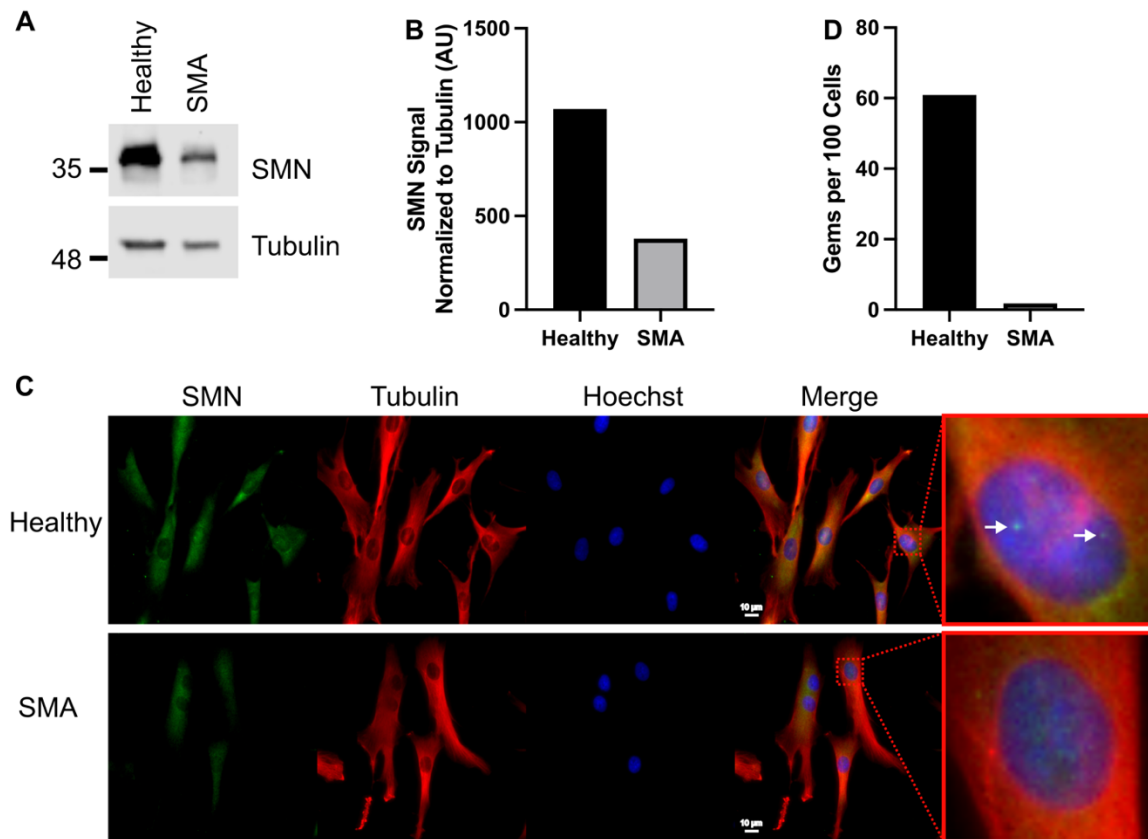
Appendix 3.2: Expanding the Availability of Onasemnogene Apeparvovec to Older Patients: The Evolving Treatment Landscape for Spinal Muscular Atrophy

- This article, of which I am first author, was published in the MDPI journal Pharmaceutics. Authors of MDPI articles retain copywrite and the articles are licensed under Creative Commons CC BY-NC-ND 4.0 license.
- Additional information: <https://www.mdpi.com/authors/rights>
- Citation: René CA, Parks RJ. Expanding the Availability of Onasemnogene Apeparvovec to Older Patients: The Evolving Treatment Landscape for Spinal Muscular Atrophy. Pharmaceutics. Jun 19 2023;15(6)doi:10.3390/pharmaceutics15061764

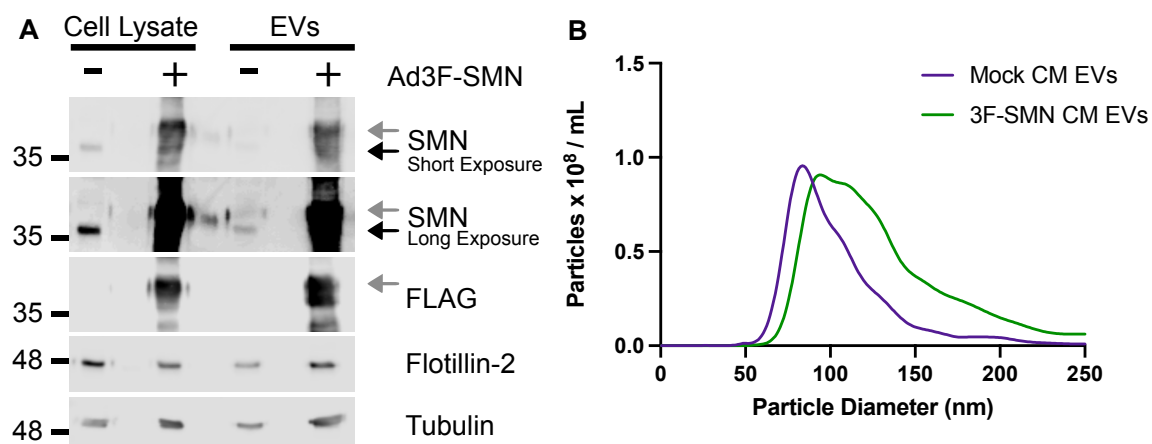
Appendix 3.3: Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency

- This article, of which I am first author, was published in the Cell Press journal Molecular Therapy Methods & Clinical Development. Authors are permitted to reuse the work as permitted by the Creative Commons CC BY-NC-ND 4.0 license.
- Citation: René CA, Parks RJ. Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency. Mol Ther Methods Clin Dev. Jun 13 2024;32(2):101259. doi:10.1016/j.omtm.2024.101259

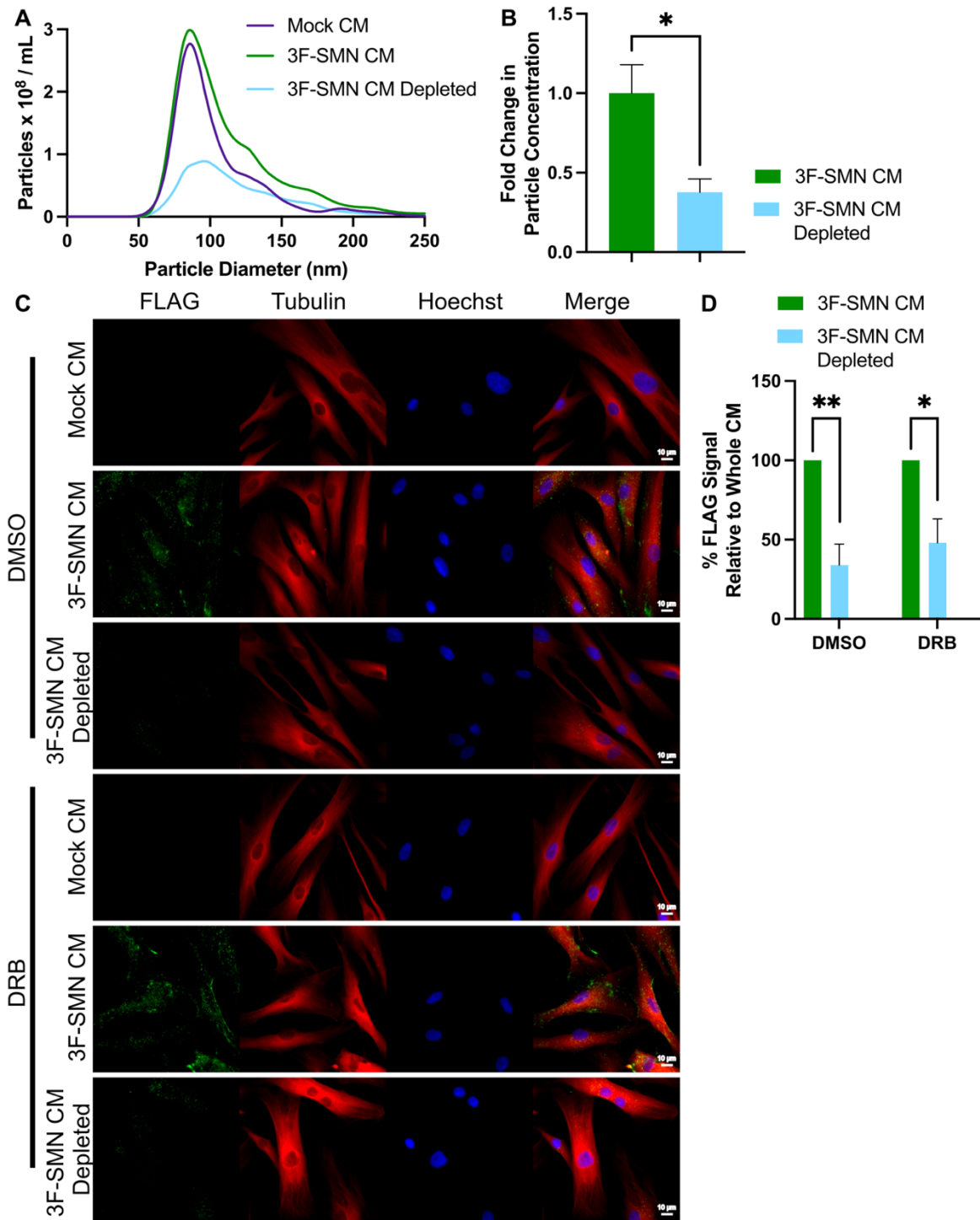
Appendix 2: Supplemental Data



Supplemental Figure S1. Characterization of fibroblast cell lines derived from an individual with SMA and healthy control. Panel A: The quantity of SMN protein in lysates from fibroblast cells derived from a healthy individual and a patient with SMA were assessed by immunoblot. Panel B: The SMN protein signal obtained in panel A was quantified and normalized to tubulin. Panel C: Epifluorescence microscopy images of fibroblast cells derived from a healthy individual and a patient with SMA, probed with antibodies to SMN and tubulin. White arrows indicate nuclear gems. Scale bars represent 10 μ m. Panel D: The number of SMN-positive nuclear gems in fibroblast cells derived from a healthy individual and a patient with SMA were enumerated and plotted per 100 cells by first counting the number of gems per nucleus in three independent fields of view each containing 15-25 cells, then converting to the number of nuclear gems per 100 cells for each field of view. The number of nuclear gems per 100 cells in the three independent fields of view was averaged and plotted.

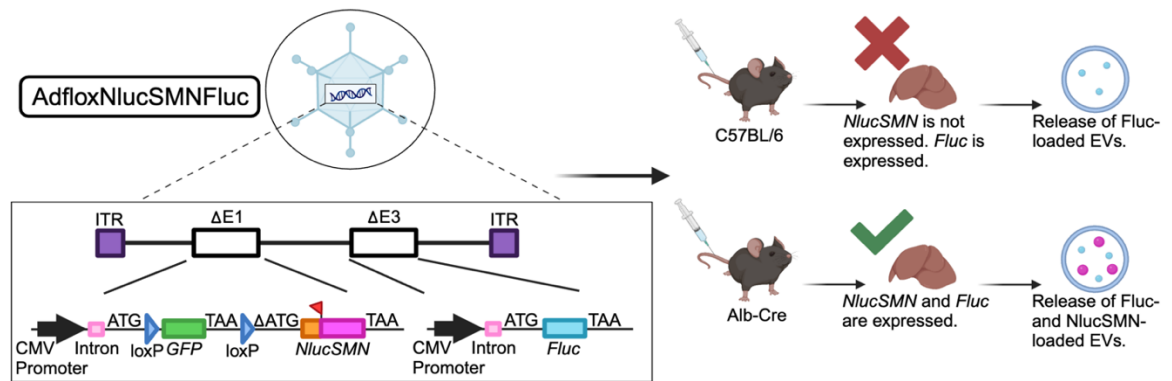


Supplemental Figure S2. Production and characterization of EVs loaded with 3F-SMN protein produced from Ad3F-SMN-transduced HepG2 cells. HepG2 cells were transduced with Ad3F-SMN at an MOI=200, or mock infected with PBS, and conditioned medium (CM) and cell lysates were collected 72 hpi. Panel A: To examine EV protein content, EVs were isolated from the CM by ultracentrifugation, and the 3F-SMN and Flotillin-2 protein, a common EV marker³⁴, was assessed by immunoblot analysis. The black arrow indicates endogenous SMN protein and the grey arrow indicates 3F-SMN protein. Immunoblotting with an SMN antibody revealed a band at approximately 38 kDa in both mock and Ad3F-SMN transduced cells, corresponding to the endogenous SMN protein. Additionally, a band at approximately 41 kDa, detected only in cells transduced with Ad3F-SMN and confirmed by FLAG immunoblotting, represented the vector-expressed 3F-SMN protein. A similar pattern was observed in EVs isolated from the cells. EVs isolated from medium from the parental cell line showed only the ~38 kDa endogenous protein, whereas EVs isolated from medium from the Ad3F-SMN-transduced cells showed bands of ~38 and ~41 kDa, the latter of which was reactive with the FLAG antibody. Panel B: A comparison of the particle diameter profile by nanoparticle tracking analysis (NTA) for EVs isolated from mock- or Ad3F-SMN-transduced cells yielded populations of particles ≤ 200 nm in diameter, which shifted slightly larger for EVs isolated from Ad-transduced cells, overall consistent with small EVs.

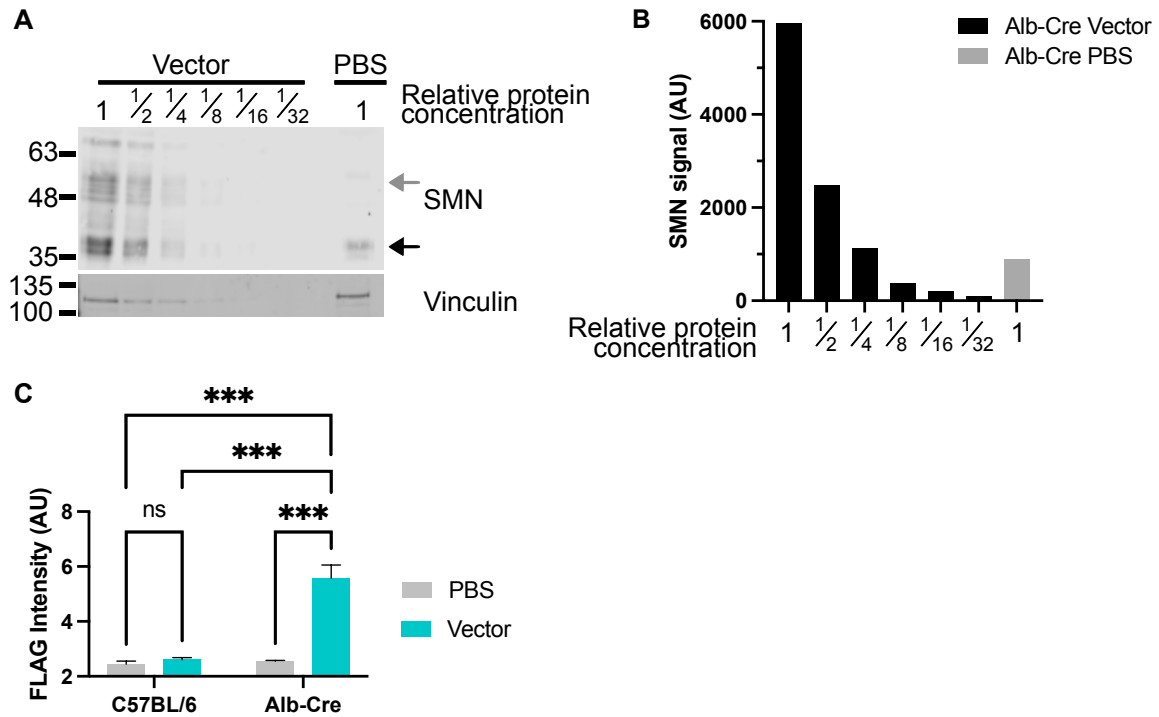


Supplemental Figure S3. 3F-SMN protein delivery to fibroblast cells is mediated by EVs. HepG2 cells were transduced with Ad3F-SMN at an MOI=200, or mock transduced (PBS), and conditioned medium (CM) was collected 72 h later. Cellular debris was removed by centrifugation. A portion of the CM was subjected to ultracentrifugation at 120 000 xg overnight at 4°C to pellet the EVs, and the resulting

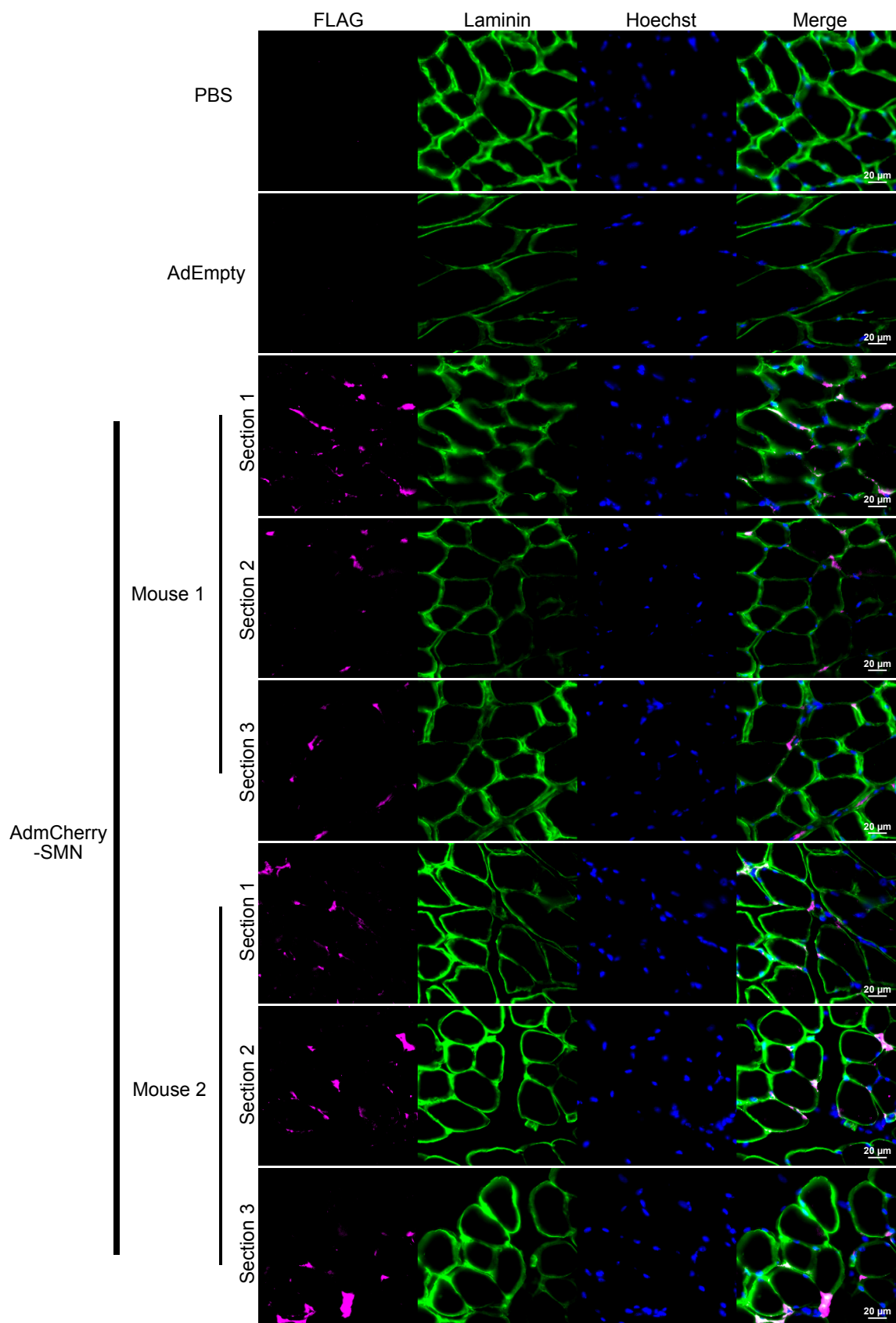
supernatant, depleted of EVs, was retained. Panel A: CM from mock-transduced cells, 3F-SMN CM and 3F-SMN CM depleted of EVs were analyzed by nanoparticle tracking analysis (NTA) for particle diameter and concentration. Panel B: Particle concentration, as assessed by NTA, was compared between 3F-SMN CM and 3F-SMN CM depleted of EVs, and fold change in particle concentration was plotted. Panel C: SMA fibroblasts were pretreated for 1 h with 100 μ M 5,6-dichloro-1- β -D-ribofuranosylbenzimidazole (DRB), or vehicle (dimethyl sulfoxide (DMSO)), then treated for 2 h with mock CM, 3F-SMN CM or 3F-SMN CM depleted of EVs in the presence or absence of DRB or vehicle (DMSO). Cells were processed for immunofluorescence staining with antibodies to FLAG and tubulin, and images were captured by epifluorescence microscopy. Scale bars represent 10 μ m. Panel D: FLAG signal intensity was quantified by CellProfiler from epifluorescence microscopy images shown in panel C. Data in panels B and D were tested for statistical significance by unpaired t-test, one asterisk (*) indicates $p < 0.05$, two asterisk (**) indicates $p < 0.01$. Data represents $n=3$.



Supplemental Figure S4. Schematic of *NlucSMN* and *Fluc* expression in liver tissue of C57BL/6 and Alb-Cre mice following tail vein injection of AdfloxNlucSMNFluc. Systemic administration of AdfloxNlucSMNFluc in C57BL/6 mice results in uptake of the vector primarily by hepatocytes, which gives rise only to expression of *Fluc*. Administration of AdfloxNlucSMNFluc to Alb-Cre mice results in uptake of the vector by hepatocytes which express Cre, resulting in excision of the floxed *GFP* from the vector, and subsequent activation of *NlucSMN* expression and protein production. Packaging of the NlucSMN protein into EVs results in distribution of the protein throughout the body of the mouse. Generated with BioRender.



Supplemental Figure S5. Quantification of expression of NlucSMN protein in the liver of Alb-Cre mice. Panel A. A liver protein lysate from an Alb-Cre mouse treated with 5×10^{12} vp/kg AdfloxNlucSMNFluc was serially diluted and the protein signal compared to lysate from an Alb-Cre mouse treated with PBS to estimate the level of over-expression of the vector-derived NlucSMN protein. The black arrow indicates endogenous SMN protein and the grey arrow indicates NlucSMN protein. Panel B: Quantified endogenous SMN and NlucSMN protein signals in panel A. Panel C: Liver tissue from the various treatment groups was sectioned and stained with antibody to FLAG, and imaged by epifluorescence microscopy. FLAG signal intensity in images was quantified by CellProfiler. Data was tested for statistical significance by two-way ANOVA, three asterisk (***) indicates $p < 0.001$. Data represents $n=3$.



Supplemental Figure S6. Visualization of liver-derived SMN protein in the TA

muscle of mice using immunofluorescence microscopy. Mice were treated with 5×10^{12} vp/kg of AdmCherry-SMN, AdEmpty, or PBS, and TA muscles were collected 72 h post-treatment. Muscle tissue sections were stained with anti-FLAG and anti-laminin antibodies and imaged by epifluorescence microscopy. Scale bars represent 20 μ m. Sections 1, 2, and 3 represent 6 μ m thick tissue sections collected ~100, 300, and 450 μ m from the proximal end of the TA muscle, respectively.

Appendix 3: Published Reviews

Appendix 3.1: Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles

Review

Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles

Charlotte A. René ^{1,2,3} and Robin J. Parks ^{1,2,3,4,*}¹ Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, ON K1H 8L6, Canada; chrene@ohri.ca² Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, ON K1N 6N5, Canada³ Centre for Neuromuscular Disease, University of Ottawa, Ottawa, ON K1N 6N5, Canada⁴ Department of Medicine, The Ottawa Hospital, Ottawa, ON K1H 8L6, Canada

* Correspondence: rparks@ohri.ca

Abstract: The central nervous system (CNS) is surrounded by the blood–brain barrier (BBB), a semipermeable border of endothelial cells that prevents pathogens, solutes and most molecules from non-selectively crossing into the CNS. Thus, the BBB acts to protect the CNS from potentially deleterious insults. Unfortunately, the BBB also frequently presents a significant barrier to therapies, impeding passage of drugs and biologicals to target cells within the CNS. This review provides an overview of different approaches to deliver therapeutics across the BBB, with an emphasis in extracellular vesicles as delivery vehicles to the CNS.

Keywords: central nervous system; blood–brain barrier; therapeutic; extracellular vesicles



Citation: René, C.A.; Parks, R.J. Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles. *Pharmaceutics* **2021**, *13*, 492. <https://doi.org/10.3390/pharmaceutics13040492>

Academic Editors: Winfried Neuhaus, Gert Fricker and Mária A. Deli

Received: 8 March 2021
Accepted: 30 March 2021
Published: 3 April 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

1. Introduction

The central nervous system (CNS) is comprised primarily of the brain and spinal cord (in addition to nerves of the olfactory and visual systems), and is tasked with interpreting, coordinating and executing most functions in the body. These functions include movement, sensation, speech, awareness, thought, and memory. Not surprisingly, given such a “central” role in orchestrating life, many of our most debilitating disorders are due to dysfunction of the CNS. Disorders of the CNS include vascular (e.g., stroke), infection (e.g., meningitis), structural (e.g., traumatic brain injury), functional (e.g., epilepsy) and degenerative (e.g., amyotrophic lateral sclerosis (ALS)). New approaches to the treatment of these varied disorders are a goal of many basic and clinical research labs.

The CNS is surrounded by a protective blockade, termed the blood–brain barrier (BBB)—or blood–spinal cord barrier (BSCB) in the case of the spinal cord—a highly selective, semipermeable border of endothelial cells (EC) that prevents pathogens, solutes and most molecules from non-selectively crossing into the CNS. While normally helping to protect the CNS, in the case of disease the BBB and BSCB also function as a “barrier” to therapies, and can prevent effective delivery of therapeutics to target cells or regions within the CNS. In the development of therapeutics for disorders of the CNS, achieving sufficient penetration of the BBB and BSCB is a major hurdle [1]. Since the BSCB shares many properties with the BBB, this review will focus on the BBB, but many of the principles also apply to the BSCB.

2. The Blood–Brain Barrier

The BBB is a complex and dynamic interface that regulates the movement of ions, molecules, and cells between the blood and the CNS [2]. Control of CNS homeostasis permits correct neuronal function, and also protects neural tissue from harm due to disease, inflammation, injury, toxins, and pathogens. The primary barrier in the BBB is EC, which

line the walls of capillaries that feed into the brain. These EC are connected through tight junctions (TJ) which restrict the passage of substances from the blood more selectively than EC elsewhere in the body. Indeed, EC in the CNS are quite distinct from those of the periphery in several ways. Specifically, EC of the CNS express BBB-specific proteins to control the entry and exit of metabolites across cells (transcellular pathway), possess highly electrical-resistant TJ to limit the flux of molecules between adjacent EC (paracellular pathway), and lack fenestrations (pores which are present in peripheral EC to allow rapid exchange of molecules between blood and tissue), all designed to limit the movement of molecules through the EC barrier [2,3].

EC are not the only cell type that contribute to BBB selectivity (Figure 1). Pericytes (PC) are embedded in the vascular basement membrane that surrounds EC [4]. CNS microvasculature has an EC:PC ratio of between 1:1 and 1:3, whereas vasculature found in, for example, striated muscle tissue has a ratio of approximately 100:1 [5]. PC are responsible for the regulation of capillary blood flow [6], by modulating capillary diameter through contraction-stimulated intracellular calcium ion (Ca^{2+}) concentration [7,8]. BBB PC function in part to maintain proper chemical composition of the surrounding environment, regulate transendothelial fluid transport and paracellular flow between cells, and also protect EC [9]. Astrocytes are major glial cells present at the abluminal side of the BBB, and help to relay signals to regulate blood flow in response to neuronal activity [10]. Neurons are present in close proximity to astrocytic end-feet at the abluminal side of the BBB, and also play a role in regulation of blood flow and microvascular permeability [11].

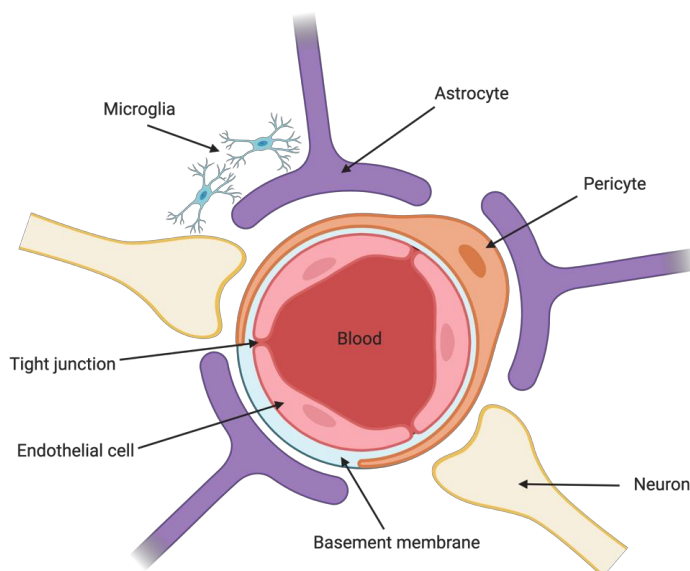


Figure 1. The blood–brain barrier. The BBB is formed by endothelial cells of the capillary wall, the dendrites of neurons and astrocyte end-feet ensheathing the capillary, and pericytes embedded in the capillary basement membrane.

While the BBB is very selective for what can pass through, the barrier is not completely impenetrable. Some small, lipophilic molecules are able to passively diffuse through EC to cross between the bloodstream and CNS [12,13] (Figure 2A). Water and some small hydrophilic molecules are able to use paracellular transport to cross the BBB [14] (Figure 2B). However, approximately 98% of all small molecules and almost all large molecules (those with a molecular weight greater than 1 kD), are unable to cross the BBB [15]. Most molecules that transit across the BBB do so by interacting with specific receptors and/or transporters

that are expressed on EC [16]. For example, small essential hydrophilic compounds, like glucose and amino acids, use transporters that are expressed on both the luminal (blood) and basolateral (brain) side of the EC [16]. Regulation of ion concentrations, including potassium (K^+), calcium (Ca^{2+}), and magnesium (Mg^{2+}), is accomplished through the presence of specific ion channels and transporters (Figure 2C) [17]. Large essential hydrophilic molecules, like hormones and lipoproteins, cross the BBB via endocytosis and transcytosis through use of specific receptors and transporters, respectively, that are highly expressed on the luminal side of EC [3] (Figure 2D,E). In short, the BBB is a very effective barrier that tightly regulates the passage of molecules into the CNS.

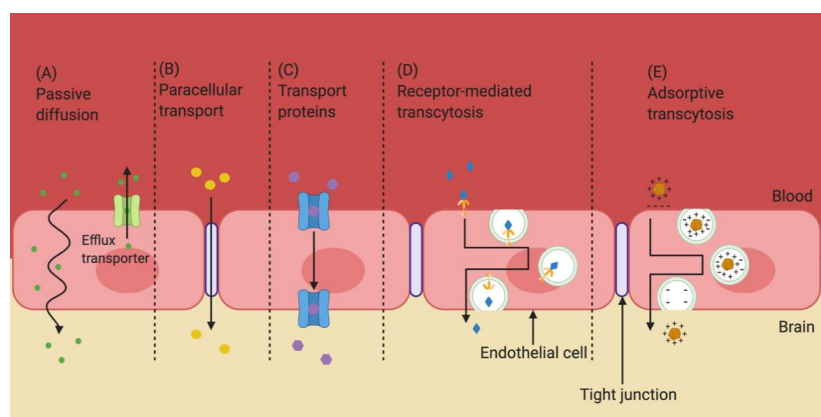


Figure 2. Transport routes across the blood–brain barrier. Molecules can cross the BBB using a variety of mechanisms. Lipophilic molecules of a low molecular weight can diffuse passively through EC (A), while hydrophilic molecules of a low molecular weight can pass between EC (B). Some solutes use transport proteins to cross the BBB (C), while others rely on receptor-mediated transcytosis (D) or adsorptive transcytosis (E). Adapted from [18], Drug Delivery and Translational Research. 2020.

3. Methods to Enhance Drug Delivery to the CNS

Invasive approaches are physical techniques that deliver a therapeutic to the CNS by mechanically breaching the BBB [19].

3.1. Invasive Approach

3.1.1. Direct Injection

Intra-cerebral injection is an invasive approach that relies on direct injection of a therapeutic into the brain, and subsequent diffusion of the therapeutic from the site of injection into surrounding regions. The injection site must be very precise for this method to be effective, as distribution of therapeutics within the brain by simple diffusion decreases significantly with distance [20]. For example, injection of chemotherapeutic agents into tumor resection cavities has been used in the treatment of brain tumors [21–24]. Direct injection of recombinant adeno-associated virus (AAV) expressing various factors has been studied for treatment of CNS disorders, such as Spinal Muscular Atrophy (SMA) [25], Niemann-Pick type C1 disease (NP-C) [26], Leigh Syndrome (LS) [27] in mouse models, as well as Parkinson’s disease (PD) in monkeys [28] and patients [29], and Alzheimer’s disease (AD) in mouse models [30,31]. Delivery to the cerebral ventricle of a self-complimentary AAV serotype 9 (AAV9) encoding a codon-optimized human survival motor neuron 1 gene (*SMN1*) led to a dose-dependent rescue of lifespan and growth [25]. Similarly, direct injection of an AAV9 encoding a human intracellular cholesterol transporter 1 gene (*NCPI*) improved lifespan, locomotor function, and disease pathology in a mouse model of NP-C [26]. The relative efficacy of direct injection into cerebral ventricles versus systemic intravenous (IV) delivery was compared in a mouse model of LS, using an therapeutic

AAV2/9 encoding the human ubiquinone oxidoreductase iron-sulfur protein 4 (*NDUFS4*) gene [27]. This study concluded that systemic IV administration was able to correct disease pathology only in peripheral organs, whereas direct injection was able to partially improve disease pathology only in the brain, and neither treatment was able to improve the lifespan of the mice. [27]. However, treatment with both methods of administration was able to significantly improve lifespan and disease pathology.

3.1.2. Intracerebroventricular Infusion

Intracerebroventricular (ICV) infusions involve prolonged administration of a therapeutic substance directly into the CSF contained in the cerebral ventricle. Administration can be achieved through use of an implanted osmotic pump or IV catheter. ICV infusions can occur over the course of several days or weeks, and delivery to the brain parenchyma is accomplished by simple diffusion [32–34].

ICV infusion of dopamine or dopamine agonists via a catheter implanted in the cerebral ventricle in a rat model of PD were found to restore dopamine levels in the CNS [35]. Similarly, ICV infusion of these compounds restored normal behavior patterns in monkeys that had been rendered akinetic through treatment with 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [35]. However, the ICV infusion was complicated due to intolerance to the pump, or frequent disconnection of the pump from the catheter [35]. Infusion of recombinant human platelet derived growth factor-BB (rhPDGF-BB) over the course of two weeks in rats with 6-hydroxydopamine-induced nigrostriatal lesions resulted in the restoration of striatal dopamine transporter binding sites and expression of nigral tyrosine hydrolase (TH), and normalized amphetamine-induced rotational behavior [36]. ICV infusion of rhPDGF-BB was used safely in a human clinical trial for treatment of PD, reducing Parkinsonian symptoms and may have increased the integrity and function of dopaminergic neurons [37]. ICV infusion of anti- β -secretase (BACE1) in non-human primates resulted in a sustained reduction of 70% of amyloid- β peptides in the CSF, suggesting a possible therapeutic avenue for neurodegenerative disorders such as AD and PD [38]. Unfortunately, ICV infusions are not an efficient method of achieving brain-wide drug delivery, but may be applicable to cases in which the target region is in close proximity to the cerebral ventricles [20].

3.1.3. Convection Enhanced Delivery

Convection enhanced delivery (CED) seeks to increase the overall area of the brain that receives exposure to a therapeutic. CED involves stereotactically guided insertion of a cannula into the brain parenchyma, followed by active pumping of a drug through the cannula over several hours or days [39]. CED is thus powered by the pressure gradient created through use of the pump, which allows for greater dispersion of a therapeutic relative to standard diffusion [40–42]. CED was used in a phase 1 clinical trial to investigate the efficacy of [124 I]-labelled 8H9 murine monoclonal antibody (MAb) for treatment of diffuse intrinsic pontine glioma in children [43]. 8H9 binds B7-H3, a surface antigen and immune modulator of natural killer and T cells, and is overexpressed in most high-grade gliomas [44]. CED delivery of [124 I]-labelled 8H9 was deemed safe, with no treatment-related deaths or grade 4 adverse events, and patient survival was extended, although this latter point may have been confounded by potential patient selection bias [43]. Limitations of CED include that optimal therapeutic delivery is contingent on proper placement of the catheter, and that some areas of the brain are difficult to saturate fully with a therapeutic by CED, particularly areas surrounding a cavity [45]. Some studies comparing CED to direct injection have noted greater distribution and efficacy with bolus injection [24,46].

