NEUROSCIENCE
2017

NEUROBIOLOGY OF DISEASE WORKSHOP

Gene Therapy to Address Unmet Needs in Neurology

Organizers: Xandra Breakefield, PhD, and Florian Eichler, MD

SfN SOCIETY *for* NEUROSCIENCE

Neurobiology of Disease Workshop

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Organized by Xandra Breakefield, PhD and Florian Eichler, MD
Friday, November 10, 2017
8 a.m.–5 p.m.
Location: Washington, DC Convention Center | Room: 146C

TIME	TOPIC	SPEAKER
7:30–8 a.m.	Check-in	
8–8:10 a.m.	Opening Remarks	Xandra Breakefield, PhD • Harvard University Florian Eichler, MD • Harvard University
8:10–8:40 a.m.	Spinal Muscular Atrophy (SMA) — Patient Presentation	Charlotte Sumner, MD • Johns Hopkins University
8:40–9:20 a.m.	Antisense Oligonucleotides as a Treatment for Spinal Muscular Atrophy	Charlotte Sumner, MD • Johns Hopkins University
9:20–10 a.m.	Gene Replacement vs. Gene Editing for Therapy of Duchenne Muscular Dystrophy	Jeff Chamberlain, PhD • University of Washington
10–10:40 a.m.	Approaches to Treating ALS with Gene Silencing	Robert Brown, MD, DPhil • University of Massachusetts
10:40–11 a.m.	Morning Break	
11–11:40 a.m.	Optimizing Gene Therapy Approaches in X-Linked Adrenoleukodystrophy	Florian Eichler, MD • Harvard University
11:40 a.m.–12:20 p.m.	CRISPR/Cas as a Novel Treatment Strategy for Neurological Disorders	Martin Ingelsson, MD, PhD • Uppsala University
12:20–1 p.m.	Neural Stem Cell–Delivered Therapies for Brain Tumors: Phase I Clinical Trials	Karen Aboody, MD • Beckman Research Institute at the City of Hope
1–2 p.m.	Lunch	Rooms 140AB

AFTERNOON BREAKOUT SESSIONS | PARTICIPANTS SELECT FIRST DISCUSSION GROUP AT 2:00 P.M.

TIME	BREAKOUT SESSIONS	SPEAKERS	ROOM
2–3:30 p.m.	Group 1: Aggressive Tactics for Brain Tumors — Oncolytic Viruses and Cell "Drones"	Paola Grandi, PhD, & Karen Aboody, MD	147A
	Group 2: Tailoring AAV Vectors for Gene Replacement	David Corey, PhD, & Jeff Chamberlain, PhD	147B
	Group 3: Gene Delivery Strategies to the Brain	Miguel Sena-Esteves, PhD, & Eloise Hudry, PhD	150B

AFTERNOON BREAKOUT SESSIONS | PARTICIPANTS SELECT SECOND DISCUSSION GROUP AT 3:30 P.M.

TIME	BREAKOUT SESSIONS	SPEAKERS	ROOM
3:30–5 p.m.	Group 4: Clinical Strategies for Rapidly Progressing Neuromuscular Diseases	Charlotte Sumner, MD, & Robert Brown, MD, DPhil	147A
	Group 5: CRISPR and Other Novel Approaches	Xandra Breakefield, PhD, & Martin Ingelsson, MD, PhD	147B

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Introduction

How Gene Therapy Will Address Unmet Needs in Neurology

Following advances in gene identification through next-generation sequencing and the Human Genome Project, gene correction may seem to be the next logical step. However, the process has been fraught with several challenges: scaling up from *in vitro* work to human application, our poor understanding of human disease, a fledgling discussion of the balance between risks and benefits, and lastly but significantly, human error. Despite these challenges, advances in single-gene correction and delivery technologies and improved understanding of biodistribution have propelled gene therapy into the clinical realm, and new medical treatment options are beginning to offer help in diseases long thought to be incurable.

The 2017 Neurobiology of Disease Workshop and companion course book describe the breadth of current gene therapy approaches being tested in promising preclinical studies and clinical trials for a variety of neurological disorders. These innovative strategies include gene replacement or correction, viral vectors, oligonucleotides for inhibition or corrective splicing of disease-causing mRNAs, reintroduction of the patient's own cells with a corrected genotype, and "armed" cells or viruses for fighting brain tumors. In many instances, model systems in preclinical studies did not predict success or failure in the clinic. Rather, the clinical scenario and unmet need in human disease appear to be key factors that forecast and drive gene therapy to the bedside.

What does all this mean? That the choice of approach for gene correction depends on a variety of factors: the nature of the disease-causing gene, the target cell or tissue, the pathophysiology of the disease on both a cellular and an organ level, and the extent of subsequent dysfunction and unmet clinical need. In addition to the many forms of gene therapy, including RNAs delivered by nanoparticles (e.g., small inhibitory RNAs and antisense oligonucleotides) that can alter splicing and inhibit translation of mRNAs; viral vectors (e.g., adeno-associated virus [AAV], lentivirus, herpes simplex virus); genetically modified cells (hematopoietic stem cells and neural stem cells); and directed changes in the genome (CRISPR technology), one needs to consider route of delivery, dosing, and speed of administration. Once these latter topics have been addressed in the context of the human disease course, more-rapid translation of preclinical studies into patient treatments will become possible.

Recent successful examples of gene therapy implemented in clinical trials include gene replacement for hereditary deafness and motor neuron degeneration using AAV vectors, as well as delivery of corrective proteins to the brain using hematopoietic stem cells engineered *ex vivo* to combat demyelination or as immunotherapy for leukemia. All these approaches benefit from a thorough understanding of the human scenario, including which cells to target and how to define the clinical window of intervention.

This workshop and syllabus span emerging technologies, promising preclinical studies, interventional strategies, and clinical trials in the context of ethical and regulatory issues, as presented by leaders in the field.

Spinal Muscular Atrophy Therapeutics: Where Do We Stand?

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Introduction

Spinal muscular atrophy (SMA) is the most common autosomal recessive cause of infant mortality, affecting one in 6000–10,000 live births. The classical pathological hallmark of SMA is the loss of motor neurons in the anterior horn of the spinal cord. Clinically, SMA patients are classified into one of five types based on age of onset and the ability to achieve motor milestones (Oskoui et al., 2016). SMA type 0 is the most severe form, with onset at neonatal stages and sometimes reduced movement *in utero*. SMA type 1 is the most common form of the disease, having a clinical onset usually before 6 months. Affected infants never acquire the ability to sit unsupported and often have no head control owing to severe hypotonia and symmetrical paralysis. SMA type 2 patients are characterized by age of onset ranging from 7 to 18 months. Although these infants can sit unsupported, they are unable to walk independently. SMA type 3 patients develop symptoms after the age of 2 years. They usually achieve all major motor milestones, although many need wheelchair assistance later in childhood or adulthood. Patients with SMA type 4 usually have an onset in the second or third decade of life. They experience mild muscle weakness and generally have no respiratory problems.

SMA Genetics

In 1995, a century after the first description of SMA, the underlying genetic defect was identified (Lefebvre et al., 1995). Genetic linkage analyses and subsequent positional cloning identified a disease-associated 140 kb region that contained the duplicated survival motor neuron (*SMN*) gene on chromosome 5q13. In SMA patients, the telomeric copy of *SMN* (*SMN^T* or *SMN1*) is mutated (usually with large deletions or, rarely, point mutations), disrupting SMN function. The highly homologous centromeric copy of *SMN* (*SMN^C* or *SMN2*) contains a critical translational silent C > T substitution in an exonic enhancer at codon 280 in exon 7 (six base pairs downstream from the 5' end of exon 7). This results in alternative splicing of *SMN2*-derived pre-mRNAs with exon 7 often removed, resulting in a truncated and nonfunctional SMN protein (Fig. 1) (Lorson et al., 1999; Monani et al., 1999). When exon 7 is retained, full-length *SMN2*-derived transcripts code for a normal SMN protein. All SMA patients harbor at least one copy of *SMN2*, and in most cases, *SMN2* copy number inversely correlates with disease severity (Lefebvre et al., 1997; Parsons et al., 1998). Rare individuals with homozygous mutations of *SMN1* and five copies of *SMN2* have been described who do not develop SMA (Prior et al., 2004).
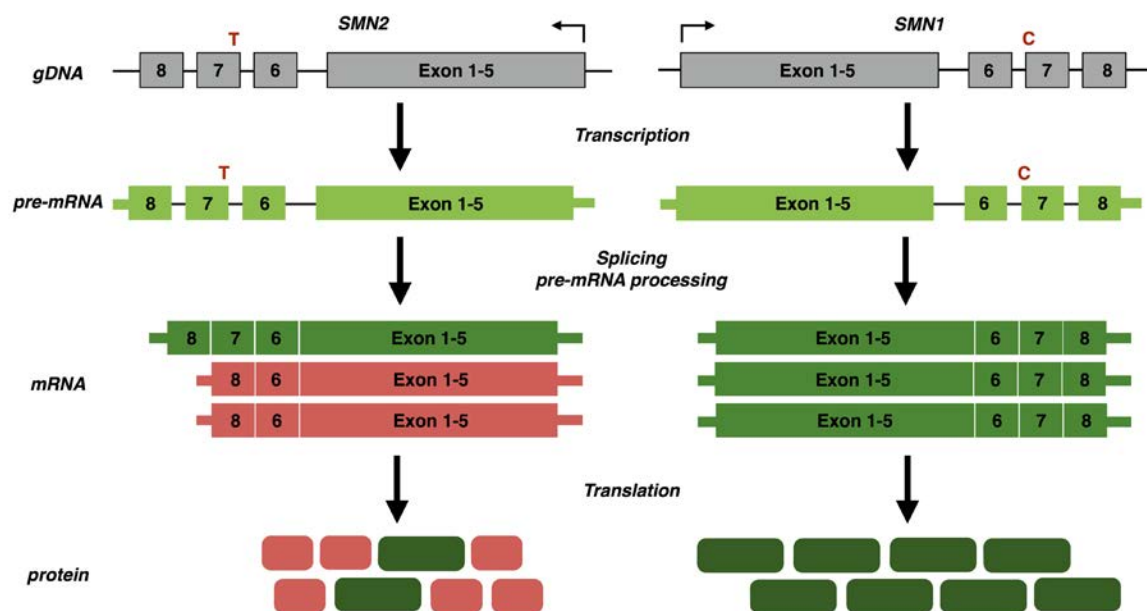

Figure 1. Genetics of SMA. SMA is caused by mutation of the *SMN1* gene and reduced SMN protein levels. All patients retain at least one copy of the highly homologous *SMN2* gene. *SMN2* harbors a translational silent C > T substitution in a splice enhancer sequence of exon 7, resulting in exon-7 skipping at the mRNA level. The alternatively spliced *SMN2* mRNA encodes a truncated, highly unstable, nonfunctional protein. A small fraction of SMN2 transcripts contain exon 7 that encodes a full-length, functional SMN protein. gDNA, genomic DNA. Reprinted with permission from d'Ydewalle and Sumner (2015), Fig. 1. Copyright 2015, Springer US.

SMN Protein

The SMN protein is a 294-amino-acid polypeptide with a predicted size of 38 kDa. It is highly conserved across species and is expressed ubiquitously, raising the question of why motor neurons are specifically vulnerable to SMN deficiency. Levels of functional full-length SMN protein that self-associate into the SMN complex inversely correlate with disease severity (Lefebvre et al., 1997). In contrast, truncated SMN protein arising from *SMN2* is unstable and less efficient at self-associating, causing reduced levels of functional SMN complex (Burnett et al., 2009). Thus, *SMN2* fails to compensate for the loss of *SMN1*, resulting in the development of SMA.

SMN localizes both to the cytoplasm and to distinct structures in the nucleus called "gems," whose function(s) are still under investigation (Liu and Dreyfuss, 1996). SMN interacts with a wide variety of known RNA-binding proteins, such as small nuclear ribonucleic particles containing small nuclear RNAs (snRNAs) and small nuclear ribonucleoproteins (snRNPs) as well as other RNA-binding proteins (Fischer et al., 1997; Liu et al., 1997). Several studies have indicated that SMN plays a crucial role in specific snRNP biogenesis and spliceosome assembly (Pellizzoni et al., 1998, 2002), and hence in pre-mRNA splicing. In line with these findings, SMN deficiency causes tissue-specific perturbations in snRNA levels and defects in splicing (Zhang et al., 2008; Lotti et al., 2012). The search is ongoing to identify specific genes whose processing is perturbed by SMN deficiency and that play a direct role in SMA pathogenesis. SMN also localizes to cytoplasmic or neuritic granules in neurons, suggesting that SMN might also play a neuron-specific role in mRNA transport or local mRNA processing (Zhang et al., 2006).

SMA Therapeutics

The correlation between *SMN2* copy number and disease severity in SMA patients suggested that increasing SMN expression could be a promising therapeutic strategy. Indeed, preclinical experiments in SMA mouse models indicated that increasing *SMN2* expression by either genetic or pharmacological means could ameliorate disease manifestations even after symptom onset (Monani et al., 2000; Avila et al., 2007; Lutz et al., 2011). Several different approaches have been explored to increase SMN expression; however, the two most successful to date are (1) to modulate splicing of pre-RNAs arising from existing *SMN2* genes using antisense oligonucleotides (ASOs) or small molecules or (2) to replace *SMN1* using viral delivery.

Modulation of splicing

Antisense oligonucleotides

Because *SMN2* gene expression results in an alternative spliced, truncated, and nonfunctional product, an approach to therapy development has focused on promoting the inclusion of exon 7 in *SMN2* mRNAs (Fig. 2). ASO technology was initially developed to downregulate gene expression by targeting mRNAs to induce their degradation or block their translation. Advances in antisense chemistry now allow application of this technology to manipulate pre-mRNA splicing (Spitali and Aartsma-Rus, 2012; Schoch and Miller, 2017). ASOs target and bind RNA via Watson–Crick base pairing. The identification of the intronic splicing silencer N1 (ISS-N1) element downstream of the 5' splice site in intron 7 of *SMN2* pre-mRNAs provided a robust target for antisense technology to modulate *SMN2* splicing (Singh et al., 2006). Significantly, deletion of ISS-N1 promoted exon-7 inclusion (Singh et al., 2006). Work from several laboratories indicated that ASOs targeting ISS-N1 dramatically promoted exon-7 inclusion to near 100% of the primary transcripts and increased SMN protein levels in transfected cell lines, patient-derived cells, and mouse models of SMA (Singh et al., 2006; Hua et al., 2007, 2008; Porensky et al., 2011). Treatment of adult heterozygous or wild-type transgenic *SMN2* mice twice per week (by tail vein injections) with the most effective ASO (ASO-10-27) resulted in a dose-dependent and time-dependent increase in exon-7 inclusion in liver and kidney (Hua et al., 2008). However, the exon-7 inclusion rate was not affected in the spinal cord of these animals because the ASO did not penetrate the blood–brain barrier (BBB) when injected systemically at this age. To investigate the effect of ASO in the spinal cord, Hua and colleagues infused ASO-10-27 continuously for 7 d in the right lateral ventricle at various doses in adult-onset mild-severity SMA mice (type 3 SMA, $Smn^{+/-}/SMN2^{+/+}$ with four copies of *SMN2*) (Hua et al., 2010). ASO treatment resulted in an almost complete rescue of exon-7 exclusion and increased SMN protein levels throughout the spinal cord. In addition, the *in vivo* half-life of the ASO appeared to be very long, as the effect of the ASO was still observable 6 months after completing the 7 d treatment.

ASO-10-27 was also examined in the more severe "Taiwanese" SMA mouse model (Hua et al., 2011). In this study, ASO was administered intracerebroventricularly on day 2 or subcutaneously on day 1 or 3. Although both routes of delivery were effective, systemic delivery was more effective,

raising the question of whether tissues other than the CNS are affected by SMN deficiency in mice and perhaps in humans.

Based on the extraordinary effects of ASO-10-27 in preclinical models, ISIS (now Ionis) Pharmaceuticals partnered with Biogen Idec to begin clinical trials of the molecule in SMA patients. Given the lack of BBB penetration, nusinersen (SPINRAZA, an SMN2-directed ASO) is delivered by intrathecal injection. The first patient received the drug in 2011, phase I trials were completed in 2013, and phase II trials were completed in 2014. These trials showed that the drug was well tolerated when administered intrathecally and that it increased SMN mRNA and protein levels in human spinal cord (Chiriboga et al., 2016; Finkel et al., 2016). Based on these data, approval-enabling phase III placebo-controlled trials were launched for type 1 SMA patients (ENDEAR: A Study to Assess the Efficacy and Safety of Nusinersen in Infants With SMA) and type 2 SMA patients (CHERISH: A Study to Assess the Efficacy and Safety of Nusinersen in Participants With Later-onset SMA). Both trials were stopped early at 13 months, having met study endpoints. For example, in ENDEAR, 51% of symptomatic type 1 infants treated before 6 months of life showed an improvement in motor milestones and a reduction in mortality (Kuntz et al., 2017).

Based on these data, on December 23, 2016, the U.S. Food and Drug Administration approved nusinersen for use in all SMA patients. This first-approved SMA treatment represents a major milestone, but as is the case for nearly all diseases, does not represent a cure. Truncal and respiratory muscles responded less well than limb muscles, and after 13 months of treatment in ENDEAR, only 8% of infants had achieved independent sitting, 49% had no improvement in motor milestones, and 16% had died. Another ongoing trial (NURTURE: A Study of Multiple Doses of Nusinersen Delivered to Infants With Genetically Diagnosed and Presymptomatic SMA), in which the drug is administered before the onset of symptoms to infants predicted to have type 1
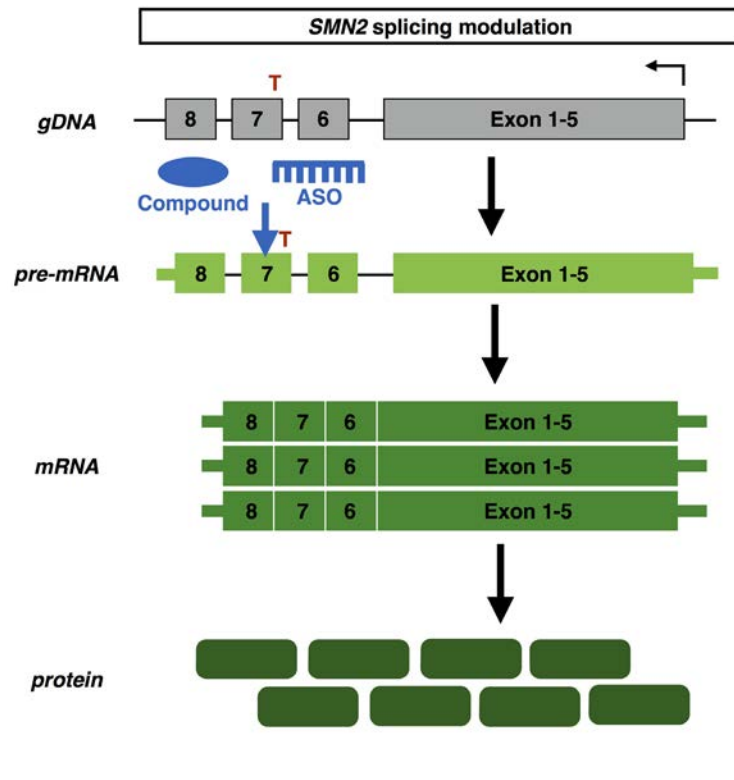

Figure 2. Mechanism of *SMN2*-splicing modulation drugs. ASOs or chemical compounds promote exon-7 retention in the *SMN2* pre-mRNA, resulting in increased full-length *SMN* mRNA and protein levels. gDNA, genomic DNA. Reprinted with permission from d'Ydewalle and Sumner (2015), Fig. 5. Copyright 2015, Springer US.

SMA, indicates that earlier administration of the drug is significantly more effective (De Vivo et al., 2017). Therefore, an effort is ongoing to institute population-wide newborn screening for SMA to increase the number of infants treated very early in the disease process. Nusinersen is now commercially available as SPINRAZA at a cost of $125,000 per dose (4 doses in the first 2 months; then once every 4 months). The cost and delivery route have raised challenges for insurance approval and timely delivery.

Small molecules
An advantage of the ASO approach is specificity because the drug itself contains the complementary sequence of ISS-N1 and thus specifically modulates *SMN2* exon-7 splicing. Disadvantages include the lack of brain penetration and the current need for an invasive delivery route (intrathecal delivery). Given these drawbacks, there has been a major effort to identify orally bioavailable small molecules that target SMN2 exon-7 splicing. Large-scale drug screens to identify such drugs were performed by two groups: (1) PTC Therapeutics and Roche,

in partnership with the SMA Foundation (Naryshkin et al., 2014), and (2) Novartis (Palacino et al., 2015). These studies mark a major breakthrough in the search for compounds that are capable of quite specifically modulating a single splicing event in the genome.

Naryshkin and colleagues demonstrated that such compounds dose-dependently increased full-length *SMN* mRNA levels and SMN protein levels in patient-derived fibroblasts (Naryshkin et al., 2014). However, the compounds did not affect widespread gene expression changes, as only six genes were upregulated or downregulated by a factor of two or more. In addition, analysis of annotated splice junctions indicated that these compounds were highly specific in promoting SMN2 exon-7 inclusion. These modifiers also increased motor function and dramatically extended survival in two different SMA mouse models when administered systemically by oral gavage and/or by intraperitoneal administration. Independently, Novartis identified another orally available small molecule that is able to modulate the splicing of *SMN2* and increase SMN protein levels in cell lines derived from SMA mouse models and SMA patients (Palacino et al., 2015). In the Novartis study, it was proposed that the molecular mechanism of action of the small molecule was to improve affinity between *SMN2* pre-mRNA and the U1 snRNP complex, thereby increasing affinity of the U1 snRNP for the 5' splice site in a sequence-dependent manner. A clinical trial of the Novartis compound in SMA type 1 patients is on clinical hold, and a second-generation Roche compound is currently in phase II/III clinical trials in Europe and the United States.

Gene therapy

Rather than increasing the expression of endogenous full-length *SMN2,* some groups have focused on replacing *SMN1* using gene therapy (Fig. 3). Groundbreaking work in 2010 showed that gene therapy had marked efficacy in severe-SMA mice (Foust et al., 2010). In this report, SMN cDNA under the control of a strong promoter was encapsulated by the self-complementary adeno-associated virus serotype-9 (scAAV9). The scAAV9 gene therapy
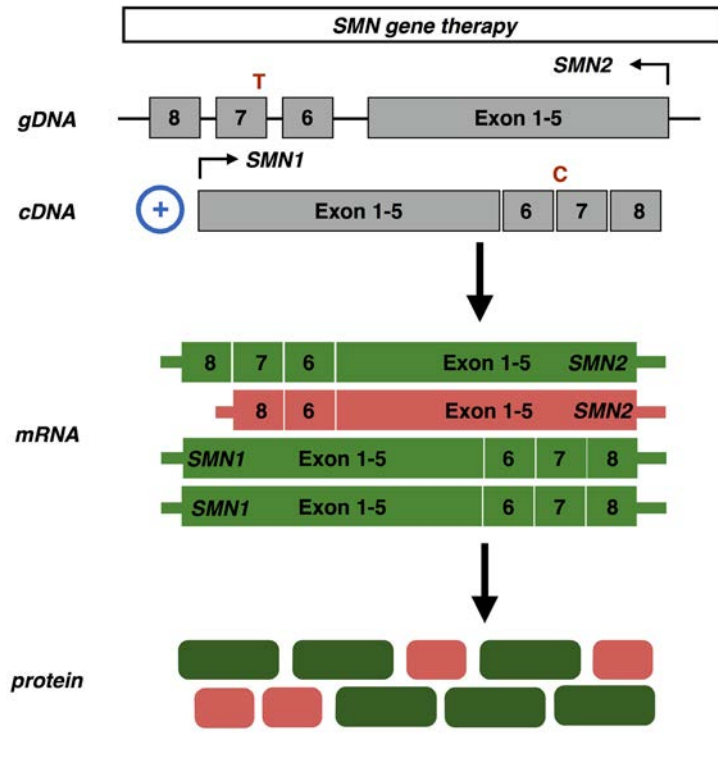

Figure 3. Mechanism of *SMN1* replacement by gene therapy. Using AAVs, *SMN1* cDNA is incorporated into the human genome, resulting in increased full-length *SMN* mRNA and protein levels. gDNA, genomic DNA. Reprinted with permission from d'Ydewalle and Sumner (2015), Fig. 6. Copyright 2015, Springer US.

delivered intravenously resulted in a marked increase in SMN protein levels in brain and in motor neurons of spinal cord, indicating that scAAV9 is able to traverse the BBB and has neurotropism. When delivered by a single-vein injection to 1- or 2-d-old *SMNΔ7* mice, they showed significant extension of survival (mean lifespan: 250 d vs 15.5 d, respectively) and improved locomotor function, weights, and neuromuscular transmission (Foust et al., 2010). Interestingly, when mice were injected at later time points, transduction with scAAV9 distributed more toward glial cells than motor neurons, and benefits were attenuated. The scAAV9 also proved to have clinical potential, as it traversed the BBB and efficiently infected cells in dorsal root ganglia and motor neurons within the whole spinal cord in a 1-d-old injected nonhuman primate (Foust et al., 2010). Other work demonstrated that transgene expression persisted in the CNS (in both motor neurons and glial cells in spinal cord) as well as skeletal muscles when nonhuman primates were systemically treated at various ages. In addition, local scAAV9 injection into the CSF of piglets efficiently transduced motor neurons throughout the spinal cord and resulted in robust transgene expression (Bevan et al., 2011).

These positive results in SMA mice and in nonhuman primates led AveXis to undertake a phase I clinical trial of AVXS-101 (ChariSMA) at the Research Institute at Nationwide Children's Hospital in Columbus, Ohio, to assess the safety and tolerability of intravenously injected scAAV9-SMN (scAAV9.CB.SMN, known as ChariSMA) in SMA type 1 patients (Lowes et al., 2017). This was the largest amount of virus delivered in a gene therapy trial. Inclusion criteria included patients with two copies of *SMN2* and disease onset before the age of 6 months. These stringent inclusion criteria were designed to target patients as early in the disease course as possible. Results reported at meetings indicate that systemic administration of scAAV9-SMN is safe and that those infants who are younger at the time of dosing and have less motor impairment show striking improvements in motor function after treatment (Lowes et al., 2017). A follow-up approval-enabling, multicenter trial is soon to start.

Future Prospects

There has been enormous progress on multiple fronts in developing "gene-targeted" therapeutics for SMA in recent years. The early promise of these novel therapeutics, including ASOs, viral gene therapy, and novel splice-switching small molecules, lends hope that they can be applied successfully to other neurodegenerative diseases. For SMA, further studies are needed to address remaining questions about optimal timing and cell targeting of SMN-induction therapeutics. In addition, SMN induction alone is likely insufficient to treat all patients optimally, so continued work will be needed to identify SMN-independent therapeutic targets.