3.1.4. Barrier Disruption

The BBB can also be physically breached by disrupting the connections between EC, causing leakage between cells [17]. One method to compromise the integrity of the BBB is through osmotic disruption, in which EC shrink due to application of osmotic shock,

thereby increasing the gap between cells [16]. Pretreatment by carotid artery injection of 25% mannitol, to induce osmotic shock and disruption of the BBB, led to significantly enhanced uptake of hydrophobically modified siRNA (hsiRNA) and subsequent target-gene silencing in rat brain [47].

Magnetic resonance imaging (MRI)-guided focused ultrasound can also be used to disrupt the BBB [16]. In animal models of cancer, disruption of the BBB using MRI-guided focused ultrasound enhanced delivery of chemotherapeutic agents such as bevacizumab, temozolomide (TMZ), and doxorubicin (DOX), slowing tumor progression and improving survival [48]. Focused ultrasound combined with viral microbubbles carrying brain-derived neurotrophic factor (BDNF) have been explored as a potential therapeutic in a rat model of AD [49]. However, this approach requires that the animal/patient is anesthetized and can be costly, and neurons can suffer permanent damage due to influx of blood component into the brain [50,51].

3.2. Lipophilic Approach

Lipophilic approaches to enhance drug delivery to the CNS involve modification of drugs at the molecular level to increase passive movement across the BBB [19]. For example, reducing the relative number of polar groups in a drug increases its transport across the BBB [52]. Covalent addition of a 1-methyl-1,4-dihydronicotinate moiety to the hydroxymethyl group of ganciclovir enhanced delivery to the brain for treatment of cytomegalovirus-induced encephalitis [53]. Ganciclovir is a prodrug and must be converted to an active form for efficacy. Direct-acting compounds can also be modified to enhance passage across the BBB, such as use of pyrrolopyrimidines to modify antioxidants to increase their ability to reach target cells in the CNS [54]. However, although these molecular changes can enhance the ability of compounds to cross the BBB, the modifications may also alter the functionality of the compound, thereby reducing effectiveness [19].

3.3. Physiological Approach

3.3.1. Receptor-Mediated Transcytosis

Large molecules that require access to the brain typically utilize receptor-mediated transcytosis (Figure 3), a multi-step process involving specific receptors expressed on the EC, such as transferrin receptors [55,56], insulin receptors [57,58], and low-density lipoprotein (LDL) receptor related proteins (LRP) [59]. The process of receptor-mediated transcytosis, also called clathrin-mediated endocytosis, begins with engagement of ligand with the receptor located on the luminal side of the BBB, followed by receptor-mediated endocytosis of the ligand-receptor complex into the EC, forming a clathrin-coated vesicle within the cell. This vesicle then fuses with the early endosome in the cytoplasm of the EC. Acidification of the endosome results in dissociation of the biomolecule from the receptor [60]. The acidified endosome then transits to the abluminal side of the EC, and fusion of the vesicle with the cell membrane releases the biomolecule into the CNS [20,61].

Transferrin receptors are responsible for facilitating transcytosis of transferrin-coupled iron to the brain parenchyma [20]. Drugs can be targeted to the transferrin receptors through conjugation to transferrin or antibodies (Ab) specific to the transferrin receptor in human tissue culture [62,63], rats [64], and primates [63]. Several studies have used this approach to delivery therapeutics to the CNS, including vasoactive intestinal peptide (VIP) [65], epidermal growth factor (EGF) [66], amyloid β 1–40 peptide (A β 1-40) [67], and β -galactosidase [68]. Recently, this approach has been used in clinical trials to lower levels of heparin sulfate in CSF, showing promise as a treatment for Hunter syndrome [69]. Indeed, several companies have now advanced transferrin-conjugated therapeutics into clinical trial for treatment of a variety of CNS disorders [69]. A similar approach has used Ab to the insulin receptor in primate studies to enhance CNS delivery of BDNF, fibroblast growth factor-2 (FGF-2), siRNA, and neurotrophin [70].

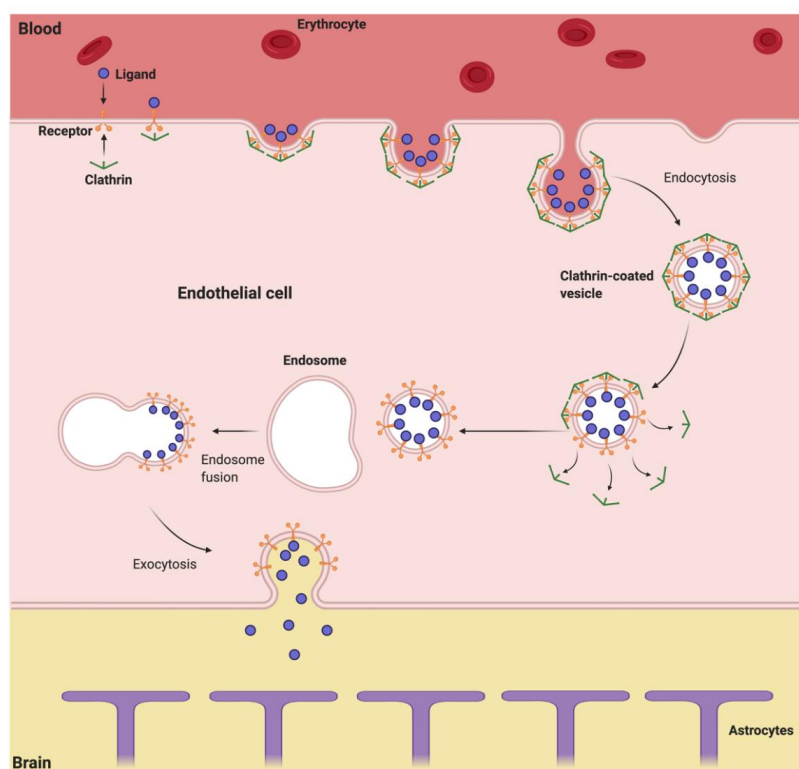


Figure 3. Receptor-mediated transcytosis. The ligand engages with the receptor at the luminal side of the BBB, followed by receptor-mediated endocytosis of the receptor-ligand complex into the EC, forming a clathrin-coated vesicle. The vesicle fuses with the endosome, after which the vesicle undergoes exocytosis, and the biomolecule is released on the abluminal side of the BBB.

The LDL receptor related proteins 1 and 2 (LRP-1 and -2) have also been used to transport target drugs to the CNS. LRP is a multi-ligand, transmembrane protein that is expressed on many cells of the CNS, including EC [59], neurons [71,72], and astrocytes [72]. LRP naturally transports apolipoprotein E and B into the CNS, and it is hypothesized that some therapeutic nanoparticles may naturally preferentially bind these apolipoproteins, which facilitates transcytosis via LRP [73]. Alternatively, fusion proteins can be created that specifically bind LRP, thus facilitating uptake. For example, fusion of the lysosomal enzyme glucocerebrosidase to the low-density lipoprotein receptor-binding domain of apolipoprotein B resulted in effective delivery of the protein to the CNS by LRP-mediated transcytosis in mice [74].

Rabies virus naturally infects neurons [75], and a peptide derived from the rabies virus glycoprotein (RVG), which binds the nicotinic acetylcholine receptor, was shown to facilitate delivery of therapeutic siRNA to the brain following systemic delivery [76]. In this approach, severe combined immunodeficient (SCID) mice were infected with Japanese Encephalitis Virus (JEV) and subsequently treated with IV injection of JEV-directed siRNA (siFvE^J) complexed with the RVG peptide. Treated mice showed 80% survival, compared to 100% lethality in untreated mice, suggesting that siFvE^J/RVG-peptide may be an effective therapeutic for treatment of JEV-induced encephalitis.

Innovative formulation technologies, such as nanoparticles that are engineered to bind receptors on the BBB, show promise as an effective platform for delivery of therapeutics to the CNS [77]. Nanoparticles bonded to transferrin or MAb against transferrin receptors

were used to successfully deliver loperamide, a model drug, into the brain [78]. Receptor-mediated transcytosis can also be used to transport plasmid DNA across the BBB [79]. For example, liposome-encapsulated DNA can be coated with polyethylene glycol (PEG), and then conjugated with MAb specific for an appropriate receptor, creating pegylated immunoliposomes (PIL) [80]. IV administration of a PIL-encapsulated plasmid encoding TH under regulation by the glial fibrillary acidic protein (GFAP) promoter resulted in an 82% reduction in apomorphine-induced rotation in a rat model of PD [81]. PIL conjugated to a murine MAb that binds the human insulin receptor were used to deliver a human EGFR antisense gene to treat a U87 human glioma cell xenograft brain tumor mouse model of cancer, and resulted in a 70–80% inhibition in cancer cell growth [82].

Although promising, receptor-mediated transcytosis does have potential drawbacks. Some studies have shown that nanoparticles have a tendency to accumulate in brain capillary EC, which reduces their effective delivery to the target tissue in rats [83,84]. As there are a finite number of receptors on both the luminal and abluminal sides of the BBB, receptor-mediated transcytosis is a saturable process [85], which may limit the concentration of the delivered drug that can be achieved in the brain parenchyma [86,87]. Finally, there is the concern of potential immunogenicity, or even toxicity, due to use of non-human Ab [88].

3.3.2. Transporter-Mediated Processes

Drugs that closely resemble a molecule that naturally transits the BBB by a transporter are able to take advantage of this transporter system [20]. For example, L-Dopa naturally has high affinity for the amino acid transporter system L [89], and has been used to deliver L-Dopa to treat PD [90]. Alternatively, a drug can be conjugated to a molecule naturally transported through this system, thus allowing co-transport of the drug. A plasmid encoding human tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) was complexed with DOX, and subsequently conjugated to a choline derivative, for transport by the choline transporter [88]. The resulting complex showed greater uptake in human neuronal glioblastoma-astrocytoma U87 MG cells and enhanced ability to induce apoptosis in vivo and in vitro compared to each component alone. To use a transporter protein to enhance drug delivery through the BBB, the structural binding requirements of the transporter and possible deleterious effects due to modification of the therapeutic compound must be considered [16].

Once in the CNS, inhibition of efflux transports can be used to maintain levels of the drug. The antibiotic cefadroxil is a substrate for multiple transporters present at the BBB, such as organic anion transporters (OATs) [91,92], organic anion transporting polypeptides (OATPs) [93], multidrug resistance-associated proteins (MRPs) [94,95], and peptide transporter 2 (PEPT2) [96], some of which can efflux the drug. Inhibition of OATs, OATPs, and MRPs with probenecid significantly increased the concentration of cefadroxil, a model drug, in brain extracellular fluid due to inhibition of cefadroxil efflux at the BBB [97]. Brain slice experiments indicated that PEPT2 is involved in uptake of cefadroxil, however inhibiting PEPT2 with Ala-Ala did not increase brain ECF cefadroxil levels.

3.3.3. Adsorptive Mediated Endocytosis

Adsorptive mediated endocytosis is triggered by the uptake of cationic molecules into endocytic vesicles on the luminal surface of the BBB, and subsequent fusion of the vesicles at the abluminal membrane, resulting in release of the endocytic contents into the brain parenchyma. Compounds such as avidin, histone, and protamine rely on adsorptive mediated endocytosis to cross the BBB [98,99]. As adsorptive mediated endocytosis requires a substance to be cationic to facilitate binding of the molecule to the negatively charged cell surface, cationizing can allow other molecules to utilize this transport process to enter the CNS. Chemical cationization of a protein is possible through the amidation of carboxylic acid groups on the protein [100]. Covalent addition of hexamethylenediamine to anti-tetanus Ab fragments cationized the fragments and allowed entry of the molecule into the

CNS [101]. This cationization did not alter the affinity of the Ab fragment for the tetanus toxin *in vitro*, and showed promise as a therapeutic strategy for treatment of tetanus. Similarly, putrescine-modified catalase was shown to increase the ability of catalase to cross the BBB in mouse models of ALS [102,103]. Limitations of chemical cationization include potential deleterious effects of the modification on protein function, and formation of immune responses against the modified protein [104]. There is also the concern of inducing immune complex-mediated glomerulonephritis, as cationic antigens and immune complexes containing cationic Ab can deposit in the glomeruli [105].

An alternative to chemical cationization is through the use of cell-penetrating peptides (CPP). CPP are small peptides with positive charge that are derived from proteins that have a natural ability to bind to and cross the cell membrane, such as the human immunodeficiency virus (HIV) transactivator of transcription (Tat) protein, first described in a human leiomyosarcoma cell line, SK-LSM [106]. CPP can be attached to biomolecules or to the surface of nanoparticles, and mediate delivery to the CNS. This approach was used to deliver ciprofloxacin, a model antibiotic, across the BBB as a potential treatment for infections of the CNS [107]. In another study, the surface of chitosan (CS)-coated nanostructured lipid carriers (NLC) were modified with Tat (CS-NLC-Tat), and used to deliver glial cell-derived neurotrophic factor (GDNF) (CS-NLC-Tat-GDNF) to a mouse model of PD [108]. Treated mice exhibited significant motor recovery, as well as increased numbers of TH+ fibers in the striatum and TH+ neurons in the substantia nigra, indicating that intranasal delivery of CS-NLC-Tat-GDNF may be a promising therapy for treatment of PD [108]. However, use of CPP have raised some concerns due to toxicity, as Tat can induce toxicity in cells in culture [109] and in rat neurons [110] and can also induce apoptosis of EC [111].

4. Extracellular Vesicles as Delivery Vehicles to the CNS

Many groups have shown that extracellular vesicles (EV) have a natural ability to transit the BBB and deliver biomolecules to the CNS. EV are tiny, membrane bound particles released from all cell types and found in all bodily fluids [112]. Although originally thought to represent cellular waste, EV actually perform an array of physiological functions in the body [113]. EV are naturally involved in intercellular communication, transferring proteins, lipids, and nucleic acids to recipient cells which may be located some distance from the site of EV origin [114]. Specific function and physical composition of the vesicle depends on the type of cell from which the EV originates [112].

4.1. EV Biogenesis

There are three main subtypes of EV that are differentiated based upon their biogenesis, release pathways, size, content, and function; exosomes, microvesicles (MV), and apoptotic bodies (Figure 4). Exosomes are the smallest, with vesicles having a diameter of between 40–100 nm [115]. Exosomes are formed within the endosomal network in multivesicular bodies (MVB) and subsequently released into the extracellular environment after fusion of the MVB with the plasma membrane [116]. MV are slightly larger, generally considered to be 100–1000 nm in diameter, and are generated by budding at the plasma membrane [112,115]. The final class of EV are apoptotic bodies, which form through blebbing from the plasma membrane of dying cells. Apoptotic bodies are the largest classification of EV, with a diameter over 1000 nm [115].

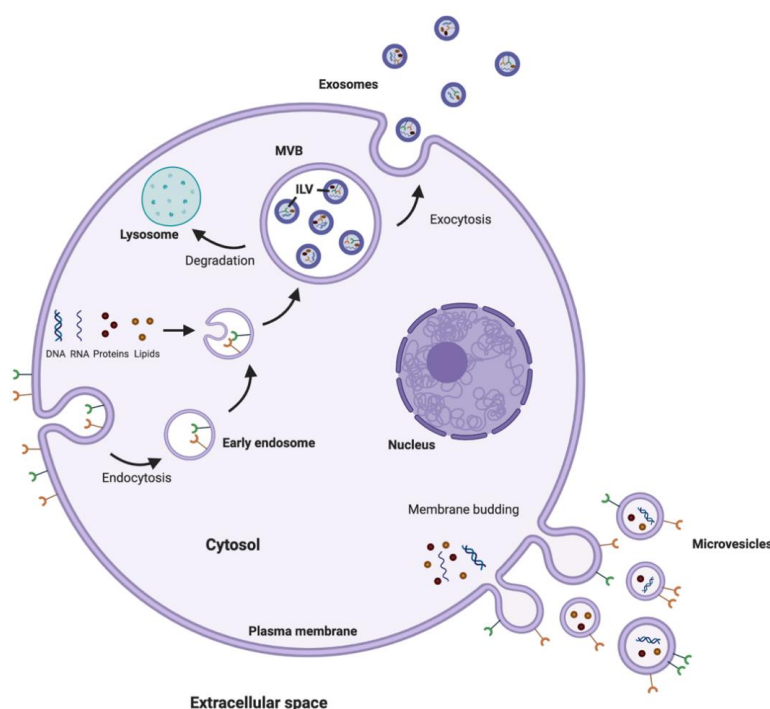


Figure 4. Biogenesis of microvesicles and exosomes. Microvesicles are generated by budding of the plasma membrane. Exosomes are formed through the endosomal network and released into the extracellular space after the MVB containing intraluminal vesicles (ILV) fuses with the plasma membrane.

Exosomes and MV are continually released under normal conditions but their release is upregulated under times of stress [112]. This enhanced release of EV may function as a method of eliminating waste and potentially toxic cellular contents from the cell, as well as a way to communicate intracellular stress to nearby or distant cells [115]. Obviously, apoptotic bodies are only released as a result of severe and terminal cellular insult.

Knowing which class of EV is used in a particular study can be somewhat challenging. Current methods of isolation are not capable of fully separating the various classes of EV, only enriching for certain size populations (e.g., “large” versus “small” EV). Moreover, the lack of standard methods for isolating EV makes it difficult to know what class of EV was actually used or to compare between studies. Finally, the term “exosome” is frequently used synonymously with EV, regardless of particle size. The International Society of Extracellular Vesicles has attempted to clarify some of these points with strong guidelines for EV terminology, isolation and characterization [117]. In the studies described below, we will utilize the terminology used in the cited work.

4.2. EV Uptake and Mechanism of Transit Across the BBB

As mentioned, one of the functions of EV appears to be to transmit signals between cells located both near and far from the cell of origin. Once bound to the plasma membrane of the target cell, EV can dissociate, remain stably associated, fuse with the plasma membrane, or be internalized by the cell. The mechanism of uptake of EV by a target cell is dependent on the recipient cell type [112], and can include endocytosis, clathrin-dependent endocytosis, caveolin-dependent endocytosis, phagocytosis, and macropinocytosis [118].

The mechanism that EV use to cross the BBB has yet to be fully elucidated. In an *in vitro* model of the BBB, purified exosomes appeared to be internalized by EC via endocytosis, either by a clathrin- or caveolin-dependent pathway, and subsequently trafficked

via endocytic mechanisms [119]. Interestingly, treatment of this model with the pro-inflammatory cytokine TNF- α , to mimic stroke-like conditions, increased the permeability of the EC monolayer, allowing passage of the EV by a transcellular route [119]. In the absence of TNF- α , EV were not found in the abluminal side of the EC. Thus, the route or efficiency of EV passage through the BBB may be strongly influenced by the inflammatory state of the host.

Transit of EV across the BBB has also been examined using tumor-derived EV [120,121]. Administration of purified MDA-MB-231 breast cancer cell-derived EV to an in vitro model of the BBB, or following cardiac injection of zebrafish in vivo, showed that EV cross the brain endothelium via transcytosis [120]. In this system, transcytosis appeared to involve caveolin-independent, clathrin-dependent endocytosis. In an in vitro study examining the mechanism of binding and internalization of exosomes derived from a brain-metastatic melanoma cell line (SK-Mel-28) in human BBB-derived EC (hCMEC/D3 cells), CD46 was determined to be a major receptor for exosome uptake in this cell type [121].

4.3. Therapeutic Applications

EV have a number of advantages as biotherapeutics [122]. Cells release EV continuously, and thus large quantities of therapeutic EV can be produced relatively easily. EV are also stable ex vivo, allowing for easy storage of therapeutic stocks. EV are resistant to breakdown in the bloodstream, and the lipid bilayer thus protects cargo from degradation. The hydrophilic shell of EV contain anti-phagocytosis surface markers, such as CD47, that enable the vesicles to evade phagocytosis by macrophages and monocytes, limiting their clearance by the reticulo-endothelial system [123,124]. Therapeutic EV induce minimal immunogenicity and toxicity in vitro and in vivo [125,126]. Finally, as mentioned, EV that are in circulation are capable of crossing the BBB, and are able to move both into and out of the CNS [122].

EV can be loaded with therapeutic cargo using either exogenous or endogenous loading. In exogenous loading, EV are first isolated and purified, and then compounds are incorporated through methods such as co-incubation, electroporation, permeabilization, freeze-thaw cycles, extrusion, or sonication [127–129]. In endogenous loading, the cells that will ultimately release the EV are manipulated, for example through engineering to overexpress a particular therapeutic protein, so that the protein is incorporated in the released EV [130,131]. EV can also be modified to enhance uptake by cells of the CNS. Expression of a fusion protein comprised of Lamp2b (normally found in the membrane of exosomes) with an RVG peptide allows presentation of the peptide on the surface of the exosomes, resulting in enhanced targeting and uptake by neurons [132].

4.3.1. EV as a Vehicle for Delivery of Small Molecules

Curcumin (Cur) is well known for its anti-inflammatory, anti-cancer, anti-microbial, anti-diabetic, and neuroprotective properties [133–138]. Unfortunately, Cur shows poor stability and bioavailability, which has limited its therapeutic use. In a mouse model of LPS-induced encephalitis, Cur loaded into exosomes showed increased stability and bioavailability compared to Cur alone, thus providing a greater protective effect in vivo [128]. To prepare the therapeutic EV, Cur was mixed with murine lymphoma cell (EL4)-derived exosomes and subjected to a sucrose concentration gradient to load Cur into the exosomes. Use of a concentration gradient alters the osmolarity of the exosomes, allowing a compound of interest, such as Cur, to flow into the exosomes. When administered intranasally, Cur-loaded exosomes induced microglial apoptosis, which in turn led to a decrease in inflammation in the brain [128]. Intranasal administration of exosomes loaded with either Cur or JSI124, an inhibitor of Stat3 signaling, protected mice against LPS-induced brain inflammation, and showed beneficial effects in an model of autoimmune encephalomyelitis (EAE) and a GL26 tumor model [133].

Drug-loaded exosomes have also been used for a combined imaging and therapeutic application. In this study, exosomes were loaded with Cur and superparamagnetic

iron oxide nanoparticles (SPION) by electroporation. SPION can function as a negative contrast agent for MRI, as well as provide a therapeutic effect through magnetic fluid hyperthermia (MFH) [139,140]. The resulting Cur/SPION exosomes were then conjugated with a neuropilin-1-targeting peptide (RGE) (RGE-Exo-SPION/Cur), to facilitate targeting to glioma cells [139]. Following tail vein delivery in a mouse model of glioma, the RGE-Exo-SPION/Cur allowed for targeted imaging of glioma tumors, which may aid in early detection of the disease. Importantly, administration of RGE-Exo-SPION/Cur significantly inhibited tumor growth, and appeared to act synergistically compared to RGE-Exo-SPION or RGE-Exo-Cur alone [139].

IV delivery of mesenchymal stromal cell (MSC)-derived exosomes conjugated to cyclo(Arg-Gly-Asp-D-Tyr-Lys) (c(RGDyK)), a peptide that has high affinity for $\alpha_v\beta_3$, was shown to target the lesion region in an ischemic brain [141]. These exosomes were loaded with Cur and administered to a transient middle cerebral artery occlusion (MCAO) mouse model of ischemic stroke. Administration of these therapeutic exosomes significantly suppressed the inflammatory response and reduced cellular apoptosis in the lesion region. Results of this study suggest that targeted exosomes loaded with Cur may serve as an effective treatment following ischemic stroke.

Exosomes have also been explored as a delivery vehicle for anti-cancer drugs to the brain. Exosomes isolated from brain endothelial bEND.2 cells were loaded with DOX by simple coincubation [142]. Zebrafish embryos that had been injected in the brain ventricle with U87 MG tumor cells were treated with the therapeutic EV by cardiac vein injection. Embryos treated with these therapeutic EV exhibited reduced tumor cell growth compared to embryos treated with the drug alone or buffer control. Exosome-delivered DOX was also found to significantly suppress vascular endothelial growth factor (VEGF) mRNA levels within the tumor, a surrogate marker of cancer growth [142]. Similarly, human endometrial stem cell-derived exosomes loaded with atorvastatin were effective in inducing apoptosis in U87 cells and also in inhibiting tumor growth in a 3D glioblastoma model [143].

4.3.2. EV as a Vehicle for Delivery of Nucleic Acids

EV can also be used to deliver therapeutic nucleic acids to the CNS. Coincubation of hsiRNAs targeted to Huntingtin mRNA (hsiRNA^{HTT}) with purified exosomes resulted in efficient loading [144]. hsiRNA^{HTT}-loaded exosomes were administered by ICV infusion over the course of 7 days, and were effective in reducing Huntingtin mRNA levels by up to 35% in the mouse brain. Cells engineered to overexpress a specific miRNA can also be used as a source of therapeutic EV. miR-133b is known to promote neurovascular plasticity [145,146], MSC transduced with a lentiviral vector to overexpress miR-133b released exosomes with increased levels of the miRNA [147]. Intra-arterially delivery of miR-133b-containing exosomes to a middle cerebral artery occlusion (MCAO) model of stroke in rats significantly improved functional recovery and increased neural plasticity. Similarly, exosomes isolated from EPC engineered to express elevated levels of miR-137 were able to provide a neuroprotective effect against apoptosis and mitochondrial dysfunction in SH-SY5Y human neuroblastoma cells [148]. Finally, stimulation of dendritic cells (DC) with IFN- γ causes release of EV enriched with miRNA-219 which, when applied intranasally, reduced oxidative stress and regulated oligodendrocyte remyelination in vivo [149], suggesting a novel therapeutic for multiple sclerosis.

As mentioned, presentation of the RVG peptide on the surface of EV can enhance homing of systemically delivered EV to the brain [132]. Electroporation-mediated loading of these CNS-targeted exosomes with siRNA to BACE1, a therapeutic target in AD, resulted in knockdown of BACE1 mRNA (60%) and protein (62%) in the brain of mice [132]. Similarly, systemic delivery of RVG-targeted exosomes loaded with siRNA to α -synuclein to a mouse model of PD resulted in a decrease in α -synuclein by 84%, and decreased α -syn-mRNA by 49% in the midbrain, 56% in the striatum, and 50% in the cortex [150].

Circular RNA (circRNA) have covalently connected 5' and 3' terminal ends, creating a closed long non-coding RNA (lncRNA) [151]. CircSHOC2, a circRNA transcribed from

the SHOC2 gene, delivered in exosomes suppressed apoptosis and ameliorated neuronal damage in an ischemic stroke model by acting through the miR-7670-3p/SIRT1 pathway to regulate autophagy [152]. In this study, ischemic-preconditioned astrocyte-derived exosomes (IPAS-Exo) were used, as these exosomes were found to have significantly increased levels of CircSHOC2 caused by ischemic treatment.

4.3.3. EV as a Vehicle for Delivery of Therapeutic Proteins

EV can also be used to deliver therapeutic proteins. Delivery of exosomes loaded with catalase promoted neuroprotective effects following intra-nasal delivery in a mouse model of PD [129]. Exosomes were loaded with catalase by several different methods, including sonication, freeze–thaw, extrusion, permeabilization with saponin, or co-incubation at room temperature. Exosomes loaded through sonication, extrusion, or saponin permeabilization were found to have the most significant loading and release of catalase. Administration of the catalase-loaded exosomes provided a significant neuroprotective effect in a mouse model of PD, with exosomes loaded utilizing saponin permeabilization providing the greatest effect. More recently, EV were used to deliver Neprilysin (NEP), an enzyme involved in the clearance of aggregated β -amyloid sheets in the brain [153,154]. Bone marrow-derived MSCs were used as a source of the EV, and NEP was loaded into the EV by freeze–thaw cycles, which interrupts the integrity of the EV membrane allowing internalization of NEP. Intranasal delivery of NEP-EV in a rat model of AD resulted in an increase in expression of B cell leukemia/lymphoma 2 (BCL2), an anti-apoptotic factor, and also a decrease in expression of both IL-1 β , an inflammation factor, and BCL2-associated X protein (BAX), a pro-apoptotic factor, in the rat brain, overall improving brain-related behavioural function [153].

More recently, ARMMs, a new class of EV [155], have been explored as a potential therapeutic vehicle. A HEK293T-based cell line was generated that stably expressed a fusion of the tumor-suppressor p53 protein to ARRDC1 (a protein naturally loaded into ARMMs), resulting in enhanced loading of the p53 fusion protein [155,156]. IV delivery of ARRDC1-p53 ARMMs resulted in protein delivery to multiple tissues, and induced significant DNA damage-dependent apoptosis in the thymus and spleen in p53-null mice. This approach may represent a novel therapeutic against many cancers in which the function of p53 is compromised [156].

5. Conclusions

In this review, current approaches for delivering therapeutics across the BBB and into the CNS have been discussed. Physiological, lipophilic, and invasive methods can effectively deliver therapeutics across the BBB, but each is associated with technical problems or safety concerns. EV have gained attention in the last several years as possible delivery vehicles for therapeutic compounds, including nucleic acids, proteins, and small molecule drugs. EV have shown great promise for delivery to the CNS in varied models of disease, and it is hoped that continued development and refinement in EV technology will further improve their utility for treating diseases of the CNS.

Author Contributions: Conceptualization, C.A.R.; literature search, C.A.R.; resources, R.J.P.; writing—original draft, C.A.R.; writing—review and editing, C.A.R., R.J.P.; supervision, R.J.P.; funding acquisition, R.J.P.; project administration, R.J.P. All authors have read and agreed to the published version of the manuscript.

Funding: Research in the Parks laboratory is supported by grants to RJP from the Canadian Institutes of Health Research (MOP-136898, MOP-142316) and the Natural Sciences and Engineering Research Council (RGPIN-2014-04810, RGPIN-2019-04786).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Acknowledgments: Figures created with BioRender.com.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Banks, W.A. From blood-brain barrier to blood-brain interface: New opportunities for CNS drug delivery. *Nat. Rev. Drug. Discov.* **2016**, *15*, 275–292. [[CrossRef](#)] [[PubMed](#)]
2. Daneman, R.; Prat, A. The blood-brain barrier. *Cold Spring Harb. Perspect. Biol.* **2015**, *7*, a020412. [[CrossRef](#)]
3. Saunders, N.R.; Liddelow, S.A.; Dziegielewska, K.M. Barrier mechanisms in the developing brain. *Front. Pharmacol.* **2012**, *3*, 46. [[CrossRef](#)] [[PubMed](#)]
4. Sims, D.E. The pericyte—A review. *Tissue Cell* **1986**, *18*, 153–174. [[CrossRef](#)]
5. Shepro, D.; Morel, N.M. Pericyte physiology. *FASEB J.* **1993**, *7*, 1031–1038. [[CrossRef](#)]
6. Hamilton, N.B.; Attwell, D.; Hall, C.N. Pericyte-mediated regulation of capillary diameter: A component of neurovascular coupling in health and disease. *Front. Neuroenergetics* **2010**, *2*. [[CrossRef](#)]
7. Sakagami, K.; Wu, D.M.; Puro, D.G. Physiology of rat retinal pericytes: Modulation of ion channel activity by serum-derived molecules. *J. Physiol.* **1999**, *521 Pt 3*, 637–650. [[CrossRef](#)]
8. Peppiatt, C.M.; Howarth, C.; Mobbs, P.; Attwell, D. Bidirectional control of CNS capillary diameter by pericytes. *Nature* **2006**, *443*, 700–704. [[CrossRef](#)]
9. Winkler, E.A.; Bell, R.D.; Zlokovic, B.V. Central nervous system pericytes in health and disease. *Nat. Neurosci.* **2011**, *14*, 1398–1405. [[CrossRef](#)]
10. Gordon, G.R.; Howarth, C.; MacVicar, B.A. Bidirectional control of arteriole diameter by astrocytes. *Exp. Physiol.* **2011**, *96*, 393–399. [[CrossRef](#)] [[PubMed](#)]
11. Zlokovic, B.V. The blood-brain barrier in health and chronic neurodegenerative disorders. *Neuron* **2008**, *57*, 178–201. [[CrossRef](#)]
12. Levin, V.A. Relationship of octanol/water partition coefficient and molecular weight to rat brain capillary permeability. *J. Med. Chem.* **1980**, *23*, 682–684. [[CrossRef](#)] [[PubMed](#)]
13. Fischer, H.; Gottschlich, R.; Seelig, A. Blood-brain barrier permeation: Molecular parameters governing passive diffusion. *J. Membr. Biol.* **1998**, *165*, 201–211. [[CrossRef](#)] [[PubMed](#)]
14. Diecke, F.P.; Cacace, V.I.; Montalbetti, N.; Ma, L.; Kuang, K.; Iserovich, P.; Fischbarg, J. Comparative permeabilities of the paracellular and transcellular pathways of corneal endothelial layers. *J. Membr. Biol.* **2011**, *242*, 41–51. [[CrossRef](#)] [[PubMed](#)]
15. Pardridge, W.M. Blood-brain barrier delivery. *Drug Discov. Today* **2007**, *12*, 54–61. [[CrossRef](#)]
16. Gabathuler, R. Approaches to transport therapeutic drugs across the blood-brain barrier to treat brain diseases. *NeuroBiol. Dis.* **2010**, *37*, 48–57. [[CrossRef](#)]
17. Abbott, N.J.; Patabendige, A.A.; Dolman, D.E.; Yusof, S.R.; Begley, D.J. Structure and function of the blood-brain barrier. *NeuroBiol. Dis.* **2010**, *37*, 13–25. [[CrossRef](#)] [[PubMed](#)]
18. Jena, L.; McErlean, E.; McCarthy, H. Delivery across the blood-brain barrier: Nanomedicine for glioblastoma multiforme. *Drug Deliv. Transl. Res.* **2020**, *10*, 304–318. [[CrossRef](#)]
19. Saint-Pol, J.; Gosselet, F.; Duban-Deweer, S.; Pottiez, G.; Karamanos, Y. Targeting and Crossing the Blood-Brain Barrier with Extracellular Vesicles. *Cells* **2020**, *9*, 851. [[CrossRef](#)] [[PubMed](#)]
20. Stanimirovic, D.B.; Sandhu, J.K.; Costain, W.J. Emerging Technologies for Delivery of Biotherapeutics and Gene Therapy Across the Blood-Brain Barrier. *BioDrugs* **2018**, *32*, 547–559. [[CrossRef](#)]
21. Weil, M.T.; Ruhwedel, T.; Möbius, W.; Simons, M. Intracerebral Injections and Ultrastructural Analysis of High-Pressure Frozen Brain Tissue. *Curr. Protoc. Neurosci.* **2017**, *78*, 2–27. [[CrossRef](#)] [[PubMed](#)]
22. Patchell, R.A.; Regine, W.F.; Ashton, P.; Tibbs, P.A.; Wilson, D.; Shapley, D.; Young, B. A phase I trial of continuously infused intratumoral bleomycin for the treatment of recurrent glioblastoma multiforme. *J. Neurooncol.* **2002**, *60*, 37–42. [[CrossRef](#)] [[PubMed](#)]
23. Hopkins, K.; Chandler, C.; Eatough, J.; Moss, T.; Kemshead, J.T. Direct injection of 90Y MoAbs into glioma tumor resection cavities leads to limited diffusion of the radioimmunoconjugates into normal brain parenchyma: A model to estimate absorbed radiation dose. *Int. J. Radiat. Oncol. Biol. Phys.* **1998**, *40*, 835–844. [[CrossRef](#)]
24. Kawakami, K.; Kawakami, M.; Kioi, M.; Husain, S.R.; Puri, R.K. Distribution kinetics of targeted cytotoxin in glioma by bolus or convection-enhanced delivery in a murine model. *J. Neurosurg.* **2004**, *101*, 1004–1011. [[CrossRef](#)]
25. Armbruster, N.; Lattanzi, A.; Jeavons, M.; Van Wittenberghe, L.; Gjata, B.; Marais, T.; Martin, S.; Vignaud, A.; Voit, T.; Mavilio, F.; et al. Efficacy and biodistribution analysis of intracerebroventricular administration of an optimized scAAV9-SMN1 vector in a mouse model of spinal muscular atrophy. *Mol. Ther. Methods Clin. Dev.* **2016**, *3*, 16060. [[CrossRef](#)]
26. Hughes, M.P.; Smith, D.A.; Morris, L.; Fletcher, C.; Colaco, A.; Huebeker, M.; Tordo, J.; Palomar, N.; Massaro, G.; Henckaerts, E.; et al. AAV9 intracerebroventricular gene therapy improves lifespan, locomotor function and pathology in a mouse model of Niemann-Pick type C1 disease. *Hum. Mol. Genet.* **2018**, *27*, 3079–3098. [[CrossRef](#)]
27. Di Meo, I.; Marchet, S.; Lamperti, C.; Zeviani, M.; Viscomi, C. AAV9-based gene therapy partially ameliorates the clinical phenotype of a mouse model of Leigh syndrome. *Gene Ther.* **2017**, *24*, 661–667. [[CrossRef](#)]