Acknowledgments

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NOTES

Gene Replacement versus Gene Editing for Therapy of Duchenne Muscular Dystrophy

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Introduction

Duchenne muscular dystrophy (DMD) and the milder form, Becker muscular dystrophy (BMD), are among the most common single-gene disorders in humans, affecting ~1 in 5000 male newborns (Mendell et al., 2012). Despite this relatively low incidence in the general population, DMD/BMD is one of the most well-known genetic disorders and has attracted enormous interest in the scientific and patient advocacy communities. A seminal series of publications by the laboratory of Louis Kunkel resulted in the identification of the *dystrophin*, or *DMD*, gene in 1986 (Monaco et al., 1986). That work enabled highly accurate prenatal diagnosis and carrier detection, clarified the tissue-specific nature of mutations, and delineated the mutational differences between DMD and BMD. The availability of the gene and the cDNA for the muscle isoform made DMD an early candidate for gene therapy (Chamberlain and Caskey, 1990). Multiple approaches have been advanced to develop genetic therapies, including antisense oligonucleotides, gene replacement, and gene editing. This chapter will focus on methods using adeno-associated virus (AAV) vectors to either replace or modify (edit) the *dystrophin* gene to restore the expression of functional dystrophin proteins.

Restoring Dystrophin Production Can Greatly Ameliorate DMD

A variety of features of DMD present enormous obstacles to development of a therapy. The gene is 2.2 MB in size, and numerous isoforms are expressed in muscle and nonmuscle tissues from seven different promoters and via alternative splicing. Fortunately, a number of discoveries suggested approaches to gene therapy that were simpler than initially envisioned. One was the identification of

rare patients with large deletions within the gene—in one case encompassing almost half the gene—that were associated with extremely mild cases of BMD (England et al., 1990). A second discovery came from isolation and expression of the muscle cDNA (11.2 kb open reading frame) in *mdx* mice, a model for DMD. From such studies, it became clear that an effective therapy could be developed if a synthetic gene based on the muscle cDNA could be delivered to striated muscles. Smaller versions of the cDNA (3.6–6.5 kb) were subsequently shown to almost completely prevent disease in the *mdx* mouse models (Fig. 1) (Phelps et al., 1995; Wells et al., 1995; Rafael et al., 1996; Yuasa et al., 1998; Crawford et al., 2000; Wang et al., 2000; Harper et al., 2002). Similar studies showed that levels of dystrophin between ~5% and 20% of normal were sufficient to alleviate or prevent dystrophy in mouse models, similar to levels found in very mildly affected BMD patients (Chamberlain, 1997).

AAV Vectors for Delivering Muscle Gene Therapy

An enormous challenge for gene therapy of muscle disorders was finding a way to deliver a gene to the striated muscles that make up nearly 40% of human body mass. To date, the only vectors shown to deliver genes bodywide are a subset of those derived from AAVs (Gregorevic et al., 2004). AAV vectors have been used in numerous clinical trials, and several pending neuromuscular disorder trials plan to use AAV vectors as well (Mendell et al., 2010, 2015; Bengtsson et al., 2016). In particular, AAV vectors derived from serotypes 6, 8, and 9 have been widely used in DMD models for systemic delivery to muscle (Gregorevic et al., 2004; Childers et al., 2014; Yue et al., 2015). However, vector tropism tends to be
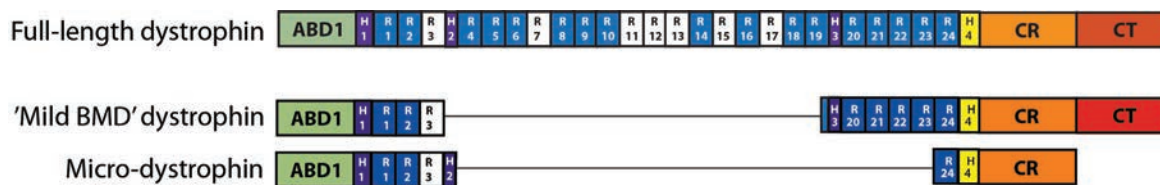

Figure 1. Comparative domain structures of full-length dystrophin (top), the mini-*dystrophin* expressed in a very mildly affected BMD patient carrying a genomic deletion that removed exons 17–48 (middle), and the structure of a micro-dystrophin protein (bottom). Domains within dystrophin are abbreviated as follows: ABD, actin-binding domain; R, spectrin-like repeats; H, hinge domains; CR, cysteine-rich domain; and CT, carboxy-terminal domain. Note that the exon 17–48 genomic deletion removes approximately two-thirds of the spectrin-like repeat 19 coding region. Numerous variants of micro-dystrophin structures have been described by different labs, although most currently planned clinical trials are using versions developed by the Chamberlain lab. Reprinted with permission from Chamberlain JR and Chamberlain JS (2017), Figure 1. Copyright 2017, Elsevier.

fairly broad, and as such, tissue-specific promoters are used to limit gene expression to the desired cell types (Salva et al., 2007). A variety of genes have been delivered to muscle using AAV vectors, including dystrophin, and components of the CRISPR/Cas9 system (CRISPR stands for clustered regularly interspaced short palindromic repeats; Cas9 is a class of RNA-guided endonucleases) (Bengtsson et al., 2017).

Dystrophin Replacement Using Micro-dystrophins

Adapting AAV vector technology for DMD still required significant innovation. AAV vectors have a carrying capacity of ~5 kb, and the early studies all used vectors derived from serotype 2, which poorly transduced striated muscles and could be administered only by intramuscular injection. However, studies in transgenic mice had revealed that highly functional "micro-dystrophin" cassettes could be generated smaller than 4 kb (Rafael et al., 1996; Crawford et al., 2000; Harper et al., 2002; Sakamoto et al., 2002). A major breakthrough occurred when it was discovered that improved vectors could be generated from newly discovered AAV serotypes (such as AAV6, AAV8, and AAV9), which, when injected into the vasculature at high dose (in the range of 10^{14} vector genomes per kg) could transduce all the striated muscles in adult mice (Gregorevic et al., 2004). This led to the demonstration that dystrophy could be almost entirely halted and largely reversed in an adult mammal via systemic deliver of AAV/micro-dystrophin vectors (Fig. 2) (Gregorevic et al., 2004, 2006). Refinement of the gene delivery

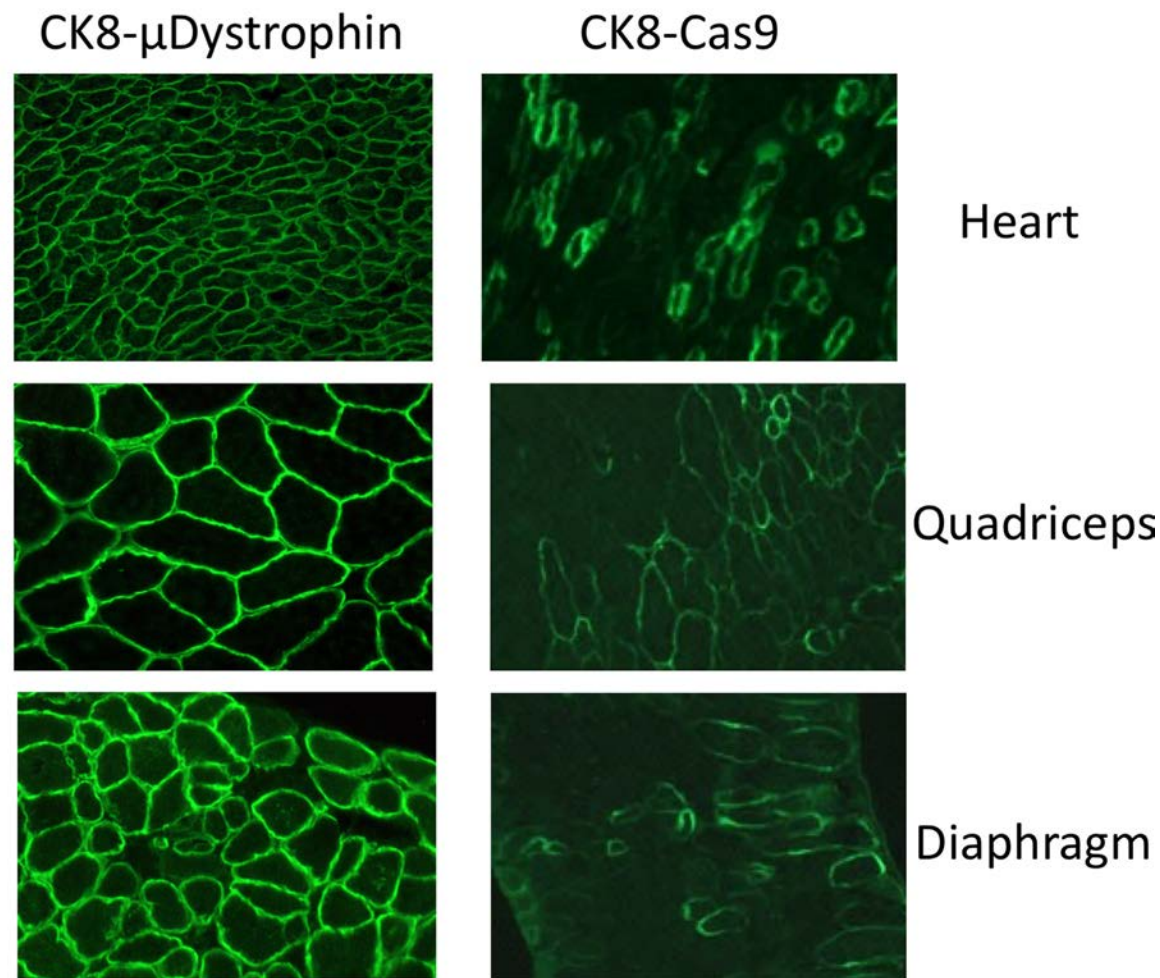**Figure 2.** Comparison of dystrophin levels obtained using AAV6 vectors to systemically deliver either micro-dystrophin (µDystrophin) or CRISPR/Cas9 (CK8-Cas9) components. Young adult *mdx⁴ᶜᵛ* mice were infused via retro-orbital injection with 4 x 10¹⁴ vector genomes/kg of AAV6, and various muscles were examined for dystrophin expression either 4 (micro-dystrophin) or 2 (CRISPR/Cas9) months after vector infusion. The results show that gene replacement is far more efficient than gene editing. Reprinted with permission from Chamberlain JR and Chamberlain JS (2017), Figure 2. Copyright 2017, Elsevier.

cassette through miniaturization of muscle-restricted gene regulatory cassettes provided greater vector functionality (Li et al., 1999; Hauser et al., 2000).

Because of considerable advances in the types of AAV vectors available, muscle-specific gene regulatory cassettes, and production and purification protocols, several groups are now planning human clinical trials involving vascular delivery of AAV/micro-dystrophin to patients. These plans are supported by extensive new data involving large-scale vector delivery to *cxmd* dogs, and for safety studies, to wild-type nonhuman primates (Rodino-Klapac et al., 2010; Toromanoff et al., 2010; Duan, 2015). Although most of the newest data remain unpublished or proprietary, several trends are emerging that are being used to support upcoming clinical trial applications to the U.S. Food and Drug Administration or the European Medicines Agency. Planned trials have many similarities but differ in details, including vector serotype, micro-dystrophin design, gene regulatory cassette usage, and the age of the patients. Depending on regulatory agency approval, some of these trials could begin within the next year.

Gene Editing of the *Dystrophin* Gene

Gene editing is attractive as a therapy because it has the potential to directly modify the mutant *DMD* gene to enable production of the dystrophin protein. In cases where the mutation is small, such as a point mutation or small deletion, this approach could lead to production of a nearly full-length protein (Long et al., 2014). The potential for this strategy was demonstrated by using AAV vectors to deliver CMV-Cas9 and single guide RNA (sgRNA) cassettes to bypass the premature stop codon in the genomes of *mdx* mice (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). More recently, multiple strategies were shown to have potential for muscle-specific editing in the more complex mutational context in *mdx⁴ᶜᵛ* mice (Bengtsson et al., 2016, 2017). However, with large deletions, editing would only enable production of smaller dystrophins, similar to approaches using antisense oligonucleotides. A method to circumvent this limitation has recently been suggested by using editing to introduce a portion of the dystrophin cDNA into the mutant gene to restore production of larger, more functional dystrophins (Young et al., 2016). Overall, multiple strategies will be needed for treating the wide variety of known *DMD* mutations.

Several issues need to be resolved before gene editing can be tested in the clinic. One is the obvious problem of low efficiency observed to date (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016; Bengtsson et al., 2017). For example, Figure 2 shows that much higher levels of dystrophin are generated following AAV-mediated delivery of a *dystrophin* gene than following delivery of CRISPR/Cas9 components. A second is the issue of expressing the bacterial Cas9 enzyme for extended periods in muscle. The studies that expressed Cas9 from the CMV enhancer/promoter are likely to lead to an immune response against the bacterial Cas9 (Hartigan-O'Connor et al., 2001; Chew et al., 2016; Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). Muscle-specific expression could reduce this concern but would still lead to long-term nuclease expression, albeit only in postmitotic cells (Long et al., 2014). Safety issues related to off-target editing also need to be addressed. Gene editing has been suggested as a method that could lead to permanent correction of dystrophin deficiency, but such a goal will require targeting the gene in myogenic stem cells. This latter issue arises because even normal muscle cells display a low rate of turnover. Current data suggest that AAV vectors are not capable of transducing myogenic stem cells *in vivo* at an efficiency needed for significant gene editing, but future modifications to the technology are likely (Arnett et al., 2014; Tabebordbar et al., 2016).

Prospects for Clinical Trials

Gene therapy using systemic delivery of AAV/micro-dystrophin vectors appears increasingly feasible and will soon be tested in clinical trials. The method as originally developed in *mdx* mice was shown to be safe and largely eliminates dystrophic pathophysiology for the lifespan of the mice. Recent and ongoing studies suggest that similar results are observed in canine models of DMD, and various types of AAV vectors have been shown to be safe in nonhuman primate studies and in clinical trials for other genetic disorders. As noted earlier, this potential for systemic gene delivery using AAV vectors is also being developed as a way to perform gene editing for various disorders, including DMD, by delivering Cas9 and sgRNA cassettes to muscle. However, widespread use of AAV vectors to deliver Cas9 must take into account a need to limit the duration of Cas9 expression, because expression of a nuclease for years in muscle cells seems likely to significantly increase the frequency of off-target gene editing. Consequently, many labs are focused on developing higher fidelity Cas9 variants and on ways to transiently express the nuclease. It seems clear that micro-dystrophin trials will enter the clinic well in advance of gene editing trials for DMD, though

both methods show promise. Whereas DMD was once viewed as an incurable disease, progress in the field suggests that successful gene therapies may soon be available.

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Gene Silencing Approaches to Amyotrophic Lateral Sclerosis

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Introduction: Pathophysiology of ALS

Twenty percent of familial amyotrophic lateral sclerosis (ALS) cases are caused by mutations in superoxide dismutase 1 (SOD1) (Rosen et al. 1993; Renton et al. 2014). Multiple pathways have been implicated in mutant SOD1–associated neurodegeneration (Taylor et al. 2016; Brown and Al-Chalabi, 2017). Transgenic *B6/SJL* mice expressing high levels of mutant human SOD1^{G93A} (hSOD1) are commonly used as an ALS mouse model (Gurney et al., 1994). Numerous approaches have been explored to silence *SOD1 in vivo* (Ralph et al., 2005; Smith et al., 2006; Wang et al., 2014; Scarrott et al., 2015). Adeno-associated virus (AAV) vectors are exceptionally efficient for gene transfer to the CNS, where they mediate long-term gene expression with no apparent toxicity (Broekman et al., 2006; Foust et al., 2009, 2013; Yang et al., 2014). In the studies reported here, we investigated the efficacy of a single-stranded AAV9 vector encoding an hSOD1-specific artificial microRNA (amiR) against human *SOD1* (amiR^{SOD1}) infused into the cerebral lateral ventricles of neonate *SOD1*^{G93A} mice. The experimental methods for this study are presented in the full version of this report (Stoica et al., 2016; available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5374859/).

Results

AAV9-delivered amiR^{SOD1} targets cells of the CNS and decreases *hSOD1* gene expression

We first analyzed the CNS transduction profile of an AAV9-GFP vector delivered intracerebroventricularly to neonatal *SOD1*^{G93A} mice at a total dose of 1×10^{11} vector genomes. Analysis of green fluorescent protein (GFP) expression at four weeks postinjection, revealed transduced neurons in the motor cortex and ventral horn of the spinal cord (Fig. 1, arrows); astrocytes or microglia were transduced in the spinal cord (Fig. 1B, arrowhead). We therefore investigated the therapeutic effectiveness of an AAV9 vector encoding an amiR against hSOD1 delivered intracerebroventricularly in neonatal *SOD1*^{G93A} mice. We injected *SOD1*^{G93A} mice at postnatal day 0–1 (P0–P1). At four weeks postinjection (Fig. 1C), hSOD1 mRNA levels in spinal cord were reduced by $\leq 50\%$ with no significant difference seen among cervical, thoracic, and lumbar regions. In peripheral tissues, hSOD1 mRNA levels were reduced by $> 80\%$ in heart and gastrocnemius muscle but were unchanged in liver or lung. The intensity of the hSOD1 mRNA signal was considerably reduced in cells expressing GFP mRNA in both motor cortex (Fig. 2A) and spinal cord (Fig. 2B).

Further, colocalization of the GFP mRNA signal with probes specific for Etv1 and ChAT mRNAs confirmed the transduction of both layer V cortical neurons (Fig. 2A, arrows) and spinal cord motor neurons (Fig. 2B, arrows) in AAV9-injected mice.

Neonatal treatment with AAV9-amiR^{SOD1} improves survival and delays the onset of paralysis in *SOD1*^{G93A} mice

We next assessed the therapeutic benefit of neonatal intracerebroventricular injection of AAV9-amiR^{SOD1} vector in *SOD1*^{G93A} mice. Litter-matched *SOD1*^{G93A} (n = 22) and nontransgenic (NTG) control mice (n = 12) were injected intracerebroventricularly with 1×10^{11} vg of AAV9-amiR^{SOD1}. This treatment extended median survival by 50%: from 137 d for untreated *SOD1*^{G93A} mice to 206 d ($p < 0.0001$; Fig. 3A). Untreated *SOD1*^{G93A} mice develop hindlimb paralysis (supplementary Movie S1A, available at https://www.ncbi.nlm.nih.gov/pmc/articles/PMC5374859), but AAV9-treated *SOD1*^{G93A} mice do not show signs of paralysis or movement impairment. Unexpectedly, AAV9-treated *SOD1*^{G93A} mice did not die from paralysis but instead had to be euthanized because of rapid weight loss ($> 15\%$ body weight) and hunched appearance (Fig. 3B). Needle EMG (Shefner et al., 1999, 2006) was used to assess several critical muscle parameters, including fibrillation potentials and the amplitude of the compound muscle action potential. The results were scored on a scale of 0 to 5, with 0 being normal and 5 being highly abnormal. The EMG scores of NTG control animals were 0, whereas untreated *SOD1*^{G93A} animals scored in the 3–5 range, corresponding to extensive acute muscle denervation (Fig. 3C). In contrast, AAV9-treated *SOD1*^{G93A} mice scored 0–2 throughout the experiment. Even at the latest time point analyzed (207–242 d), some AAV9-treated *SOD1*^{G93A} mice had normal EMG scores (0). AAV9-treated *SOD1*^{G93A} mice maintained a normal number of motor units, and only a few mice, which had scored 2 on the EMG scale, showed a decrease at older ages (Fig. 3D). Untreated *SOD1*^{G93A} mice showed an increase in motor unit size compared with NTG controls, whereas AAV9-treated *SOD1*^{G93A} mice maintained a normal motor unit size (Fig. 3E).

We assessed pulmonary function in awake, spontaneously breathing animals. At 127 d, the AAV9-treated and untreated *SOD1*^{G93A} mice had greater minute ventilation (MV) than NTG mice ($p < 0.05$). However, when subjected to a respiratory challenge using hypercapnia, both AAV9-treated and untreated *SOD1*^{G93A} mice had a significantly attenuated MV response compared with NTG
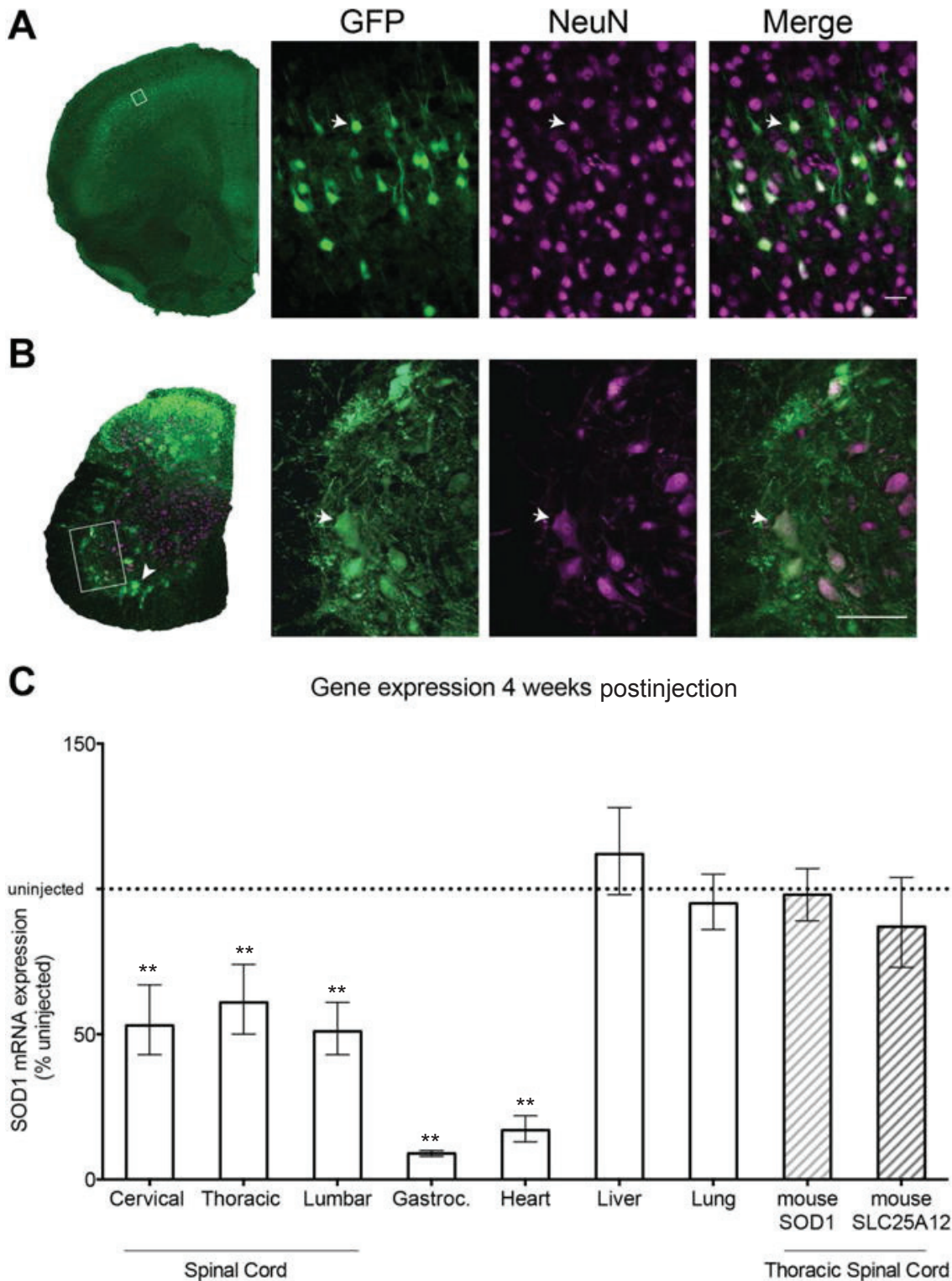
Figure 1. A single neonate intracerebroventricular injection of AAV9 vectors transduces neurons in motor cortex and spinal cord and reduces human SOD1 mRNA. Immunofluorescence staining of brain (*A*) and spinal cord (*B*) sections with antibodies to GFP and NeuN reveal broad neuronal transduction. Arrows indicate double-labeled cells. Arrowhead indicates nonneuronal transduced cells. *C,* RT-qPCR analysis of gene expression. Dotted line sindicates uninjected *SOD1*^{G93A} mice. Scale bars, *A,* 25 μm; *B,* 50 μm. Data are represented as mean ± error; **$p < 0.005$. Unpaired two-tailed *t*-test was used for statistical comparison. Gastroc., gastrocnemius muscle. Reprinted with permission from Stoica et al. (2016), Fig. 1. Copyright 2016, John Wiley and Sons.
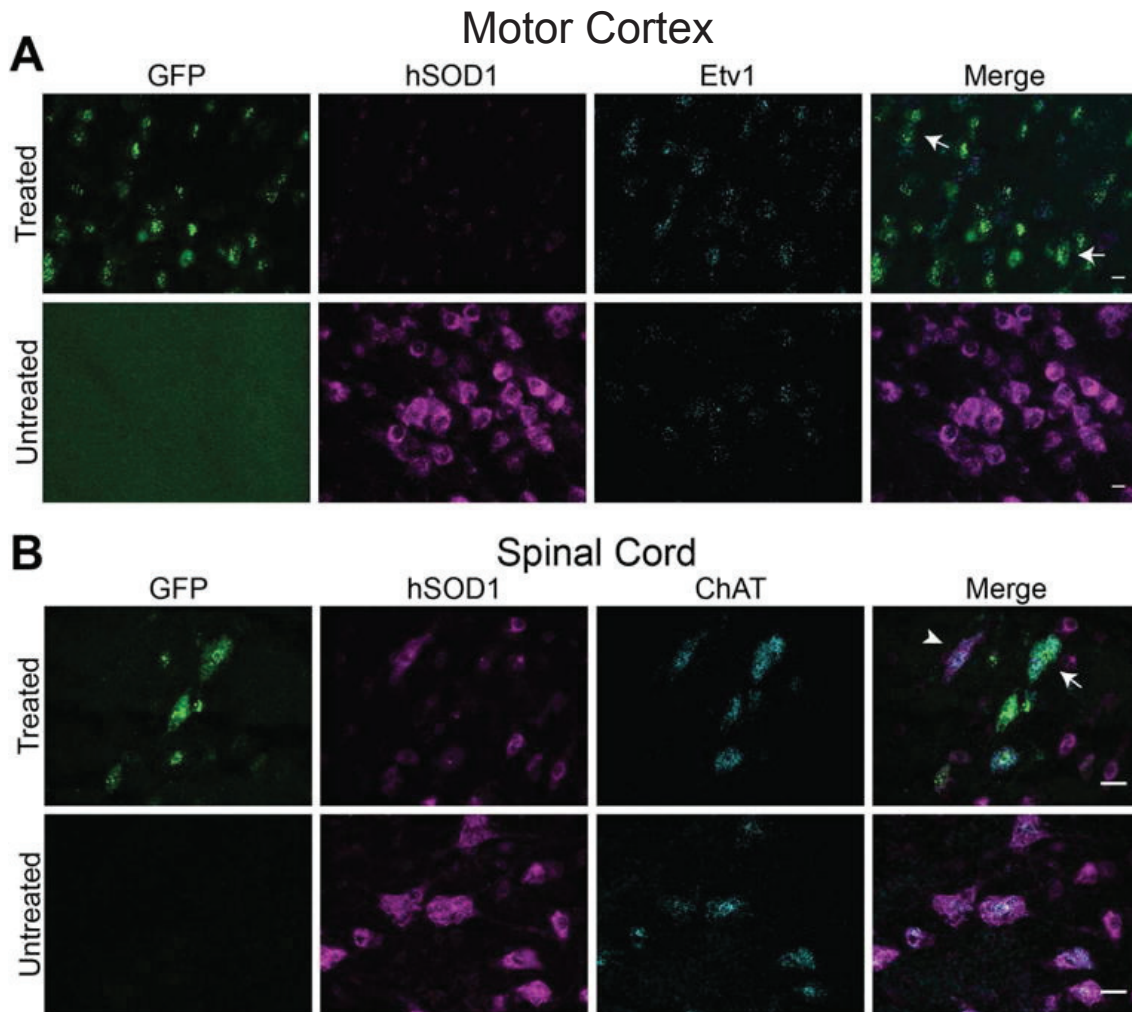
Figure 2. SOD1 mRNA expression is reduced in AAV9-amiR^SOD1^–transduced cortical and spinal motor neurons. Multiplex fluorescent *in situ* hybridization was used to assess changes in human SOD1 mRNA at the cellular level in the brain and spinal cord of AAV9-treated and untreated *SOD1*^G93A^ mice at 4 weeks of age. Probes for GFP (green), and human SOD1 mRNA (magenta) were multiplexed with probes to genes in cortical (Etv1) or spinal cord (ChAT) motor neurons (cyan). Cells expressing GFP mRNA, indicating transduced cells, had reduced levels of hSOD1 mRNA in both the brain (*A*) and spinal cord (*B*). Arrows indicate AAV9-transduced cells with reduced hSOD1 mRNA signal. Arrowhead indicates nontransduced cells, lacking GFP mRNA, and retaining a high SOD1 mRNA signal in the spinal cord. Scale bars: *A*, 10 µm; *B*, 25 µm. Reprinted with permission from Stoica et al. (2016), Fig. 2. Copyright 2016, John Wiley and Sons.