28. Herzog, C.D.; Dass, B.; Holden, J.E.; Stansell, J.; Gasmi, M.; Tuszyński, M.H.; Bartus, R.T.; Kordower, J.H. Striatal delivery of CERE-120, an AAV2 vector encoding human neurturin, enhances activity of the dopaminergic nigrostriatal system in aged monkeys. *Mov. Disord.* **2007**, *22*, 1124–1132. [\[CrossRef\]](#)
29. Marks, W.J.; Ostrem, J.L.; Verhagen, L.; Starr, P.A.; Larson, P.S.; Bakay, R.A.; Taylor, R.; Cahn-Weiner, D.A.; Stoessl, A.J.; Olanow, C.W.; et al. Safety and tolerability of intraputamin delivery of CERE-120 (adeno-associated virus serotype 2-neurturin) to patients with idiopathic Parkinson's disease: An open-label, phase I trial. *Lancet Neurol.* **2008**, *7*, 400–408. [\[CrossRef\]](#)
30. Zhao, L.; Gottesdiener, A.J.; Parmar, M.; Li, M.; Kaminsky, S.M.; Chiuchio, M.J.; Sondhi, D.; Sullivan, P.M.; Holtzman, D.M.; Crystal, R.G.; et al. Intracerebral adeno-associated virus gene delivery of apolipoprotein E2 markedly reduces brain amyloid pathology in Alzheimer's disease mouse models. *Neurobiol. Aging* **2016**, *44*, 159–172. [\[CrossRef\]](#) [\[PubMed\]](#)
31. Jaworski, T.; Dewachter, I.; Lechat, B.; Croes, S.; Termont, A.; Demedts, D.; Borghgraef, P.; Devijver, H.; Filipkowski, R.K.; Kaczmarek, L.; et al. AAV-tau mediates pyramidal neurodegeneration by cell-cycle re-entry without neurofibrillary tangle formation in wild-type mice. *PLoS ONE* **2009**, *4*, e7280. [\[CrossRef\]](#) [\[PubMed\]](#)
32. Gómez-Sánchez, E.P.; Venkataraman, M.T.; Thwaites, D.; Fort, C. ICV infusion of corticosterone antagonizes ICV-aldosterone hypertension. *Am. J. Physiol.* **1990**, *258*, E649–E653. [\[CrossRef\]](#) [\[PubMed\]](#)
33. Slavc, I.; Schuller, E.; Falger, J.; Günes, M.; Pillwein, K.; Czech, T.; Dietrich, W.; Rössler, K.; Dieckmann, K.; Prayer, D.; et al. Feasibility of long-term intraventricular therapy with mafosfamide (n = 26) and etoposide (n = 11): Experience in 26 children with disseminated malignant brain tumors. *J. Neurooncol.* **2003**, *64*, 239–247. [\[CrossRef\]](#)
34. Peyrl, A.; Chocholous, M.; Azizi, A.A.; Czech, T.; Dorfer, C.; Mitteregger, D.; Gojo, J.; Minichmayr, E.; Slavc, I. Safety of Ommaya reservoirs in children with brain tumors: A 20-year experience with 5472 intraventricular drug administrations in 98 patients. *J. Neurooncol.* **2014**, *120*, 139–145. [\[CrossRef\]](#)
35. Garcia de Yebenes, J.; Fahn, S.; Mena, M.A.; Pardo, B.; Casarejos, M.J. Intracerebroventricular infusion of dopamine and its agonists in rodents and primates. An experimental approach to the treatment of Parkinson's disease. *ASAIO Trans.* **1988**, *34*, 951–957. [\[PubMed\]](#)
36. Zachrisson, O.; Zhao, M.; Andersson, A.; Dannaeus, K.; Häggblad, J.; Isacson, R.; Nielsen, E.; Patrone, C.; Rönnholm, H.; Wikström, L.; et al. Restorative effects of platelet derived growth factor-BB in rodent models of Parkinson's disease. *J. Parkinsons Dis.* **2011**, *1*, 49–63. [\[CrossRef\]](#) [\[PubMed\]](#)
37. Paul, G.; Zachrisson, O.; Varrone, A.; Almqvist, P.; Jerling, M.; Lind, G.; Rehnström, S.; Linderöth, B.; Bjartmarz, H.; Shafer, L.L.; et al. Safety and tolerability of intracerebroventricular PDGF-BB in Parkinson's disease patients. *J. Clin. Invest.* **2015**, *125*, 1339–1346. [\[CrossRef\]](#)
38. Yadav, D.B.; Maloney, J.A.; Wildsmith, K.R.; Fuji, R.N.; Meilandt, W.J.; Solanoy, H.; Lu, Y.; Peng, K.; Wilson, B.; Chan, P.; et al. Widespread brain distribution and activity following i.c.v. infusion of anti- β -secretase (BACE1) in nonhuman primates. *Br. J. Pharmacol.* **2017**, *174*, 4173–4185. [\[CrossRef\]](#)
39. Bobo, R.H.; Laske, D.W.; Akbasak, A.; Morrison, P.F.; Dedrick, R.L.; Oldfield, E.H. Convection-enhanced delivery of macromolecules in the brain. *Proc. Natl. Acad. Sci. USA* **1994**, *91*, 2076–2080. [\[CrossRef\]](#)
40. Lonser, R.R.; Walbridge, S.; Garmestani, K.; Butman, J.A.; Walters, H.A.; Vortmeyer, A.O.; Morrison, P.F.; Brechbiel, M.W.; Oldfield, E.H. Successful and safe perfusion of the primate brainstem: In vivo magnetic resonance imaging of macromolecular distribution during infusion. *J. Neurosurg.* **2002**, *97*, 905–913. [\[CrossRef\]](#)
41. Lieberman, D.M.; Laske, D.W.; Morrison, P.F.; Bankiewicz, K.S.; Oldfield, E.H. Convection-enhanced distribution of large molecules in gray matter during interstitial drug infusion. *J. Neurosurg.* **1995**, *82*, 1021–1029. [\[CrossRef\]](#) [\[PubMed\]](#)
42. Lonser, R.R.; Corthésy, M.E.; Morrison, P.F.; Gogate, N.; Oldfield, E.H. Convection-enhanced selective excitotoxic ablation of the neurons of the globus pallidus internus for treatment of parkinsonism in nonhuman primates. *J. Neurosurg.* **1999**, *91*, 294–302. [\[CrossRef\]](#)
43. Souweidane, M.M.; Kramer, K.; Pandit-Taskar, N.; Zhou, Z.; Haque, S.; Zanzonico, P.; Carrasquillo, J.A.; Lyashchenko, S.K.; Thakur, S.B.; Donzelli, M.; et al. Convection-enhanced delivery for diffuse intrinsic pontine glioma: A single-centre, dose-escalation, phase 1 trial. *Lancet Oncol.* **2018**, *19*, 1040–1050. [\[CrossRef\]](#)
44. Modak, S.; Kramer, K.; Gultekin, S.H.; Guo, H.F.; Cheung, N.K. Monoclonal antibody 8H9 targets a novel cell surface antigen expressed by a wide spectrum of human solid tumors. *Cancer Res.* **2001**, *61*, 4048–4054.
45. Vandergrift, W.A.; Patel, S.J.; Nicholas, J.S.; Varma, A.K. Convection-enhanced delivery of immunotoxins and radioisotopes for treatment of malignant gliomas. *Neurosurg. Focus* **2006**, *20*, E13. [\[CrossRef\]](#) [\[PubMed\]](#)
46. Groothuis, D.R.; Benalcázar, H.; Allen, C.V.; Wise, R.M.; Dills, C.; Dobrescu, C.; Rothholtz, V.; Levy, R.M. Comparison of cytosine arabinoside delivery to rat brain by intravenous, intrathecal, intraventricular and intraparenchymal routes of administration. *Brain Res.* **2000**, *856*, 281–290. [\[CrossRef\]](#)
47. Godinho, B.M.D.C.; Henninger, N.; Bouley, J.; Alterman, J.F.; Haraszti, R.A.; Gilbert, J.W.; Sapp, E.; Coles, A.H.; Biscans, A.; Nikan, M.; et al. Transvascular Delivery of Hydrophobically Modified siRNAs: Gene Silencing in the Rat Brain upon Disruption of the Blood-Brain Barrier. *Mol. Ther.* **2018**, *26*, 2580–2591. [\[CrossRef\]](#)
48. Bunevicius, A.; McDannold, N.J.; Golby, A.J. Focused Ultrasound Strategies for Brain Tumor Therapy. *Oper. Neurosurg. (Hagerstown)* **2020**, *19*, 9–18. [\[CrossRef\]](#) [\[PubMed\]](#)
49. Wang, F.; Wei, X.X.; Chang, L.S.; Dong, L.; Wang, Y.L.; Li, N.N. Ultrasound Combined With Microbubbles Loading BDNF Retrovirus to Open Blood-Brain Barrier for Treatment of Alzheimer's Disease. *Front. Pharmacol.* **2021**, *12*, 615104. [\[CrossRef\]](#)

50. Tamaki, K.; Sadoshima, S.; Heistad, D.D. Increased susceptibility to osmotic disruption of the blood-brain barrier in chronic hypertension. *Hypertension* **1984**, *6*, 633–638. [\[CrossRef\]](#) [\[PubMed\]](#)
51. Joshi, S.; Ergin, A.; Wang, M.; Reif, R.; Zhang, J.; Bruce, J.N.; Bigio, I.J. Inconsistent blood brain barrier disruption by intraarterial mannitol in rabbits: Implications for chemotherapy. *J. Neurooncol.* **2011**, *104*, 11–19. [\[CrossRef\]](#)
52. Pardridge, W.M. Blood-brain barrier biology and methodology. *J. Neurovirol.* **1999**, *5*, 556–569. [\[CrossRef\]](#)
53. Brewster, M.E.; Raghavan, K.; Pop, E.; Bodor, N. Enhanced delivery of ganciclovir to the brain through the use of redox targeting. *Antimicrob. Agents Chemother.* **1994**, *38*, 817–823. [\[CrossRef\]](#) [\[PubMed\]](#)
54. Sawada, G.A.; Williams, L.R.; Lutzke, B.S.; Raub, T.J. Novel, highly lipophilic antioxidants readily diffuse across the blood-brain barrier and access intracellular sites. *J. Pharmacol. Exp. Ther.* **1999**, *288*, 1327–1333.
55. Jefferies, W.A.; Brandon, M.R.; Hunt, S.V.; Williams, A.F.; Gatter, K.C.; Mason, D.Y. Transferrin receptor on endothelium of brain capillaries. *Nature* **1984**, *312*, 162–163. [\[CrossRef\]](#) [\[PubMed\]](#)
56. Roberts, R.L.; Fine, R.E.; Sandra, A. Receptor-mediated endocytosis of transferrin at the blood-brain barrier. *J. Cell Sci.* **1993**, *104 Pt 2*, 521–532.
57. King, G.L.; Johnson, S.M. Receptor-mediated transport of insulin across endothelial cells. *Science* **1985**, *227*, 1583–1586. [\[CrossRef\]](#) [\[PubMed\]](#)
58. Baura, G.D.; Foster, D.M.; Porte, D.; Kahn, S.E.; Bergman, R.N.; Cobelli, C.; Schwartz, M.W. Saturable transport of insulin from plasma into the central nervous system of dogs in vivo. A mechanism for regulated insulin delivery to the brain. *J. Clin. Investig.* **1993**, *92*, 1824–1830. [\[CrossRef\]](#) [\[PubMed\]](#)
59. Rebeck, G.W.; Reiter, J.S.; Strickland, D.K.; Hyman, B.T. Apolipoprotein E in sporadic Alzheimer's disease: Allelic variation and receptor interactions. *Neuron* **1993**, *11*, 575–580. [\[CrossRef\]](#)
60. Chen, Y.; Liu, L. Modern methods for delivery of drugs across the blood-brain barrier. *Adv. Drug Deliv. Rev.* **2012**, *64*, 640–665. [\[CrossRef\]](#)
61. Gao, H.; Shi, W.; Freund, L.B. Mechanics of receptor-mediated endocytosis. *Proc. Natl. Acad. Sci. USA* **2005**, *102*, 9469–9474. [\[CrossRef\]](#)
62. Cordon-Cardo, C.; O'Brien, J.P.; Casals, D.; Rittman-Grauer, L.; Biedler, J.L.; Melamed, M.R.; Bertino, J.R. Multidrug-resistance gene (P-glycoprotein) is expressed by endothelial cells at blood-brain barrier sites. *Proc. Natl. Acad. Sci. USA* **1989**, *86*, 695–698. [\[CrossRef\]](#) [\[PubMed\]](#)
63. Pardridge, W.M.; Kang, Y.S.; Buciak, J.L.; Yang, J. Human insulin receptor monoclonal antibody undergoes high affinity binding to human brain capillaries in vitro and rapid transcytosis through the blood-brain barrier in vivo in the primate. *Pharm. Res.* **1995**, *12*, 807–816. [\[CrossRef\]](#) [\[PubMed\]](#)
64. Friden, P.M.; Walus, L.R.; Musso, G.F.; Taylor, M.A.; Malfroy, B.; Starzyk, R.M. Anti-transferrin receptor antibody and antibody-drug conjugates cross the blood-brain barrier. *Proc. Natl. Acad. Sci. USA* **1991**, *88*, 4771–4775. [\[CrossRef\]](#)
65. Bickel, U.; Yoshikawa, T.; Landaw, E.M.; Faull, K.F.; Pardridge, W.M. Pharmacologic effects in vivo in brain by vector-mediated peptide drug delivery. *Proc. Natl. Acad. Sci. USA* **1993**, *90*, 2618–2622. [\[CrossRef\]](#)
66. Kurihara, A.; Pardridge, W.M. Imaging brain tumors by targeting peptide radiopharmaceuticals through the blood-brain barrier. *Cancer Res.* **1999**, *59*, 6159–6163. [\[PubMed\]](#)
67. Lee, H.J.; Zhang, Y.; Zhu, C.; Duff, K.; Pardridge, W.M. Imaging brain amyloid of Alzheimer disease in vivo in transgenic mice with an Abeta peptide radiopharmaceutical. *J. Cereb. Blood Flow Metab.* **2002**, *22*, 223–231. [\[CrossRef\]](#)
68. Zhang, Y.; Pardridge, W.M. Delivery of beta-galactosidase to mouse brain via the blood-brain barrier transferrin receptor. *J. Pharmacol. Exp. Ther.* **2005**, *313*, 1075–1081. [\[CrossRef\]](#) [\[PubMed\]](#)
69. Patricia Whitehead. Agents shuttle across blood-brain barrier to treat Hunter syndrome. *Nat. Biotechnol.* **2021**, *39*. [\[CrossRef\]](#)
70. Coloma, M.J.; Lee, H.J.; Kurihara, A.; Landaw, E.M.; Boado, R.J.; Morrison, S.L.; Pardridge, W.M. Transport across the primate blood-brain barrier of a genetically engineered chimeric monoclonal antibody to the human insulin receptor. *Pharm. Res.* **2000**, *17*, 266–274. [\[CrossRef\]](#)
71. Bu, G.; Maksymovitch, E.A.; Nerbonne, J.M.; Schwartz, A.L. Expression and function of the low density lipoprotein receptor-related protein (LRP) in mammalian central neurons. *J. Biol. Chem.* **1994**, *269*, 18521–18528. [\[CrossRef\]](#)
72. Moestrup, S.K.; Gliemann, J.; Pallesen, G. Distribution of the alpha 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell Tissue Res.* **1992**, *269*, 375–382. [\[CrossRef\]](#) [\[PubMed\]](#)
73. Kreuter, J.; Shamenkov, D.; Petrov, V.; Ramge, P.; Cychutek, K.; Koch-Brandt, C.; Alyautdin, R. Apolipoprotein-mediated transport of nanoparticle-bound drugs across the blood-brain barrier. *J. Drug Target.* **2002**, *10*, 317–325. [\[CrossRef\]](#) [\[PubMed\]](#)
74. Spencer, B.J.; Verma, I.M. Targeted delivery of proteins across the blood-brain barrier. *Proc. Natl. Acad. Sci. USA* **2007**, *104*, 7594–7599. [\[CrossRef\]](#) [\[PubMed\]](#)
75. Lentz, T.L.; Burrage, T.G.; Smith, A.L.; Crick, J.; Tignor, G.H. Is the acetylcholine receptor a rabies virus receptor? *Science* **1982**, *215*, 182–184. [\[CrossRef\]](#)
76. Kumar, P.; Wu, H.; McBride, J.L.; Jung, K.E.; Kim, M.H.; Davidson, B.L.; Lee, S.K.; Shankar, P.; Manjunath, N. Transvascular delivery of small interfering RNA to the central nervous system. *Nature* **2007**, *448*, 39–43. [\[CrossRef\]](#) [\[PubMed\]](#)
77. Bors, L.A.; Erdő, F. Overcoming the Blood-Brain Barrier: Challenges and Tricks for CNS Drug Delivery. *Sci. Pharm.* **2019**, *87*, 6. [\[CrossRef\]](#)

78. Ulbrich, K.; Hekmatara, T.; Herbert, E.; Kreuter, J. Transferrin- and transferrin-receptor-antibody-modified nanoparticles enable drug delivery across the blood-brain barrier (BBB). *Eur. J. Pharm. Biopharm.* **2009**, *71*, 251–256. [\[CrossRef\]](#)
79. Shi, N.; Pardridge, W.M. Noninvasive gene targeting to the brain. *Proc. Natl. Acad. Sci. USA* **2000**, *97*, 7567–7572. [\[CrossRef\]](#) [\[PubMed\]](#)
80. Shi, N.; Boado, R.J.; Pardridge, W.M. Receptor-mediated gene targeting to tissues in vivo following intravenous administration of pegylated immunoliposomes. *Pharm. Res.* **2001**, *18*, 1091–1095. [\[CrossRef\]](#)
81. Zhang, Y.; Schlachetzki, F.; Zhang, Y.F.; Boado, R.J.; Pardridge, W.M. Normalization of striatal tyrosine hydroxylase and reversal of motor impairment in experimental parkinsonism with intravenous nonviral gene therapy and a brain-specific promoter. *Hum. Gene Ther.* **2004**, *15*, 339–350. [\[CrossRef\]](#) [\[PubMed\]](#)
82. Zhang, Y.; Jeong Lee, H.; Boado, R.J.; Pardridge, W.M. Receptor-mediated delivery of an antisense gene to human brain cancer cells. *J. Gene Med.* **2002**, *4*, 183–194. [\[CrossRef\]](#) [\[PubMed\]](#)
83. Moos, T.; Morgan, E.H. Transferrin and transferrin receptor function in brain barrier systems. *Cell Mol. Neurobiol.* **2000**, *20*, 77–95. [\[CrossRef\]](#)
84. Gosk, S.; Vermehren, C.; Storm, G.; Moos, T. Targeting anti-transferrin receptor antibody (OX26) and OX26-conjugated liposomes to brain capillary endothelial cells using in situ perfusion. *J. Cereb. Blood Flow Metab.* **2004**, *24*, 1193–1204. [\[CrossRef\]](#) [\[PubMed\]](#)
85. Xiao, G.; Gan, L.S. Receptor-mediated endocytosis and brain delivery of therapeutic biologics. *Int. J. Cell Biol.* **2013**, *2013*, 703545. [\[CrossRef\]](#)
86. Pulgar, V.M. Transcytosis to Cross the Blood Brain Barrier, New Advancements and Challenges. *Front. Neurosci.* **2018**, *12*, 1019. [\[CrossRef\]](#) [\[PubMed\]](#)
87. Paris-Robidas, S.; Brouard, D.; Emond, V.; Parent, M.; Calon, F. Internalization of targeted quantum dots by brain capillary endothelial cells in vivo. *J. Cereb. Blood Flow Metab.* **2016**, *36*, 731–742. [\[CrossRef\]](#)
88. Li, J.; Guo, Y.; Kuang, Y.; An, S.; Ma, H.; Jiang, C. Choline transporter-targeting and co-delivery system for glioma therapy. *Biomaterials* **2013**, *34*, 9142–9148. [\[CrossRef\]](#)
89. Kageyama, T.; Nakamura, M.; Matsuo, A.; Yamasaki, Y.; Takakura, Y.; Hashida, M.; Kanai, Y.; Naito, M.; Tsuruo, T.; Minato, N.; et al. The 4F2hc/LAT1 complex transports L-DOPA across the blood-brain barrier. *Brain Res.* **2000**, *879*, 115–121. [\[CrossRef\]](#)
90. Dorszewska, J.; Prendecki, M.; Lianeri, M.; Kozubski, W. Molecular Effects of L-dopa Therapy in Parkinson's Disease. *Curr. Genom.* **2014**, *15*, 11–17. [\[CrossRef\]](#)
91. Khamdang, S.; Takeda, M.; Babu, E.; Noshiro, R.; Onozato, M.L.; Tojo, A.; Enomoto, A.; Huang, X.L.; Narikawa, S.; Anzai, N.; et al. Interaction of human and rat organic anion transporter 2 with various cephalosporin antibiotics. *Eur. J. Pharmacol.* **2003**, *465*, 1–7. [\[CrossRef\]](#)
92. Takeda, M.; Babu, E.; Narikawa, S.; Endou, H. Interaction of human organic anion transporters with various cephalosporin antibiotics. *Eur. J. Pharmacol.* **2002**, *438*, 137–142. [\[CrossRef\]](#)
93. Nakakariya, M.; Shimada, T.; Irokawa, M.; Maeda, T.; Tamai, I. Identification and species similarity of OATP transporters responsible for hepatic uptake of beta-lactam antibiotics. *Drug. Metab. Pharmacokinet.* **2008**, *23*, 347–355. [\[CrossRef\]](#) [\[PubMed\]](#)
94. Akanuma, S.; Uchida, Y.; Ohtsuki, S.; Kamiie, J.; Tachikawa, M.; Terasaki, T.; Hosoya, K. Molecular-weight-dependent, anionic-substrate-preferential transport of β -lactam antibiotics via multidrug resistance-associated protein 4. *Drug. Metab. Pharmacokinet.* **2011**, *26*, 602–611. [\[CrossRef\]](#) [\[PubMed\]](#)
95. Kato, Y.; Takahara, S.; Kato, S.; Kubo, Y.; Sai, Y.; Tamai, I.; Yabuuchi, H.; Tsuji, A. Involvement of multidrug resistance-associated protein 2 (Abcc2) in molecular weight-dependent biliary excretion of beta-lactam antibiotics. *Drug Metab. Dispos.* **2008**, *36*, 1088–1096. [\[CrossRef\]](#)
96. Shen, H.; Smith, D.E.; Keep, R.F.; Brosius, F.C. Immunolocalization of the proton-coupled oligopeptide transporter PEPT2 in developing rat brain. *Mol. Pharm.* **2004**, *1*, 248–256. [\[CrossRef\]](#)
97. Chen, X.; Loryan, I.; Payan, M.; Keep, R.F.; Smith, D.E.; Hammarlund-Udenaes, M. Effect of transporter inhibition on the distribution of cefadroxil in rat brain. *Fluids Barriers CNS* **2014**, *11*, 25. [\[CrossRef\]](#)
98. Pardridge, W.M.; Boado, R.J. Enhanced cellular uptake of biotinylated antisense oligonucleotide or peptide mediated by avidin, a cationic protein. *FEBS Lett.* **1991**, *288*, 30–32. [\[CrossRef\]](#)
99. Pardridge, W.M.; Triguero, D.; Buciak, J. Transport of histone through the blood-brain barrier. *J. Pharmacol. Exp. Ther.* **1989**, *251*, 821–826.
100. Kumagai, A.K.; Eisenberg, J.B.; Pardridge, W.M. Absorptive-mediated endocytosis of cationized albumin and a beta-endorphin-cationized albumin chimeric peptide by isolated brain capillaries. Model system of blood-brain barrier transport. *J. Biol. Chem.* **1987**, *262*, 15214–15219. [\[CrossRef\]](#)
101. Girod, J.; Fenart, L.; Régina, A.; Dehouck, M.P.; Hong, G.; Scherrmann, J.M.; Cecchelli, R.; Roux, F. Transport of cationized anti-tetanus Fab'2 fragments across an in vitro blood-brain barrier model: Involvement of the transcytosis pathway. *J. Neurochem.* **1999**, *73*, 2002–2008.
102. Reinholz, M.M.; Merkle, C.M.; Poduslo, J.F. Therapeutic benefits of putrescine-modified catalase in a transgenic mouse model of familial amyotrophic lateral sclerosis. *Exp. Neurol.* **1999**, *159*, 204–216. [\[CrossRef\]](#)

103. Poduslo, J.F.; Whelan, S.L.; Curran, G.L.; Wengenack, T.M. Therapeutic benefit of polyamine-modified catalase as a scavenger of hydrogen peroxide and nitric oxide in familial amyotrophic lateral sclerosis transgenics. *Ann. Neurol.* **2000**, *48*, 943–947. [\[CrossRef\]](#)
104. Muckerheide, A.; Apple, R.J.; Pesce, A.J.; Michael, J.G. Cationization of protein antigens. I. Alteration of immunogenic properties. *J. Immunol.* **1987**, *138*, 833–837.
105. Nell, L.J.; Thomas, J.W. Frequency and specificity of protamine antibodies in diabetic and control subjects. *Diabetes* **1988**, *37*, 172–176. [\[CrossRef\]](#)
106. Vogel, B.E.; Lee, S.J.; Hildebrand, A.; Craig, W.; Pierschbacher, M.D.; Wong-Staal, F.; Ruoslahti, E. A novel integrin specificity exemplified by binding of the alpha v beta 5 integrin to the basic domain of the HIV Tat protein and vitronectin. *J. Cell Biol.* **1993**, *121*, 461–468. [\[CrossRef\]](#) [\[PubMed\]](#)
107. Liu, L.; Guo, K.; Lu, J.; Venkatraman, S.S.; Luo, D.; Ng, K.C.; Ling, E.A.; Mochhala, S.; Yang, Y.Y. Biologically active core/shell nanoparticles self-assembled from cholesterol-terminated PEG-TAT for drug delivery across the blood-brain barrier. *Biomaterials* **2008**, *29*, 1509–1517. [\[CrossRef\]](#) [\[PubMed\]](#)
108. Hernando, S.; Herran, E.; Figueiro-Silva, J.; Pedraz, J.L.; Igartua, M.; Carro, E.; Hernandez, R.M. Intranasal Administration of TAT-Conjugated Lipid Nanocarriers Loading GDNF for Parkinson's Disease. *Mol. Neurobiol.* **2018**, *55*, 145–155. [\[CrossRef\]](#) [\[PubMed\]](#)
109. Vivès, E.; Brodin, P.; Lebleu, B. A truncated HIV-1 Tat protein basic domain rapidly translocates through the plasma membrane and accumulates in the cell nucleus. *J. Biol. Chem.* **1997**, *272*, 16010–16017. [\[CrossRef\]](#)
110. Bruce-Keller, A.J.; Chauhan, A.; Dimayuga, F.O.; Gee, J.; Keller, J.N.; Nath, A. Synaptic transport of human immunodeficiency virus-Tat protein causes neurotoxicity and gliosis in rat brain. *J. Neurosci.* **2003**, *23*, 8417–8422. [\[CrossRef\]](#)
111. Jia, H.; Lohr, M.; Jezequel, S.; Davis, D.; Shaikh, S.; Selwood, D.; Zachary, I. Cysteine-rich and basic domain HIV-1 Tat peptides inhibit angiogenesis and induce endothelial cell apoptosis. *Biochem Biophys Res. Commun.* **2001**, *283*, 469–479. [\[CrossRef\]](#) [\[PubMed\]](#)
112. Raposo, G.; Stoorvogel, W. Extracellular vesicles: Exosomes, microvesicles, and friends. *J. Cell Biol.* **2013**, *200*, 373–383. [\[CrossRef\]](#) [\[PubMed\]](#)
113. Johnstone, R.M.; Adam, M.; Hammond, J.R.; Orr, L.; Turbide, C. Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* **1987**, *262*, 9412–9420. [\[CrossRef\]](#)
114. Mathivanan, S.; Ji, H.; Simpson, R.J. Exosomes: Extracellular organelles important in intercellular communication. *J. Proteom.* **2010**, *73*, 1907–1920. [\[CrossRef\]](#)
115. György, B.; Szabó, T.G.; Pásztói, M.; Pál, Z.; Misják, P.; Aradi, B.; László, V.; Pállinger, E.; Pap, E.; Kittel, A.; et al. Membrane vesicles, current state-of-the-art: Emerging role of extracellular vesicles. *Cell Mol. Life Sci.* **2011**, *68*, 2667–2688. [\[CrossRef\]](#) [\[PubMed\]](#)
116. Yáñez-Mó, M.; Siljander, P.R.; Andreu, Z.; Zavec, A.B.; Borràs, F.E.; Buzas, E.I.; Buzas, K.; Casal, E.; Cappello, F.; Carvalho, J.; et al. Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* **2015**, *4*, 27066. [\[CrossRef\]](#)
117. Théry, C.; Witwer, K.W.; Aikawa, E.; Alcaraz, M.J.; Anderson, J.D.; Andriantsitohaina, R.; Antoniou, A.; Arab, T.; Archer, F.; Atkin-Smith, G.K.; et al. Minimal information for studies of extracellular vesicles 2018 (MISEV2018): A position statement of the International Society for Extracellular Vesicles and update of the MISEV2014 guidelines. *J. Extracell. Vesicles* **2018**, *7*, 1535750. [\[CrossRef\]](#)
118. Mulcahy, L.A.; Pink, R.C.; Carter, D.R. Routes and mechanisms of extracellular vesicle uptake. *J. Extracell. Vesicles* **2014**, *3*. [\[CrossRef\]](#)
119. Chen, C.C.; Liu, L.; Ma, F.; Wong, C.W.; Guo, X.E.; Chacko, J.V.; Farhoodi, H.P.; Zhang, S.X.; Zimak, J.; Ségaliny, A.; et al. Elucidation of Exosome Migration across the Blood-Brain Barrier Model In Vitro. *Cell Mol. Bioeng.* **2016**, *9*, 509–529. [\[CrossRef\]](#)
120. Morad, G.; Carman, C.V.; Hagedorn, E.J.; Perlin, J.R.; Zon, L.I.; Mustafaoglu, N.; Park, T.E.; Ingber, D.E.; Daisy, C.C.; Moses, M.A. Tumor-Derived Extracellular Vesicles Breach the Intact Blood-Brain Barrier. *ACS Nano* **2019**, *13*, 13853–13865. [\[CrossRef\]](#)
121. Kuroda, H.; Tachikawa, M.; Yagi, Y.; Umetsu, M.; Nurdin, A.; Miyauchi, E.; Watanabe, M.; Uchida, Y.; Terasaki, T. Cluster of Differentiation 46 Is the Major Receptor in Human Blood-Brain Barrier Endothelial Cells for Uptake of Exosomes Derived from Brain-Metastatic Melanoma Cells (SK-Mel-28). *Mol. Pharm.* **2019**, *16*, 292–304. [\[CrossRef\]](#)
122. Wiklander, O.P.B.; Brennan, M.; Lötvall, J.; Breakefield, X.O.; El Andaloussi, S. Advances in therapeutic applications of extracellular vesicles. *Sci. Transl. Med.* **2019**, *11*. [\[CrossRef\]](#)
123. Chauhan, S.; Danielson, S.; Clements, V.; Edwards, N.; Ostrand-Rosenberg, S.; Fenselau, C. Surface Glycoproteins of Exosomes Shed by Myeloid-Derived Suppressor Cells Contribute to Function. *J. Proteome Res.* **2017**, *16*, 238–246. [\[CrossRef\]](#) [\[PubMed\]](#)
124. Haraszti, R.A.; Didiot, M.C.; Sapp, E.; Leszyk, J.; Shaffer, S.A.; Rockwell, H.E.; Gao, F.; Narain, N.R.; DiFiglia, M.; Kiebish, M.A.; et al. High-resolution proteomic and lipidomic analysis of exosomes and microvesicles from different cell sources. *J. Extracell. Vesicles* **2016**, *5*, 32570. [\[CrossRef\]](#)
125. Saleh, A.F.; Lázaro-Ibáñez, E.; Forsgård, M.A.; Shatnyeva, O.; Osteikoetxea, X.; Karlsson, F.; Heath, N.; Ingelsten, M.; Rose, J.; Harris, J.; et al. Extracellular vesicles induce minimal hepatotoxicity and immunogenicity. *Nanoscale* **2019**, *11*, 6990–7001. [\[CrossRef\]](#) [\[PubMed\]](#)

126. Zhu, X.; Badawi, M.; Pomeroy, S.; Sutaria, D.S.; Xie, Z.; Baek, A.; Jiang, J.; Elgamal, O.A.; Mo, X.; Perle, K.; et al. Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *J. Extracell. Vesicles* **2017**, *6*, 1324730. [\[CrossRef\]](#) [\[PubMed\]](#)
127. Antimisariar, S.G.; Mourtas, S.; Marazioti, A. Exosomes and Exosome-Inspired Vesicles for Targeted Drug Delivery. *Pharmaceutics* **2018**, *10*, 218. [\[CrossRef\]](#) [\[PubMed\]](#)
128. Sun, D.; Zhuang, X.; Xiang, X.; Liu, Y.; Zhang, S.; Liu, C.; Barnes, S.; Grizzle, W.; Miller, D.; Zhang, H.G. A novel nanoparticle drug delivery system: The anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol. Ther.* **2010**, *18*, 1606–1614. [\[CrossRef\]](#) [\[PubMed\]](#)
129. Haney, M.J.; Klyachko, N.L.; Zhao, Y.; Gupta, R.; Plotnikova, E.G.; He, Z.; Patel, T.; Piroyan, A.; Sokolsky, M.; Kabanov, A.V.; et al. Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J. Control. Release* **2015**, *207*, 18–30. [\[CrossRef\]](#) [\[PubMed\]](#)
130. Armstrong, J.P.; Holme, M.N.; Stevens, M.M. Re-Engineering Extracellular Vesicles as Smart Nanoscale Therapeutics. *ACS Nano* **2017**, *11*, 69–83. [\[CrossRef\]](#)
131. Nash, L.A.; McFall, E.R.; Perozzo, A.M.; Turner, M.; Poulin, K.L.; De Repentigny, Y.; Burns, J.K.; McMillan, H.J.; Warman Chardon, J.; Burger, D.; et al. Survival Motor Neuron Protein is Released from Cells in Exosomes: A Potential Biomarker for Spinal Muscular Atrophy. *Sci. Rep.* **2017**, *7*, 13859. [\[CrossRef\]](#) [\[PubMed\]](#)
132. Alvarez-Erviti, L.; Seow, Y.; Yin, H.; Betts, C.; Lakhal, S.; Wood, M.J. Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* **2011**, *29*, 341–345. [\[CrossRef\]](#) [\[PubMed\]](#)
133. Zhuang, X.; Xiang, X.; Grizzle, W.; Sun, D.; Zhang, S.; Axtell, R.C.; Ju, S.; Mu, J.; Zhang, L.; Steinman, L.; et al. Treatment of brain inflammatory diseases by delivering exosome encapsulated anti-inflammatory drugs from the nasal region to the brain. *Mol. Ther.* **2011**, *19*, 1769–1779. [\[CrossRef\]](#) [\[PubMed\]](#)
134. Jennings, M.R.; Parks, R.J. Curcumin as an Antiviral Agent. *Viruses* **2020**, *12*, 1242. [\[CrossRef\]](#) [\[PubMed\]](#)
135. Ashrafizadeh, M.; Zarrabi, A.; Hushmandi, K.; Zarrin, V.; Moghadam, E.R.; Hashemi, F.; Makvandi, P.; Samarghandian, S.; Khan, H.; Najafi, M.; et al. Toward Regulatory Effects of Curcumin on Transforming Growth Factor-Beta Across Different Diseases: A Review. *Front. Pharmacol.* **2020**, *11*, 585413. [\[CrossRef\]](#)
136. Unlu, A.; Nayir, E.; Dogukan Kalenderoglu, M.; Kirca, O.; Ozdogan, M. Curcumin (Turmeric) and cancer. *J. BUON* **2016**, *21*, 1050–1060.
137. Nabavi, S.F.; Thiagarajan, R.; Rastrelli, L.; Daglia, M.; Sobarzo-Sánchez, E.; Alinezhad, H.; Nabavi, S.M. Curcumin: A natural product for diabetes and its complications. *Curr. Top. Med. Chem.* **2015**, *15*, 2445–2455. [\[CrossRef\]](#)
138. Forouzanfar, F.; Read, M.I.; Barreto, G.E.; Sahebkar, A. Neuroprotective effects of curcumin through autophagy modulation. *IUBMB Life* **2020**, *72*, 652–664. [\[CrossRef\]](#)
139. Jia, G.; Han, Y.; An, Y.; Ding, Y.; He, C.; Wang, X.; Tang, Q. NRP-1 targeted and cargo-loaded exosomes facilitate simultaneous imaging and therapy of glioma in vitro and in vivo. *Biomaterials* **2018**, *178*, 302–316. [\[CrossRef\]](#)
140. Mondal, S.; Manivasagan, P.; Bharathiraja, S.; Santha Moorthy, M.; Nguyen, V.T.; Kim, H.H.; Nam, S.Y.; Lee, K.D.; Oh, J. Hydroxyapatite Coated Iron Oxide Nanoparticles: A Promising Nanomaterial for Magnetic Hyperthermia Cancer Treatment. *Nanomaterials* **2017**, *7*, 426. [\[CrossRef\]](#)
141. Tian, T.; Zhang, H.X.; He, C.P.; Fan, S.; Zhu, Y.L.; Qi, C.; Huang, N.P.; Xiao, Z.D.; Lu, Z.H.; Tannous, B.A.; et al. Surface functionalized exosomes as targeted drug delivery vehicles for cerebral ischemia therapy. *Biomaterials* **2018**, *150*, 137–149. [\[CrossRef\]](#) [\[PubMed\]](#)
142. Yang, T.; Martin, P.; Fogarty, B.; Brown, A.; Schurman, K.; Phipps, R.; Yin, V.P.; Lockman, P.; Bai, S. Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in Danio rerio. *Pharm. Res.* **2015**, *32*, 2003–2014. [\[CrossRef\]](#) [\[PubMed\]](#)
143. Nooshabadi, V.T.; Khanmohammadi, M.; Shafei, S.; Banafshe, H.R.; Malekshahi, Z.V.; Ebrahimi-Barough, S.; Ai, J. Impact of atorvastatin loaded exosome as an anti-glioblastoma carrier to induce apoptosis of U87 cancer cells in 3D culture model. *Biochem. Biophys. Rep.* **2020**, *23*, 100792. [\[CrossRef\]](#) [\[PubMed\]](#)
144. Didiot, M.C.; Hall, L.M.; Coles, A.H.; Haraszti, R.A.; Godinho, B.M.; Chase, K.; Sapp, E.; Ly, S.; Alterman, J.F.; Hassler, M.R.; et al. Exosome-mediated Delivery of Hydrophobically Modified siRNA for Huntingtin mRNA Silencing. *Mol. Ther.* **2016**, *24*, 1836–1847. [\[CrossRef\]](#) [\[PubMed\]](#)
145. Kim, J.; Inoue, K.; Ishii, J.; Vanti, W.B.; Voronov, S.V.; Murchison, E.; Hannon, G.; Abeliovich, A. A MicroRNA feedback circuit in midbrain dopamine neurons. *Science* **2007**, *317*, 1220–1224. [\[CrossRef\]](#)
146. Sanchez-Simon, F.M.; Zhang, X.X.; Loh, H.H.; Law, P.Y.; Rodriguez, R.E. Morphine regulates dopaminergic neuron differentiation via miR-133b. *Mol. Pharmacol.* **2010**, *78*, 935–942. [\[CrossRef\]](#)
147. Xin, H.; Wang, F.; Li, Y.; Lu, Q.E.; Cheung, W.L.; Zhang, Y.; Zhang, Z.G.; Chopp, M. Secondary Release of Exosomes From Astrocytes Contributes to the Increase in Neural Plasticity and Improvement of Functional Recovery After Stroke in Rats Treated With Exosomes Harvested From MicroRNA 133b-Overexpressing Multipotent Mesenchymal Stromal Cells. *Cell Transplant.* **2017**, *26*, 243–257. [\[CrossRef\]](#)
148. Li, Y.; Wang, J.; Chen, S.; Wu, P.; Xu, S.; Wang, C.; Shi, H.; Bihl, J. miR-137 boosts the neuroprotective effect of endothelial progenitor cell-derived exosomes in oxyhemoglobin-treated SH-SY5Y cells partially via COX2/PGE2 pathway. *Stem. Cell Res. Ther.* **2020**, *11*, 330. [\[CrossRef\]](#)
149. Pusic, A.D.; Pusic, K.M.; Clayton, B.L.; Kraig, R.P. IFN γ -stimulated dendritic cell exosomes as a potential therapeutic for remyelination. *J. Neuroimmunol.* **2014**, *266*, 12–23. [\[CrossRef\]](#)