controls. By day 192, the AAV9-treated *SOD1*^G93A^ mice had a further decline in their MV response (Fig. 3F) and peak inspiratory flow (PIF) (Fig. 3G) during hypercapnia. Additionally, chest CT scans of > 200-d-old animals showed decreased chest volume in AAV9-treated *SOD1*^G93A^ mice compared with NTG controls (Fig. 3H).

AAV9-treated *SOD1*^G93A^ mice have improved axonal integrity and motor neuron numbers

We observed extensive axonal loss in untreated *SOD1*^G93A^ mice, whereas the sciatic nerves of AAV9-treated *SOD1*^G93A^ and NTG mice were indistinguishable.

The numbers of large- and small-diameter fibers in the ventral roots of AAV9-treated *SOD1*^G93A^ mice were significantly different from untreated *SOD1*^G93A^ mice and NTG controls. Thus, AAV9-treated *SOD1*^G93A^ mice display remarkably preserved axonal integrity. AAV9-treated *SOD1*^G93A^ mice at the humane endpoint revealed variable degrees of mild neuromuscular junction denervation and, overall, distinctly less disorganization than was detected in untreated *SOD1*^G93A^ mice. The end-stage spinal cords of untreated *SOD1*^G93A^ mice had significantly fewer ChAT^+^ neurons than did control NTG mice ($p < 0.005$); no statistical difference was seen between end-stage AAV9-treated *SOD1*^G93A^ mice and NTG mice. We analyzed the motor cortex of mice
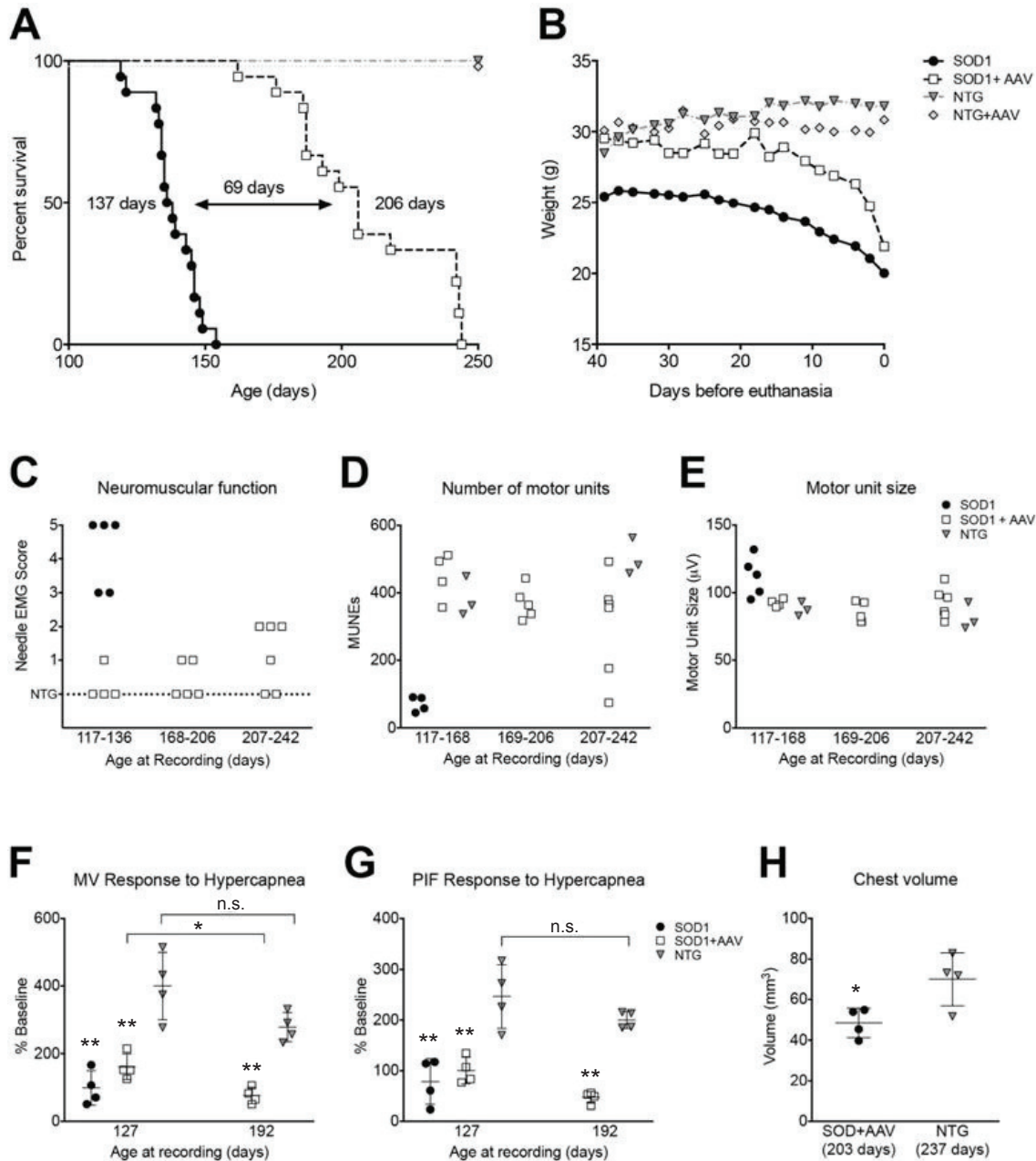
Figure 3. AAV9-amiR[SOD1] treatment increases lifespan and improves neuromuscular function of *SOD1[G93A]* mice. *A,* Kaplan–Meier survival plot shows a 69-d increase in median survival for AAV9-treated mice compared with untreated *SOD1[G93A]* littermates. Log-rank test; $p < 0.001$. *B,* Average weights during the last 40 d before euthanasia show a sharp decline in the weight of AAV9-treated *SOD1[G93A]* mice compared with the steady decline in untreated *SOD1[G93A]* mice. Electrophysiological recordings revealed remarkable preservation of motor neuron function in AAV9-treated *SOD1[G93A]* mice as assessed by (*C*) needle EMG scores, (*D*) number of motor units, and (*E*) motor unit size. Plethysmography recordings show a drop in response to hypercapnea in (*F*) MV and (*G*) PIF in both AAV9-treated and untreated *SOD1[G93A]* mice compared with age-matched NTG mice, indicating breathing impairment due to diaphragm dysfunction. Near the humane endpoint, there is a significant decrease in (*H*) the chest volume of AAV9-treated *SOD1[G93A]* mice compared with NTG mice. Each data point in *C–H* represents an individual animal. In *F–H,* horizontal lines and vertical bars represent mean ± SD. $*p < 0.05$, $**p < 0.01$. Unpaired two-tailed *t*-test was used for statistical comparison. n.s., not significant. Reprinted with permission from Stoica et al. (2016), Fig. 3. Copyright 2016, John Wiley and Sons.

in all three cohorts for the presence of layer V motor neurons identified by immunofluorescence staining with a Ctip2-specific antibody (Arlotta et al., 2005). A qualitative assessment suggests that there are lower numbers of neurons in the untreated $SOD1^{G93A}$ mice at endpoint compared with NTG mice, and that AAV9 treatment had a modest impact on the survival of cortical layer V motor neurons.

AAV9-treated $SOD1^{G93A}$ mice show delayed onset of inflammation in the spinal cord

We assessed inflammatory markers in the lumbar spinal cord of our ALS mouse cohorts. The AAV9-mediated silencing of hSOD1 in the $SOD1^{G93A}$ mice markedly delayed the onset of microgliosis and astrocytosis. The spinal cord of AAV9-treated $SOD1^{G93A}$ 135-d-old mice showed a marginal increase in activated Iba1+ microglia and GFAP-positive reactive astrocytes compared with NTG control animals at 260 d. A considerable increase in these markers was apparent by age ~200–210 d in AAV9-treated $SOD1^{G93A}$ mice. We performed RT-qPCR (reverse-transcriptase quantitative PCR) for genes upregulated in activated microglia (Tyrobp, Cybb) and reactive astrocytes (GFAP). All three genes were significantly increased in the spinal cord of untreated $SOD1^{G93A}$ mice and, to a lesser extent, in AAV9-treated $SOD1^{G93A}$ animals at 135 d old but reached comparable levels by the humane endpoint. The expression levels of these three genes were unchanged by AAV9-delivered treatment of NTG animals.

Discussion

Delivering AAV9-amiR^{SOD1} vector intracerebroventricularly in neonatal animals achieved a 50% increase in median survival and remarkable preservation of motor function. The end stage for these animals was determined by rapid weight loss and severe kyphosis rather than hindlimb paralysis. The histological outcome measures in this study document that AAV9 treatment delayed but did not arrest disease progression. Physiological measurements of breathing during a challenge with hypercapnea revealed a respiratory phenotype in the $SOD1^{G93A}$ mice at endpoint that is similar to the restrictive lung disease of ALS patients.

It is conceivable that the greatly delayed but persistent disease process reflects the residual level of mutant hSOD1 expression in brain and spinal motor neurons. It is also likely a consequence of unabated hSOD1 expression in nonneuronal cells. Studies indicate that the expression of this protein in nonneuronal cells (e.g., microglia, astrocytes, oligodendroglia) is

sufficient to drive disease progression in this mouse model (Lobsiger and Cleveland, 2007; Nagai et al., 2007; Papadeas et al., 2011; Kang et al., 2013).

Studies in large animals have already shown the ability of AAV9 to transduce the CNS using alternative delivery approaches, such as infusion into cisterna magna and intrathecal infusions. Although our particular amiR will reduce the levels of both wild-type and mutant alleles, it is unlikely that reducing overall levels of SOD1 will be detrimental to patients, since SOD1 knock-out mice do not display overt toxicities (Reaume et al., 1996). A phase I study using non-allele-specific antisense oligonucleotides for ALS has proven safe (Miller et al., 2013). Moreover, it is possible to design allele-specific amiRs using single nucleotide differences between mutant and wild-type alleles. Because AAV delivery to the CNS has shown consistent safety in multiple clinical trials (Tardieu et al., 2014), our approach using AAV-mediated CSF delivery of a therapeutic amiR for SOD1 is directly translatable to the clinic.

Acknowledgments

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NOTES

Optimizing Gene Therapy Approaches in X-Linked Adrenoleukodystrophy

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Introduction

X-linked adrenoleukodystrophy (ALD) is a single-gene disorder caused by mutations in the peroxisomal half-transporter *ABCD1*. This half-transporter needs to homodimerize or heterodimerize to be functional. All disease-causing mutations lead to elevations in very-long-chain fatty acids, causing a variety of clinical phenotypes ranging from adrenal dysfunction to acute brain inflammation and chronic spinal cord axonal degeneration. Levels of very-long-chain fatty acids do not predict phenotype or severity. Like other Mendelian disorders, ALD is a single-gene disorder with several distinct manifestations impacting treatment approaches and raising questions around pathobiology.

Gene therapy should account for the differences in affected tissue across various phenotypes. In this chapter, we argue that different phenotypes require different approaches for gene correction. Considerations range from modality (*ex vivo* vs *in vivo*) and capsid type to delivery route and speed of infusion.

Phenotypes in X-Linked Adrenoleukodystrophy

To understand why different gene therapy approaches for the same single-gene disorder are needed, it is necessary to understand the various phenotypes of ALD (Ferrer et al., 2010). Sixty percent of ALD patients develop adrenomyeloneuropathy (AMN) due to an axonal degeneration of the spinal cord. This rate contrasts with the 35–40% of boys with ALD who develop fatal cerebral ALD (CALD), characterized by progressive cerebral demyelination and inflammation in the white matter of the brain. So far, hematopoietic stem cell transplant (HSCT) is the only modality that is able to halt the progressive cerebral demyelination. However, HSCT has several limitations, discussed below. Engraftment problems and graft-versus-host disease remain significant downsides to the current HSCT approach.

Notably, the most common phenotype, AMN, currently has no treatment options available. Several thousand AMN patients live across the United States with progressive gait difficulties and bladder and bowel problems that are caused by chronic axonal degeneration affecting the corticospinal tracts and dorsal columns. These axons are under duress owing to the importance of *ABCD1* in maintaining the longest axons of the human nervous system. Interestingly, this degeneration does not apply in the same way to the lower motor neuron and its projections, as these are spared in AMN.

Cellular Expression of ABCD1 Across the Nervous System

Because the corticospinal tracts and the dorsal columns are affected in AMN, one would think that ABCD1 is important to neurons' function. Yet in the wild-type CNS, ABCD1 is barely expressed in most neuronal populations, except for certain neurons in the hypothalamus, the basal nucleus of Meynert, the periaqueductal gray matter, and the locus ceruleus (Fouquet et al 1997; Höftberger et al 2007). The highest expression of ABCD1 is found in microglia, astrocytes, and endothelial cells, whereas variable levels of ABCD1 are expressed in different populations of oligodendrocytes in subcortical white matter and cerebellum.

Currently, the exact mechanism of axonal degeneration in ALD and AMN disease pathology is not known. Disturbed oligodendrocyte–axon interaction may in large part be responsible for axonal degeneration because *Abcd1* knockdown in oligodendrocytes contributes to disrupted redox equilibrium and oxidative stress. However, microglial apoptosis is also observed in perilesional white matter in ALD and represents an early stage in lesion evolution. As we will see, this cell type plays an important role in the development of CALD and possibly determines the impact of HSCT and *ex vivo* gene therapy.

Therapeutic Techniques for ALD

Allogeneic HSCT in CALD

The only modality that can treat and halt CALD, the most devastating form of ALD manifesting mostly in childhood, is allogeneic HSCT. HSCT with donor cells performed during the early stages of cerebral disease dramatically improves survival (Mahmood et al., 2007). Allogeneic HSCT is optimally performed using an unaffected human leukocyte antigen (HLA)–matched sibling hematopoietic stem cell donor. However, a matched sibling donor is available for only ≤ 30% of patients (Miller et al., 2011). Given that a matched sibling donor is often unavailable, alternative options include HSCT with cells derived from an HLA-mismatched related donor, a matched unrelated donor, or transplant with cells derived from banked cord blood (umbilical cord blood transplant).

Why does HSCT work? It turns out that surrounding the lesion, there is a zone of microglial cell death that is likely the culprit of the spreading demyelination seen in this disorder (Eichler et al., 2008). During HSCT, bone-marrow-derived monocytes enter the CNS and differentiate into microglia-like cells expressing normal ALD protein. Regardless of

the exact mechanism, which remains unknown, introduction of bone marrow cells expressing the corrected gene appears effective.

However, allogeneic bone marrow transplant also carries significant risks and limitations. The donor search can be time-consuming and thus delay treatment. At the earliest, the progression of CNS lesions is halted 6–12 months after engraftment. In addition, more than 10% of patients suffer from treatment-related mortality, and close to 50% of the patients experience moderate to severe graft-versus-host disease. The best outcomes are achieved with a full HLA donor match. Unfortunately, almost half of ALD transplants are performed with mismatched unrelated donors.

Ex vivo gene therapy to target brain inflammation

In 2009, Natalie Cartier and Patrick Aubourg performed a crucial proof-of-concept study (Cartier et al., 2009). In two patients for whom a well-matched donor could not be found, stabilization occurred after administering autologous CD34+ cells that had been transfected *ex vivo* with a lentivirus delivering the corrected *ABCD1* gene. This approach was further tested in a multicenter trial (the Starbeam Study) using a similar self-inactivating lentiviral vector delivering a functional copy of the ABCD1 cDNA to autologous hematopoetic stem cells in boys with early CALD.

The results of the Starbeam Study indicate that early treatment with lentiviral gene therapy may halt neuroinflammation and demyelination in most CALD patients. The clear majority of patients treated remain free from major functional disabilities to date (median follow-up, 29.4 months; range, 21.6–42.0 months), and lesions on imaging have stabilized in ~80% of patients (Eichler et al., in press). Even so, the subtle reemergence of gadolinium enhancement seen on brain magnetic resonance imaging in a handful of patients is reason for caution, and may indicate that fully halting the active demyelination requires further optimization. Important to note, no deaths, graft failure, or graft-versus-host disease was seen using *ex vivo* lentiviral gene therapy (Eichler et al., in press). Therefore, this approach may offer an alternative to allogeneic bone marrow transplant, particularly for patients with no matched sibling donor. All in all, additional follow-up is needed to fully assess efficacy, durability of effect, and long-term safety.

AAV-mediated gene correction to target neurodegeneration in the spinal cord

Although results of Starbeam, the first trial of single-gene addition in ALD, represent a milestone in the field, they have significant limitations. Delays in engraftment lead to loss in brain function over time. Adverse events during myeloablation remain significant. More significantly, patients with ALD also develop the late-onset form of the disease even after HSCT. Thus, it appears that adrenomyeloneuropathy requires broad gene delivery to the entire spinal cord via a different approach.

What other approach could be used? It turns out that adeno-associated virus (AAV) allows for direct transduction, offering fast and robust transgene expression in the CNS. AAV9 capsid was initially cloned by Jim Wilson's lab (Gao et al., 2004). Then in 2009, Brian Kaspar's group showed that AAV9 efficiently transduces murine CNS after systemic delivery (Foust et al., 2009). Intravenous delivery led to transduction of neurons, astrocytes, and endothelial cells. These observations have been consistent across many species, including nonhuman primates.

AAV9-mediated gene correction has also been applied to the spinal cord in young infants with spinal muscular atrophy. Owing to the success of AAV9 in transducing structures of the spinal cord, a similar approach was employed in the mouse model of adrenomyeloneuropathy.

In 2015, we packaged the human ABCD1 cDNA into an AAV9 vector under control of a chicken β-actin promoter and delivered it to the mouse CNS via intracerebroventricular and intravenous routes (Gong et al., 2015). To our surprise, the gene not only was delivered to the correct intracellular compartment, i.e., the peroxisome, but also led to functional very-long-chain fatty acid degradation.

The next key question was whether this biochemical correction led to any improvement in behavior. To understand the phenotype of the mouse is critical. The *Abcd1* homozygous knock-out mouse does not develop symptoms until one year of age. From then on, the mouse develops sensory symptoms and excessive hind limb clasping. To impact this behavior, we next needed to know how to optimally target the spinal cord.

Targeted delivery to the nervous system via the intrathecal route

Where does this leave us? We decided to continue developing the AAV9 capsid system that was already in use in clinical trials and that the U.S. Food and Drug Administration had approved. We optimized AAV9-hABCD1 delivery for AMN treatment by pursuing an intrathecal osmotic pump. This approach increased spinal cord targeting and led to a reduction in peripheral leakage of our construct.

There are several advantages to the intrathecal delivery approach. First, within this compartment, AAV is less likely to be neutralized by circulating anti-AAV antibodies (~33% of the population is seropositive for anti-AAV9 antibodies) (Gray et al., 2011). Second, compared with intravenous delivery, a log lower dose is required—a huge advantage given the challenges to vector production when scaling up to humans. Third, intrathecal delivery causes less exposure of peripheral organs to the vector. This reduces the risks of peripheral vector-related toxicities (e.g., transgene overexpression, cytotoxic T-lymphocytes against the vector).

Across species, intrathecal delivery has been found to be more efficient than intravenous delivery (Meyer et al., 2015). Even when using a 10× lower dose compared with the single intravenous application, widespread transgene expression throughout the spinal cord in mice and nonhuman primates was achieved. Together with others, we have shown that AAV9 delivery of green fluorescent protein (GFP) targets key structures of the spinal cord, such as dorsal root ganglia neurons.

Various strategies should be considered when performing intrathecal AAV9-mediated gene correction. Moreover, transduction efficacy is further improved when subjects are kept in the Trendelenburg position to facilitate spreading of the vector (Meyer et al., 2015). When considering intrathecal delivery, both cisterna magna and lumbar approaches have been explored in rodents and primates. Recently, lumbar delivery of AAV9 delivery was reported to lead to greater diffusion throughout the entire spinal cord and to GFP expression mainly in the cerebellum, cortex, and olfactory bulb (Bey et al., 2017). In contrast, intracisternal delivery led to strong GFP expression throughout the entire brain.

Although largely unexplored, we think that infusion speed is a further important determinant in optimizing the delivery of AAV-mediated gene correction. Over the coming years, more information on the mechanics of flow and barrier function within the CSF compartment will help shape studies in humans. Much can be gained by building on the experience in the field of anesthesia, which has employed intrathecal pumps for more than a decade.

Conclusion

In conclusion, gene therapy trials in the leukodystrophies, such as ALD, have provided reassuring safety data as well as encouraging efficacy data. Specific phenotypes within an individual leukodystrophy may require different approaches, e.g., *ex vivo* versus *in vivo* gene therapy. Despite the potentially transformative impact of gene therapy, a multimodal approach will be required over time. In this endeavor, preclinical assessments and basic science insights into pathobiology will be key factors.

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CRISPR/Cas As a Novel Treatment Strategy for Neurological Disorders

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Gene Therapy for Neurological Disorders

Very recently, the first gene therapy against any neurological disorder was approved by the U.S. Food and Drug Administration and the European Medicines Agency for the treatment of certain forms of spinal muscular atrophy. The antisense oligonucleotide–based drug nusinersen (SPINRAZA) targets the *SMN2* gene and alters the splicing of SMN2 pre-mRNA in order to increase production of the full-length protein.

In addition, a range of promising gene therapeutic approaches are being evaluated. Most of the strategies in past and current clinical trials are based on virus vector–mediated delivery of a gene that can substitute for a deficit, e.g., of a growth factor or enzyme implicated in the pathogenesis of a particular disorder. Currently, adeno-associated virus (AAV) vector–mediated gene replacement therapy is being assessed in a clinical trial for spinal muscular atrophy (ClinicalTrials.gov identifier NCT02122952), and a lentivirus-mediated replacement strategy is under way in a phase II/III clinical trial for X-linked adrenoleukodystrophy (X-ALD) (ClinicalTrials.gov identifier NCT01896102).

Patients suffering from acquired neurological diseases might also benefit from gene therapy. For example, the use of an AAV-based gene delivery vector expressing human NGF delivered stereotactically into the nucleus basalis of Meynert has been evaluated for the treatment of Alzheimer's disease (AD). A phase I trial has been completed and a multicenter phase II trial is being planned (Mandel, 2010).

For Parkinson's disease (PD), the direct administration of a lentiviral vector expressing three of the rate-limiting enzymes for dopamine synthesis has shown promise in a phase I/II trial (Palfi et al., 2014). In addition, neurotrophic factors (Marks et al., 2016), or other enzymes involved in the pathogenesis (Eberling et al., 2009), expressed via viral vectors have begun to be assessed on either nonhuman primates or PD patients.

Other neurodegenerative disorders, such as amyotrophic lateral sclerosis and Huntington's disease (HD), have been approached in similar ways. Delivery via AAV vectors of neurotrophic factors (e.g., BDNF, GDNF, VEGF) (McBride et al., 2003; Kells et al., 2004; Dodge et al., 2010) and other compounds (e.g., IGF-1) (Franz et al., 2009) with neuroprotective properties has shown promise in preclinical studies, although translation of such data to the clinical stage has yet to be achieved.

Experimental Development of Novel Gene Therapy Strategies

RNA interference

The development of efficient, tissue-specific, and nonimmunogenic delivery technologies offers new possibilities for gene therapy of CNS pathology. In particular, systemic administration of endogenous nanovesicles (known as exosomes) can be used for delivering DNA or RNA sequences to target and correct pathogenic processes in mouse brain (Andaloussi et al., 2013). By mimicking the naturally occurring mechanism of RNA interference (RNAi), using the application of short interfering RNA (siRNA) or short hairpin RNA (shRNA), the expression of a certain gene can be efficiently downregulated.

The RNAi technology also offers the possibility of selectively silencing the pathogenic allele by designing the siRNA or shRNA molecules to have a mismatch at the mutated site. Such a strategy has already been successfully applied to models of CNS disorders, such as dystonia (Gonzalez-Alegre et al., 2003), spinocerebellar ataxia (Evers et al., 2013; Ramachandran et al., 2014; Scholefield et al., 2014), and frontotemporal dementia (Miller et al., 2003).

Gene Editing Strategies

In recent years, novel methods that can potentially alter the chromosomal DNA have been developed. These systems are collectively referred to as "gene editing" or "genome engineering" systems (Table 1). These include zinc-finger nucleases (ZFNs), transcription activator–like effector nucleases (TALENs), and the CRISPR system (CRISPR stands for clustered regularly interspaced short palindromic repeats).

With all three techniques, DSBs can be induced and, depending on the conditions and cell type, such breaks will lead to either nonhomologous end-joining (NHEJ) or homology directed repair (HDR) (Sander and Joung, 2014). Of these, NHEJ is the predominant mechanism and will, because it is prone to errors, result in insertions or deletions (indels) at the targeted site (Maruyama et al., 2015). Such indels will often lead to coding sequence frameshifts that will disrupt gene expression (Fig. 1). Thus, the combination of a targeted DSB and NHEJ constitutes a potential tool to disrupt disease-causing alleles and thereby knock out or possibly correct the gene in question (Ran et al., 2013).

Table 1. Comparison of different genome editing techniques.