150. Cooper, J.M.; Wiklander, P.B.; Nordin, J.Z.; Al-Shawi, R.; Wood, M.J.; Vithlani, M.; Schapira, A.H.; Simons, J.P.; El-Andaloussi, S.; Alvarez-Erviti, L. Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice. *Mov. Disord.* **2014**, *29*, 1476–1485. [[CrossRef](#)]
151. Yu, C.Y.; Kuo, H.C. The emerging roles and functions of circular RNAs and their generation. *J. Biomed. Sci.* **2019**, *26*, 29. [[CrossRef](#)] [[PubMed](#)]
152. Chen, W.; Wang, H.; Zhu, Z.; Feng, J.; Chen, L. Exosome-Shuttled circSHOC2 from IPASs Regulates Neuronal Autophagy and Ameliorates Ischemic Brain Injury via the miR-7670-3p/SIRT1 Axis. *Mol. Ther. Nucleic Acids* **2020**, *22*, 657–672. [[CrossRef](#)] [[PubMed](#)]
153. Izadpanah, M.; Dargahi, L.; Ai, J.; Asgari Taei, A.; Ebrahimi Barough, S.; Mowla, S.J.; TavoosiDana, G.; Farahmandfar, M. Extracellular Vesicles as a Neprilysin Delivery System Memory Improvement in Alzheimer's Disease. *Iran. J. Pharm Res.* **2020**, *19*, 45–60. [[CrossRef](#)] [[PubMed](#)]
154. Iwata, N.; Tsubuki, S.; Takaki, Y.; Watanabe, K.; Sekiguchi, M.; Hosoki, E.; Kawashima-Morishima, M.; Lee, H.J.; Hama, E.; Sekine-Aizawa, Y.; et al. Identification of the major Abeta1-42-degrading catabolic pathway in brain parenchyma: Suppression leads to biochemical and pathological deposition. *Nat. Med.* **2000**, *6*, 143–150. [[CrossRef](#)] [[PubMed](#)]
155. Nabhan, J.F.; Hu, R.; Oh, R.S.; Cohen, S.N.; Lu, Q. Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. *Proc. Natl. Acad. Sci. USA* **2012**, *109*, 4146–4151. [[CrossRef](#)] [[PubMed](#)]
156. Wang, Q.; Yu, J.; Kadungure, T.; Beyene, J.; Zhang, H.; Lu, Q. ARMMs as a versatile platform for intracellular delivery of macromolecules. *Nat. Commun.* **2018**, *9*, 960. [[CrossRef](#)]

**Appendix 3.2: Expanding the Availability of
Onasemnogene Abeparvovec to Older Patients: The
Evolving Treatment Landscape for Spinal Muscular
Atrophy**

Review

Expanding the Availability of Onasemnogene Apeparvovec to Older Patients: The Evolving Treatment Landscape for Spinal Muscular Atrophy

Charlotte A. René ^{1,2,3} and Robin J. Parks ^{1,2,3,4,*}

¹ Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, ON K1H 8L6, Canada; chrene@ohri.ca

² Department of Biochemistry, Microbiology, and Immunology, University of Ottawa, Ottawa, ON K1H 8M5, Canada

³ Centre for Neuromuscular Disease, University of Ottawa, Ottawa, ON K1H 8M5, Canada

⁴ Department of Medicine, The Ottawa Hospital, Ottawa, ON K1H 8L6, Canada

* Correspondence: rparks@ohri.ca



Citation: René, C.A.; Parks, R.J. Expanding the Availability of Onasemnogene Apeparvovec to Older Patients: The Evolving Treatment Landscape for Spinal Muscular Atrophy. *Pharmaceutics* **2023**, *15*, 1764. <https://doi.org/10.3390/pharmaceutics15061764>

Academic Editor: Alicia Rodríguez-Gascón

Received: 14 May 2023

Revised: 6 June 2023

Accepted: 15 June 2023

Published: 19 June 2023



Copyright: © 2023 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<https://creativecommons.org/licenses/by/4.0/>).

Abstract: Spinal muscular atrophy (SMA) is a devastating neuromuscular disorder caused by mutations in the survival of motor neuron 1 (*SMN1*) gene, which leads to a reduced level in the SMN protein within cells. Patients with SMA suffer from a loss of alpha motor neurons in the spinal cord leading to skeletal muscle atrophy in addition to deficits in other tissues and organs. Patients with severe forms of the disease require ventilator assistance and typically succumb to the disease due to respiratory failure. Onasemnogene apeparvovec is an adeno-associated virus (AAV)-based gene therapeutic that has been approved for infants and young children with SMA, and it is delivered through intravenous administration using a dose based on the weight of the patient. While excellent outcomes have been observed in treated patients, the greater viral dose necessary to treat older children and adults raises legitimate safety concerns. Recently, onasemnogene apeparvovec use was investigated in older children through a fixed dose and intrathecal administration, a route that provides a more direct delivery to affected cells in the spinal cord and central nervous system. The promising results observed in the STRONG trial may support approval of onasemnogene apeparvovec for a greater proportion of patients with SMA.

Keywords: gene therapy; onasemnogene apeparvovec; neuromuscular disease; motor neuron disease; spinal muscular atrophy

1. Introduction

Spinal muscular atrophy (SMA) is a genetic neuromuscular disorder that was initially described at the end of the nineteenth century by Werdnig [1] and by Hoffmann [2]. SMA is characterized by a degeneration of the alpha motor neurons of the spinal cord, which leads to atrophy of skeletal muscle, although primary defects also occur in many other tissues and organs [3–5]. SMA is caused by the homozygous mutation or deletion of the survival of motor neuron 1 (*SMN1*) gene [6], resulting in inadequate levels of survival of motor neuron (SMN) protein within cells [7]. Humans have a paralogous gene, termed *SMN2*, that contains a key C-to-T base transition relative to *SMN1* that induces a skipping of exon 7 in the majority of the messenger RNA (mRNA) transcripts that are derived from this gene, leading to the production of only ~10% full-length SMN protein [8,9]. *SMN2* is variably amplified in humans, and more copies of the gene result in the production of a greater amount of the full-length SMN protein within cells [10]. Thus, more copies of *SMN2* commonly leads to less severe disease for a patient with SMA [11].

Fortunately, several treatments have been approved for this devastating disorder, including nusinersen and risdiplam, both of which promote the inclusion of exon 7 in the

SMN2-derived mRNA, and thus a greater production of the full-length SMN protein [12–14]. Arguably, the most promising therapy, onasemnogene abeparvovec, an adeno-associated virus (AAV)-based vector that encodes the *SMN1* gene, has only been approved for use in infants and young children [15–18]. Onasemnogene abeparvovec is currently administered intravenously (IV) using a dose based on patient weight, and it is presently unavailable to older children with SMA due to safety concerns related to high absolute doses of vector [15–19]. However, a recent trial has explored the use of administering fixed-dose onasemnogene abeparvovec in children who are up to 5 years of age through intrathecal (IT) administration [20]—a more direct route through which to deliver the therapy to the cells of the spinal cord and central nervous system (CNS). In this review, we will provide background and context for this recent trial.

2. Onasemnogene Abeparvovec

Onasemnogene abeparvovec, marketed as Zolgensma, is a self-complementary AAV serotype 9 (scAAV9) vector that encodes a functional copy of *SMN1* that is under the control of a hybrid cytomegalovirus (CMV) enhancer-chicken beta-actin promoter (CBA) [21]. The safety and efficacy of onasemnogene abeparvovec has been explored in several clinical trials, as detailed in Table 1.

Table 1. Onasemnogene abeparvovec clinical trials.

Clinical Trial	Patient Age	SMN2 Copy #	<i>n</i>	Administration Method	Dose	Status *
START Phase 1 NCT02122952	<8 months	2	15	Intravenous	Low: 6.7×10^{13} vg/kg High: 1.1×10^{14} vg/kg	Completed
STRIVE-US Phase 3 NCT03306277	<6 months	1 or 2	22	Intravenous	1.1×10^{14} vg/kg	Completed
STRIVE-EU Phase 3 NCT03461289	<6 months	1 or 2	33	Intravenous	1.1×10^{14} vg/kg	Completed
SPRINT Phase 3 NCT03505099	<6 weeks	1, 2, or 3	30	Intravenous	1.1×10^{14} vg/kg	Completed
STRONG Phase 1 NCT03381729	6–60 months	3	32	Intrathecal	Low: 6.0×10^{13} vg Medium: 1.2×10^{14} vg High: 2.4×10^{14} vg	Terminated
SMART Phase 3b NCT04851873	<17 years	N/A	24	Intravenous	N/A (likely 1.1×10^{14} vg/kg)	Active
STEER Phase 3 NCT05089656	≥ 2 –<18 years	N/A	125 (estimated)	Intrathecal	1.2×10^{14} vg	Recruiting

* as of June 2023.

Initially, the safety and efficacy of a single IV infusion of onasemnogene abeparvovec was examined in the phase 1 START trial (NCT02122952), which involved symptomatic infants <8 months of age with SMA type 1 and who possessed two copies of *SMN2*. A total of 15 infants received either a low (6.7×10^{13} viral genomes (vg)/kg; $n = 3$) or a high (1.1×10^{14} vg/kg; $n = 12$) dose of IV onasemnogene abeparvovec. All 15 infants were alive without the need for mechanical ventilation at 20 months of age, a dramatic improvement from the 8% survival rate observed in the historical control cohort. Among 11/12 children in the high-dose cohort, an increase in survival was accompanied by improvements in the Children’s Hospital of Philadelphia Infant Test of Neuromuscular Dis-

orders (CHOP-INTEND) scores compared to their baseline. A total of 13 patients from the START trial were enrolled in a long-term follow-up (LTFU) study (NCT03421977) in which all 10 children from the high-dose group were alive without the need for permanent ventilation and maintained previously achieved motor milestones for up to 7.5 years post-treatment, demonstrating the longevity of onasemnogene abeparvovec [22,23]. Without treatment, children with SMA type 1 with two copies of *SMN2* rarely survive past the age of two, typically succumbing to respiratory failure [24,25].

The follow-up phase 3 trials, STRIVE-US (NCT03306277) and STRIVE-EU (NCT03461289), treated children < 6 months of age with SMA type I and up to two copies of *SMN2* with the high dose used in the START trial [21,26,27]. Both of these trials demonstrated that over 90% of infants survived free of the need for permanent ventilation at 14 months compared to 26% in the natural history cohort; furthermore, approximately half achieved independent sitting at 18 months, a milestone that was not achieved in the natural history cohort. Both STRIVE trials showed a very good benefit–risk profile for the IV administration of onasemnogene abeparvovec in symptomatic infants with SMA that were younger than 6 months, thus supporting the case for the drug’s approval [15]. This favourable benefit–risk profile was also demonstrated in the SPRINT trial (NCT03505099), which treated pre-symptomatic infants less than 6 weeks of age with 2 ($n = 14$) or 3 ($n = 15$) copies of *SMN2* [28,29]. The START, STRIVE, and SPRINT trials examined the safety of onasemnogene abeparvovec in both symptomatic and pre-symptomatic infants with SMA; however, all the infants who participated in these studies weighed < 8.5 kg. The Global Managed Access Program (GMAP) launched by Novartis in January 2020 provided treatment to all patients with SMA under the age of 24 months and weighing ≤ 21 kg [30]. As of 2022, 5 out of 102 children who were treated through this program were ≥ 13.5 kg, the heaviest of which was 17 kg (this patient received a dose of 1.87×10^{15} total vg). The GMAP data showed that the safety findings of patients that were ≥ 8.5 kg at the time of infusion were consistent with the previous data of patients who were < 8.5 kg.

As of January 2023, IV onasemnogene abeparvovec was approved in 45 countries [31], although the maximum patient age and/or weight varies between countries. In the United States and Japan, the first countries to approve onasemnogene abeparvovec, the drug is approved for use in children with SMA up to two years old, regardless of weight [15,16]. In Canada and Europe, onasemnogene abeparvovec is used for the treatment of pediatric patients with 5q SMA that have bi-allelic mutations in the *SMN1* gene, and who are either clinically diagnosed as SMA type 1, or possess three or fewer copies of the *SMN2* gene [17,18,32]. This regulatory approval includes dosing guidance for babies and young children up to 21 kg. Based on natural history studies, the majority of untreated children 5 years old or younger with SMA type 1 or type 2 would likely be ≤ 21 kg, and this could include children as old as 8 years of age [33].

To further explore the efficacy and safety of IV onasemnogene abeparvovec in heavier children, the SMART phase 3b trial (NCT04851873) is investigating the use of IV onasemnogene abeparvovec in children with SMA who were < 18 years of age and who weighed ≥ 8.5 kg to ≤ 21 kg. Although recruitment criteria for this trial permit participants to be up to 17 years of age, it is unlikely that participants over 8 years of age would meet the weight requirement of ≤ 21 kg at the time of infusion [33].

Following the success of the STRIVE and SPRINT trials, there was an obvious desire to examine the efficacy of onasemnogene abeparvovec in an older patient population. However, there are inherent challenges in treating older, and thus larger, patients with SMA. To date, the therapeutic dose has been calculated based on weight at 1.1×10^{14} vg/kg. Larger patients would require larger absolute doses of vector. A very high dose of vector is required because IV administration is a relatively inefficient method through which to deliver vector to the CNS—only a small portion of the vector actually transits through the blood–brain barrier (BBB) and transduces the target tissues located within the CNS [34,35]. The majority of the IV-administered vector actually accumulates in the liver and peripheral

tissues [36]. No gene therapy vector is completely “safe”; thus, administering a greater quantity of vector raises legitimate safety concerns. Use of AAV, such as onasemnogene abeparvovec, can cause serious adverse events, including acute liver failure in large animal models [34] and in human trials [19], as well as thrombotic microangiopathy (TMA) in human trials [37]—the risk of which increases as more viral genomes are administered. Indeed, trends toward greater elevations in liver transaminases have been reported amongst heavier children who were administered onasemnogene abeparvovec [38,39]; however, this was not the case in all studies [30]. Tragically, two children have died of acute liver failure within 5 to 6 weeks following IV administration of onasemnogene abeparvovec [40,41]. Novartis released a statement following the patient fatalities, highlighting that acute liver failure is a known side effect of onasemnogene abeparvovec; however, the benefit-risk profile is still favourable [41]. Similarly, four children in a clinical trial for the genetic neuromuscular disorder X-linked myotubular myopathy (XLMTM) died following the IV administration of AAV8-MTM1, three of which received a dose of 3.5×10^{14} vg/kg and were confirmed to have died from complications of liver failure [42–45]. AAV may not have been the sole cause of liver failure in these children; it is possible that the administration of the vector could have exacerbated pre-existing liver dysfunction, as intrahepatic cholestasis has been found to be a clinically significant feature of XLMTM [46]. Finally, although the majority of delivered AAV genomes remain episomal, AAV can insert in the cell’s genome, which has been associated with hepatocellular carcinoma in humans [47] and mice [48–50] (although not in all studies [51–54]). In short, although AAV-mediated gene therapy can be very effective, it does not come without risk. For SMA, an alternative approach to achieve efficient delivery to the CNS using less vector is greatly desired.

3. Intrathecal Administration of Onasemnogene Abeparvovec

To circumvent the need for the extremely high numbers of vg required for IV treatment in older, and thus larger, patients, IT administration of onasemnogene abeparvovec has been explored with the aim of providing a more efficient transduction of the CNS. In mice and nonhuman primates, IT administration of scAAV9-SMN results in widespread transgene expression throughout the spinal cord, and this is achieved using 10-fold less vector compared to IV administration [55].

Initiated in 2017, the phase 1 STRONG trial (NCT03381729) evaluated a single IT fixed-dose administration of onasemnogene abeparvovec in non-ambulatory children with SMA type 2 and who were 6 to <60-months of age with three copies of SMN2. Unlike the previous trials that investigated onasemnogene abeparvovec, there was no weight limit in the eligibility criteria for the STRONG trial. This phase I dose escalation study used doses ranging from 6.0×10^{13} to 2.4×10^{14} total vg, which is significantly less than the highest dose administered to patients in the IV phase 1 START trial (which administered 1.69×10^{15} total vg to the largest patient [21]). Unfortunately, a partial clinical hold was placed on the STRONG trial by the FDA in October 2019, citing concern due to dorsal root ganglia (DRG) mononuclear cell inflammation observed in animals treated with IT onasemnogene abeparvovec [56,57]. Following further long-term preclinical safety studies, the partial clinical hold was lifted in August of 2021 [58].

Recently, results of the completed STRONG trial were published [20]. 32 children were divided into two groups based on age at dosing: younger (6 to <24 months) and older (24 to <60 months). Patients in the younger children grouping were enrolled into one of three fixed-dose cohorts: low- (6.0×10^{13} total vg, $n = 3$), medium- (1.2×10^{14} total vg, $n = 13$), and high-dose (2.4×10^{14} total vg, $n = 4$). The older group all received the medium dose (1.2×10^{14} total vg, $n = 12$). Amongst the younger patients, 1/3 (33.3%) in the low-dose cohort and 1/13 (7.7%) in the medium-dose cohort (or 12.5% (2/16 treated patients) when combining the two dosing groups) achieved independent standing. Based on natural history studies, 13.7% would have been predicted to achieve this milestone. Again, amongst the younger children cohort, two children that received the low dose gained a total of four motor Bayley-III milestones (crawls, $n = 2$; pulls to stand, $n = 1$;

and stands alone, $n = 1$), while six children in the medium-dose cohort gained a total of 15 motor milestones (rolls, $n = 4$; crawls, $n = 2$; pulls to a stand $n = 2$; stands with assistance, $n = 3$; stands alone, $n = 1$; walks with assistance $n = 2$; and walks alone, $n = 1$). Three children in the high-dose cohort achieved 3 motor milestones (rolls, $n = 1$; pulls to a stand, $n = 1$; and stands with assistance $n = 1$). Older children who received the medium dose (1.2×10^{14} total vg, $n = 12$) showed a mean change of 6.0 points in their Hammersmith Functional Motor Scale Expanded (HFMSE) scores at month 12 when compared to baseline, which was significantly different from the natural history cohort, who only saw a 0.5-point change. Furthermore, 11/12 (91.7%) of treated children achieved a clinically meaningful (≥ 3 -point) increase in their HFMSE score compared to their initial score at baseline visit, which is far greater than the 13.3% who achieved a similar improvement in the natural history cohort. Three children in this group achieved a total of four Bayley-III motor milestones (rolling, $n = 1$; standing with assistance, $n = 2$; and walking with assistance, $n = 1$). No deaths were reported in this study. Several adverse events of special interest (AESI) were reported, including nine hepatotoxicity events in seven children (21.9%), including one patient in the medium-dose cohort who experienced elevated alanine aminotransferase (ALT) and aspartate aminotransferase (AST)—which was deemed a treatment-related serious adverse event. There were also five thrombocytopenia events observed in five children (15.6%, one of which was deemed probably treatment related), twelve cardiac events in nine children (28.1%, including one instance each of sinus tachycardia and increased creatinine phosphokinase-MB, which were deemed to possibly be treatment related), and one instance of hepatomegaly (deemed probably treatment related). Overall, the investigators concluded that IT onasemnogene abeparvovec is safe and well-tolerated, and the therapy showed efficacy in children aged 2 to 5 years. Unfortunately, the primary efficacy endpoint of independent standing for ≥ 3 s in children aged 6 to 24 months was not met during the post-dose observation period of this study.

Patients enrolled in the STRONG trial could participate in LT-002 (NCT04042025), a voluntary phase 4, 15-year follow-up safety and efficacy study, which also enrolled patients from the STRIVE and SPRINT studies. Novartis provided an update on LT-002 at the 2023 Muscular Dystrophy Association Clinical and Scientific Conference. All children who participated in the STRONG trial had maintained or achieved new developmental gains at a mean follow-up length of 3.6 years [23]. In addition, 5/16 (31.3%) had achieved a new motor milestone; 6 of 12 (50%) had a ≥ 3 -point increase in HFMSE score; and 3 of 12 (25%) had a >10 -point increase [59]. However, interpretation of the data is somewhat complicated by the fact that some of the patients also received add-on therapy (nusinersen or risdiplam) [23,59]. Of the 18 patients from the STRONG trial who were enrolled in LT-002, 50% were also receiving add-on therapy [59]. This is far greater than the 24.6% of patients who received add-on therapy following treatment with IV onasemnogene abeparvovec in the STRIVE or SPRINT trials. It is unclear why a larger proportion of patients that received the IT treatment also sought add-on therapy compared to the IV-treated group. Perhaps the systemic distribution of onasemnogene abeparvovec to all tissues of the body during IV treatment provides a better outcome than delivering vector only to the CNS. While combining therapies may be in the best interest of the patient, it does complicate interpretation of the data from the clinical trial as any improvement in motor function may be attributed to more than just onasemnogene abeparvovec. As more data become available, it will be interesting to see if there is a correlation between dose, age, and performance in tests of motor function among those who received add-on therapy compared to onasemnogene abeparvovec alone.

As the medium-dose of IT onasemnogene abeparvovec was well-tolerated and demonstrated efficacy, it was selected for use in the ongoing phase 3 STEER trial (NCT05089656), which is exploring the use of IT fixed-dose onasemnogene abeparvovec in patients aged ≥ 2 to <18 years old. Unlike the SMART trial—in which patients were only eligible if they were ≤ 21 kg in weight at the time of infusion—all treated patients in the STEER trial will be administered 1.2×10^{14} total vg, regardless of weight. Exploring the

efficacy of onasemnogene abeparvovec in the oldest population to date through an IT administration of a fixed dose has the potential to satisfy the unmet need for gene therapy for children and adolescent patients with SMA.

Although IT onasemnogene abeparvovec may be a promising therapeutic option for the treatment of SMA in older children, it is important to note that the most dramatic improvement with IV onasemnogene abeparvovec were observed in pre-symptomatic infants treated at less than 6 weeks of age [28,29]. In the SPR1NT trial, infants with three copies of *SMN2* all achieved independent sitting, and most achieved independent walking, within the normal development window, which is typically not seen in patients with SMA [29]. Amongst infants with two copies of *SMN2*, most sat independently for ≥ 30 s within the normal development window, and all achieved this milestone by 18 months, while none in the natural history cohort achieved this milestone [28]. In the STRONG trial, children in the younger group gained more Bayley-III motor milestones than those in the older group [20]. Treatment is likely most effective when supplied before the irreversible loss of motor neurons has occurred. These data emphasize the urgent need for universal newborn screening to identify at-risk children as early as possible in order to initiate treatment early. Fortunately, newborn screening has been adopted in many countries around the world [60]. The early treatment of individuals at high risk of developing SMA, prior to symptom onset, results in improved outcomes and greater functional independence, leading to improved quality of life.

4. Onasemnogene Abeparvovec versus Nusinersen or Risdiplam

The results of the STRONG trial should be compared to similar trials involving nusinersen, marketed as Spinraza, which is currently available for the treatment of SMA in patients of all ages. Nusinersen is an antisense oligonucleotide (ASO) delivered IT that modulates the splicing of *SMN2* pre-mRNA to promote the inclusion of exon 7, thereby increasing the production of the full-length SMN protein [13,61,62]. As opposed to onasemnogene abeparvovec, which is delivered with a one-time infusion, nusinersen is administered IT through a series of four loading doses over the course of the first two months, followed by maintenance doses every four months [15,63]. All patients receive the same dose (12 mg of the ASO in a 5 mL volume), regardless of age, size, or severity of disease. The CHERISH trial (NCT02292537) used nusinersen to treat 84 children with SMA between 2 and 12 years of age and who had between two and four copies of *SMN2*, with 42 children in a sham control group [64]. At 15 months, 57% of children in the treated group had achieved a change in their HFMSE score of ≥ 3 points compared to 27% in the sham control group [64]. This is far less than the 91.7% of children who were 2 to 5 years of age and were treated with the medium dose of onasemnogene abeparvovec in the STRONG trial, and who achieved the same increase in HFMSE score at month 12 [20]. Overall, the mean increases in HFMSE scores were not as pronounced in the CHERISH trial at 15 months than in the STRONG trial at 12 months [20,64]. Nevertheless, the CHERISH trial concluded that significant improvement in motor function was observed in children with later-onset SMA compared to the sham control group [64]. Complications associated with lumbar puncture were significantly higher in the treated group than in the control group (15% versus 3%, respectively), and these included back pain, headache, and vomiting. Pyrexia and epistaxis were also ≥ 5 percentage points higher in the treated group relative to the control group (pyrexia: 43% versus 36%; epistaxis: 7% versus 0%, respectively). This study reported no elevations in ALT or AST as adverse events, unlike in some patients who were treated with onasemnogene abeparvovec [19,20,64].

The use of ASOs such as nusinersen comes with some associated risks, such as renal toxicity, thrombocytopenia, and coagulation abnormalities [63]. Recently, delivery of ASOs into the cerebrospinal fluid (CSF) have been linked to neurotoxicity in mice, which is caused by changes in the intercellular levels of calcium [65]. In several studies in mice, ASO delivery to the CNS has led to behavioural abnormalities, such as decreased desire to explore the cage, reduced responsiveness to stimulation, unusual postures, disrupted

breathing patterns, and reduced consciousness [65–68]. However, this phenomenon has not been reported in any human patients to date.

Importantly, up to 50% of patients in some clinical trials have failed to respond to nusinersen for unknown reasons [64]. Regardless of these drawbacks, nusinersen is considered to be reasonably safe, reasonably effective, and is approved for a wider age group than onasemnogene abeparvovec. Indeed, treatment with nusinersen has demonstrated a stabilization of disease progression and even clinically meaningful improvements in the motor function in some adult patients with SMA [69,70].

Risdiplam, marketed as Evrysdi, is a small molecule splicing modifier approved to treat patients with SMA of all ages [71,72]. It also acts on *SMN2* by stabilizing the *SMN2* transcript and promoting the inclusion of exon 7 to increase the cellular levels of full-length SMN protein [14]. The drug is taken once daily in an oral solution and is dosed based on patient age and weight at 0.15 mg–0.25 mg/kg up to 20 kg, and 5 mg is the maximum recommended dose for all patients above 20 kg [73]. In clinical trials, the use of risdiplam improved HMFSE scores, although to a lesser degree than that observed for nusinersen and IT onasemnogene abeparvovec [20,64,74,75]. Patients between 2 to 11 years of age only achieved a mean 1.7-point increase in HMFSE score at month 24 following treatment with risdiplam [74]. Unlike nusinersen, which is designed to interact with only *SMN2* pre-mRNA, risdiplam does not necessarily target only *SMN2*. Indeed, in animal models risdiplam has been associated with alternative splicing of the off-target genes *FOXMI* and *MADD*, which are associated with the cell cycle and apoptosis, respectively [14]. Whether the off-target splicing of *FOXMI*, *MADD*, or other genes has long-term, adverse consequences in treated patients has yet to be determined. The degree of off-target splicing (if any) in patients has not yet been reported [74,75]. The easy administration of risdiplam makes the drug attractive to patients with SMA; however, the clinical benefits do not appear to be as pronounced as onasemnogene abeparvovec or nusinersen.

5. Conclusions

For children with SMA between the ages of 2 and 5, the results of the STRONG trial propose treatment with IT onasemnogene abeparvovec as a therapeutic option as it has a favourable benefit-risk profile. Although there have been some adverse events associated with onasemnogene abeparvovec, this treatment appears more effective than nusinersen in older children with SMA (91.7% versus 57% of children attaining a ≥ 3 -point increase in HFMSE score for IT onasemnogene abeparvovec versus nusinersen, respectively) [20,64]. A ≥ 3 -point change in HFMSE score is considered a clinically meaningful change involving two or three skills [76,77]. Attaining these skills may translate into significant improvements in the quality of life for children with SMA. For example, an improvement in the HFMSE test item “one hand to head in sitting task” may mean that a patient could assist in their feeding or do so independently. IV onasemnogene abeparvovec has been proven to be effective for at least 7 years [22,23]; thus, it is possible the older children who were IT-treated may also maintain achieved motor milestones for many years after treatment. All of the approved treatments for SMA are exceedingly expensive, but a single dose of onasemnogene abeparvovec is reported to be more cost effective than the chronic dosing of nusinersen [78], and thus may ultimately save health care costs.

Some patients with SMA who previously were treated with onasemnogene abeparvovec have started an additional add-on therapy (nusinersen or risdiplam) [23,59]. In fact, there are dedicated clinical trials exploring the safety and efficacy of treatment with either nusinersen (NCT04488133) or risdiplam (NCT05861986) following treatment with onasemnogene abeparvovec. In most cases, add-on therapy is initiated due to a perceived or real plateauing of motor function improvement following the initial onasemnogene abeparvovec treatment [79–81]. Results from combinatorial therapies have previously been reported, but these are typically of small sample size or have relatively short follow-up duration [79–81]. Onasemnogene abeparvovec, nusinersen, and risdiplam all aim to increase SMN protein concentration, but they use different mechanisms of action to do

so [12–18]. Although the use of multiple drugs will obviously increase the already high cost of treatment, a combinatorial approach to treatment may provide an improved outcome compared to monotherapy for some patients with SMA.

In recent years, the prognosis for SMA has been transformed by the availability of life-changing therapeutics. Most notably, the availability of a gene therapy drug restoring a functional copy of *SMN1* through a single intravenous infusion has allowed infants to not only achieve previously unattainable motor milestones, but to also live far beyond the average lifespan of untreated infants. Now, efforts are focusing on expanding the availability of this transformative drug to other age groups. The results of the STRONG clinical trial investigating IT administration of onasemnogene abeparvovec in older children provides optimism for the field, and positive outcomes from the STEER trial may support broadening the availability of onasemnogene abeparvovec for patients up to the age of 18. Clinically meaningful improvements in motor function from this and other therapies will hopefully improve the quality of life for a wider group of patients with SMA.

Author Contributions: Conceptualization, C.A.R. and R.J.P.; literature search, C.A.R.; resources, R.J.P.; writing—original draft, C.A.R.; writing—review and editing, C.A.R. and R.J.P.; supervision, R.J.P.; funding acquisition, C.A.R. and R.J.P.; project administration, R.J.P. All authors have read and agreed to the published version of the manuscript.

Funding: Research in the Parks lab was supported by grants to R.J.P. from the Canadian Institutes of Health Research (FRN-142316, PJT-178120, FRN-175428, PJT-186267), the Natural Sciences and Engineering Research Council (RGPIN-2019-04786), and Cure SMA/Cure SMA Canada. C.A.R. was supported by a Queen Elizabeth II Graduate Scholarship in Science and Technology from the Ontario Provincial Government (Canada).