	Zinc-Finger Nuclease	TALEN	CRISPR/Cas
Recognition site	Typically 9–18 bp per ZFN monomer, 18–36 bp per ZFN pair	Typically 14–20 bp per TALEN monomer, 28–40 bp per TALEN pair	20-23 bp guide sequence and 3-8 bp PAM [3 bp for *Streptococcus pyogenes* (Cas9)]
Specificity	Small number of positional mismatches tolerated	Small number of positional mismatches tolerated	Positional and multiple consecutive mismatches tolerated (most likely at the distal end of PAM site)
Targeting constraints	Difficult to target non-G-rich sequences	Five targeted base must be a T for each TALEN monomer	Targeted sequence must precede or follow a PAM
Ease of engineering	Difficult; may require substantial protein engineering	Moderate; requires complex molecular cloning methods	Easily retargeted using standard cloning procedures and oligo synthesis
Immunogenicity	Likely low, as zinc fingers are based on human protein scaffold	Unknown; protein derived from *Xanthomonas* spp.	Unknown; protein derived from various bacterial species
Ease of *ex vivo* delivery	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction	Relatively easy through methods such as electroporation and viral transduction
Ease of *in vivo* delivery	Relatively easy as small size of ZFN expression cassettes allows use in a variety of viral vectors	Difficult owing to the large size of each TALEN and repetitive nature of DNA encoding TALENs, leading to unwanted recombination events when packaged into lentiviral vectors	Moderate: the commonly used Cas9 from *S. pyogenes* is large and may impose packaging problems for viral vectors such as AAV, but smaller orthologues exist
Ease of multiplexing	Low	Low	High

Adapted from Wang et al. (2016), Table 1. Copyright 2016, Annual Reviews.

Zinc-finger nucleases

Zinc-finger nucleases are artificial restriction enzymes generated by fusing a zinc-finger DNA-binding domain to a DNA-cleavage domain (Wirt and Porteus, 2012). The fusion protein induces a double-strand break (DSB) in the DNA, which usually results in gene disruption due to NHEJ action. However, if ZFNs are used in the presence of an additional "donor" DNA sequence that encodes the correct gene sequence, the cell can use the donor as a template to achieve precise DNA repair via HDR, leading to specific changes in the gene (e.g., corrections, insertions, or deletions). The usefulness of this approach was shown in a mouse model of hemophilia in which ZFNs were codelivered with a gene-targeting vector and resulted in a successful correction through a targeted gene insertion. Notably, the level of gene targeting was sufficient to correct the prolonged clotting time that the actual transgenic mice otherwise suffer from (Li et al., 2011).

Transcription activator–like effector-based nucleases

TALENs are naturally occurring proteins found in the *Xanthomonas* plant pathogen species combined with a nuclease-effector domain (e.g., FokI) (Niu et al., 2014a). These proteins contain DNA-binding domains that recognize a single base pair through a conserved 34-amino-acid motif, except for positions 12 and 13 (repeat variable di-residues), which determine base specificity. Much like ZFNs, TALENs can be directed to any sequence in the genome and induce DSBs (resulting in deletions, insertions, or substitutions at the target site). TALENs have been successfully used for the editing of several genomes, including the human, in terms of gene expression (activation or suppression), gene knock-out, and gene correction (with the use of a donor template) (Miller et al., 2011).

The CRISPR system

In recent years, the CRISPR system has become a widely used tool to create, disrupt, or replace pathological changes in the DNA (Hsu et al., 2014; Wang et al., 2016). With this strategy, genomic DNA sequences with certain protospacer adjacent motif (PAM) sites (NGG in the case of *Streptococcus pyogenes* Cas9) can be targeted with single guide RNAs (sgRNAs) that recognize this DNA sequence and mediate interaction with an RNA-guided endonuclease (Fig. 1). Cas9 from *S. pyogenes* has been mainly used (Ran et al., 2013), but novel enzymes are now being introduced, either by discovering new variants or by engineering existing ones (Kleinstiver et al., 2015).
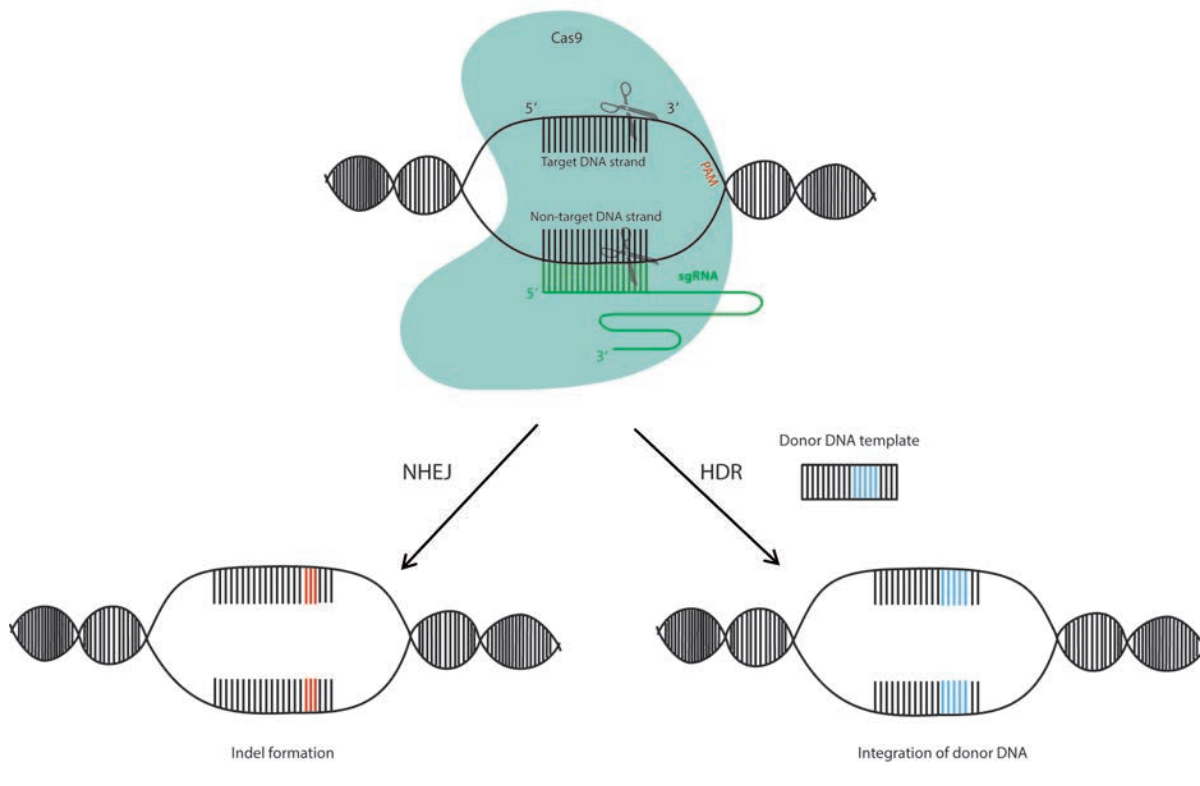
Figure 1. Overview of the CRISPR/Cas system.

CRISPR as a Tool for Studying Pathogenic Mechanisms of Neurological Disorders

Novel genome engineering techniques hold great potential for the study and modeling of neurological disorders. Application of the CRISPR system to human induced pluripotent stem cells (hiPSCs) provides researchers with tools to develop easier and more efficient *in vitro* models to decipher the complex pathogenic molecular mechanisms of CNS disorders (Takahashi et al., 2007). For example, in the case of AD, mutations in the *presenilin 1* (*PSEN1*) and *presenilin 2* (*PSEN2*) genes, as well as disease-associated genetic risk factors (e.g., *APOEε4*), can be introduced in established cell lines, thereby allowing for studies of their monogenic influence (Yagi et al., 2011). Moreover, differentiation of such genetically engineered hiPSCs into neurons or other cell types can provide a better understanding of how the mutated genes affect a particular cellular environment (Poon et al., 2017). With the help of CRISPR, several induced pluripotent stem cell (iPSC)–based models have already been developed for a number of neurological disorders, such as AD (Yagi et al., 2011), PD (Soldner et al., 2011), and HD (Jeon et al., 2012). Although several parameters still need to be optimized, these models have shown that it is possible to mimic the molecular characteristics of a CNS disorder "in a dish" which, hopefully, can point to novel targets for drug screening.

Apart from *in vitro* models, the CRISPR system has been used for generating animal models that can more closely mimic the human disease phenotype. Genetic manipulation of single-cell embryos has already given rise to transgenic nonhuman primate models, potentially bringing researchers one step closer to unraveling the mysteries of brain disorders (Niu et al., 2014b).

CRISPR as a Novel Treatment Strategy for Neurological Disorders

Duchenne muscular dystrophy and Becker muscular dystrophy

Duchenne muscular dystrophy (DMD) is an X-linked genetic muscle-wasting disease that occurs in approximately 1 out of 3500 males and mainly affects skeletal and cardiac muscles (Gee et al., 2017). Most *DMD* cases are caused by dysfunctional forms of dystrophin, a protein that anchors the cytoskeleton

of muscle cells to the extracellular matrix. Severe disease forms are caused by deletions at various sites throughout the 79 exons in the large *DMD* gene that result in dysfunctional, truncated forms of dystrophin. Other dystrophin mutations that do not cause DNA frameshifts lead to the more benign phenotype of Becker muscular dystrophy (BMD).

Four different experimental strategies have been taken to restore dystrophin expression and function via CRISPR-mediated editing. First, exon skipping was adopted to eliminate the entire exon 45 from iPSCs harboring the BMD-associated Δ44-associated exon. In such cells, by CRISPR action, the coding sequence of exon 43 could come into frame with exon 46, whereby the major part of the protein could be restored (Li et al., 2015). Second, exon deletion has been conducted to restore the function of dystrophin in *mdx* mice. Three different groups demonstrated that CRISPR-mediated deletion of exon 23 (in which these mice have a premature stop codon) restores the open reading frame and functionality of dystrophin (Long et al., 2016; Nelson et al., 2016; Tabebordbar et al., 2016). Third, CRISPR was used to target a premature stop codon in exon 45 in iPSCs from DMD patients harboring the Δ44 mutation. When successful, the CRISPR action resulted in NHEJ creating indels that restored the open reading frame and generated a functional dystrophin (Li et al., 2015). In the same study, yet another approach was reported. By using CRISPR to knock in exon 44 in the iPSCs, the authors were able to demonstrate the expression of full-length dystrophin in differentiated myoblasts (Li et al., 2015).

Huntington's disease
Huntington's disease is caused by a trinucleotide repeat expansion in the gene for huntingtin (*HTT*) and, among the neurodegenerative disorders, has been the most widely studied condition in the context of CRISPR/Cas gene editing. In a recent study, sgRNAs against single nucleotide polymorphisms (SNPs) in the regulatory elements within exon 1 of *HTT* were designed and applied both *ex vivo* and *in vivo* (Monteys et al., 2017). Initially, the human genome was screened for the presence of HTT exon-1 SNPs that had a frequency of ≥ 5%. A total of 47 such SNPs were identified and six of those were found to be located in proximity to a Cas9 PAM site. After demonstrating that sgRNAs against these targets could affect the expression of *HTT* in human embryonic kidney (HEK) 293 cells, the approach was evaluated on cultured HD patient fibroblasts. Twenty-three individual cell lines were screened for presence of the actual SNPs, and two were chosen for the subsequent experiments. The use of one sgRNA/Cas9 pair, corresponding to one of

the SNPs, resulted in an effective disruption of the mutant *HTT* allele. Moreover, intracranial injection of one sgRNA/Cas9 pair in an *HTT* transgenic mouse resulted in a 40% reduction in the expression of the transgene (Monteys et al., 2017).

In another recent study, Yang and colleagues evaluated treatment strategies in knock-in (KI) mouse models (Yang et al., 2017). A set of sgRNAs was designed against the region flanking the CAG repeat containing human *HTT* exon 1. Homozygous *HD140QKI* mice, in which the human *HTT* exon 1 with 140 CAG repeats has replaced the corresponding mouse sequence, were treated with intrastriatal injections of AAV-*HTT*-sgRNA/AAV-CMV-Cas9. A significant decrease in *HTT* expression, together with a dramatic decrease in the nuclear aggregation and accumulation of huntingtin, could be observed in treated mice. When transducing striatum with the same vectors under a neuron-specific promoter on 9-month-old heterozygous HD140QKI mice, the authors found that the treatment could reverse both neuropathology and the behavioral phenotype these mice typically exhibit (Yang et al., 2017).

Fragile X syndrome
Another disorder caused by abnormal trinucleotide repeat expansions is the fragile X syndrome (FXS). Subjects with this disorder suffer from intellectual disability, behavioral and learning challenges, as well as various abnormal physical characteristics. A CGG repeat expansion in the 5'-untranslated region of the *fragile X mental retardation 1* (*FMR1*) gene results in epigenetic silencing and, consequently, decreased levels of *FMR1* mRNA and FMR1 protein. No therapies are available for this condition.

In an initial study, CRISPR was adopted to target *FMR1* upstream of the repeat region in iPSCs from an FXS patient. In one successful experiment, a large deletion was created, leading to the removal of CGG repeats and the reactivation of *FMR1* (Park et al., 2015). In a subsequent study, two sgRNAs flanking the repeat regions were used. The DSBs obtained resulted in more precise and efficient removal of the CGG repeat and, thus, an increased expression of *FMR1* (Xie et al., 2016).

Parkinson's disease
Another example of CRISPR-based gene editing has been described in an experimental model of PD. CRISPR-mediated knock-in of designer receptors exclusively activated by designer drugs (DREADDs) can enable precise regulation of genes in human pluripotent stem cell (hPSC)–derived neurons by

chemical compounds. Chen and colleagues showed that hPSC-derived human midbrain dopaminergic neurons transplanted into a PD mouse model could reverse both pathology and symptoms when treated with a DREADD ligand (Chen et al., 2016).