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

References

1. Werdnig, G. Zwei frühinfantile hereditäre Fälle von progressiver Muskelatrophie unter dem Bilde der Dystrophie, aber anf neurotischer Grundlage. *Arch. Psychiatr. Nervenkrankh.* **1891**, *22*, 437–480. [\[CrossRef\]](#)
2. Hoffmann, J. Ueber chronische spinale Muskelatrophie im Kindesalter, auf familiärer Basis. *Dtsch. Z. Nervenheilkd.* **1893**, *3*, 427–470. [\[CrossRef\]](#)
3. Yeo, C.J.J.; Darras, B.T. Overturning the Paradigm of Spinal Muscular Atrophy as Just a Motor Neuron Disease. *Pediatr. Neurol.* **2020**, *109*, 12–19. [\[CrossRef\]](#)
4. Deguise, M.O.; Baranello, G.; Mastella, C.; Beauvais, A.; Michaud, J.; Leone, A.; De Amicis, R.; Battezzati, A.; Dunham, C.; Selby, K.; et al. Abnormal fatty acid metabolism is a core component of spinal muscular atrophy. *Ann. Clin. Transl. Neurol.* **2019**, *6*, 1519–1532. [\[CrossRef\]](#) [\[PubMed\]](#)
5. Nery, F.C.; Siranosian, J.J.; Rosales, I.; Deguise, M.O.; Sharma, A.; Muhtaseb, A.W.; Nwe, P.; Johnstone, A.J.; Zhang, R.; Fatouraei, M.; et al. Impaired kidney structure and function in spinal muscular atrophy. *Neurol. Genet.* **2019**, *5*, e353. [\[CrossRef\]](#) [\[PubMed\]](#)
6. Lefebvre, S.; Bürglen, L.; Reboullet, S.; Clermont, O.; Burlet, P.; Viollet, L.; Benichou, B.; Cruaud, C.; Millasseau, P.; Zeviani, M. Identification and characterization of a spinal muscular atrophy-determining gene. *Cell* **1995**, *80*, 155–165. [\[CrossRef\]](#)
7. Lefebvre, S.; Burlet, P.; Liu, Q.; Bertrand, S.; Clermont, O.; Munnich, A.; Dreyfuss, G.; Melki, J. Correlation between severity and SMN protein level in spinal muscular atrophy. *Nat. Genet.* **1997**, *16*, 265–269. [\[CrossRef\]](#)
8. Lorson, C.L.; Hahnen, E.; Androphy, E.J.; Wirth, B. A single nucleotide in the SMN gene regulates splicing and is responsible for spinal muscular atrophy. *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6307–6311. [\[CrossRef\]](#)
9. Monani, U.R.; Lorson, C.L.; Parsons, D.W.; Prior, T.W.; Androphy, E.J.; Burghes, A.H.; McPherson, J.D. A single nucleotide difference that alters splicing patterns distinguishes the SMA gene SMN1 from the copy gene SMN2. *Hum. Mol. Genet.* **1999**, *8*, 1177–1183. [\[CrossRef\]](#)
10. Wadman, R.I.; Jansen, M.D.; Stam, M.; Wijngaarde, C.A.; Curial, C.A.D.; Medic, J.; Sodaar, P.; Schouten, J.; Vijzelaar, R.; Lemmink, H.H.; et al. Intragenic and structural variation in the SMN locus and clinical variability in spinal muscular atrophy. *Brain Commun.* **2020**, *2*, fcaa075. [\[CrossRef\]](#)

11. Feldkötter, M.; Schwarzer, V.; Wirth, R.; Wienker, T.F.; Wirth, B. Quantitative analyses of SMN1 and SMN2 based on real-time lightCycler PCR: Fast and highly reliable carrier testing and prediction of severity of spinal muscular atrophy. *Am. J. Hum. Genet.* **2002**, *70*, 358–368. [CrossRef] [PubMed]
12. Singh, R.N.; Singh, N.N. Mechanism of Splicing Regulation of Spinal Muscular Atrophy Genes. *Adv. Neurobiol.* **2018**, *20*, 31–61. [CrossRef] [PubMed]
13. Chiriboga, C.A.; Swoboda, K.J.; Darras, B.T.; Iannaccone, S.T.; Montes, J.; De Vivo, D.C.; Norris, D.A.; Bennett, C.F.; Bishop, K.M. Results from a phase 1 study of nusinersen (ISIS-SMN(Rx)) in children with spinal muscular atrophy. *Neurology* **2016**, *86*, 890–897. [CrossRef] [PubMed]
14. Ratni, H.; Ebeling, M.; Baird, J.; Bendels, S.; Bylund, J.; Chen, K.S.; Denk, N.; Feng, Z.; Green, L.; Guerard, M.; et al. Discovery of Risdiplam, a Selective Survival of Motor Neuron-2 (SMN2) Gene Splicing Modifier for the Treatment of Spinal Muscular Atrophy (SMA). *J. Med. Chem.* **2018**, *61*, 6501–6517. [CrossRef]
15. AveXis. AveXis Receives FDA Approval for Zolgensma®, the First and Only Gene Therapy for Pediatric Patients with Spinal Muscular Atrophy (SMA). 2019. Available online: <https://www.novartis.com/news/media-releases/avexis-receives-fda-approval-zolgensma-first-and-only-gene-therapy-pediatric-patients-spinal-muscular-atrophy-sma> (accessed on 11 October 2022).
16. Novartis. Novartis Receives Approval from Japanese Ministry of Health, Labour and Welfare for Zolgensma® the Only Gene Therapy for Patients with Spinal Muscular Atrophy (SMA). 2020. Available online: [https://www.novartis.com/news/media-releases/novartis-receives-approval-from-japanese-ministry-health-labour-and-welfare-zolgensma-only-gene-therapy-patients-spinal-muscular-atrophy-sma#:~:text=\(%E2%80%9CNovartis%20Pharma%E2%80%9D\)%20today,are%20pre%2Dsymptomatic%20at%20diagnosis](https://www.novartis.com/news/media-releases/novartis-receives-approval-from-japanese-ministry-health-labour-and-welfare-zolgensma-only-gene-therapy-patients-spinal-muscular-atrophy-sma#:~:text=(%E2%80%9CNovartis%20Pharma%E2%80%9D)%20today,are%20pre%2Dsymptomatic%20at%20diagnosis) (accessed on 18 April 2023).
17. Health-Canada. Summary Basis of Decision—Zolgensma—Health Canada. 2021. Available online: <https://hpr-rps.hres.ca/reg-content/summary-basis-decision-detailTwo.php?linkID=SBD00521#AClinBasisTitle> (accessed on 18 April 2023).
18. European-Medicines-Agency. New Gene Therapy to Treat Spinal Muscular Atrophy. 2020. Available online: <https://www.ema.europa.eu/en/news/new-gene-therapy-treat-spinal-muscular-atrophy-corrected> (accessed on 14 June 2023).
19. Chand, D.; Mohr, F.; McMillan, H.; Tukov, F.F.; Montgomery, K.; Kleyn, A.; Sun, R.; Tauscher-Wisniewski, S.; Kaufmann, P.; Kullak-Ublick, G. Hepatotoxicity following administration of onasemnogene abeparvovec (AVXS-101) for the treatment of spinal muscular atrophy. *J. Hepatol.* **2021**, *74*, 560–566. [CrossRef]
20. Finkel, R.S.; Darras, B.T.; Mendell, J.R.; Day, J.W.; Kuntz, N.L.; Connolly, A.M.; Zaidman, C.; Crawford, T.O.; Butterfield, R.J.; Shieh, P.B.; et al. Intrathecal Onasemnogene Abeparvovec for Sitting, Nonambulatory Patients with Spinal Muscular Atrophy: Phase I Ascending-Dose Study (STRONG). *J. Neuromuscul. Dis.* **2023**, *10*, 389–404. [CrossRef]
21. Mendell, J.R.; Al-Zaidy, S.; Shell, R.; Arnold, W.D.; Rodino-Klapac, L.R.; Prior, T.W.; Lowes, L.; Alfano, L.; Berry, K.; Church, K.; et al. Single-Dose Gene-Replacement Therapy for Spinal Muscular Atrophy. *N. Engl. J. Med.* **2017**, *377*, 1713–1722. [CrossRef] [PubMed]
22. Mendell, J.R.; Al-Zaidy, S.A.; Lehman, K.J.; McCollly, M.; Lowes, L.P.; Alfano, L.N.; Reash, N.F.; Iammarino, M.A.; Church, K.R.; Kleyn, A.; et al. Five-Year Extension Results of the Phase 1 START Trial of Onasemnogene Abeparvovec in Spinal Muscular Atrophy. *JAMA Neurol.* **2021**, *78*, 834–841. [CrossRef]
23. Novartis. Novartis Shares Zolgensma Long-Term Data Demonstrating Sustained Durability up to 7.5 Years Post-Dosing; 100% Achievement of All Assessed Milestones in Children Treated Prior to SMA Symptom Onset. 2023. Available online: <https://www.novartis.com/news/media-releases/novartis-shares-zolgensma-long-term-data-demonstrating-sustained-durability-75-years-post-dosing-100-achievement-all-assessed-milestones-children-treated-prior-sma-symptom-onset> (accessed on 25 March 2023).
24. Kolb, S.J.; Coffey, C.S.; Yankey, J.W.; Krossschell, K.; Arnold, W.D.; Rutkove, S.B.; Swoboda, K.J.; Reyna, S.P.; Sakonju, A.; Darras, B.T.; et al. Natural history of infantile-onset spinal muscular atrophy. *Ann. Neurol.* **2017**, *82*, 883–891. [CrossRef]
25. Finkel, R.S.; McDermott, M.P.; Kaufmann, P.; Darras, B.T.; Chung, W.K.; Sproule, D.M.; Kang, P.B.; Foley, A.R.; Yang, M.L.; Martens, W.B.; et al. Observational study of spinal muscular atrophy type I and implications for clinical trials. *Neurology* **2014**, *83*, 810–817. [CrossRef] [PubMed]
26. Day, J.W.; Finkel, R.S.; Chiriboga, C.A.; Connolly, A.M.; Crawford, T.O.; Darras, B.T.; Iannaccone, S.T.; Kuntz, N.L.; Peña, L.D.M.; Shieh, P.B.; et al. Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy in patients with two copies of SMN2 (STRIVE): An open-label, single-arm, multicentre, phase 3 trial. *Lancet Neurol.* **2021**, *20*, 284–293. [CrossRef]
27. Mercuri, E.; Muntoni, F.; Baranello, G.; Masson, R.; Boespflug-Tanguy, O.; Bruno, C.; Corti, S.; Daron, A.; Deconinck, N.; Servais, L.; et al. Onasemnogene abeparvovec gene therapy for symptomatic infantile-onset spinal muscular atrophy type 1 (STRIVE-EU): An open-label, single-arm, multicentre, phase 3 trial. *Lancet Neurol.* **2021**, *20*, 832–841. [CrossRef] [PubMed]
28. Strauss, K.A.; Farrar, M.A.; Muntoni, F.; Saito, K.; Mendell, J.R.; Servais, L.; McMillan, H.J.; Finkel, R.S.; Swoboda, K.J.; Kwon, J.M.; et al. Onasemnogene abeparvovec for presymptomatic infants with two copies of SMN2 at risk for spinal muscular atrophy type 1: The Phase III SPR1NT trial. *Nat. Med.* **2022**, *28*, 1381–1389. [CrossRef] [PubMed]
29. Strauss, K.A.; Farrar, M.A.; Muntoni, F.; Saito, K.; Mendell, J.R.; Servais, L.; McMillan, H.J.; Finkel, R.S.; Swoboda, K.J.; Kwon, J.M.; et al. Onasemnogene abeparvovec for presymptomatic infants with three copies of SMN2 at risk for spinal muscular atrophy: The Phase III SPR1NT trial. *Nat. Med.* **2022**, *28*, 1390–1397. [CrossRef] [PubMed]

30. Chand, D.H.; Mitchell, S.; Sun, R.; LaMarca, N.; Reyna, S.P.; Sutter, T. Safety of Onasemnogene Apeparvovec for Patients With Spinal Muscular Atrophy 8.5 kg or Heavier in a Global Managed Access Program. *Pediatr. Neurol.* **2022**, *132*, 27–32. [CrossRef]
31. Novartis. Zolgensma Global Managed Access Program in 2023. 2023. Available online: <https://www.novartis.com/news/zolgensma-global-managed-access-program-2023> (accessed on 25 March 2023).
32. European-Medicines-Agency. Summary of the Risk Management Plan for Zolgensma®.(Onasemnogene abeparvovec). 2023. Available online: https://www.ema.europa.eu/en/documents/rmp-summary/zolgensma-epar-risk-management-plan-summary_en.pdf (accessed on 18 April 2023).
33. De Amicis, R.; Baranello, G.; Foppiani, A.; Leone, A.; Battezzati, A.; Bedogni, G.; Ravella, S.; Giaquinto, E.; Mastella, C.; Agosto, C.; et al. Growth patterns in children with spinal muscular atrophy. *Orphanet J. Rare Dis.* **2021**, *16*, 375. [CrossRef]
34. Hinderer, C.; Katz, N.; Buza, E.L.; Dyer, C.; Goode, T.; Bell, P.; Richman, L.K.; Wilson, J.M. Severe Toxicity in Nonhuman Primates and Piglets Following High-Dose Intravenous Administration of an Adeno-Associated Virus Vector Expressing Human SMN. *Hum. Gene Ther.* **2018**, *29*, 285–298. [CrossRef]
35. René, C.A.; Parks, R.J. Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles. *Pharmaceutics* **2021**, *13*, 492. [CrossRef]
36. Thomsen, G.; Burghes, A.H.M.; Hsieh, C.; Do, J.; Chu, B.T.T.; Perry, S.; Barkho, B.; Kaufmann, P.; Sproule, D.M.; Feltner, D.E.; et al. Biodistribution of onasemnogene abeparvovec DNA, mRNA and SMN protein in human tissue. *Nat. Med.* **2021**, *27*, 1701–1711. [CrossRef]
37. Yazaki, K.; Sakuma, S.; Hikita, N.; Fujimaru, R.; Hamazaki, T. Child Neurology: Pathologically Confirmed Thrombotic Microangiopathy Caused by Onasemnogene Apeparvovec Treatment for SMA. *Neurology* **2022**, *98*, 808–813. [CrossRef]
38. Waldrop, M.A.; Karingada, C.; Storey, M.A.; Powers, B.; Iammarino, M.A.; Miller, N.F.; Alfano, L.N.; Noritz, G.; Rossman, I.; Ginsberg, M.; et al. Gene Therapy for Spinal Muscular Atrophy: Safety and Early Outcomes. *Pediatrics* **2020**, *146*, e20200729. [CrossRef]
39. Weiß, C.; Ziegler, A.; Becker, L.L.; Johannsen, J.; Brennenstuhl, H.; Schreiber, G.; Flotats-Bastardas, M.; Stoltenburg, C.; Hartmann, H.; Illsinger, S.; et al. Gene replacement therapy with onasemnogene abeparvovec in children with spinal muscular atrophy aged 24 months or younger and bodyweight up to 15 kg: An observational cohort study. *Lancet Child Adolesc. Health* **2022**, *6*, 17–27. [CrossRef] [PubMed]
40. Silverman, E. Novartis Reports Two Children Died from Acute Liver Failure after Treatment with Zolgensma Gene Therapy. 2022. Available online: <https://www.statnews.com/pharmalot/2022/08/11/novartis-zolgensma-liver-failure-gene-therapy-death/> (accessed on 11 October 2022).
41. Novartis. Zolgensma Acute Liver Failure Update. 2022. Available online: <https://www.novartis.com/news/zolgensma-acute-liver-failure-update> (accessed on 11 October 2022).
42. Astellas. Astellas Reports Update to September 1 Announcement on the ASPIRO Clinical Trial of AT132 in Patients with X-Linked Myotubular Myopathy. 2021. Available online: <https://www.astellas.com/en/news/17161> (accessed on 19 October 2022).
43. Astellas. Audent Therapeutics Provides Update on the ASPIRO Clinical Trial Evaluating AT132 in Patients with X-linked Myotubular Myopathy. 2020. Available online: <https://www.astellas.com/en/news/22726> (accessed on 19 October 2022).
44. Philippidis, A. Fourth Boy Dies in Trial of Astellas Gene Therapy Candidate. 2021. Available online: <https://www.genengnews.com/news/fourth-boy-dies-in-trial-of-astellas-gene-therapy-candidate/> (accessed on 19 October 2022).
45. Morales, L.; Gambhir, Y.; Bennett, J.; Stedman, H.H. Broader Implications of Progressive Liver Dysfunction and Lethal Sepsis in Two Boys following Systemic High-Dose AAV. *Mol. Ther.* **2020**, *28*, 1753–1755. [CrossRef]
46. Molera, C.; Sarishvili, T.; Nascimento, A.; Rtskhiladze, I.; Muñoz Bartolo, G.; Fernández Cebrián, S.; Valverde Fernández, J.; Muñoz Cabello, B.; Graham, R.J.; Miller, W.; et al. Intrahepatic Cholestasis Is a Clinically Significant Feature Associated with Natural History of X-Linked Myotubular Myopathy (XLMTM): A Case Series and Biopsy Report. *J. Neuromuscul. Dis.* **2022**, *9*, 73–82. [CrossRef]
47. Nault, J.C.; Datta, S.; Imbeaud, S.; Franconi, A.; Mallet, M.; Couchy, G.; Letouzé, E.; Pilati, C.; Verret, B.; Blanc, J.F.; et al. Recurrent AAV2-related insertional mutagenesis in human hepatocellular carcinomas. *Nat. Genet.* **2015**, *47*, 1187–1193. [CrossRef]
48. Zhong, L.; Malani, N.; Li, M.; Brady, T.; Xie, J.; Bell, P.; Li, S.; Jones, H.; Wilson, J.M.; Flotte, T.R.; et al. Recombinant adeno-associated virus integration sites in murine liver after ornithine transcarbamylase gene correction. *Hum. Gene Ther.* **2013**, *24*, 520–525. [CrossRef]
49. Donsante, A.; Vogler, C.; Muzyczka, N.; Crawford, J.M.; Barker, J.; Flotte, T.; Campbell-Thompson, M.; Daly, T.; Sands, M.S. Observed incidence of tumorigenesis in long-term rodent studies of rAAV vectors. *Gene. Ther.* **2001**, *8*, 1343–1346. [CrossRef]
50. Dalwadi, D.A.; Torrens, L.; Abril-Fornaguera, J.; Pinyol, R.; Willoughby, C.; Posey, J.; Llovet, J.M.; Lanciault, C.; Russell, D.W.; Grompe, M.; et al. Liver Injury Increases the Incidence of HCC following AAV Gene Therapy in Mice. *Mol. Ther.* **2021**, *29*, 680–690. [CrossRef]
51. Bell, P.; Moscioni, A.D.; McCarter, R.J.; Wu, D.; Gao, G.; Hoang, A.; Sanmiguel, J.C.; Sun, X.; Wivel, N.A.; Raper, S.E.; et al. Analysis of tumors arising in male B6C3F1 mice with and without AAV vector delivery to liver. *Mol. Ther.* **2006**, *14*, 34–44. [CrossRef]
52. Bell, P.; Wang, L.; Lebherz, C.; Flieder, D.B.; Bove, M.S.; Wu, D.; Gao, G.P.; Wilson, J.M.; Wivel, N.A. No evidence for tumorigenesis of AAV vectors in a large-scale study in mice. *Mol. Ther.* **2005**, *12*, 299–306. [CrossRef]

53. Gil-Farina, I.; Fronza, R.; Kaepfel, C.; Lopez-Franco, E.; Ferreira, V.; D'Avola, D.; Benito, A.; Prieto, J.; Petry, H.; Gonzalez-Aseguinolaza, G.; et al. Recombinant AAV Integration Is Not Associated With Hepatic Genotoxicity in Nonhuman Primates and Patients. *Mol. Ther.* **2016**, *24*, 1100–1105. [CrossRef]
54. Sabatino, D.E.; Bushman, F.D.; Chandler, R.J.; Crystal, R.G.; Davidson, B.L.; Dolmetsch, R.; Eggan, K.C.; Gao, G.; Gil-Farina, I.; Kay, M.A.; et al. Evaluating the state of the science for adeno-associated virus integration: An integrated perspective. *Mol. Ther.* **2022**, *30*, 2646–2663. [CrossRef] [PubMed]
55. Meyer, K.; Ferraiuolo, L.; Schmelzer, L.; Braun, L.; McGovern, V.; Likhite, S.; Michels, O.; Govoni, A.; Fitzgerald, J.; Morales, P.; et al. Improving single injection CSF delivery of AAV9-mediated gene therapy for SMA: A dose-response study in mice and nonhuman primates. *Mol. Ther.* **2015**, *23*, 477–487. [CrossRef] [PubMed]
56. Tukov, F.F.; Mansfield, K.; Milton, M.; Meseck, E.; Penraat, K.; Chand, D.; Hartmann, A. Single-Dose Intrathecal Dorsal Root Ganglia Toxicity of Onasemnogene Apeparvovec in Cynomolgus Monkeys. *Hum. Gene Ther.* **2022**, *33*, 740–756. [CrossRef]
57. Novartis. Novartis Announces AVXS-101 Intrathecal Study Update. 2019. Available online: <https://www.novartis.com/news/media-releases/novartis-announces-avxs-101-intrathecal-study-update> (accessed on 11 October 2022).
58. Novartis. Novartis Announces Lift of Partial Clinical Trial Hold and Plans to Initiate a New, Pivotal Phase 3 Study of Intrathecal OAV-101 in Older Patients with SMA. 2021. Available online: <https://www.novartis.com/news/media-releases/novartis-announces-lift-partial-clinical-trial-hold-and-plans-initiate-new-pivotal-phase-3-study-intrathecal-oav-101-older-patients-sma> (accessed on 11 October 2022).
59. Connolly, A.M.; Mercuri, E.; Strauss, K.A.; Day, J.W.; Chien, Y.-H.; Masson, R.; Widgerson, M.; Alecu, I.; Ballarini, N.; Mehl, L.; et al. Intravenous and Intrathecal Onasemnogene Apeparvovec Gene Therapy in Symptomatic and Presymptomatic Spinal Muscular Atrophy: Long-Term Follow-Up Study. In Proceedings of the 2023 MDA Clinical & Scientific Conference, Dallas, TX, USA, 19–23 March 2023.
60. Dangouloff, T.; Vrščaj, E.; Servais, L.; Osredkar, D.; Group, S.N.W.S. Newborn screening programs for spinal muscular atrophy worldwide: Where we stand and where to go. *Neuromuscul. Disord.* **2021**, *31*, 574–582. [CrossRef]
61. Finkel, R.S.; Mercuri, E.; Darras, B.T.; Connolly, A.M.; Kuntz, N.L.; Kirschner, J.; Chiriboga, C.A.; Saito, K.; Servais, L.; Tizzano, E.; et al. Nusinersen versus Sham Control in Infantile-Onset Spinal Muscular Atrophy. *N. Engl. J. Med.* **2017**, *377*, 1723–1732. [CrossRef]
62. Passini, M.A.; Bu, J.; Richards, A.M.; Kinnecom, C.; Sardi, S.P.; Stanek, L.M.; Hua, Y.; Rigo, F.; Matson, J.; Hung, G.; et al. Antisense oligonucleotides delivered to the mouse CNS ameliorate symptoms of severe spinal muscular atrophy. *Sci. Transl. Med.* **2011**, *3*, 72ra18. [CrossRef]
63. Biogen. Spinraza Prescribing Information. 2020. Available online: https://www.spinraza.com/content/dam/commercial/spinraza/caregiver/en_us/pdf/spinraza-prescribing-information.pdf (accessed on 20 October 2022).
64. Mercuri, E.; Darras, B.T.; Chiriboga, C.A.; Day, J.W.; Campbell, C.; Connolly, A.M.; Iannaccone, S.T.; Kirschner, J.; Kuntz, N.L.; Saito, K.; et al. Nusinersen versus Sham Control in Later-Onset Spinal Muscular Atrophy. *N. Engl. J. Med.* **2018**, *378*, 625–635. [CrossRef]
65. Jia, C.; Lei Mon, S.S.; Yang, Y.; Katsuyama, M.; Yoshida-Tanaka, K.; Nagata, T.; Yoshioka, K.; Yokota, T. Change of intracellular calcium level causes acute neurotoxicity by antisense oligonucleotides via CSF route. *Mol. Ther. Nucleic Acids* **2023**, *31*, 182–196. [CrossRef]
66. Southwell, A.L.; Skotte, N.H.; Kordasiewicz, H.B.; Østergaard, M.E.; Watt, A.T.; Carroll, J.B.; Doty, C.N.; Villanueva, E.B.; Petoukhov, E.; Vaid, K.; et al. In vivo evaluation of candidate allele-specific mutant huntingtin gene silencing antisense oligonucleotides. *Mol. Ther.* **2014**, *22*, 2093–2106. [CrossRef]
67. Toonen, L.J.A.; Casaca-Carreira, J.; Pellisé-Tintoré, M.; Mei, H.; Temel, Y.; Jahanshahi, A.; van Roon-Mom, W.M.C. Intracerebroventricular Administration of a 2'-O-Methyl Phosphorothioate Antisense Oligonucleotide Results in Activation of the Innate Immune System in Mouse Brain. *Nucleic Acid Ther.* **2018**, *28*, 63–73. [CrossRef] [PubMed]
68. Hagedorn, P.H.; Brown, J.M.; Easton, A.; Pierdomenico, M.; Jones, K.; Olson, R.E.; Mercer, S.E.; Li, D.; Loy, J.; Høg, A.M.; et al. Acute Neurotoxicity of Antisense Oligonucleotides after Intracerebroventricular Injection into Mouse Brain Can Be Predicted from Sequence Features. *Nucleic Acid Ther.* **2022**, *32*, 151–162. [CrossRef]
69. Pechmann, A.; Behrens, M.; Dörmbrack, K.; Tassoni, A.; Wenzel, F.; Stein, S.; Vogt, S.; Zöller, D.; Bernert, G.; Hagenacker, T.; et al. Improvements in Walking Distance during Nusinersen Treatment—A Prospective 3-year SMARTCARE Registry Study. *J. Neuromuscul. Dis.* **2023**, *10*, 29–40. [CrossRef]
70. Arslan, D.; Inan, B.; Kilinc, M.; Bekircan-Kurt, C.E.; Erdem-Ozdamar, S.; Tan, E. Nusinersen for adults with spinal muscular atrophy. *Neurol. Sci.* **2023**, *44*, 2393–2400. [CrossRef]
71. Dhillon, S. Risdiplam: First Approval. *Drugs* **2020**, *80*, 1853–1858. [CrossRef]
72. Wire, B. FDA Approves Genentech's Evrysdi (Risdiplam) for Use in Babies under Two Months with Spinal Muscular Atrophy (SMA). 2022. Available online: <https://www.businesswire.com/news/home/20220530005033/en/FDA-Approves-Genentech> (accessed on 11 October 2022).
73. Genentech. Evrysdi Prescribing Information. 2022. Available online: <https://www.gene.com/download/pdf/evrysdi-prescribing.pdf> (accessed on 3 April 2023).

74. Mercuri, E.; Baranello, G.; Boespflug-Tanguy, O.; De Waele, L.; Goemans, N.; Kirschner, J.; Masson, R.; Mazzone, E.S.; Pechmann, A.; Pera, M.C.; et al. Risdiplam in types 2 and 3 spinal muscular atrophy: A randomised, placebo-controlled, dose-finding trial followed by 24 months of treatment. *Eur. J. Neurol.* **2022**, *30*, 1945–1956. [\[CrossRef\]](#)
75. Mercuri, E.; Deconinck, N.; Mazzone, E.S.; Nascimento, A.; Oskoui, M.; Saito, K.; Vuillerot, C.; Baranello, G.; Boespflug-Tanguy, O.; Goemans, N.; et al. Safety and efficacy of once-daily risdiplam in type 2 and non-ambulant type 3 spinal muscular atrophy (SUNFISH part 2): A phase 3, double-blind, randomised, placebo-controlled trial. *Lancet Neurol.* **2022**, *21*, 42–52. [\[CrossRef\]](#)
76. Glanzman, A.M.; O'Hagen, J.M.; McDermott, M.P.; Martens, W.B.; Flickinger, J.; Riley, S.; Quigley, J.; Montes, J.; Dunaway, S.; Deng, L.; et al. Validation of the Expanded Hammersmith Functional Motor Scale in spinal muscular atrophy type II and III. *J. Child Neurol.* **2011**, *26*, 1499–1507. [\[CrossRef\]](#)
77. O'Hagen, J.M.; Glanzman, A.M.; McDermott, M.P.; Ryan, P.A.; Flickinger, J.; Quigley, J.; Riley, S.; Sanborn, E.; Irvine, C.; Martens, W.B.; et al. An expanded version of the Hammersmith Functional Motor Scale for SMA II and III patients. *Neuromuscul. Disord.* **2007**, *17*, 693–697. [\[CrossRef\]](#)
78. Malone, D.C.; Dean, R.; Arjunji, R.; Jensen, I.; Cyr, P.; Miller, B.; Maru, B.; Sproule, D.M.; Feltner, D.E.; Dabbous, O. Cost-effectiveness analysis of using onasemnogene abeparvocec (AVXS-101) in spinal muscular atrophy type 1 patients. *J. Mark. Access Health Policy* **2019**, *7*, 1601484. [\[CrossRef\]](#)
79. Oechsl, K.F.; Cartwright, M.S. Combination therapy with onasemnogene and risdiplam in spinal muscular atrophy type 1. *Muscle Nerve* **2021**, *64*, 487–490. [\[CrossRef\]](#) [\[PubMed\]](#)
80. Mirea, A.; Shelby, E.S.; Axente, M.; Badina, M.; Padure, L.; Leanca, M.; Dima, V.; Sporea, C. Combination Therapy with Nusinersen and Onasemnogene Abeparvocec-xioi in Spinal Muscular Atrophy Type I. *J. Clin. Med.* **2021**, *10*, 5540. [\[CrossRef\]](#) [\[PubMed\]](#)
81. Chiriboga, C.A.; Bruno, C.; Duong, T.; Fischer, D.; Mercuri, E.; Kirschner, J.; Kostera-Pruszczyk, A.; Jaber, B.; Gorni, K.; Kletzl, H.; et al. Risdiplam in Patients Previously Treated with Other Therapies for Spinal Muscular Atrophy: An Interim Analysis from the JEWELFISH Study. *Neurol. Ther.* **2023**, *12*, 543–557. [\[CrossRef\]](#)

Disclaimer/Publisher's Note: The statements, opinions and data contained in all publications are solely those of the individual author(s) and contributor(s) and not of MDPI and/or the editor(s). MDPI and/or the editor(s) disclaim responsibility for any injury to people or property resulting from any ideas, methods, instructions or products referred to in the content.

Appendix 3.3: Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency

Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency

Charlotte A. René^{1,2,3} and Robin J. Parks^{1,2,3,4}

¹Regenerative Medicine Program, Ottawa Hospital Research Institute, Ottawa, ON K1H 8L6, Canada; ²Department of Biochemistry, Microbiology and Immunology, University of Ottawa, Ottawa, ON K1H 8M5, Canada; ³Centre for Neuromuscular Disease, University of Ottawa, Ottawa, ON K1H 8M5, Canada; ⁴Department of Medicine, The Ottawa Hospital and University of Ottawa, Ottawa, ON K1H 8L6, Canada

Extracellular vesicles (EVs) have the innate ability to carry proteins, lipids, and nucleic acids between cells, and thus these vesicles have gained much attention as potential therapeutic delivery vehicles. Many strategies have been explored to enhance the loading of specific cargoes of interest into EVs, which could result in the delivery of more therapeutic to recipient cells, thus enhancing therapeutic efficacy. In this review, we discuss the natural biogenesis of EVs, the mechanism by which proteins and nucleic acids are selected for inclusion in EVs, and novel methods that have been employed to enhance loading of specific cargoes into EVs. As well, we discuss biodistribution of administered EVs *in vivo* and summarize clinical trials that have attempted to harness the therapeutic potential of EVs.

INTRODUCTION

Extracellular vesicles (EVs) are small membrane-bound particles secreted from cells into the extracellular space.¹ Initially, EVs were thought to be involved only in the elimination of cellular waste, but EVs actually perform an array of physiological functions in the body.^{2,3} These vesicles shuttle proteins, lipids, and nucleic acids between cells to mediate intercellular communication.⁴ The specific function and physical composition of EVs is determined by both the type and physiological state of the cell from which the EVs are released.¹

EVs are a heterogeneous population of particles, of which there are three main subtypes: exosomes, microvesicles (MVs), and apoptotic bodies. Exosomes have a diameter of between approximately 40 and 150 nm and are the smallest of the three subtypes.^{5,6} Exosomes are formed through invagination of the membrane of multivesicular bodies (MVBs) and are subsequently released into the extracellular environment following fusion of the MVB with the plasma membrane of the cell.³ In contrast, MVs, which are approximately 40–1000 nm in diameter, are generated by budding at the plasma membrane.^{1,6,7} The largest subtype of EVs are apoptotic bodies, >1,000-nm-diameter particles generated by dying cells through blebbing from the plasma membrane.⁶ Many other subtypes of EVs have been described, such as large oncosomes and arrestin domain-containing protein 1 (ARRDC1)-mediated MVs (ARMs),⁷ but these particles are less-well characterized than the three main EVs. Differentiating between the various subtypes of EVs can be challenging, as

the subtypes frequently have common cargo content and overlapping particle diameter.⁸ In the absence of actually observing the biogenesis of the vesicle (i.e., whether the particle arises from fusion of the MVB with the plasma membrane or directly from budding at the plasma membrane), it is more accurate in an experimental context to refer to EVs as either small (≤ 200 nm) or large (>200 nm) EVs. This review uses the term that was used by the original publication, whether that is “EV,” “exosomes,” or “MVs.”

EVs have many attractive features as therapeutics for the treatment of disease. The innate ability of EVs to shuttle cargo between cells makes the vesicles attractive therapeutic vehicles. Some EVs can target their uptake to specific cell types based on proteins presented on the surface of the EV and a complementary “receptor” on the surface of the target cell.⁹ Many studies have shown EVs administered to an allogeneic recipient induce minimal immunogenicity *in vitro* or *in vivo*.^{10–12} Some populations of EVs even have transmembrane proteins, such as CD24,^{13,14} CD47,¹⁵ and programmed death-ligand 1 (PD-L1),^{16,17} that allow the EVs to avoid phagocytosis by macrophages, thus providing the EVs with greater opportunity to deliver cargo to the desired target cell. Given these distinct advantages, it is unsurprising that many groups are exploring the use of EVs as therapeutic delivery vehicles and also developing methods to target specific proteins or nucleic acids into the EVs.

NATURAL BIOLOGY OF EVs

Biogenesis of exosomes and MVs

Exosomes and MVs are generated through different mechanisms of biogenesis, which each involve several sequential steps (Figure 1). For exosomes, the process begins with invagination of the plasma membrane, resulting in the formation of early endosomes inside the cell. The early endosome matures into the late endosome in a process mediated by Ras-associated binding (Rab) GTPases, notably those involved in the conversion of Rab5⁺ endosomes to Rab7⁺ endosomes.^{18,19} This endosome maturation step is followed by formation of intraluminal vesicles (ILVs) within the MVB through invagination

<https://doi.org/10.1016/j.omtm.2024.101259>

Correspondence: Robin J. Parks, Ottawa Hospital Research Institute, Room C4415 - Regenerative Medicine Program, 501 Smyth Road, Ottawa, ON K1H 8L6, Canada. E-mail: rparks@ohri.ca



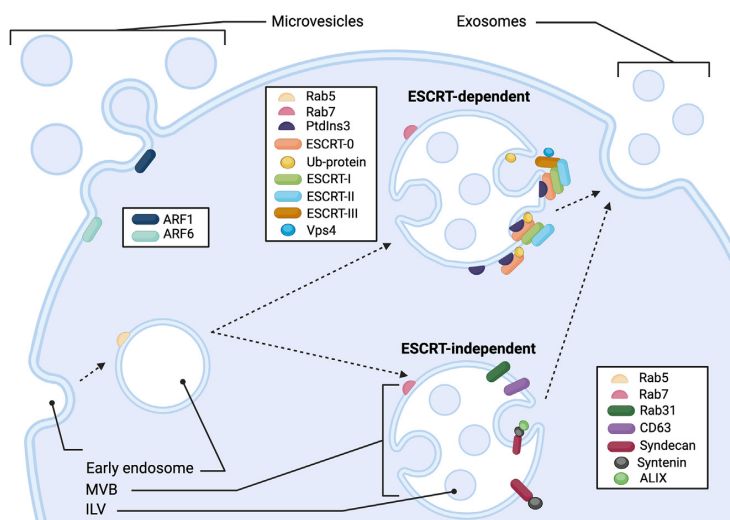


Figure 1. Biogenesis of exosomes and microvesicles

Invagination of the plasma membrane of the cell leads to development of the early endosome. The early endosome matures into the MVB, at which point Rab5 is replaced by Rab7. In ESCRT-dependent exosome biogenesis, ESCRT-0 interacts with Ub-proteins and binds PtdIns3P on the membrane of the endosome. ESCRT-I and ESCRT-II are then recruited, driving the invagination of the endosomal membrane. ESCRT-III completes ILV formation and is dissociated by Vps4. In ESCRT-independent exosome biogenesis, ILV formation can be driven by Rab31, CD63, or through the syndecan-syntenin-ALIX pathway. In both types of biogenesis, the MVB fuses with the cell membrane and leads to the release of ILV as exosomes. In microvesicle biogenesis, outward budding of the cell membrane and pinching off of the membrane results in the release of microvesicles. This process involves ARF1 and ARF6. ESCRT, endosomal sorting complex required for transport; ILV, intraluminal vesicle; MVB, multivesicular body; PtdIns3, phosphoinositide; Rab, ras-associated GTPases; Ub, ubiquitinated.

of the endosomal membrane, mediated by the endosomal sorting complex required for transport (ESCRT) machinery, composed of the ESCRT-0, -I, -II, and -III multiprotein complexes.²⁰ Hepatocyte growth factor-regulated tyrosine kinase (HRS), a subunit of the ESCRT-0 complex, is recruited to the endosomal membrane where it binds to phosphoinositide (PtdIns3P) on the endosome membrane, initiating the process of ILV formation.^{21–24} The tumor susceptibility gene 101 (TSG101) subunit of the ESCRT-I complex binds to HRS and, with ESCRT-II, drives the invagination of the endosomal membrane.^{25,26} ILV formation within the MVB is completed through membrane scission mediated by ESCRT-III, which is composed of vacuolar protein-sorting-associated protein 20 (Vps20), sucrose non-fermenting protein 7 (Snf7), Vsp24, and Vps2.^{27,28} The AAA ATPase VPS4 disassembles the ESCRT-III complex, dissociating its subunits from the MVB and enabling their reuse.^{29,30}

Exosome biogenesis can still occur in the absence of the ESCRT machinery,³¹ and several cellular proteins have been implicated in mediating this ESCRT-independent pathway. Rab31 can be recruited to the endosome, where it is phosphorylated by epidermal growth factor receptor (EGFR) present on the surface of the endosome.³² Rab31 then interacts with flotillin proteins, thus driving ILV formation. Similarly, tetraspanins, such as CD9, CD81, and CD63, are thought to be involved in ESCRT-independent mechanisms of exosome biogenesis.^{33–35} Indeed, depletion of CD9 leads to a reduction in formation of early endosomes and decreased exosome secretion.³⁶ Similar to HRS, CD63 can engage the endosomal membrane, permitting ILV formation even in the absence of ESCRT-0.³⁷ The syndecan-syntenin-ALG-associated protein X (ALIX) pathway for exosome biogenesis also does not require ESCRT-0; syndecan in the membrane of the MVB interacts with syntenin, which promotes ILV biogenesis through interaction with ALIX.^{38,39}

In both ESCRT-dependent and -independent exosome biogenesis, the MVB ultimately fuses with the plasma membrane of the cell to release ILVs into the extracellular space as exosomes.^{40,41} A portion of the exosomes released from the ILVs may remain associated with the plasma membrane of the cell, mediated by tetherin located on the surface of both the cell and exosome.⁴² Alternatively, the MVB may fuse with a lysosome, leading to degradation of the MVB contents due to the action of hydrolases and proteases contained in the fused vesicle.⁴³

MVs differ from exosomes in that they originate from the outward budding and fission of the cell plasma membrane, but much less is known about their mechanism of biogenesis.⁴⁴ The outward budding of the plasma membrane is accompanied by distinct changes in protein and lipid content of the membrane that leads to a change in the curvature and rigidity of the membrane. ADP-ribosylation factor 1 (ARF1) can use myosin light-chain kinase (MLCK) to activate contractile machinery to allow for pinching off of the plasma membrane to release MVs into the extracellular environment.⁴⁵ ARF6 can also activate the contractile machinery via MLCK, and can regulate the selective recruitment of some proteins into MVs.⁴⁶ ARRDC1 can interact with TSG101 to release a subpopulation of MVs called ARMMs.⁷ ARRDC1 is localized to the plasma membrane, and recruits TSG101 to the membrane from the endosome. ARMMs are released into the extracellular space following ubiquitination of ARRDC1.⁷

Cargo loading

Thousands of proteins have been identified as EV cargo, and these proteins are cataloged in databases such as Vesiclepedia (<http://www.microvesicles.org>). Some of these proteins are actively loaded into EVs, such as through the processes detailed below, but many may be passively loaded, as any protein or nucleic acid that is in

the general vicinity of EV biogenesis may ultimately be incorporated into the EVs.

Many proteins involved in the biogenesis of EVs are retained within mature EVs. These same proteins also frequently mediate the selective loading of other cellular proteins, which are recruited to EVs as a consequence of natural protein-protein interactions. As mentioned, one of the possible fates of the MVB is to fuse with the lysosome, leading to degradation of the MVB contents.⁴⁷ Indeed, some of the protein contents of the ILV are ubiquitinated—a cellular protein modification commonly used to tag a protein for degradation and disposal.^{48,49} The ESCRT machinery, along with a number of accessory proteins, plays a role in biomolecule sorting of exosomal cargo and can specifically interact with ubiquitinated proteins to facilitate their loading into EVs.⁵⁰ The HRS and signal transducing adaptor molecule (STAM) subunits of the ESCRT-0 complex both contain ubiquitin identification motifs, and can recruit ubiquitinated protein cargo to the endosomal membrane, leading to their incorporation into exosomes.⁵¹ Similarly, the TSG101 and ubiquitin-associated protein 1 (UBAP1) subunits of ESCRT-I each contain a ubiquitin binding domain that can also recruit ubiquitinated cargo to the endosomal membrane.^{52,53} The Vps45 domain of ESCRT-II also can bind ubiquitinated proteins and plays a role in loading of ubiquitinated cargo.⁵⁴ EVs are enriched in many tetraspanins, including CD9, CD37, CD63, CD81, and CD82.^{55,56} CD9 and CD63 can facilitate the incorporation of major histocompatibility complex (MHC) class II proteins into exosomes.^{57,58}

Proteins may be selectively included into MVs during biogenesis, such as proteins transported via the ARF6-regulated endosomal recycling pathway, including β 1 integrin receptors and vesicle-associated membrane protein 3 (VAMP3).^{59,60} RAB22A, which is overexpressed in many breast cancer cells and can selectively recruit proteins to MVs, facilitates an increased production of MVs under hypoxic conditions.⁶¹ These hypoxia-induced MVs were found to stimulate focal adhesion formation, extracellular matrix deposition, and enhance lung colonization by breast cancer cells in an RAB22A-dependent manner *in vitro*.

Ribonucleic acid (RNA)-binding proteins (RBPs) make up approximately 25% of the total proteins contained within EVs, and thus RNAs are also common cargo within EVs.⁶² There is a lack of consensus on whether full-length messenger RNA (mRNA) can be incorporated into EVs, as evidence suggests that fragmented transcripts,^{63,64} full-length transcripts,^{65,66} or both forms^{67–69} are loaded into EVs. Many proteins are thought to mediate the loading of microRNA (miRNA) into EVs, including various heterogeneous nuclear ribonucleoproteins (hnRNPs),^{70–72} major vault protein (MVP),⁷³ and Y-box-binding protein-1 (YBX-1).^{74,75} Several sequence motifs in RNA have been identified that are thought to lead to the enrichment of specific RNAs within EVs.^{76–78} One such motif is CGGGAG that can be recognized by two RBPs, fused in sarcoma (Fus) and Alyref, promoting the loading of miRNA into EVs.⁷⁷ Exploitation of these natural pathways for cargo loading can be used to selectively enhance uptake of RNA into EVs, as discussed below.

Table 1. Engineered cargo loading strategies

Cargo	Strategy	Reference
Protein	Overexpression of protein	Nash et al. ⁸³
	Fusion proteins	
	CD63	McCann et al. ⁸⁸
	LAMP2B	Alvarez-Erviti et al. ⁸⁹
	GPI	Kooijmans et al. ⁹⁰
	PDGFR	Ohno et al. ⁹¹
	Lactadherin	Rountree et al. ⁹²
	PTGFRN	Dooley et al. ⁹³
	BASP1	Dooley et al. ⁹³
	Nef	Manfredi et al. ⁹⁴
	Protein:protein association	
	KFERQ motif	Ferreira et al. ⁹⁵
	EXPLORs system	Yim et al. ⁹⁶
	Rapamycin-FKBP-FRB system	Somiya and Kuroda ⁹⁷
	WW-domain	Sterzenbach et al. ⁹⁸
	Ub tag	Cheng and Schorey ⁹⁹
	EXOmotifs	Villarroya-Beltri et al. ⁷⁰
	Incorporation of pre-miR-451 backbone	Reshke et al. ¹⁰⁰
	GUGCC-containing motif	Bolukbasi et al. ⁷⁶
	TAMEL system	Hung and Leonard ¹⁰¹
	G58T peptide	Dar et al. ¹⁰²
RNA		

ENGINEERED EV CARGO LOADING STRATEGIES

EVs can be loaded with cargo of interest using either exogenous^{79–81} or endogenous^{82,83} loading. In exogenous loading, EVs are first harvested from a biofluid, and then cargo is loaded. Many different methods have been used to achieve exogenous loading, including electroporation, sonication, freeze-thaw cycles, extrusion, and the use of pore-forming agents.^{81,84–86} Unfortunately, these approaches can result in permanent damage of the EV membrane, as many of these methods rely on transient disruption of the membrane to load the cargo. The efficiency of electroporation was found to be extremely low, with less than 0.05% encapsulation efficiency.⁸⁷ In endogenous loading, EVs are isolated from cells that have been manipulated in some way, for example through engineering to overexpress a particular therapeutic protein, so that the protein is incorporated into the EVs during their natural formation.^{82,83} Several strategies have been developed to increase the efficiency of endogenous cargo loading, thus increasing the concentration of the cargo of interest within EVs (Table 1).

Protein

EV cargo content usually reflects the state of the cell from which they originate, and simply overexpressing a protein of interest within donor cells can lead to release of EVs loaded with high levels of that protein.^{103,104} For example, we showed that plasmid- or adenovirus-mediated overexpression of survival motor neuron (SMN)

protein in donor cells led to release of exosomes with elevated concentrations of SMN protein.⁸³ Countless studies have shown a similar effect with numerous different proteins.

Fusion proteins

A popular approach to enhance uptake of a desired protein into EVs is to create a fusion protein with a second protein that is naturally incorporated into EVs. Fusion of protein cargo to CD63 can increase the presentation of that cargo on the surface of the EVs. CD63 is a tetraspanin found on the surface of cells, and is consequently also found in endosomes and on the surface of EVs.⁵⁵ HEK293T cells transfected with a plasmid encoding a CD63-emerald green fluorescent protein (emGFP) fusion protein incorporated more emGFP into EVs compared with cells transfected with plasmid expressing emGFP alone.⁸⁸ GFP has been conjugated to several other EV proteins, and vesicle association of GFP was more efficient when coupled to membrane-associated proteins, such as CD9, CD63, and CD81, compared with proteins normally found in the cytosol of EVs, such as ALIX, Flotillin-2, or native-uncoupled GFP.^{105,106} Conjugation of ovalbumin (OVA) antigen (Ag) to CD63 (CD63-OVA) dramatically increased the presence of OVA on the EV surface.¹⁰⁷ Moreover, CD63-OVA EVs elicited a stronger Ag-specific immune response than OVA EVs or OVA alone following immunization of mice. Fusion of the neuron-specific rabies viral glycoprotein (RVG) peptide to the EV membrane protein lysosome-associated membrane glycoprotein 2B (LAMP2B) resulted in presentation of RVG peptide on the surface of exosomes, which was used to enhance targeting of the vesicles to neurons *in vivo*.^{89,108} Glycosylphosphatidylinositol (GPI),⁹⁰ platelet-derived growth factor receptors (PDGFRs),⁹¹ and lactadherin (C1C2 domain),⁹² all of which are naturally found in the membrane of EVs, have also been used to display proteins of interest on the surface of EVs.

Proteins prostaglandin F2 receptor inhibitor (PTGFRN), a member of the immunoglobulin superfamily, and brain acid soluble protein 1 (BASP1), a member of the myristoylated alanine-rich C-kinase substrate protein family, are enriched in EVs compared with non-EV fractions of conditioned medium as assessed by liquid chromatography mass spectrometry.⁹³ Cargo can be loaded into EVs with high efficiency when fused to the PTGFRN or BASP1. Fusion of GFP to the intracellular domains of PTGFRN or BASP1 resulted in enhanced loading of GFP into EVs compared with GFP alone.⁹³ These same fusion proteins led to higher levels of association of GFP with EVs than similar fusion proteins with LAMP2B, CD9, CD63, or CD81, which may simply reflect the relative abundance of the various membrane proteins naturally found in EVs. As PTGFRN is a single-pass transmembrane protein, target proteins can be fused to the C or N terminus, resulting in the presence of the target protein on either the extra- or intravesicular side of the EV membrane, respectively. Fusion to PTGFRN permitted display of several target molecules on the surface of EVs, including cytokines and antigens.⁹³ Fusion to BASP1 increased loading of several proteins into the lumen of EVs, including clustered regularly interspaced short palindromic repeat (CRISPR)-associated protein 9 (Cas9) protein.⁹³

The human immunodeficiency virus (HIV)-1 negative regulatory factor (Nef) protein is naturally incorporated into exosomes at high levels.⁹⁴ Nef promotes viral replication of HIV-1 by enabling immune escape of infected cells.^{109,110} Use of an engineered mutant of Nef (Nef^{mut}), deficient in most functions of intact Nef protein, was able to efficiently load several fusion proteins into exosomes, including GFP and Ag from several viruses.^{94,111} These Ag-loaded exosomes effectively primed cytotoxic T lymphocyte immunity in mice.¹¹¹

Protein:protein association

Cargo loading can also be increased through use of peptide motifs that mediate non-covalent interaction with proteins normally recruited into EVs. Proteomic and bioinformatic analysis of EVs derived from cells either expressing or lacking LAMP2A, an EV scaffolding protein, showed that proteins containing a KFERQ motif were under-represented in the LAMP2A-deficient EVs.⁹⁵ Fusion of the KFERQ motif to mCherry led to binding of mCherry to LAMP2A, as assessed by co-immunoprecipitation, and enhanced uptake of the protein into EVs compared with mCherry alone. The authors also showed that proteins tagged with KFERQ motifs were loaded into EVs independent of the ESCRT machinery.

An elegant exosomal loading technique was developed called exosomes for protein loading via optically reversible protein-protein interactions (EXPLORs).⁹⁶ This system is composed of two components. First, photoreceptor cryptochrome 2 (CRY2) protein was fused to target cargo, such as mCherry. Second, truncated CRY-interacting basic-helix-loop-helix 1 (CIBN) protein was fused to the intraluminal side of CD9, which resulted in presentation of CIBN on the external surface of the MVB. Application of blue light induced association of CIB1 and CRY2, recruiting the mCherry-CRY2 to the membrane of the MVB to transiently interact with CIBN-CD9, thus drawing the protein into the exosome during ILV formation. Once the blue light was turned off, mCherry-CRY2 disassociated from CIBN-CD9, resulting in release of the protein into the intraluminal space of the exosomes, ensuring that the target protein was not irreversibly stuck to the exosome membrane, and ultimately allowing efficient delivery of the target protein to recipient cells. This study further demonstrated functional delivery of various proteins, such as Bax-mCherry:EXPLORs to HeLa cells, which caused a release of cytochrome *c* from the mitochondria, leading to caspase-dependent apoptosis. Delivery of srIκB-mCherry:EXPLORs (super-repressor IκB [srIκB], an inhibitor of nuclear factor [NF]-κB) to HeLa cells reduced tumor necrosis factor-α (TNF-α)-induced translocation of NF-κB to the nucleus of the cells, thereby decreasing the expression of genes related to survival and inflammation of cancerous cells.¹¹² Yim et al.⁹⁶ used EXPLORs to deliver Cre protein to a transgenic enhanced yellow fluorescent protein (EYFP) reporter mouse. Ventrolateral injection of the brain with Cre:EXPLORs led to EYFP protein expression mainly in neurons in the ventrolateral part of the brain of these mice as assessed 96 h post-injection.

Recently, a system similar to EXPLORs was developed that used rapamycin to induce interaction of specific fusion proteins to enhance their incorporation into EVs.⁹⁷ Rapamycin naturally induces

heterodimerization of FK506 binding protein (FKBP) and FKBO12-rapamycin-binding (FRB) domains.¹¹³ CD81 (naturally found in the membrane of exosomes) was fused to FKBP, and FRB was fused to the tetracycline transactivator (tTa) protein. HEK293T cells were transfected with pCD81-FKBP, pFRB-tTa, and pVSV-G, a vesicular stomatitis virus fusogenic glycoprotein known to enhance delivery efficiency of EVs,^{114,115} and the cells were cultured in the presence or absence of rapamycin. The FRB-tTa fusion protein was found in much higher quantity in EVs isolated from cells cultured in rapamycin, and these EVs delivered more tTa to recipient cells, compared with EVs isolated from cells not treated with rapamycin, as measured by induction of expression from a tTa-controlled reporter gene present in the target cells. Cytoplasmic delivery of an FRB-fused protein was also demonstrated using a split NanoLuc luciferase reporter, in which one part of NanoLuc was fused to FRB and the complementing fragment was expressed in the recipient cells, thus emitting luminescence only if the FRB-fused protein was successfully internalized and was available to complement NanoLuc activity. In place of CD81, other EV-enriched proteins were tested using the EXPLORs approach. CD63-FKBP and HIV Gag-FKBP showed weaker reporter gene expression in recipient cells than CD81-FKBP as detected by the split NanoLuc reporter, but were still significantly greater than control. The rapamycin-FKBP-FRB EV cargo loading method was also used to package Cas9 protein and single guide RNA (sgRNA) into EVs to induce therapeutic exon skipping in reporter mice and tissue culture, including in Duchenne's muscular dystrophy (DMD) patient-induced pluripotent stem cells (iPSCs).¹¹⁶ In that study, exon 45 of dystrophin was removed using this method to restore the reading frame and dystrophin protein expression in the DMD patient-derived iPSCs containing exon 44 or 46–47 deletions. A VGV-G-free method of rapamycin-FKBP-FRB EV cargo loading has been developed through the use of Syncytin-1, an endogenous human fusogen protein.¹¹⁷

A ubiquitin-based technique has also been used to incorporate target proteins into exosomes.⁹⁸ Fusion of a WW-domain (a 35-amino acid motif containing two conserved tryptophans) onto a protein allows the protein to interact with Nedd4 family interacting protein 1 (Ndfip1), which facilitates Nedd4-mediated ubiquitination of the target protein, and subsequent preferential loading into EVs.^{98,118,119} For example, transient expression of Cre protein alone within the producer cells did not result in appreciable loading of the Cre protein into exosomes, whereas WW-Cre protein was loaded into exosomes, but only if Ndfip1 was expressed in the producer HEK293 cells.⁹⁸ The requirement for Ndfip1 was further demonstrated through the use of murine embryonic fibroblasts (MEFs) isolated from Ndfip1 knockout (KO) mice; WW-Cre was not effectively loaded into exosomes in the Ndfip1-KO MEFs. Intranasal delivery of WW-Cre exosomes in transgenic reporter mice that can inducibly express tdTomato led to Cre-mediated recombination in the brain.

Another method that took advantage of the natural ubiquitin-based protein sorting system involved use of an engineered tag that mimics ubiquitin.⁹⁹ This method used a modified ubiquitin that was deleted

of the two C-terminal glycine residues of ubiquitin (designated cUb), which improves the half-life of the protein.¹²⁰ Expression of a fusion protein composed of cUb and the *Mycobacterium tuberculosis* proteins Ag85B and early secreted antigen target 6 kDa (ESAT6) led to a 10-fold increase in loading of the fusion protein into exosomes compared with protein without the cUb tag.⁹⁹ Exosomes containing the Ag85B-ESAT6-cUb fusion protein were able to induce a stronger antigen-specific immune response in naive mice compared with exosomes isolated from cells expressing Ag85B-ESAT6 lacking the cUb tag. One caveat of this study is that it was not determined whether the cUb tag increases the immunogenicity of the fusion protein.

RNA

As mentioned, both deoxyribonucleic acid (DNA) and RNA, including miRNA, are found naturally in EVs. Numerous studies have analyzed the miRNA composition of EVs isolated from specific cell types or pathological conditions, such as cancer,^{121,122} to gain insight into the disease state or identify potential new biomarkers. miRNAs released in cancer cell-derived EVs can have profound effects on neighboring cells, including promoting angiogenesis and tumorigenesis.^{123,124} However, quantitative studies have shown that miRNAs are only present at very low levels in EVs, ranging from one copy of a given miRNA per EV to one copy per 10⁶ EV,¹²⁵ and thus techniques must be developed to increase the efficiency of miRNA loading. Several proteins have been implicated in facilitating or chaperoning the loading of miRNAs into EVs. Sumoylated heterogeneous nuclear ribonucleoprotein A2B1 (hnRNPA2B1) binds a subset of miRNA through recognition of specific sequence motifs, called EXOmotifs, mediating their loading into exosomes.⁷⁰ Annexin A2 (AXA2) has also been shown to play a role in loading of miRNA into EVs.¹²⁶ Small interfering RNA (siRNA) silencing of AXA2 significantly decreased the amount of miRNA loaded into EVs in tissue culture.

Bioinformatic analysis of all miRNAs found in EVs isolated from MEFs, MDA-MB-231 human breast cancer cells, and NSC-34 motor neuron-like cells showed that pre-miR-451 is selectively enriched within EVs, suggesting it may contain an RNA motif that mediates preferential loading.¹⁰⁰ Indeed, incorporation of other miRNA sequences into the pre-miR-451 hairpin enhanced their loading into EVs.^{100,127} Incorporation of siRNAs targeting GFP, tetracycline repressor, or superoxide dismutase 1 (SOD1) into the pre-miR-451 backbone caused these siRNAs to be enriched in EVs.¹⁰⁰ Incorporation in the pre-miR-451 backbone of siRNA targeting GFP led to an ~3,700-fold increase in incorporation of the modified siRNA into EVs and was very effective in silencing GFP expression in primary motor neurons isolated from GFP transgenic mice. When these EVs were administered to GFP transgenic mice by tail vein injection, ~50% GFP knockdown was observed in the small intestine and liver of the animals, while no knockdown was noted in the heart, lungs, or brown fat. In the G93A SOD1 mouse model of amyotrophic lateral sclerosis, systemic administration of EVs loaded with siRNA targeting SOD1 in the pre-miR-451 backbone resulted in knockdown of SOD1 by ~60% in the liver and ~75% in the small intestine. This study clearly shows the promise of this approach in improving

incorporation of a desired siRNA into EVs, but also highlights the challenge of uneven distribution of EVs following systemic delivery.

Glyceraldehyde-3-dehydrogenase (GAPDH) can associate with the surface of EVs through a phosphatidylserine-binding domain, termed the G58 peptide.¹⁰² G58T, a fusion protein consisting of the G58 peptide and the RNA-binding domain of TAR RNA-binding protein 2 (TARBP2), was able to load siRNA onto EVs with high efficiency without the need to incorporate a targeting sequence into the siRNA. Delivery of EVs loaded with siRNA targeting GAPDH was able to reduce the levels of the protein to ~15% of untreated N2a recipient cells. These researchers also utilized the G58T system to develop EVs that contained a mixture of siRNA directed toward Huntingtin protein (Htt), designated G58TF/siRNA-RVG-EVs. The EVs also had an RVG peptide on the surface to target EVs to neurons. The G58T chimeric protein was further engineered to include a flock house nodovirus (FHV) domain,¹⁰² which has been shown to enhance release of cargo from late endosomes.¹²⁸ In the Q140 mouse model of Huntington's disease, administration of G58TF/siRNA-RVG-EVs once a week for 4 weeks resulted in ~40% reduction in Htt expression in the mouse brain cortex.¹⁰²

For mRNAs, a 25-nucleotide sequence in the 3' untranslated region (UTR) of some mRNA appears to be involved in EV loading.⁷⁶ This sequence binds miR-1289 and includes a GUGCC core presented on a stem-loop structure. Incorporation of this sequence into the 3' UTR of a reporter mRNA led to increased loading into MVs, although the protein or proteins responsible for the increased loading of GUGCC motif-containing mRNA remains unknown.

A novel technique for loading RNA into EVs has been developed called Targeted and Modular EV Loading (TAMEL).¹⁰¹ The coat protein from bacteriophage MS2 naturally binds to a stem-loop structure present in the viral RNA genome.¹²⁹ For the TAMEL system, the MS2 coat protein was fused to LAMP2B, a protein naturally recruited to EVs, and the stem-loop structure from the MS2 genome was engineered into the cargo RNA.¹⁰¹ The TAMEL approach was able to enhance the loading of cargo RNA into EVs up to 6-fold. When the TAMEL approach was used in cells also expressing VSV-G, a 40-fold increase in cargo RNA loading into EVs was observed. The authors hypothesized that this increase in loading may be due to the ability of VSV-G to augment uptake of cargo into EVs via binding to the low-density lipoprotein (LDL) receptor, which normally also plays a role in VGV-G-mediated lentiviral delivery.^{130,131}

Taken together, these studies show that there are many elegant techniques that can be exploited to load a specific nucleic acid or protein into EVs. However, once a product is developed, the next hurdle is producing and purifying sufficient product to perform *in vitro* and/or *in vivo* characterization of efficacy.

ISOLATION AND PURIFICATION OF EVs

There are several methods currently in use for isolation of EVs. The most common methods include differential ultracentrifugation, size-

exclusion chromatography, filter concentration, polyethylene glycol (PEG) precipitation, density gradients, and tangential flow filtration (TFF).⁸ Each method has a different degree of specificity and recovery, and unique advantages and disadvantages. Many insightful reviews have discussed these different isolation methods in depth, as well as challenges associated with large-scale manufacturing of therapeutic EVs.^{132–136} The method of EV isolation employed for a particular study in part depends on the intended application. For example, while PEG-based methods may be ideal for isolation of EVs from small volumes of biological fluids for biomarker studies, TFF may be more appropriate for isolation of EVs from large volumes of tissue culture medium for use in human clinical trials. Regardless of the method used to isolate the EVs, the resulting product must be fully characterized to determine the purity, quality and composition (e.g., particle size distribution, concentration) of the sample. The International Society for Extracellular Vesicles has released a series of consensus papers entitled “Minimal information for studies of extracellular vesicles (MISEV),” most recently in 2023 (MISEV2023),⁸ that outline preferred methods to evaluate EV samples. If adopted throughout the field, the MISEV guidelines should lead to greater clarity and reproducibility of all EV studies.

BIODISTRIBUTION OF EVs

EVs are released by virtually every cell type and found in all bodily fluids, including blood,¹³⁷ urine,¹³⁸ saliva,¹³⁹ and cerebrospinal fluid (CSF).¹⁴⁰ EVs from different cell types will have specificity for different target tissues, based on a combination of the composition of proteins present on the EV surface and complementary receptors found on the recipient cell.^{141,142} This is also true for EVs mass-produced in tissue culture for *in vivo* studies. However, the method of EV administration will also influence where delivered EVs accumulate.⁹

Intravenous administration

Many groups have demonstrated that EVs isolated from a variety of cell types accumulate in liver, spleen, kidney, and lungs following tail vein injection in mice, although the primary tissue in which the EVs accumulated varied between studies.^{143–146} In one study, intravenous (i.v.) administration of EVs resulted in preferential uptake by macrophages in the liver and spleen and endothelial cells in the lungs in mice.¹⁴⁷ In another study, EVs isolated from mesenchymal stem cells (MSCs) labeled with fluorescent dye were found in not only the liver and spleen, but also in the femur, tibia, and spinal cord following i.v. administration in a radiation injury bone marrow mouse model.¹⁴⁸ Accumulation of MSC-derived EVs at the site of injured bone marrow could be detected as soon as 1 h post-injection (hpi) and fluorescence was statistically unchanged between 2 and 24 hpi. The authors interpreted this as maintenance in concentration of therapeutic EVs at the target tissue over time. Many of the studies listed below compared i.v. administration with intraperitoneal (i.p.) or intranasal administration, and further demonstrate the wide variety of tissues EVs distribute to following i.v. administration.

Intraperitoneal administration

The i.p. administration of therapeutic EVs has also been investigated in mouse models. MSC-derived EVs were administered i.p. in healthy

and pancreatic tumor-bearing mice.¹⁴⁹ In both treatment groups at 6 hpi, EVs accumulated in the pancreas, but showed especially high accumulation within the tumors in the pancreas. The study reported that accumulation of EVs in the pancreas was greater than EV accumulation within the liver, spleen, or lungs in both tumor-bearing mice and controls. EVs were also present within pancreatic tumors following i.v. administration of EVs, although to a lesser extent than EVs administered i.p., and greater signal intensity was found in the liver, lungs, and spleen compared with EVs administered i.p.. Accumulation of signal intensity in the pancreatic tumors at 24 and 48 hpi suggested tumor-specific EV retention. In another study that compared methods of administration, EVs isolated from HEK293T cells were labeled with the fluorescent lipophilic tracer DiR and administered i.p., i.v., or subcutaneously in mice.⁹ All treatment groups had significant accumulation of EVs in the liver, but i.v. administration led to the highest level of liver uptake of the three delivery routes examined. The i.v. administration group also had the greatest accumulation of EVs in the spleen. Accumulation of EVs in the lungs was similar in mice treated subcutaneously or i.v., but lower in mice treated i.p. In contrast, EV accumulation in the pancreas was greatest in mice treated i.p. and lowest in mice treated i.v. Significant accumulation of EVs was noted in the gastrointestinal tract of mice treated i.p. and subcutaneously, and to a lesser extent in mice treated i.v. Low levels of EV accumulation were also noted in brain, heart, kidney, and quadriceps muscle of all treated mice.

Intranasal administration

Although EVs are naturally capable of transiting the blood-brain barrier,^{150,151} intranasal delivery of EVs has been used in an attempt to increase delivery of EVs to the brain. Following intranasal delivery, particles, such as EVs, are thought to be transported to the brain via transcytosis of olfactory receptor neurons, which connects the nasal cavity and olfactory bulb.¹⁵² In a study comparing methods of administration, EVs isolated from MSC were administered intranasally or i.v. to mice, although mice treated i.v. were administered a four times higher dose of EVs than the intranasal group.¹⁵³ Twenty-four hours after administration, the greatest accumulation of EVs was in the liver, lungs, and spleen of i.v.-treated mice, with some accumulation in the heart, kidney, and brain. In contrast, relatively high accumulation of EVs was noted in the brain of mice treated intranasally, with relatively low levels in the heart and gut. In a similar study, increasing doses of EVs containing luciferase were administered to non-human primates (NHPs) either i.v. or intranasally.¹⁵⁴ Intranasal administration at all doses resulted in little-to-no detectable luminescence in plasma at a variety of time points spanning from 1 min to 24 h post-administration. In contrast, doses administered i.v. produced a luminescent signal in plasma but, surprisingly, higher doses of EVs were cleared more rapidly from plasma than lower doses, for reasons that are unclear. In CSF, luminescence was detected with high doses of EVs delivered i.v., whereas no luminescence was detected in mice treated intranasally. Also in this study, NHPs were administered a fixed-dose of EVs either intranasally or i.v., and tissues were collected 1 hpi. Greater luminescence was noted in liver, spleen, lung, and colon tissue of i.v.-treated NHPs compared

with the intranasal administration group, whereas similar luminescence was noted in kidney and heart tissue of both administration groups. In general, similar luminescence was detected in the brain of intranasally and i.v. treated NHPs; however, greater luminescence was noted in the cerebellum of i.v.-treated NHPs, and in the midbrain of intranasally treated NHPs. These researchers also examined biodistribution of EVs in mice, and observed enhanced uptake of EVs in the liver, spleen, kidney, brain, and colon of i.v.-treated mice compared with mice treated intranasally. Thus, the hypothesis that intranasal delivery of EVs may lead to enhanced delivery to the brain may not be supported by the experimental data. Overall, these studies highlight the impact of method of administration on biodistribution of therapeutic EVs.

EV-BASED THERAPEUTICS IN HUMANS

Although this review has focused exclusively on EVs derived from mammalian systems, the first significant use of EV-like particles in humans was the development and application of anti-meningitis type B vaccines derived from bacterial outer membrane vesicles (OMVs).¹⁵⁵ These OMVs are isolated from *Neisseria meningitis* type B bacteria, and are naturally released through blebbing of the outer membrane.^{156,157} The OMVs contain many bacterial proteins and lipids, and can stimulate anti-bacterial immunity when administered as a vaccine in humans.^{158,159} One such vaccine, VAMENGOC-BC15, has been administered in over 60 million doses in Latin America since it was first developed in 1989, and has proven both safe and effective.^{155,156} EVs derived from mammalian sources have not yet been approved for any human disease indication, but have been studied in several clinical trials, as outlined below.

Cancer therapy

EVs have gained significant attention as potential therapeutic vehicles to efficiently deliver chemotherapeutic agents to cancer cells. MV isolated from tumor cells *in vitro* have the capacity to effectively deliver chemotherapeutic agents to the tumor microenvironment *in vivo*.^{160–162} One clinical trial examined the ability of MVs containing chemotherapeutic drugs to reverse drug resistance of tumor-repopulating cells (TRCs).¹⁶¹ Six end-stage lung cancer patients with metastatic malignant pleural effusion were included in the study. Primary tumor cells collected from malignant fluids prior to MV treatment showed resistance to the chemotherapeutic drug cisplatin (Cis) due in part to upregulated expression of multidrug-resistance genes. In this study, A549 cells, a lung carcinoma cell line, were co-cultured with Cis, during which Cis was taken up by these cells, thus releasing MVs loaded with Cis, which were subsequently isolated by centrifugation. Three patients were treated with these Cis-loaded MVs (MV-Cis), administered by intrathoracic injection, while the remaining three patients were treated with intrapleural Cis injections. Both treatment groups received daily injections; however, the dose of MVs administered to the patients was not indicated by the authors. After 7 days of treatment, the MV-Cis treatment group had a >95% decrease in tumor cells in pleural fluid, whereas patients treated with Cis alone did not show a significant reduction in tumor cells. These data indicate encapsulation of Cis within MVs may reverse TRC drug resistance.

In another similar clinical trial, patients were treated with A549 cell-derived MVs loaded with the chemotherapeutic drug methotrexate (MTX) (MV-MTX).¹⁶² Eleven patients with advanced lung cancer and malignant pleural effusion were included in this trial. MV-MTX was administered by intrapleural infusion every other day for 12 days, totaling six infusions. Response to treatment was determined by objective clinical responses as per the British Thoracic Society Pleural Effusion guidelines.¹⁶³ Decreased pleural effusion volume was observed in 10 of 11 patients (91%) at 28 days post-treatment.¹⁶² Four of 11 patients (36%) showed a complete response, six of 11 (54%) showed a partial response, and one of 11 (9%) showed no response. Analysis of pleural fluid collected pre- and post-treatment from eight patients showed a reduction in tumor cells.

Another study examined the safety and efficacy of MV-MTX combined with the chemotherapeutics pemetrexed and Cis for treatment of malignant pleural effusion in advanced non-squamous non-small cell lung cancer (NSCLC).¹⁶⁴ A total of 86 chemotherapy-naïve patients with non-squamous NSCLC and malignant pleural effusion were enrolled in this study. All 86 patients received pemetrexed and Cis chemotherapy. Five days post-treatment, 43 patients received MV-MTX by intrapleural infusion every 48 h, six times over the course of 12 days, while the remaining 48 patients received saline placebo. Forty patients from the MV-MTX group and 39 patients from the placebo group were included in the final analysis. Pleural effusion volume was significantly decreased in the MV-MTX group compared with control. At 1 year post-treatment, survival was 77.5% for the MV-MTX group and 59.0% for the placebo group, although these differences were not statistically significant. Together, these data demonstrate EVs may serve as effective delivery vehicles of chemotherapeutic drugs for cancer treatment.

Treatment of COVID-19

MSCs are multipotent progenitor cells that differentiate into a variety of different cell types and support hematopoiesis through the release of a variety of molecules integral to the proliferation and differentiation of hematopoietic stem and progenitor cells.^{165–167} Several clinical trials have demonstrated the efficacy of MSCs for treatment of COVID-19 symptoms, as MSCs can modulate the immune response and inhibit production of pro-inflammatory cytokines that can lead to a cytokine storm.^{168–171} EVs isolated from MSCs retain many of the natural properties of MSCs,^{172,173} and have been investigated as a cell-free alternative for treatment of COVID-19 symptoms. The safety and efficacy of exosomes derived from allogeneic bone marrow MSCs (designated ExoFlo) was examined in a clinical trial as a potential treatment for acute respiratory distress syndrome (ARDS) caused by severe COVID-19.¹⁷⁴ Unfortunately, details about how the exosomes were prepared were not included in the study. ExoFlos were not loaded with exogenous cargo, rather these exosomes were predicted to innately convey the regenerative and immunomodulatory properties of MSCs. Twenty-four patients were administered a one-time, 15-mL i.v. infusion of ExoFlo and monitored for 14 days. Seventeen of 24 (71%) patients recovered, three of 24 (13%) patients remained critically ill but stable, and four of 24 (16%) patients suc-

cumbed to COVID-19. These results can be compared with historical data, in which patients with COVID-19-induced ARDS experience a 39% mortality rate.¹⁷⁵ A significant decrease in C-reactive protein (CRP), ferritin, and D-dimer, and significant increase in oxygenation, lymphocyte counts, and neutrophil count was reported.¹⁷⁴ This pilot study was followed by a phase 2 placebo-controlled clinical trial in which ExoFlo was again administered to patients with ARDS caused by COVID-19.¹⁷⁶ A total of 102 patients were included in the study, and patients received an i.v. infusion of 100 mL of saline ($n = 34$), 90 mL saline with 10 mL of ExoFlo ($n = 34$), or 85 mL saline with 15 mL ExoFlo ($n = 34$) on day 1 and day 4, for a total of two treatments. The mortality rate at 60 days post-treatment was 48.4% in the placebo group, 46.6% in the ExoFlo 10 mL group, and 30.4% in the ExoFlo 15 mL group. This placebo-controlled study further demonstrated the efficacy of the 15-mL dose of ExoFlo for COVID-19-induced ARDS.

Another trial examined the safety and efficacy of allogeneic adipose tissue MSC-derived exosomes for treatment of COVID-19 pneumonia.¹⁷⁷ In this study, seven patients with severe COVID-19-induced ARDS were treated with nebulized MSC-derived exosomes for 5 days. All patients tolerated the treatment well, and different degrees of resolution of pulmonary lesions were observed, with four of seven (57%) patients attaining the most pronounced resolution as assessed by computed tomography image scores. One patient demonstrated complete resolution of pulmonary lesions following treatment. A similar study examined the safety of umbilical cord MSC-derived exosomes for treatment of COVID-19-induced pneumonia.¹⁷⁸ In this pilot study, seven patients with COVID-19-induced pneumonia were treated with nebulized MSC-derived exosomes, twice a day, over the course of 7–16 days. This small trial concluded that nebulization of MSC-derived exosomes promoted absorption of pulmonary lesions in mild cases of COVID-19-induced pneumonia.

A clinical trial compared the efficacy of MSC therapy and MSCs combined with MSC-derived EVs for treatment of COVID-19-induced ARDS.¹⁷⁹ One study group ($n = 11$) received two consecutive i.v. doses of umbilical cord or placental MSCs, while the other study group ($n = 8$) received one i.v. dose of the MSCs followed by one i.v. dose of 1,600 μ g of MSC-derived EVs, both compared with a control group ($n = 24$). Forty-eight hours post-dosing, eight of 11 (72%) patients in the MSCs-alone group, eight of eight (100%) in the MSCs and MSCs + EVs group, and 16 of 24 (66%) patients in the control group were alive. Causes for mortality were reported to be multifactorial and attributed to several causes including acute ischemic cerebral stroke, congestive heart failure, multi-organ failure, myocardial infarction, pulmonary dysfunction, and septic shock. Changes in interleukin (IL)-6, interferon (IFN)- γ , and CRP were most pronounced in the MSCs + EVs group, while changes in TNF- α was most pronounced in the MSCs-alone group, and decrease in plasma IL-6 concentration was also noted in this treatment group. Partial thromboplastin time was significantly decreased in the MSCs-alone group, which is associated with a lower risk of thrombotic and disseminated intravascular coagulation.^{179,180} The results from this

study suggest that MSCs and MSCs + MSCs-derived EVs are beneficial for treatment of COVID-19-induced ARDS, and the addition of MSC-derived EVs as part of a therapeutic regimen may allow for more robust changes in specific inflammatory cytokines.

Other therapeutics

EVs isolated from platelets (pEVs) have been used in a clinical trial to examine their potential as a treatment for delayed wound healing.¹⁸¹ pEVs were shown to contain proteins essential in the wound-healing process, such as insulin growth factor and transforming growth factor β (TGF- β). In a double-blind, placebo-controlled clinical trial in healthy adults who had punch biopsy-induced wounds, injection of pEVs was shown to be safe and well tolerated. No difference in wound healing was observed between healthy and control participants. However, the healthy participants were not deficient in any aspect of wound healing and thus would be expected to undergo efficient, normal wound healing. A phase II study examining therapeutic efficacy in patients with disrupted or delayed wound healing is required to make a definitive conclusion about the utility of pEVs for this application.

Exosomes isolated from human placental MSCs have been explored as a possible treatment of perianal fistulas in multiple clinical trials.^{182,183} In one study, 11 patients without Crohn's disease who had perianal fistulas for a minimum of 1 year that had persisted despite standard medical and surgical treatment were injected with MSC-derived exosomes along the fistula tract once a week for 3 weeks.¹⁸² Patients were followed for 6 months post-treatment, and 10 of 11 patients (91%) showed improvement, and five of 11 patients (45%) demonstrated complete resolution of the fistulas. In a similar study, MSC-derived exosomes were injected along the fistula tract once a week for 3 weeks in five patients with Crohn's disease with perianal fistulas that had persisted for 6 months following TNF- α therapy. Patients were followed for 6 months post-treatment, and improvement was observed in four of five patients (80%), with three of five patients (60%) completely healed. Thus, the cargo innately carried within EVs derived from MSCs holds promise for treatment of a number of diseases.

CONCLUSION

Pre-clinical studies of EVs have investigated numerous engineering strategies to increase the loading of cargoes into EVs, including proteins, mRNAs, miRNAs, and siRNAs. Upon systemic delivery *in vivo*, EVs show a wide biodistribution, including to the brain, which suggests EVs may be a promising therapeutic vehicle for diseases of the central nervous system, an area of the body that has been historically difficult to reach. Success of EV-based therapeutics has already been demonstrated in early clinical trials. Thus, although the characterization and therapeutic use of EVs is still in its infancy, these particles have a strong potential to provide successful treatments for many different diseases.

ACKNOWLEDGMENTS

Figure 1 was generated using [Biorender.com](https://biorender.com). Research in the Parks lab is supported by grants to R.J.P. from the Canadian Institutes of

Health Research (FRN-142316, PJT-178120, FRN-175428, PJT-186267), the Natural Sciences and Engineering Research Council of Canada (RGPIN-2019-04786), and Cure SMA/Cure SMA Canada. C.A.R. was supported by a Queen Elizabeth II Graduate Scholarship in Science and Technology from the Government of Ontario (Canada).

AUTHOR CONTRIBUTIONS

Conceptualization, C.A.R.; literature search, C.A.R.; resources, R.J.P.; writing—original draft, C.A.R.; writing—review and editing, C.A.R. and R.J.P.; supervision, R.J.P.; funding acquisition, R.J.P.; project administration, R.J.P. All authors have read and agreed to the published version of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

- Raposo, G., and Stoorvogel, W. (2013). Extracellular vesicles: exosomes, microvesicles, and friends. *J. Cell Biol.* 200, 373–383. <https://doi.org/10.1083/jcb.201211138>.
- Johnstone, R.M., Adam, M., Hammond, J.R., Orr, L., and Turbide, C. (1987). Vesicle formation during reticulocyte maturation. Association of plasma membrane activities with released vesicles (exosomes). *J. Biol. Chem.* 262, 9412–9420. [https://doi.org/10.1016/S0021-9258\(18\)48095-7](https://doi.org/10.1016/S0021-9258(18)48095-7).
- Yáñez-Mó, M., Siljander, P.R.M., Andreu, Z., Zavec, A.B., Borràs, F.E., Buzas, E.I., Buzas, K., Casal, E., Cappello, F., Carvalho, J., et al. (2015). Biological properties of extracellular vesicles and their physiological functions. *J. Extracell. Vesicles* 4, 27066. <https://doi.org/10.3402/jev.v4.27066>.
- Mathivanan, S., Ji, H., and Simpson, R.J. (2010). Exosomes: extracellular organelles important in intercellular communication. *J. Proteomics* 73, 1907–1920. <https://doi.org/10.1016/j.jprot.2010.06.006>.
- Paulaitis, M., Agarwal, K., and Nana-Sinkam, P. (2018). Dynamic Scaling of Exosome Sizes. *Langmuir* 34, 9387–9393. <https://doi.org/10.1021/acs.langmuir.7b04080>.
- György, B., Szabó, T.G., Pásztói, M., Pál, Z., Mészáros, P., Aradi, B., László, V., Pállinger, E., Pap, E., Kittel, A., et al. (2011). Membrane vesicles, current state-of-the-art: emerging role of extracellular vesicles. *Cell. Mol. Life Sci.* 68, 2667–2688. <https://doi.org/10.1007/s00018-011-0689-3>.
- Nabhan, J.F., Hu, R., Oh, R.S., Cohen, S.N., and Lu, Q. (2012). Formation and release of arrestin domain-containing protein 1-mediated microvesicles (ARMMs) at plasma membrane by recruitment of TSG101 protein. *Proc. Natl. Acad. Sci. USA* 109, 4146–4151. <https://doi.org/10.1073/pnas.1200448109>.
- Welsh, J.A., Goberdhan, D.C.I., O'Driscoll, L., Buzas, E.I., Blenkiron, C., Bussolati, B., Cai, H., Di Vizio, D., Driedonks, T.A.P., Erdbrügger, U., et al. (2024). Minimal information for studies of extracellular vesicles (MISEV2023): From basic to advanced approaches. *J. Extracell. Vesicles* 13, e12404. <https://doi.org/10.1002/jev2.12404>.
- Wiklander, O.P.B., Nordin, J.Z., O'Loughlin, A., Gustafsson, Y., Corso, G., Mäger, I., Vader, P., Lee, Y., Sork, H., Seow, Y., et al. (2015). Extracellular vesicle *in vivo* biodistribution is determined by cell source, route of administration and targeting. *J. Extracell. Vesicles* 4, 26316. <https://doi.org/10.3402/jev.v4.26316>.
- Saleh, A.F., Lázaro-Ibáñez, E., Forsgard, M.A.M., Shatnyeva, O., Osteikoetxea, X., Karlsson, F., Heath, N., Ingelsten, M., Rose, J., Harris, J., et al. (2019). Extracellular vesicles induce minimal hepatotoxicity and immunogenicity. *Nanoscale* 11, 6990–7001. <https://doi.org/10.1039/c8nr08720b>.
- Zhu, X., Badawi, M., Pomeroy, S., Sutar, D.S., Xie, Z., Baek, A., Jiang, J., Elgamal, O.A., Mo, X., Perle, K.L., et al. (2017). Comprehensive toxicity and immunogenicity studies reveal minimal effects in mice following sustained dosing of extracellular vesicles derived from HEK293T cells. *J. Extracell. Vesicles* 6, 1324730. <https://doi.org/10.1080/20013078.2017.1324730>.

12. Kordelas, L., Rebmann, V., Ludwig, A.K., Radtke, S., Ruesing, J., Doeppner, T.R., Eppler, M., Horn, P.A., Beelen, D.W., and Giebel, B. (2014). MSC-derived exosomes: a novel tool to treat therapy-refractory graft-versus-host disease. *Leukemia* 28, 970–973. <https://doi.org/10.1038/leu.2014.41>.
13. Barkal, A.A., Brewer, R.E., Markovic, M., Kowarsky, M., Barkal, S.A., Zaro, B.W., Krishnan, V., Hatakeyama, J., Dorigo, O., Barkal, L.J., and Weissman, I.L. (2019). CD24 signalling through macrophage Siglec-10 is a target for cancer immunotherapy. *Nature* 572, 392–396. <https://doi.org/10.1038/s41586-019-1456-0>.
14. Ayre, D.C., Chute, I.C., Joy, A.P., Barnett, D.A., Hogan, A.M., Grüll, M.P., Peña-Castillo, L., Lang, A.S., Lewis, S.M., and Christian, S.L. (2017). CD24 induces changes to the surface receptors of B cell microvesicles with variable effects on their RNA and protein cargo. *Sci. Rep.* 7, 8642. <https://doi.org/10.1038/s41598-017-08094-8>.
15. Kamberkar, S., LeBleu, V.S., Sugimoto, H., Yang, S., Ruivo, C.F., Melo, S.A., Lee, J.J., and Kalluri, R. (2017). Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* 546, 498–503. <https://doi.org/10.1038/nature22341>.
16. Gordon, S.R., Maute, R.L., Dulken, B.W., Hutter, G., George, B.M., McCracken, M.N., Gupta, R., Tsai, J.M., Sinha, R., Corey, D., et al. (2017). PD-1 expression by tumour-associated macrophages inhibits phagocytosis and tumour immunity. *Nature* 545, 495–499. <https://doi.org/10.1038/nature22396>.
17. Yang, Y., Li, C.W., Chan, L.C., Wei, Y., Hsu, J.M., Xia, W., Cha, J.H., Hou, J., Hsu, J.L., Sun, L., and Hung, M.C. (2018). Exosomal PD-L1 harbors active defense function to suppress T cell killing of breast cancer cells and promote tumor growth. *Cell Res.* 28, 862–864. <https://doi.org/10.1038/s41422-018-0060-4>.
18. Rink, J., Ghigo, E., Kalaidzidis, Y., and Zerial, M. (2005). Rab conversion as a mechanism of progression from early to late endosomes. *Cell* 122, 735–749. <https://doi.org/10.1016/j.cell.2005.06.043>.
19. Poteryaev, D., Datta, S., Ackema, K., Zerial, M., and Spang, A. (2010). Identification of the switch in early-to-late endosome transition. *Cell* 141, 497–508. <https://doi.org/10.1016/j.cell.2010.03.011>.
20. Hessvik, N.P., and Llorente, A. (2018). Current knowledge on exosome biogenesis and release. *Cell. Mol. Life Sci.* 75, 193–208. <https://doi.org/10.1007/s00018-017-2595-9>.
21. Bilodeau, P.S., Urbanowski, J.L., Winistorfer, S.C., and Piper, R.C. (2002). The Vps27p Hsc1p complex binds ubiquitin and mediates endosomal protein sorting. *Nat. Cell Biol.* 4, 534–539. <https://doi.org/10.1038/ncb815>.
22. Raiborg, C., Bache, K.G., Gillooly, D.J., Madhus, I.H., Stang, E., and Stenmark, H. (2002). Hrs sorts ubiquitinated proteins into clathrin-coated microdomains of early endosomes. *Nat. Cell Biol.* 4, 394–398. <https://doi.org/10.1038/ncb791>.
23. Odorizzi, G., Babst, M., and Emr, S.D. (1998). Fab1p PtdIns(3)P 5-kinase function essential for protein sorting in the multivesicular body. *Cell* 95, 847–858. [https://doi.org/10.1016/s0092-8674\(00\)81707-9](https://doi.org/10.1016/s0092-8674(00)81707-9).
24. Wegner, C.S., Schink, K.O., Stenmark, H., and Brech, A. (2014). Monitoring phosphatidylinositol 3-phosphate in multivesicular endosome biogenesis. *Methods Enzymol.* 534, 3–23. <https://doi.org/10.1016/B978-0-12-397926-1.00001-9>.
25. Teo, H., Gill, D.J., Sun, J., Perisic, O., Veprintsev, D.B., Vallis, Y., Emr, S.D., and Williams, R.L. (2006). ESCRT-I core and ESCRT-II GLUE domain structures reveal role for GLUE in linking to ESCRT-I and membranes. *Cell* 125, 99–111. <https://doi.org/10.1016/j.cell.2006.01.047>.
26. Gill, D.J., Teo, H., Sun, J., Perisic, O., Veprintsev, D.B., Emr, S.D., and Williams, R.L. (2007). Structural insight into the ESCRT-II link and its role in MVB trafficking. *EMBO J.* 26, 600–612. <https://doi.org/10.1038/sj.emboj.7601501>.
27. Teis, D., Saksena, S., and Emr, S.D. (2008). Ordered assembly of the ESCRT-III complex on endosomes is required to sequester cargo during MVB formation. *Dev. Cell* 15, 578–589. <https://doi.org/10.1016/j.devcel.2008.08.013>.
28. Wollert, T., Wunder, C., Lippincott-Schwartz, J., and Hurley, J.H. (2009). Membrane scission by the ESCRT-III complex. *Nature* 458, 172–177. <https://doi.org/10.1038/nature07836>.
29. Saksena, S., Wahlman, J., Teis, D., Johnson, A.E., and Emr, S.D. (2009). Functional reconstitution of ESCRT-III assembly and disassembly. *Cell* 136, 97–109. <https://doi.org/10.1016/j.cell.2008.11.013>.
30. Jackson, C.E., Scruggs, B.S., Schaffer, J.E., and Hanson, P.I. (2017). Effects of Inhibiting VPS4 Support a General Role for ESCRTs in Extracellular Vesicle Biogenesis. *Biophys. J.* 113, 1342–1352. <https://doi.org/10.1016/j.bpj.2017.05.032>.
31. Stuffers, S., Sem Wegner, C., Stenmark, H., and Brech, A. (2009). Multivesicular endosome biogenesis in the absence of ESCRTs. *Traffic* 10, 925–937. <https://doi.org/10.1111/j.1600-0854.2009.00920.x>.
32. Wei, D., Zhan, W., Gao, Y., Huang, L., Gong, R., Wang, W., Zhang, R., Wu, Y., Gao, S., and Kang, T. (2021). RAB31 marks and controls an ESCRT-independent exosome pathway. *Cell Res.* 31, 157–177. <https://doi.org/10.1038/s41422-020-00409-1>.
33. Mazurov, D., Barbashova, L., and Filatov, A. (2013). Tetraspanin protein CD9 interacts with metalloprotease CD10 and enhances its release via exosomes. *FEBS J.* 280, 1200–1213. <https://doi.org/10.1111/febs.12110>.
34. Perez-Hernandez, D., Gutiérrez-Vázquez, C., Jorge, I., López-Martín, S., Ursa, A., Sánchez-Madrid, F., Vázquez, J., and Yáñez-Mó, M. (2013). The intracellular interactome of tetraspanin-enriched microdomains reveals their function as sorting machineries toward exosomes. *J. Biol. Chem.* 288, 11649–11661. <https://doi.org/10.1074/jbc.M112.445304>.
35. Verweij, F.J., van Eijndhoven, M.A.J., Hopmans, E.S., Vendrig, T., Wurdinger, T., Cahir-McFarland, E., Kieff, E., Geerts, D., van der Kant, R., Neefjes, J., et al. (2011). LMP1 association with CD63 in endosomes and secretion via exosomes limits constitutive NF-κB activation. *EMBO J.* 30, 2115–2129. <https://doi.org/10.1038/emboj.2011.123>.
36. Suárez, H., Andreu, Z., Mazzeo, C., Toribio, V., Pérez-Rivera, A.E., López-Martín, S., García-Silva, S., Hurtado, B., Morato, E., Peláez, L., et al. (2021). CD9 inhibition reveals a functional connection of extracellular vesicle secretion with mitophagy in melanoma cells. *J. Extracell. Vesicles* 10, e12082. <https://doi.org/10.1002/jev2.12082>.
37. Edgar, J.R., Eden, E.R., and Futter, C.E. (2014). Hrs- and CD63-dependent competing mechanisms make different sized endosomal intraluminal vesicles. *Traffic* 15, 197–211. <https://doi.org/10.1111/tra.12139>.
38. Baietti, M.F., Zhang, Z., Mortier, E., Melchior, A., Degeest, G., Geeraerts, A., Ivarsson, Y., Depoortere, F., Coomans, C., Vermeiren, E., et al. (2012). Syndecan-syntenin-ALIX regulates the biogenesis of exosomes. *Nat. Cell Biol.* 14, 677–685. <https://doi.org/10.1038/ncb2502>.
39. Matsui, T., Osaki, F., Hiragi, S., Sakamaki, Y., and Fukuda, M. (2021). ALIX and ceramide differentially control polarized small extracellular vesicle release from epithelial cells. *EMBO Rep.* 22, e51475. <https://doi.org/10.15252/embr.202051475>.
40. Morandi, M.I., Busko, P., Ozer-Partuk, E., Khan, S., Zarfati, G., Elbaz-Alon, Y., Abou Karam, P., Napso Shogan, T., Ginini, L., Gil, Z., et al. (2022). Extracellular vesicle fusion visualized by cryo-electron microscopy. *PNAS Nexus* 1, pgac156. <https://doi.org/10.1093/pnasnexus/pgac156>.
41. Bebelman, M.P., Bun, P., Huveners, S., van Niel, G., Pegtel, D.M., and Verweij, F.J. (2020). Real-time imaging of multivesicular body-plasma membrane fusion to quantify exosome release from single cells. *Nat. Protoc.* 15, 102–121. <https://doi.org/10.1038/s41596-019-0245-4>.
42. Edgar, J.R., Manna, P.T., Nishimura, S., Banting, G., and Robinson, M.S. (2016). Tetherin is an exosomal tether. *Elife* 5, e17180. <https://doi.org/10.7554/eLife.17180>.
43. Karim, M.A., Samyn, D.R., Mattie, S., and Brett, C.L. (2018). Distinct features of multivesicular body-lysosome fusion revealed by a new cell-free content-mixing assay. *Traffic* 19, 138–149. <https://doi.org/10.1111/tra.12543>.
44. Cocucci, E., Racchetti, G., and Meldolesi, J. (2009). Shedding microvesicles: artefacts no more. *Trends Cell Biol.* 19, 43–51. <https://doi.org/10.1016/j.tcb.2008.11.003>.
45. Schlienger, S., Campbell, S., and Claing, A. (2014). ARF1 regulates the Rho/MLC pathway to control EGF-dependent breast cancer cell invasion. *Mol. Biol. Cell* 25, 17–29. <https://doi.org/10.1091/mbc.E13-06-0335>.
46. Muralidharan-Chari, V., Clancy, J., Plou, C., Romao, M., Chavrier, P., Raposo, G., and D'Souza-Schorey, C. (2009). ARF6-regulated shedding of tumor cell-derived plasma membrane microvesicles. *Curr. Biol.* 19, 1875–1885. <https://doi.org/10.1016/j.cub.2009.09.059>.
47. Futter, C.E., Pearce, A., Hewlett, L.J., and Hopkins, C.R. (1996). Multivesicular endosomes containing internalized EGF-EGF receptor complexes mature and then fuse directly with lysosomes. *J. Cell Biol.* 132, 1011–1023. <https://doi.org/10.1083/jcb.132.6.1011>.

48. Chau, V., Tobias, J.W., Bachmair, A., Marriott, D., Ecker, D.J., Gonda, D.K., and Varshavsky, A. (1989). A multiubiquitin chain is confined to specific lysine in a targeted short-lived protein. *Science* 243, 1576–1583. <https://doi.org/10.1126/science.2538923>.
49. Hershko, A. (1999). Mechanisms and regulation of the degradation of cyclin B. *Philos. Trans. R. Soc. Lond. B Biol. Sci.* 354, 1571–1576. <https://doi.org/10.1098/rstb.1999.0500>.
50. Frankel, E.B., and Audhya, A. (2018). ESCRT-dependent cargo sorting at multivesicular endosomes. *Semin. Cell Dev. Biol.* 74, 4–10. <https://doi.org/10.1016/j.semcdb.2017.08.020>.
51. Takahashi, H., Mayers, J.R., Wang, L., Edwardson, J.M., and Audhya, A. (2015). Hrs and STAM function synergistically to bind ubiquitin-modified cargoes *in vitro*. *Biophys. J.* 108, 76–84. <https://doi.org/10.1016/j.bpj.2014.11.004>.
52. Agromayor, M., Soler, N., Caballe, A., Kueck, T., Freund, S.M., Allen, M.D., Bycroft, M., Perisic, O., Ye, Y., McDonald, B., et al. (2012). The UBA1 subunit of ESCRT-I interacts with ubiquitin via a SOUBA domain. *Structure* 20, 414–428. <https://doi.org/10.1016/j.str.2011.12.013>.
53. Strickland, M., Watanabe, S., Bonn, S.M., Camara, C.M., Starich, M.R., Fushman, D., Carter, C.A., and Tjandra, N. (2022). Tsg101/ESCRT-I recruitment regulated by the dual binding modes of K63-linked diubiquitin. *Structure* 30, 289–299.e6. <https://doi.org/10.1016/j.str.2021.09.006>.
54. Slagsvold, T., Aasland, R., Hirano, S., Bache, K.G., Raiborg, C., Trambaiolo, D., Wakatsuki, S., and Stenmark, H. (2005). Eap45 in mammalian ESCRT-II binds ubiquitin via a phosphoinositide-interacting GLUE domain. *J. Biol. Chem.* 280, 19600–19606. <https://doi.org/10.1074/jbc.M501510200>.
55. Escola, J.M., Kleijmeer, M.J., Stoorvogel, W., Griffith, J.M., Yoshie, O., and Geuze, H.J. (1998). Selective enrichment of tetraspan proteins on the internal vesicles of multivesicular endosomes and on exosomes secreted by human B-lymphocytes. *J. Biol. Chem.* 273, 20121–20127. <https://doi.org/10.1074/jbc.273.32.20121>.
56. Mizenko, R.R., Brostoff, T., Rojalin, T., Koster, H.J., Swindell, H.S., Leiserowitz, G.S., Wang, A., and Carney, R.P. (2021). Tetraspanins are unevenly distributed across single extracellular vesicles and bias sensitivity to multiplexed cancer biomarkers. *J. Nanobiotechnol.* 19, 250. <https://doi.org/10.1186/s12951-021-00987-1>.
57. Rocha-Perugini, V., Martínez Del Hoyo, G., González-Granado, J.M., Ramírez-Huesca, M., Zorita, V., Rubinstein, E., Boucheix, C., and Sánchez-Madrid, F. (2017). CD9 Regulates Major Histocompatibility Complex Class II Trafficking in Monocyte-Derived Dendritic Cells. *Mol. Cell Biol.* 37, e00202-17. <https://doi.org/10.1128/MCB.00202-17>.
58. Petersen, S.H., Odintsova, E., Haigh, T.A., Rickinson, A.B., Taylor, G.S., and Berditchevski, F. (2011). The role of tetraspanin CD63 in antigen presentation via MHC class II. *Eur. J. Immunol.* 41, 2556–2561. <https://doi.org/10.1002/eji.201141438>.
59. D'Souza-Schorey, C., and Chavrier, P. (2006). ARF proteins: roles in membrane traffic and beyond. *Nat. Rev. Mol. Cell Biol.* 7, 347–358. <https://doi.org/10.1038/nrm1910>.
60. Donaldson, J.G. (2003). Multiple roles for Arf6: sorting, structuring, and signaling at the plasma membrane. *J. Biol. Chem.* 278, 41573–41576. <https://doi.org/10.1074/jbc.R300026200>.
61. Wang, T., Gilkes, D.M., Takano, N., Xiang, L., Luo, W., Bishop, C.J., Chaturvedi, P., Green, J.J., and Semenza, G.L. (2014). Hypoxia-inducible factors and RAB22A mediate formation of microvesicles that stimulate breast cancer invasion and metastasis. *Proc. Natl. Acad. Sci. USA* 111, E3234–E3242. <https://doi.org/10.1073/pnas.1410041111>.
62. Sork, H., Corso, G., Krjtskov, K., Johansson, H.J., Nordin, J.Z., Wiklander, O.P.B., Lee, Y.X.F., Westholm, J.O., Lehtö, J., Wood, M.J.A., et al. (2018). Heterogeneity and interplay of the extracellular vesicle small RNA transcriptome and proteome. *Sci. Rep.* 8, 10813. <https://doi.org/10.1038/s41598-018-28485-9>.
63. Pérez-Boza, J., Lion, M., and Struman, I. (2018). Exploring the RNA landscape of endothelial exosomes. *RNA* 24, 423–435. <https://doi.org/10.1261/rna.064352.117>.
64. Batagov, A.O., and Kurochkin, I.V. (2013). Exosomes secreted by human cells transport largely mRNA fragments that are enriched in the 3'-untranslated regions. *Biol. Direct* 8, 12. <https://doi.org/10.1186/1745-6150-8-12>.
65. Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J.J., and Lötvall, J.O. (2007). Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659. <https://doi.org/10.1038/ncb1596>.
66. Morhayim, J., van de Peppel, J., Dudakovic, A., Chiba, H., van Wijnen, A.J., and van Leeuwen, J.P. (2017). Molecular characterization of human osteoblast-derived extracellular vesicle mRNA using next-generation sequencing. *Biochim. Biophys. Acta Mol. Cell Res.* 1864, 1133–1141. <https://doi.org/10.1016/j.bbamcr.2017.03.011>.
67. Hinger, S.A., Cha, D.J., Franklin, J.L., Higginbotham, J.N., Dou, Y., Ping, J., Shu, L., Prasad, N., Levy, S., Zhang, B., et al. (2018). Diverse Long RNAs Are Differentially Sorted into Extracellular Vesicles Secreted by Colorectal Cancer Cells. *Cell Rep.* 25, 715–725.e4. <https://doi.org/10.1016/j.celrep.2018.09.054>.
68. Wei, Z., Batagov, A.O., Schinelli, S., Wang, J., Wang, Y., El Fatimy, R., Rabinovsky, R., Balaj, L., Chen, C.C., Hochberg, F., et al. (2017). Coding and noncoding landscape of extracellular RNA released by human glioma stem cells. *Nat. Commun.* 8, 1145. <https://doi.org/10.1038/s41467-017-01196-x>.
69. Li, Y., Zhao, J., Yu, S., Wang, Z., He, X., Su, Y., Guo, T., Sheng, H., Chen, J., Zheng, Q., et al. (2019). Extracellular Vesicles Long RNA Sequencing Reveals Abundant mRNA, circRNA, and lncRNA in Human Blood as Potential Biomarkers for Cancer Diagnosis. *Clin. Chem.* 65, 798–808. <https://doi.org/10.1373/clinchem.2018.301291>.
70. Villarroya-Beltri, C., Gutiérrez-Vázquez, C., Sánchez-Cabo, F., Pérez-Hernández, D., Vázquez, J., Martín-Cofreces, N., Martínez-Herrera, D.J., Pascual-Montano, A., Mittelbrunn, M., and Sánchez-Madrid, F. (2013). Sumoylated hnRNP A2B1 controls the sorting of miRNAs into exosomes through binding to specific motifs. *Nat. Commun.* 4, 2980. <https://doi.org/10.1038/ncomms3980>.
71. Santangelo, L., Giurato, G., Cicchini, C., Montaldo, C., Mancone, C., Tarallo, R., Battistelli, C., Alonzi, T., Weisz, A., and Tripodi, M. (2016). The RNA-Binding Protein SYNCRIP Is a Component of the Hepatocyte Exosomal Machinery Controlling MicroRNA Sorting. *Cell Rep.* 17, 799–808. <https://doi.org/10.1016/j.celrep.2016.09.031>.
72. Zhang, H., Deng, T., Liu, R., Ning, T., Yang, H., Liu, D., Zhang, Q., Lin, D., Ge, S., Bai, M., et al. (2020). CAF secreted miR-522 suppresses ferroptosis and promotes acquired chemo-resistance in gastric cancer. *Mol. Cancer* 19, 43. <https://doi.org/10.1186/s12943-020-01168-8>.
73. Teng, Y., Ren, Y., Hu, X., Mu, J., Samykutty, A., Zhuang, X., Deng, Z., Kumar, A., Zhang, L., Merchant, M.L., et al. (2017). MVP-mediated exosomal sorting of miR-193a promotes colon cancer progression. *Nat. Commun.* 8, 14448. <https://doi.org/10.1038/ncomms14448>.
74. Lin, F., Zeng, Z., Song, Y., Li, L., Wu, Z., Zhang, X., Li, Z., Ke, X., and Hu, X. (2019). YBX-1 mediated sorting of miR-133 into hypoxia/reoxygenation-induced EPC-derived exosomes to increase fibroblast angiogenesis and MEndoT. *Stem Cell Res. Ther.* 10, 263. <https://doi.org/10.1186/s13287-019-1377-8>.
75. Shurtleff, M.J., Temoche-Diaz, M.M., Karfilis, K.V., Ri, S., and Schekman, R. (2016). Y-box protein 1 is required to sort microRNAs into exosomes in cells and in a cell-free reaction. *Elife* 5, e19276. <https://doi.org/10.7554/eLife.19276>.
76. Bolukbasi, M.F., Mizrak, A., Ozdener, G.B., Madlener, S., Ströbel, T., Erkan, E.P., Fan, J.B., Breakefield, X.O., and Saydam, O. (2012). miR-1289 and "Zipcode"-like Sequence Enrich mRNAs in Microvesicles. *Mol. Ther. Nucleic Acids* 1, e10. <https://doi.org/10.1038/mtna.2011.2>.
77. Garcia-Martin, R., Wang, G., Brandão, B.B., Zanotto, T.M., Shah, S., Kumar Patel, S., Schilling, B., and Kahn, C.R. (2022). MicroRNA sequence codes for small extracellular vesicle release and cellular retention. *Nature* 601, 446–451. <https://doi.org/10.1038/s41586-021-04234-3>.
78. Ahadi, A., Brennan, S., Kennedy, P.J., Hutvagner, G., and Tran, N. (2016). Long non-coding RNAs harboring miRNA seed regions are enriched in prostate cancer exosomes. *Sci. Rep.* 6, 24922. <https://doi.org/10.1038/srep24922>.
79. Antimisiaris, S.G., Mourtas, S., and Marazioti, A. (2018). Exosomes and Exosome-Inspired Vesicles for Targeted Drug Delivery. *Pharmaceutics* 10, 218. <https://doi.org/10.3390/pharmaceutics10040218>.
80. Sun, D., Zhuang, X., Xiang, X., Liu, Y., Zhang, S., Liu, C., Barnes, S., Grizzle, W., Miller, D., and Zhang, H.G. (2010). A novel nanoparticle drug delivery system: the anti-inflammatory activity of curcumin is enhanced when encapsulated in exosomes. *Mol. Ther.* 18, 1606–1614. <https://doi.org/10.1038/mt.2010.105>.

81. Haney, M.J., Klyachko, N.L., Zhao, Y., Gupta, R., Plotnikova, E.G., He, Z., Patel, T., Piroyan, A., Sokolsky, M., Kabanov, A.V., and Batrakova, E.V. (2015). Exosomes as drug delivery vehicles for Parkinson's disease therapy. *J. Contr. Release* 207, 18–30. <https://doi.org/10.1016/j.jconrel.2015.03.033>.
82. Armstrong, J.P.K., Holme, M.N., and Stevens, M.M. (2017). Re-Engineering Extracellular Vesicles as Smart Nanoscale Therapeutics. *ACS Nano* 11, 69–83. <https://doi.org/10.1021/acsnano.6b07607>.
83. Nash, L.A., McFall, E.R., Perozzo, A.M., Turner, M., Poulin, K.L., De Repentigny, Y., Burns, J.K., McMillan, H.J., Warman Chardon, J., Burger, D., et al. (2017). Survival Motor Neuron Protein is Released from Cells in Exosomes: A Potential Biomarker for Spinal Muscular Atrophy. *Sci. Rep.* 7, 13859. <https://doi.org/10.1038/s41598-017-14313-z>.
84. Walker, S., Busatto, S., Pham, A., Tian, M., Suh, A., Carson, K., Quintero, A., Lafrence, M., Malik, H., Santana, M.X., and Wolfram, J. (2019). Extracellular vesicle-based drug delivery systems for cancer treatment. *Theranostics* 9, 8001–8017. <https://doi.org/10.7150/thno.37097>.
85. Fuhrmann, G., Serio, A., Mazo, M., Nair, R., and Stevens, M.M. (2015). Active loading into extracellular vesicles significantly improves the cellular uptake and photodynamic effect of porphyrins. *J. Contr. Release* 205, 35–44. <https://doi.org/10.1016/j.jconrel.2014.11.029>.
86. Kim, M.S., Haney, M.J., Zhao, Y., Mahajan, V., Deygen, I., Klyachko, N.L., Inskoe, E., Piroyan, A., Sokolsky, M., Okolie, O., et al. (2016). Development of exosome-encapsulated paclitaxel to overcome MDR in cancer cells. *Nanomedicine* 12, 655–664. <https://doi.org/10.1016/j.nano.2015.10.012>.
87. Kooijmans, S.A.A., Stremersch, S., Braeckmans, K., de Smedt, S.C., Hendrix, A., Wood, M.J.A., Schifferleers, R.M., Raemdonck, K., and Vader, P. (2013). Electroporation-induced siRNA precipitation obscures the efficiency of siRNA loading into extracellular vesicles. *J. Contr. Release* 172, 229–238. <https://doi.org/10.1016/j.jconrel.2013.08.014>.
88. McCann, J.V., Bischoff, S.R., Zhang, Y., Cowley, D.O., Sanchez-Gonzalez, V., Daaboul, G.D., and Dudley, A.C. (2020). Reporter mice for isolating and auditing cell type-specific extracellular vesicles *in vivo*. *Genesis* 58, e23369. <https://doi.org/10.1002/dvg.23369>.
89. Alvarez-Erviti, L., Seow, Y., Yin, H., Betts, C., Lakhai, S., and Wood, M.J.A. (2011). Delivery of siRNA to the mouse brain by systemic injection of targeted exosomes. *Nat. Biotechnol.* 29, 341–345. <https://doi.org/10.1038/nbt.1807>.
90. Kooijmans, S.A.A., Aleza, C.G., Roffler, S.R., van Solinge, W.W., Vader, P., and Schifferleers, R.M. (2016). Display of GPI-anchored anti-EGFR nanobodies on extracellular vesicles promotes tumour cell targeting. *J. Extracell. Vesicles* 5, 31053. <https://doi.org/10.3402/jev.v5.31053>.
91. Ohno, S.I., Takanashi, M., Sudo, K., Ueda, S., Ishikawa, A., Matsuyama, N., Fujita, K., Mizutani, T., Ohgi, T., Ochiya, T., et al. (2013). Systemically injected exosomes targeted to EGFR deliver antitumor microRNA to breast cancer cells. *Mol. Ther.* 21, 185–191. <https://doi.org/10.1038/mt.2012.180>.
92. Rountree, R.B., Mandl, S.J., Nachtwey, J.M., Dalpozzo, K., Do, L., Lombardo, J.R., Schoonmaker, P.L., Brinkmann, K., Dirmeier, U., Laus, R., and Delcayre, A. (2011). Exosome targeting of tumor antigens expressed by cancer vaccines can improve antigen immunogenicity and therapeutic efficacy. *Cancer Res.* 71, 5235–5244. <https://doi.org/10.1158/0008-5472.CAN-10-4076>.
93. Dooley, K., McConnell, R.E., Xu, K., Lewis, N.D., Haupt, S., Youniss, M.R., Martin, S., Sia, C.L., McCoy, C., Moniz, R.J., et al. (2021). A versatile platform for generating engineered extracellular vesicles with defined therapeutic properties. *Mol. Ther.* 29, 1729–1743. <https://doi.org/10.1016/j.ymthe.2021.01.020>.
94. Manfredi, F., Di Bonito, P., Arenaccio, C., Anticoli, S., and Federico, M. (2016). Incorporation of Heterologous Proteins in Engineered Exosomes. *Methods Mol. Biol.* 1448, 249–260. https://doi.org/10.1007/978-1-4939-3753-0_18.
95. Ferreira, J.V., da Rosa Soares, A., Ramalho, J., Máximo Carvalho, C., Cardoso, M.H., Pintado, P., Carvalho, A.S., Beck, H.C., Matthiesen, R., Zuzarte, M., et al. (2022). LAMP2A regulates the loading of proteins into exosomes. *Sci. Adv.* 8, eabm1140. <https://doi.org/10.1126/sciadv.abm1140>.
96. Yim, N., Ryu, S.W., Choi, K., Lee, K.R., Lee, S., Choi, H., Kim, J., Shaker, M.R., Sun, W., Park, J.H., et al. (2016). Exosome engineering for efficient intracellular delivery of soluble proteins using optically reversible protein-protein interaction module. *Nat. Commun.* 7, 12277. <https://doi.org/10.1038/ncomms12277>.
97. Somiya, M., and Kuroda, S. (2022). Engineering of Extracellular Vesicles for Small Molecule-Regulated Cargo Loading and Cytoplasmic Delivery of Bioactive Proteins. *Mol. Pharm.* 19, 2495–2505. <https://doi.org/10.1021/acs.molpharmaceut.2c00192>.
98. Sterzenbach, U., Putz, U., Low, L.H., Silke, J., Tan, S.S., and Howitt, J. (2017). Engineered Exosomes as Vehicles for Biologically Active Proteins. *Mol. Ther.* 25, 1269–1278. <https://doi.org/10.1016/j.ymthe.2017.03.030>.
99. Cheng, Y., and Schorey, J.S. (2016). Targeting soluble proteins to exosomes using a ubiquitin tag. *Biotechnol. Bioeng.* 113, 1315–1324. <https://doi.org/10.1002/bit.25884>.
100. Reshke, R., Taylor, J.A., Savard, A., Guo, H., Rhym, L.H., Kowalski, P.S., Trung, M.T., Campbell, C., Little, W., Anderson, D.G., and Gibbins, D. (2020). Reduction of the therapeutic dose of silencing RNA by packaging it in extracellular vesicles via a pre-microRNA backbone. *Nat. Biomed. Eng.* 4, 52–68. <https://doi.org/10.1038/s41551-019-0502-4>.
101. Hung, M.E., and Leonard, J.N. (2016). A platform for actively loading cargo RNA to elucidate limiting steps in EV-mediated delivery. *J. Extracell. Vesicles* 5, 31027. <https://doi.org/10.3402/jev.v5.31027>.
102. Dar, G.H., Mendes, C.C., Kuan, W.L., Speciale, A.A., Conceição, M., Görgens, A., Uliyakina, I., Lobo, M.J., Lim, W.F., El Andaloussi, S., et al. (2021). GAPDH controls extracellular vesicle biogenesis and enhances the therapeutic potential of EV mediated siRNA delivery to the brain. *Nat. Commun.* 12, 6666. <https://doi.org/10.1038/s41467-021-27056-3>.
103. Roberto, J., Poulin, K.L., Parks, R.J., and Vacratsis, P.O. (2021). Label-free quantitative proteomic analysis of extracellular vesicles released from fibroblasts derived from patients with spinal muscular atrophy. *Proteomics* 21, e2000301. <https://doi.org/10.1002/pmic.202000301>.
104. Rontogianni, S., Synadaki, E., Li, B., Liefwaard, M.C., Lips, E.H., Wesseling, J., Wu, W., and Altelaar, M. (2019). Proteomic profiling of extracellular vesicles allows for human breast cancer subtyping. *Commun. Biol.* 2, 325. <https://doi.org/10.1038/s42003-019-0570-8>.
105. Corso, G., Heusermann, W., Trojer, D., Görgens, A., Steib, E., Voshol, J., Graff, A., Genoud, C., Lee, Y., Hean, J., et al. (2019). Systematic characterization of extracellular vesicle sorting domains and quantification at the single molecule - single vesicle level by fluorescence correlation spectroscopy and single particle imaging. *J. Extracell. Vesicles* 8, 1663043. <https://doi.org/10.1080/20013078.2019.1663043>.
106. Stickney, Z., Losacco, J., McDevitt, S., Zhang, Z., and Lu, B. (2016). Development of exosome surface display technology in living human cells. *Biochem. Biophys. Res. Commun.* 472, 53–59. <https://doi.org/10.1016/j.bbrc.2016.02.058>.
107. Kanuma, T., Yamamoto, T., Kobiyama, K., Moriishi, E., Masuta, Y., Kusakabe, T., Ozasa, K., Kuroda, E., Jounai, N., and Ishii, K.J. (2017). CD63-Mediated Antigen Delivery into Extracellular Vesicles via DNA Vaccination Results in Robust CD8. *J. Immunol.* 198, 4707–4715. <https://doi.org/10.4049/jimmunol.1600731>.
108. Cooper, J.M., Wiklander, P.B.O., Nordin, J.Z., Al-Shawi, R., Wood, M.J., Vithlani, M., Schapira, A.H.V., Simons, J.P., El-Andaloussi, S., and Alvarez-Erviti, L. (2014). Systemic exosomal siRNA delivery reduced alpha-synuclein aggregates in brains of transgenic mice. *Mov. Disord.* 29, 1476–1485. <https://doi.org/10.1002/mds.25978>.
109. Kirchhoff, F., Greenough, T.C., Brettler, D.B., Sullivan, J.L., and Desrosiers, R.C. (1995). Brief report: absence of intact nef sequences in a long-term survivor with nonprogressive HIV-1 infection. *N. Engl. J. Med.* 332, 228–232. <https://doi.org/10.1056/NEJM199501263320405>.
110. Watkins, R.L., Foster, J.L., and Garcia, J.V. (2015). *In vivo* analysis of Nef's role in HIV-1 replication, systemic T cell activation and CD4(+) T cell loss. *Retrovirology* 12, 61. <https://doi.org/10.1186/s12977-015-0187-z>.
111. Anticoli, S., Manfredi, F., Chiozzini, C., Arenaccio, C., Olivetta, E., Ferrantelli, F., Capocello, A., Falcone, E., Ruggieri, A., and Federico, M. (2018). An Exosome-Based Vaccine Platform Imparts Cytotoxic T Lymphocyte Immunity Against Viral Antigens. *Biotechnol. J.* 13, e1700443. <https://doi.org/10.1002/biot.201700443>.
112. Basak, S., Kim, H., Kearns, J.D., Tergaonkar, V., O'Dea, E., Werner, S.L., Benedict, C.A., Ware, C.F., Ghosh, G., Verma, I.M., and Hoffmann, A. (2007). A fourth

- IkappaB protein within the NF-kappaB signaling module. *Cell* 128, 369–381. <https://doi.org/10.1016/j.cell.2006.12.033>.
113. Chen, J., Zheng, X.F., Brown, E.J., and Schreiber, S.L. (1995). Identification of an 11-kDa FKBP12-rapamycin-binding domain within the 289-kDa FKBP12-rapamycin-associated protein and characterization of a critical serine residue. *Proc. Natl. Acad. Sci. USA* 92, 4947–4951. <https://doi.org/10.1073/pnas.92.11.4947>.
 114. Somya, M., and Kuroda, S. (2021). Real-Time Luminescence Assay for Cytoplasmic Cargo Delivery of Extracellular Vesicles. *Anal. Chem.* 93, 5612–5620. <https://doi.org/10.1021/acs.analchem.1c00339>.
 115. Zhang, X., Xu, Q., Zi, Z., Liu, Z., Wan, C., Crisman, L., Shen, J., and Liu, X. (2020). Programmable Extracellular Vesicles for Macromolecule Delivery and Genome Modifications. *Dev. Cell* 55, 784–801.e9. <https://doi.org/10.1016/j.devcel.2020.11.007>.
 116. Gee, P., Lung, M.S.Y., Okuzaki, Y., Sasakawa, N., Iguchi, T., Makita, Y., Hozumi, H., Miura, Y., Yang, L.F., Iwasaki, M., et al. (2020). Extracellular nanovesicles for packaging of CRISPR-Cas9 protein and sgRNA to induce therapeutic exon skipping. *Nat. Commun.* 11, 1334. <https://doi.org/10.1038/s41467-020-14957-y>.
 117. Bui, S., Dancourt, J., and Lavie, G. (2023). Virus-Free Method to Control and Enhance Extracellular Vesicle Cargo Loading and Delivery. *ACS Appl. Bio Mater.* 6, 1081–1091. <https://doi.org/10.1021/acsabm.2c00955>.
 118. Sudol, M., Chen, H.I., Bougeret, C., Einbond, A., and Bork, P. (1995). Characterization of a novel protein-binding module—the WW domain. *FEBS Lett.* 369, 67–71. [https://doi.org/10.1016/0014-5793\(95\)00550-s](https://doi.org/10.1016/0014-5793(95)00550-s).
 119. Putz, U., Howitt, J., Lackovic, J., Foot, N., Kumar, S., Silke, J., and Tan, S.S. (2008). Nedd4 family-interacting protein 1 (Ndfip1) is required for the exosomal secretion of Nedd4 family proteins. *J. Biol. Chem.* 283, 32621–32627. <https://doi.org/10.1074/jbc.M804120200>.
 120. Hadari, T., Warmes, J.V., Rose, I.A., and Hershko, A. (1992). A ubiquitin C-terminal isopeptidase that acts on polyubiquitin chains. Role in protein degradation. *J. Biol. Chem.* 267, 719–727.
 121. Kim, M.W., Park, S., Lee, H., Gwak, H., Hyun, K.A., Kim, J.Y., Jung, H.I., and Il Kim, S. (2021). Multi-miRNA panel of tumor-derived extracellular vesicles as promising diagnostic biomarkers of early-stage breast cancer. *Cancer Sci.* 112, 5078–5087. <https://doi.org/10.1111/cas.15155>.
 122. Figueira, I., Godinho-Pereira, J., Galego, S., Maia, J., Haskó, J., Molnár, K., Malhó, R., Costa-Silva, B., Wilhelm, I., Krizbai, I.A., and Brito, M.A. (2021). MicroRNAs and Extracellular Vesicles as Distinctive Biomarkers of Precocious and Advanced Stages of Breast Cancer Brain Metastases Development. *Int. J. Mol. Sci.* 22, 5214. <https://doi.org/10.3390/ijms22105214>.
 123. Melo, S.A., Sugimoto, H., O'Connell, J.T., Kato, N., Villanueva, A., Vidal, A., Qiu, L., Vitkin, E., Perelman, L.T., Melo, C.A., et al. (2014). Cancer exosomes perform cell-independent microRNA biogenesis and promote tumorigenesis. *Cancer Cell* 26, 707–721. <https://doi.org/10.1016/j.ccr.2014.09.005>.
 124. Lawson, J., Dickman, C., Towle, R., Jabalee, J., Javer, A., and Garnis, C. (2019). Extracellular vesicle secretion of miR-142-3p from lung adenocarcinoma cells induces tumor promoting changes in the stroma through cell-cell communication. *Mol. Carcinog.* 58, 376–387. <https://doi.org/10.1002/mc.22935>.
 125. Chevillet, J.R., Kang, Q., Ruf, I.K., Briggs, H.A., Vojtech, L.N., Hughes, S.M., Cheng, H.H., Arroyo, J.D., Meredith, E.K., Gallichotte, E.N., et al. (2014). Quantitative and stoichiometric analysis of the microRNA content of exosomes. *Proc. Natl. Acad. Sci. USA* 111, 14888–14893. <https://doi.org/10.1073/pnas.1408301111>.
 126. Hagiwara, K., Katsuda, T., Gailhouse, L., Kosaka, N., and Ochiya, T. (2015). Commitment of Annexin A2 in recruitment of microRNAs into extracellular vesicles. *FEBS Lett.* 589, 4071–4078. <https://doi.org/10.1016/j.febslet.2015.11.036>.
 127. Yang, J.S., Maurin, T., Robine, N., Rasmussen, K.D., Jeffrey, K.L., Chandwani, R., Papapetrou, E.P., Sadelain, M., O'Carroll, D., and Lai, E.C. (2010). Conserved vertebrate mir-451 provides a platform for Dicer-independent, Ago2-mediated microRNA biogenesis. *Proc. Natl. Acad. Sci. USA* 107, 15163–15168. <https://doi.org/10.1073/pnas.1006432107>.
 128. Nakase, I., Hirose, H., Tanaka, G., Tadokoro, A., Kobayashi, S., Takeuchi, T., and Futaki, S. (2009). Cell-surface accumulation of flock house virus-derived peptide leads to efficient internalization via macropinocytosis. *Mol. Ther.* 17, 1868–1876. <https://doi.org/10.1038/mt.2009.192>.
 129. Peabody, D.S., and Ely, K.R. (1992). Control of translational repression by protein-protein interactions. *Nucleic Acids Res.* 20, 1649–1655. <https://doi.org/10.1093/nar/20.7.1649>.
 130. Sekine, Y., Koike, H., Nakano, T., Nakajima, K., Takahashi, S., and Suzuki, K. (2009). Remnant lipoproteins induced proliferation of human prostate cancer cell, PC-3 but not LNCaP, via low density lipoprotein receptor. *Cancer Epidemiol.* 33, 16–23. <https://doi.org/10.1016/j.canep.2009.04.004>.
 131. Finkelshtein, D., Werman, A., Novick, D., Barak, S., and Rubinstein, M. (2013). LDL receptor and its family members serve as the cellular receptors for vesicular stomatitis virus. *Proc. Natl. Acad. Sci. USA* 110, 7306–7311. <https://doi.org/10.1073/pnas.1214441110>.
 132. Pincela Lins, P.M., Pirllet, E., Szymonik, M., Bronckaers, A., and Nelissen, I. (2023). Manufacture of extracellular vesicles derived from mesenchymal stromal cells. *Trends Biotechnol.* 41, 965–981. <https://doi.org/10.1016/j.tibtech.2023.01.003>.
 133. Giancaterino, S., and Boi, C. (2023). Alternative biological sources for extracellular vesicles production and purification strategies for process scale-up. *Biotechnol. Adv.* 63, 108092. <https://doi.org/10.1016/j.biotechadv.2022.108092>.
 134. Lai, J.J., Hill, J.J., Huang, C.Y., Lee, G.C., Mai, K.W., Shen, M.Y., and Wang, S.K. (2024). Unveiling the Complex World of Extracellular Vesicles: Novel Characterization Techniques and Manufacturing Considerations. *Chonnam Med. J.* 60, 1–12. <https://doi.org/10.4068/cmj.2024.60.1.1>.
 135. Omrani, M., Beyrampour-Basmenj, H., Jahanban-Esfahlan, R., Talebi, M., Raesi, M., Serej, Z.A., Akbar-Gharalari, N., Khodakarimi, S., Wu, J., and Ebrahimi-Kalan, A. (2024). Global trend in exosome isolation and application: an update concept in management of diseases. *Mol. Cell. Biochem.* 479, 679–691. <https://doi.org/10.1007/s11010-023-04756-6>.
 136. Wang, W., Sun, H., Duan, H., Sheng, G., Tian, N., Liu, D., and Sun, Z. (2024). Isolation and usage of exosomes in central nervous system diseases. *CNS Neurosci. Ther.* 30, e14677. <https://doi.org/10.1111/cns.14677>.
 137. Caby, M.P., Lankar, D., Vincendeau-Scherrer, C., Raposo, G., and Bonnerot, C. (2005). Exosomal-like vesicles are present in human blood plasma. *Int. Immunol.* 17, 879–887. <https://doi.org/10.1093/intimm/dxh267>.
 138. Pisitkun, T., Shen, R.F., and Knepper, M.A. (2004). Identification and proteomic profiling of exosomes in human urine. *Proc. Natl. Acad. Sci. USA* 101, 13368–13373. <https://doi.org/10.1073/pnas.0403453101>.
 139. Palanisamy, V., Sharma, S., Deshpande, A., Zhou, H., Gimzewski, J., and Wong, D.T. (2010). Nanostructural and transcriptomic analyses of human saliva derived exosomes. *PLoS One* 5, e8577. <https://doi.org/10.1371/journal.pone.0008577>.
 140. Street, J.M., Barran, P.E., Mackay, C.L., Weidt, S., Balmforth, C., Walsh, T.S., Chalmers, R.T.A., Webb, D.J., and Dear, J.W. (2012). Identification and proteomic profiling of exosomes in human cerebrospinal fluid. *J. Transl. Med.* 10, 5. <https://doi.org/10.1186/1479-5876-10-5>.
 141. Rana, S., Yue, S., Stadel, D., and Zöller, M. (2012). Toward tailored exosomes: the exosomal tetraspanin web contributes to target cell selection. *Int. J. Biochem. Cell Biol.* 44, 1574–1584. <https://doi.org/10.1016/j.biocel.2012.06.018>.
 142. Jurgielewicz, B.J., Yao, Y., and Stice, S.L. (2020). Kinetics and Specificity of HEK293T Extracellular Vesicle Uptake using Imaging Flow Cytometry. *Nanoscale Res. Lett.* 15, 170. <https://doi.org/10.1186/s11671-020-03399-6>.
 143. Wu, A.Y.T., Sung, Y.C., Chen, Y.J., Chou, S.T.Y., Guo, V., Chien, J.C.Y., Ko, J.J.S., Yang, A.L., Huang, H.C., Chuang, J.C., et al. (2020). Multiresolution Imaging Using Bioluminescence Resonance Energy Transfer Identifies Distinct Biodistribution Profiles of Extracellular Vesicles and Exosomes with Redirected Tropism. *Adv. Sci.* 7, 2001467. <https://doi.org/10.1002/advsc.202001467>.
 144. Takahashi, Y., Nishikawa, M., Shinotsuka, H., Matsui, Y., Ohara, S., Imai, T., and Takakura, Y. (2013). Visualization and *in vivo* tracking of the exosomes of murine melanoma B16-BL6 cells in mice after intravenous injection. *J. Biotechnol.* 165, 77–84. <https://doi.org/10.1016/j.jbiotec.2013.03.013>.
 145. Gangadaran, P., Li, X.J., Lee, H.W., Oh, J.M., Kalimuthu, S., Rajendran, R.L., Son, S.H., Baek, S.H., Singh, T.D., Zhu, L., et al. (2017). A new bioluminescent reporter system to study the biodistribution of systemically injected tumor-derived bioluminescent extracellular vesicles in mice. *Oncotarget* 8, 109894–109914. <https://doi.org/10.18632/oncotarget.22493>.

146. Grange, C., Tapparo, M., Bruno, S., Chatterjee, D., Quesenberry, P.J., Tetta, C., and Camussi, G. (2014). Biodistribution of mesenchymal stem cell-derived extracellular vesicles in a model of acute kidney injury monitored by optical imaging. *Int. J. Mol. Med.* 33, 1055–1063. <https://doi.org/10.3892/ijmm.2014.1663>.
147. Imai, T., Takahashi, Y., Nishikawa, M., Kato, K., Morishita, M., Yamashita, T., Matsumoto, A., Charoenviriyakul, C., and Takakura, Y. (2015). Macrophage-dependent clearance of systemically administered B16BL6-derived exosomes from the blood circulation in mice. *J. Extracell. Vesicles* 4, 26238. <https://doi.org/10.3402/jev.v4.26238>.
148. Wen, S., Dooner, M., Papa, E., Del Tatto, M., Pereira, M., Borgovan, T., Cheng, Y., Goldberg, L., Liang, O., Camussi, G., and Quesenberry, P. (2019). Biodistribution of Mesenchymal Stem Cell-Derived Extracellular Vesicles in a Radiation Injury Bone Marrow Murine Model. *Int. J. Mol. Sci.* 20, 5468. <https://doi.org/10.3390/ijms20215468>.
149. Mendt, M., Kamerkar, S., Sugimoto, H., McAndrews, K.M., Wu, C.C., Gagea, M., Yang, S., Blanko, E.V.R., Peng, Q., Ma, X., et al. (2018). Generation and testing of clinical-grade exosomes for pancreatic cancer. *JCI Insight* 3, e99263. <https://doi.org/10.1172/jci.insight.99263>.
150. Chen, C.C., Liu, L., Ma, F., Wong, C.W., Guo, X.E., Chacko, J.V., Farhoodi, H.P., Zhang, S.X., Zimak, J., Ségalliny, A., et al. (2016). Elucidation of Exosome Migration across the Blood-Brain Barrier Model *In Vitro*. *Cell. Mol. Bioeng.* 9, 509–529. <https://doi.org/10.1007/s12195-016-0458-3>.
151. René, C.A., and Parks, R.J. (2021). Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles. *Pharmaceutics* 13, 492. <https://doi.org/10.3390/pharmaceutics13040492>.
152. Ali, J., Ali, M., Baboota, S., Sahani, J.K., Ramassamy, C., Dao, L., and Bhavna. (2010). Potential of nanoparticulate drug delivery systems by intranasal administration. *Curr. Pharmaceut. Des.* 16, 1644–1653. <https://doi.org/10.2174/138161210791164108>.
153. Tolomeo, A.M., Zuccolotto, G., Malvicini, R., De Lazzari, G., Penna, A., Franco, C., Caicci, F., Magarotto, F., Quarta, S., Pozzobon, M., et al. (2023). Biodistribution of Intratracheal, Intranasal, and Intravenous Injections of Human Mesenchymal Stromal Cell-Derived Extracellular Vesicles in a Mouse Model for Drug Delivery Studies. *Pharmaceutics* 15, 548. <https://doi.org/10.3390/pharmaceutics15020548>.
154. Driedonks, T., Jiang, L., Carlson, B., Han, Z., Liu, G., Queen, S.E., Shirk, E.N., Golobova, O., Liao, Z., Nyberg, L.H., et al. (2022). Pharmacokinetics and biodistribution of extracellular vesicles administered intravenously and intranasally to J. Extracell. Biol. 1, e59. <https://doi.org/10.1002/jex2.59>.
155. Sierra-González, V.G. (2019). Cuban Meningococcal Vaccine VA-MENGOC-BC:30 Years of Use and Future Potential. *MEDICC Rev.* 21, 19–27. <https://doi.org/10.37757/MR2019.V21.N44>.
156. Sierra, G.V., Campa, H.C., Varcacel, N.M., Garcia, I.L., Izquierdo, P.L., Sotolongo, P.F., Casanueva, G.V., Rico, C.O., Rodriguez, C.R., and Terry, M.H. (1991). Vaccine against group B *Neisseria meningitidis*: protection trial and mass vaccination results in Cuba. *NIPH (Natl. Inst. Public Health) Ann. (Oslo)* 14, 195–210.
157. Schwechheimer, C., Sullivan, C.J., and Kuehn, M.J. (2013). Envelope control of outer membrane vesicle production in Gram-negative bacteria. *Biochemistry* 52, 3031–3040. <https://doi.org/10.1021/bi400164t>.
158. Sartorio, M.G., Pardue, E.J., Feldman, M.F., and Haurat, M.F. (2021). Bacterial Outer Membrane Vesicles: From Discovery to Applications. *Annu. Rev. Microbiol.* 75, 609–630. <https://doi.org/10.1146/annurev-micro-052821-031444>.
159. Toyofuku, M., Schild, S., Kparakis-Liascos, M., and Eberl, L. (2023). Composition and functions of bacterial membrane vesicles. *Nat. Rev. Microbiol.* 21, 415–430. <https://doi.org/10.1038/s41579-023-00875-5>.
160. Tang, K., Zhang, Y., Zhang, H., Xu, P., Liu, J., Ma, J., Lv, M., Li, D., Katirai, F., Shen, G.X., et al. (2012). Delivery of chemotherapeutic drugs in tumour cell-derived microparticles. *Nat. Commun.* 3, 1282. <https://doi.org/10.1038/ncomms2282>.
161. Ma, J., Zhang, Y., Tang, K., Zhang, H., Yin, X., Li, Y., Xu, P., Sun, Y., Ma, R., Ji, T., et al. (2016). Reversing drug resistance of soft tumor-repopulating cells by tumor cell-derived chemotherapeutic microparticles. *Cell Res.* 26, 713–727. <https://doi.org/10.1038/cr.2016.53>.
162. Guo, M., Wu, F., Hu, G., Chen, L., Xu, J., Xu, P., Wang, X., Li, Y., Liu, S., Zhang, S., et al. (2019). Autologous tumor cell-derived microparticle-based targeted chemotherapy in lung cancer patients with malignant pleural effusion. *Sci. Transl. Med.* 11, eaat5690. <https://doi.org/10.1126/scitranslmed.aat5690>.
163. Roberts, M.E., Neville, E., Berrisford, R.G., Antunes, G., and Ali, N.J.; BTS Pleural Disease Guideline Group (2010). Management of a malignant pleural effusion: British Thoracic Society Pleural Disease Guideline 2010. *Thorax* 65, ii32–40. <https://doi.org/10.1136/thx.2010.136994>.
164. Dong, X., Huang, Y., Yi, T., Hu, C., Gao, Q., Chen, Y., Zhang, J., Chen, J., Liu, L., Meng, R., et al. (2022). Intrapleural infusion of tumor cell-derived microparticles packaging methotrexate or saline combined with pemetrexed-cisplatin chemotherapy for the treatment of malignant pleural effusion in advanced non-squamous non-small cell lung cancer: A double-blind, randomized, placebo-controlled study. *Front. Immunol.* 13, 1002938. <https://doi.org/10.3389/fimmu.2022.1002938>.
165. Dominici, M., Le Blanc, K., Mueller, I., Slaper-Cortenbach, I., Marini, F., Krause, D., Deans, R., Keating, A., Prockop, D., and Horwitz, E. (2006). Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 8, 315–317. <https://doi.org/10.1080/14653240600855905>.
166. Reinisch, A., Etchart, N., Thomas, D., Hofmann, N.A., Fruehwirth, M., Sinha, S., Chan, C.K., Senarath-Yapa, K., Seo, E.Y., Wearda, T., et al. (2015). Epigenetic and *in vivo* comparison of diverse MSC sources reveals an endochondral signature for human hematopoietic niche formation. *Blood* 125, 249–260. <https://doi.org/10.1182/blood-2014-04-572255>.
167. Aqmasheh, S., Shamsasanjan, K., Akbarzadehlaleh, P., Pashoutan Sarvar, D., and Timari, H. (2017). Effects of Mesenchymal Stem Cell Derivatives on Hematopoiesis and Hematopoietic Stem Cells. *Adv. Pharmaceut. Bull.* 7, 165–177. <https://doi.org/10.15171/apb.2017.021>.
168. Meng, F., Xu, R., Wang, S., Xu, Z., Zhang, C., Li, Y., Yang, T., Shi, L., Fu, J., Jiang, T., et al. (2020). Human umbilical cord-derived mesenchymal stem cell therapy in patients with COVID-19: a phase 1 clinical trial. *Signal Transduct. Targeted Ther.* 5, 172. <https://doi.org/10.1038/s41392-020-00286-5>.
169. Dilogo, I.H., Aditiansih, D., Sugianto, A., Burhan, E., Damayanti, T., Sitompul, P.A., Mariana, N., Antarianto, R.D., Liem, I.K., Kispas, T., et al. (2021). Umbilical cord mesenchymal stromal cells as critical COVID-19 adjuvant therapy: A randomized controlled trial. *Stem Cells Transl. Med.* 10, 1279–1287. <https://doi.org/10.1002/sctm.21-0046>.
170. Wei, F., Kong, D., Li, T., Li, A., Tan, Y., Fang, J., Zhuang, X., Lai, C., Xu, W., Dong, H., et al. (2021). Efficacy and safety of umbilical cord mesenchymal stem cells for the treatment of patients with COVID-19. *Clinics* 76, e2604. <https://doi.org/10.6061/clinics/2021/e2604>.
171. Feng, Y., Huang, J., Wu, J., Xu, Y., Chen, B., Jiang, L., Xiang, H., Peng, Z., and Wang, X. (2020). Safety and feasibility of umbilical cord mesenchymal stem cells in patients with COVID-19 pneumonia: A pilot study. *Cell Prolif.* 53, e12947. <https://doi.org/10.1111/cpr.12947>.
172. Yu, B., Zhang, X., and Li, X. (2014). Exosomes derived from mesenchymal stem cells. *Int. J. Mol. Sci.* 15, 4142–4157. <https://doi.org/10.3390/ijms15034142>.
173. Lee, J.H., Park, J., and Lee, J.W. (2019). Therapeutic use of mesenchymal stem cell-derived extracellular vesicles in acute lung injury. *Transfusion* 59, 876–883. <https://doi.org/10.1111/trf.14838>.
174. Sengupta, V., Sengupta, S., Lazo, A., Woods, P., Nolan, A., and Bremer, N. (2020). Exosomes Derived from Bone Marrow Mesenchymal Stem Cells as Treatment for Severe COVID-19. *Stem Cells Dev.* 29, 747–754. <https://doi.org/10.1089/scd.2020.0080>.
175. Hasan, S.S., Capstick, T., Ahmed, R., Kow, C.S., Mazhar, F., Merchant, H.A., and Zaidi, S.T.R. (2020). Mortality in COVID-19 patients with acute respiratory distress syndrome and corticosteroids use: a systematic review and meta-analysis. *Expert Rev. Respir. Med.* 14, 1149–1163. <https://doi.org/10.1080/17476348.2020.1804365>.
176. Lightner, A.L., Sengupta, V., Qian, S., Ransom, J.T., Suzuki, S., Park, D.J., Melson, T.L., Williams, B.P., Walsh, J.J., and Awili, M. (2023). Bone Marrow Mesenchymal Stem Cell-Derived Extracellular Vesicle Infusion for the Treatment of Respiratory Failure From COVID-19: A Randomized, Placebo-Controlled Dosing Clinical Trial. *Chest* 164, 1444–1453. <https://doi.org/10.1016/j.chest.2023.06.024>.
177. Zhu, Y.G., Shi, M.M., Monsel, A., Dai, C.X., Dong, X., Shen, H., Li, S.K., Chang, J., Xu, C.L., Li, P., et al. (2022). Nebulized exosomes derived from allogenic adipose

Appendix 4: Curriculum Vitae

Charlotte A. René

EDUCATION

Doctor of Philosophy

2019-2025

University of Ottawa, Ottawa, Ontario

- PhD Microbiology and Immunology
- Supervised by Dr. Robin J. Parks, PhD at the Ottawa Hospital Research Institute
- Thesis: Extracellular vesicle-mediated delivery of survival motor neuron protein: A potential therapeutic strategy for Spinal Muscular Atrophy
- Transferred to PhD program from MSc program: 2021

Bachelor of Science

2015-2019

Bishop's University, Lennoxville, Quebec

- Major: Biology – Health Science Concentration
- Minor: Psychology

AWARDS

- Stem Cell Network Travel Award (\$2 650 total) 2023
- Queen Elizabeth II Graduate Scholarship in Science and Technology (QEII-GSST) (\$15 000 total) 2022-2023
- University of Ottawa PhD Entrance Scholarship (\$36 000 total) 2021-2025
- University of Ottawa MSc Entrance Scholarship (\$15 000 total) 2019-2021
- Bishop's University Richard Tomlinson Scholarship for Academic Excellence (\$6 000 total) 2018-2019

PUBLICATIONS

René, C. A., Poulin, K. L., Petryk, J., Bell, J.C., Burger, D., Vacratsis, O. P., Warman-Chardon, J., Kothary, R., and Parks, R. J. (2025). Use of gene therapy to engineer the liver to release extracellular vesicles that distribute a therapeutic protein throughout the body of mice. (Under Review).

Poulin, K. L., **René, C. A.**, Smith, I. C., Vacratsis, O. P., Burger, D., McMillian, H. J., Kothary, R., Perkins, T. J., Warman-Chardon, J., and Parks, R. J. (2025). Extracellular vesicles as a biomarker for patients with spinal muscular atrophy disease and response to therapy. (Under Review).

René, C. A. and Parks, R. J. (2025). Efficient extracellular vesicle-mediated delivery of survival motor neuron protein to cells in culture. *Scientific Reports*. 15(1):5674.

René, C. A. and Parks, R. J. (2024). Bioengineering extracellular vesicle cargo for optimal therapeutic efficiency. *Molecular Therapy - Methods & Clinical Development*. 32(2):101259.

René, C. A. and Parks, R. J. (2023). Expanding the Availability of Onasemnogene Apeparvovec to Older Patients: The Evolving Treatment Landscape for Spinal Muscular Atrophy. *Pharmaceutics*. 15(6):764.

René, C. A. and Parks, R. J. (2021). Delivery of Therapeutic Agents to the Central Nervous System and the Promise of Extracellular Vesicles. *Pharmaceutics*. 13(4):492.

PUBLICATIONS IN PREPARATION

René, C. A., Kothary, R., and Parks, R. J. (2024). Survival motor neuron protein is preferentially loaded into extracellular vesicles. (In preparation).

COMMITTEES

- Ottawa Hospital Research Institute Trainee Committee 2023-2024

SOCIETY MEMBERSHIP

- Canadian Society of Extracellular Vesicles (CanSEV) 2023-
- Neuromuscular Disease Network for Canada (NMD4C) 2023-

PROFESSIONAL DEVELOPMENT

- American Society of Gene & Cell Therapy (ASGCT) Class Consideration Immunogenicity for AAV Gene Therapy Products 2025
- Patient-Oriented Research Training for Neuromuscular Disease 2024
- Canadian Institutes of Health Research (CIHR) Institute of Musculoskeletal Health and Arthritis (IMHA) and Institute of Genetics (IG) Patient Engagement 101 Workshop 2024
- CIHR-IMHA Patient Engagement in Research Training Program 2024
- SickKids Gene Therapy Workshop: TOGETHER (The Ontario Gene Therapy) Network 2024
- adMare BioInnovation Scientist Program: Foundations 2023
- BioTalent Canada BioSkills Recognition Program 2023
- Mayo Clinic Center for Regenerative Biotherapeutics Regenerative Medicine Course 2023
- Stem Cell Network Synthetic Biology for Regenerative Medicine Workshop 2023
- CIHR Science Communication Workshop Series 2023
- Collaborative Institutional Training Initiative (CITI) Canada Privacy Course 2021
- University of Ottawa Faculty of Medicine Biostatistics Workshop 2021
- CIHR Integrating Sex and Gender in Health Research Training 2019

CERTIFICATIONS

- Canada Good Clinical Practices (GCP) 2024
- Health Canada Division 5 – Drugs for Clinical Trials Involving Human Subjects 2021
- Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans Course on Research Ethics (TCPS 2: CORE) 2021
- Transportation of Dangerous Goods (TDG/IATA) 2021

- Responsible Conduct of Research (RCR) (Life Science – Student) 2021

SEMINAR PRESENTATIONS

University of Ottawa Biochemistry, Microbiology and Immunology 2024

Seminar Day

Seminar: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy”
(René, C. A., Kothary, R., and Parks, R. J.)

66th Annual Canadian Society for Molecular Biosciences Meeting 2023

Flash Talk and Poster: “Preferential loading of SMN protein into extracellular vesicles”
(René, C. A., Kothary, R., and Parks, R. J.)

2nd Annual Canadian Extracellular Vesicle Symposium 2023

Virtual Seminar/Poster: “Preferential loading of SMN protein into extracellular vesicles”
(René, C. A., Kothary, R., and Parks, R. J.)

Ottawa Hospital Research Institute Research Day 2022 2022

Virtual Seminar/Poster: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

University of Ottawa Department of Medicine Research Day 2022 2022

Virtual Seminar: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

University of Ottawa Biochemistry, Microbiology and Immunology 2022

Seminar Day

Virtual Seminar: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

Canadian Extracellular Vesicle Symposium 2022

Virtual Seminar/Poster: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

Ottawa Hospital Research Institute Research Day 2021 2021

Virtual Seminar/Poster: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

University of Ottawa Faculty of Medicine Research Day 2021

Virtual Seminar/Poster: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

University of Ottawa Biochemistry, Microbiology and Immunology 2021

Poster/Seminar Day

Virtual Seminar: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

Ottawa Hospital Research Institute Research Day 2020 2020
Virtual Seminar/Poster: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

University of Ottawa Biochemistry, Microbiology and Immunology 2020
Poster/Seminar Day
Virtual Seminar: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

POSTER PRESENTATIONS

Canadian Society for Extracellular Vesicles (CanSEV) Symposium 2025 2025
“Use of a gene therapy approach to engineer the liver to release extracellular vesicles that distribute a therapeutic protein throughout the body and central nervous system of mice” (René, C. A., Poulin, K. L., Petryk, J., Bell, J. C., Burger, D., Vacratsis, P. O., Warman-Chardon, J., Kothary, R., and Parks, R. J.)

Ottawa Hospital Research Institute Research Day 2024
“Use of a gene therapy approach to engineer the liver to release extracellular vesicles that distribute a therapeutic protein throughout the body and central nervous system of mice” (René, C. A., Poulin, K. L., Petryk, J., Bell, J. C., Burger, D., Vacratsis, P. O., Warman-Chardon, J., Kothary, R., and Parks, R. J.)

Ottawa Hospital Research Institute Research Day 2023 2023
Poster: “Preferential loading of SMN protein into extracellular vesicles” (René, C. A., Kothary, R., and Parks, R. J.)

6th Annual Ottawa International Conference on Neuromuscular Disease and Biology 2023
Poster: “Preferential loading of SMN protein into extracellular vesicles” (René, C. A., Kothary, R., and Parks, R. J.)

University of Ottawa Brain and Mind Institute Brain Health Research Day 2023
Poster: “Preferential loading of SMN protein into extracellular vesicles” (René, C. A., Kothary, R., and Parks, R. J.)

University of Ottawa Department of Medicine Research Day 2023 2023
Poster: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

University of Ottawa Brain and Mind Research Institute Research Day 2022 2022
Poster: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C. A., Kothary, R., and Parks, R. J.)

University of Ottawa Biochemistry, Microbiology and Immunology Symposium 2022
“Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy” (René, C.A., Kothary, R., and Parks, R.J.)

Ottawa Hospital Research Institute Research Day 2019 2019
Poster: “Extracellular Vesicles as a Novel Biotherapeutic for Spinal Muscular Atrophy”
(René, C. A., Kothary, R., and Parks, R. J.)

SELECT CONFERENCE ATTENDANCE

- 2025 MDA Clinical & Scientific Conference 2025
- 2024 Annual SMA Research & Clinical Care Meeting 2024
- 2024 MDA Clinical & Scientific Conference 2024
- 2023 Annual SMA Research & Clinical Care Meeting 2023
- Canadian National SMA Conference 2022
- 5th Ottawa International Conference on Neuromuscular Disease and Biology 2019