Alzheimer's disease

Alzheimer's disease is the most common age-related neurodegenerative disorder, which affects approximately 20–30 million individuals worldwide. According to the amyloid hypothesis, AD is caused by the accumulation and aggregation of amyloid-β (Aβ) peptides in the brain. Early-onset familial AD cases result from dominantly inherited mutations in the *amyloid-beta precursor protein* (*AβPP*) gene, as well as in *PSEN1* and *PSEN2*. Some of the *AβPP* mutations lead to an increased generation of Aβ, whereas *PSEN1/PSEN2* mutations do not increase Aβ levels in general, but instead shift the generation from Aβ40 to the more aggregation-prone Aβ42. (Bertram and Tanzi, 2012). Twenty-six *APP*, 291 *PSEN1*, and 18 *PSEN2* pathogenic AD mutations have been described to date (http://www.alzforum.org/mutations).

However, mutations in any of these three genes seem to account for less than 5% of the total number of disease cases. The remaining 95%, known as sporadic cases, most likely result from several different genetic risk factors together with as of yet unknown environmental factors. So far, the only known substantial genetic risk factor for AD is the gene for apolipoprotein E (*APOE*); subjects who are heterozygous for *APOEε4* have a three to four times increased disease risk, whereas *APOEε4* homozygotes are 10–15 times more likely to develop disease than non-ε4 carriers (Corder et al., 1993). The exact mechanism is incompletely understood, but *APOEε4* seems to promote Aβ brain accumulation, possibly by affecting its transport over the blood–brain barrier.

Thus, disease-causing gene mutations as well as the *APOEε4* polymorphism could be potential targets for CRISPR therapy. By selectively knocking out the alleles harboring the mutated allele (and leaving the nonmutated allele intact), or editing the *APOEε4* alleles to become either *APOEε2* or *APOEε3*, the dysregulation of Aβ could hopefully be counteracted. Ongoing and future studies will clarify the potential of such therapeutic approaches for AD.

Concluding Remarks

Because of easy *in vivo* delivery, the CRISPR system has advantages in terms of clinical applicability over other gene editing techniques, such as ZFNs or TALENs (Table 1). Moreover, CRISPR allows precise editing of a disease-causing genetic locus that will be permanent in the targeted cells. These are desirable features whenever the deletion or correction have occurred at the intended site. However, the technique may also cause off-target effects that can lead to unwanted side effects. Another concern relates to the fact that the Cas proteins are of bacterial origin and thus could elicit an immune response in the human brain. If such a reaction were to happen, edited cells could be effectively eliminated, mitigating the positive consequences of the induced genetic alterations.

In spite of these concerns, CRISPR has for the first time been applied to humans. A research team at Sichuan University in Chengdu, China, used the technique to disable *PD-1*, a gene involved in cellular immune responses, in a patient with metastatic non-small-cell lung cancer. The patient's own blood immune cells were isolated, CRISPR/Cas9 treated, and delivered back to the patient (Cyranoski, 2016). A rigorous scientific evaluation of this type of intervention is needed before we can assess whether the strategy can be of clinical use.

Regardless of the outcome of such trials, further development is needed to derive tools that can better assess the risk of unwanted off-target effects for a particular sgRNA/Cas combination. Also, efforts should be made to ensure that the RNA-guided endonucleases—and probably also the sgRNAs—are only transiently expressed in the targeted cells.

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NOTES

Neural Stem Cell–Delivered Therapies for Brain Tumors: Phase I Clinical Trials

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Introduction

Despite advances in surgical, radiotherapeutic, and chemotherapeutic interventions, glioblastoma multiforme (GBM) remains a fatal brain tumor primarily because of its highly invasive nature. Residual cells act as "moving targets" that migrate away from local treatments such as drug-eluting hydrogels and nanoparticle infusions, causing recurrence at multiple sites. Restricted drug passage through the blood–brain barrier (BBB) renders systemic chemotherapy ineffective, leading to a dismal prognosis. The same limitations exist for brain metastases from melanoma, breast cancer, and lung cancer. Development of more-effective brain tumor–targeted treatments is critical to improving clinical outcomes.

Neural stem cells (NSCs) hold great promise for brain tumor therapy owing to their inherent tumor-tropic and minimally immunogenic properties, making them an ideal vehicle to selectively deliver therapeutic payloads to infiltrative CNS tumor foci. NSCs migrate to invasive primary and secondary brain tumor sites in orthotopic preclinical models, whether delivered intracerebrally in the same or opposite hemisphere, the lateral ventricle, or intravenously—demonstrating the ability of NSCs to cross the BBB (Aboody et al., 2000). They are also attracted to hypoxic tumor areas (Zhao et al., 2008). NSCs can serve as an ideal platform to deliver and distribute various anticancer payloads to tumor sites, including prodrug-converting enzymes (Aboody et al., 2006; Metz et al., 2013); antibodies (Frank et al., 2009); nanoparticles (Mooney et al., 2014a,b); and oncolytic viruses (Ahmed et al., 2013; Morshed et al., 2015). NSCs can therefore increase the concentration of anticancer agents specifically at tumor foci while sparing toxicity to normal tissues—potentially improving patient quality of life.

At City of Hope, we have brought two novel NSC-mediated enzyme/prodrug gene therapies "from bench to bedside" with ongoing phase I dose-escalation trials for recurrent glioma patients. In collaboration with Northwestern University, we also brought to the clinic a novel NSC-delivered oncolytic virotherapy for newly diagnosed glioma patients. Here, we describe various NSC-mediated brain tumor strategies, our preclinical pipeline, their path to the clinic, and early clinical trial results.

NSC-Mediated Brain Tumor Therapies

Treating invasive brain tumors is complicated by the need to preserve as much normal brain tissue as possible in order to avoid devastating impairments.

Tumor-tropic NSCs can be genetically modified to deliver anticancer agents selectively to tumor foci throughout the brain (Fig. 1). These NSC delivery strategies, discussed in the following sections, include:

1. Enzyme/prodrug gene therapy, in which NSCs are engineered to express enzymes that convert systemically delivered inactive prodrugs to chemotherapeutic agents;

2. Oncolytic virotherapy, in which NSCs are engineered to produce an oncolytic virus designed to replicate selectively in tumor cells;

3. Antibody delivery, in which NSCs are engineered to produce full or single-chain antibodies or minibodies (Frank et al., 2010);

4. Nanoparticle (NP) delivery, in which NSCs are conjugated to drug-loaded NPs, or loaded with gold NPs or nanorods (AuNPs/AuNRs) exposed to near infrared (NIR) laser light for thermal-ablative therapy; and

5. Exosome or extracellular vesicle (ECV) oligonucleotide NSC delivery.

The advantages of NSC delivery of these cancer therapies include more-effective and selective delivery to and distribution through tumor foci, minimal to no immunogenicity, and limited off-target effects resulting in decreased toxicity to normal tissues.

NSC-mediated enzyme/prodrug therapy

Enzyme/prodrug therapy involves using the NSCs to deliver prodrug-activating enzymes throughout tumor foci to convert inactive prodrugs into tumor-toxic effector drugs. Once generated, each effector molecule can affect multiple surrounding tumor cells through diffusion, intercellular gap junctions, or endocytosis of apoptotic bodies released from dying cells—all contributing to their "bystander effect." Genetically engineering NSCs to express prodrug-converting enzymes also provides a critical safety switch that can eliminate the cells after their therapeutic effect has been actualized (Li and Xiang, 2013). Cells have been used to enhance the efficacy of five of the more than 50 different enzyme–prodrug combinations that have been developed during the past two decades (Greco and Dachs, 2001). Our lab has performed the preclinical efficacy and safety/toxicity studies—enabling Investigational New Drug (IND) application to the U.S. Food and Drug
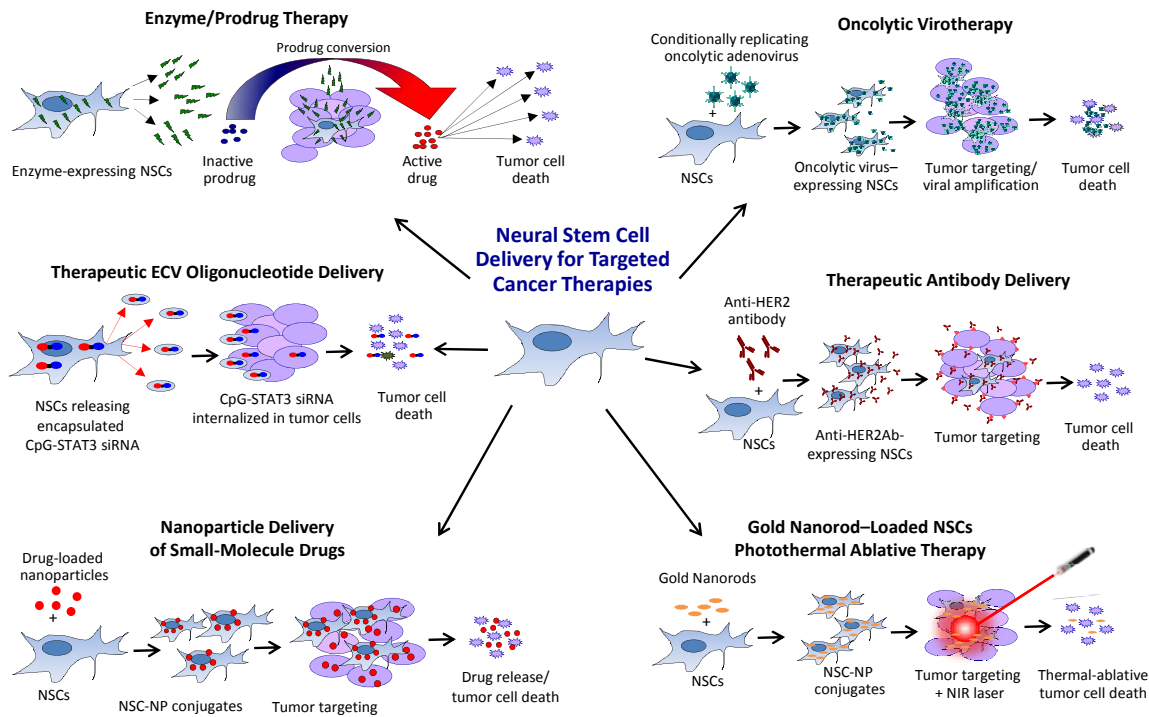
Figure 1. Engineering NSCs to deliver targeted anticancer payloads. Tumor-tropic NSCs can be genetically modified to deliver anticancer agents selectively to tumor foci throughout the brain. Methods include enzyme/prodrug gene therapy, in which NSCs are engineered to express enzymes that convert systemically delivered inactive prodrugs to chemotherapeutic agents; oncolytic virotherapy, in which NSCs are engineered to produce an oncolytic virus designed to replicate selectively in tumor cells; antibody delivery, in which NSCs are engineered to produce full or single-chain antibodies or minibodies; NP delivery, in which NSCs are conjugated to drug-loaded NPs, or loaded with gold NPs or nanorods exposed to NIR laser light for thermal-ablative therapy; and exosome or extracellular vesicle oligonucleotide NSC delivery.

Administration (FDA)—for NSCs engineered to express two prodrug-converting enzymes for tumor-localized chemotherapy production (Aboody et al., 2013; Metz et al., 2013).

First-in-human NSC enzyme/prodrug therapy for GBM

A first-in-human pilot safety/feasibility study, completed in 2013, assessed the safety of using genetically modified allogeneic NSCs for tumor-selective enzyme/prodrug therapy (ClinicalTrials.gov Identifier: NCT01172964). An immortalized, clonal NSC line was retrovirally transduced to stably express *Escherichia coli* cytosine deaminase (HB1.F3.CD21; CD-NSCs), which converts the prodrug 5-fluorocytosine (5-FC) to the active chemotherapeutic 5-fluorouracil (5-FU). Fifteen patients with recurrent high-grade gliomas received an intracerebral dose of CD-NSCs at the time of resection or biopsy, followed by a 7 d course of oral 5-FC. Results

demonstrated safety, nonimmunogenicity, and proof of concept (using intracerebral microdialysis) for brain tumor–localized conversion of 5-FC to 5-FU by CD-NSCs (Portnow et al., 2017). Brain autopsy data documented NSC migration to distant tumor sites and nontumorigenicity of NSCs. This first-in-human study demonstrated safety and proof of concept regarding the ability of NSCs to target tumor foci in the brain and locally produce chemotherapy. We are currently conducting a phase I dose-escalation, multiple-treatment-round study of CD-NSCs in combination with 5-FC and folinic acid (Leucovorin) to determine the maximum tolerated NSC dose (ClinicalTrials.gov Identifier: NCT02015819).

Second-generation NSCs

These same NSCs were further modified to secrete a modified human carboxylesterase (hCE1m6; CE-NSCs), which converts the prodrug irinotecan (IRN;

CPT-11) to its active metabolite SN-38, a potent topoisomerase I inhibitor. A second phase I clinical trial for recurrent GBM patients is ongoing, similar to the previous protocol, using intracerebral CE-NSCs combined with intravenous IRN (ClinicalTrials.gov Identifier: NCT02192359). Using a 3 + 3 dose-escalation schema, the primary objective of both studies is to define the phase II–recommended doses of these NSC-based treatments in patients with recurrent high-grade gliomas. Secondary objectives include pharmacokinetics, immunogenicity, and cell-fate correlative studies.

NSC oncolytic virotherapy

Oncolytic viruses can induce cancer-cell death regardless of radiotherapy or chemotherapy resistance, and can stimulate immune system recognition of cancer cells as a result of exposure of tumor antigens on lysis (Ding, 2014). Although GBM clinical trials to date have demonstrated the safety of oncolytic viruses (Hartkopf et al., 2012), the efficacy of this approach has been limited by delivery hurdles such as rapid immune system inactivation, poor viral penetration of tumors, and an inability of the viruses to reach invasive foci that are separated from the main tumor mass by normal tissue (Ahmed et al., 2013). In collaboration with Dr. M. Lesniak's group at the University of Chicago, we engineered the CD-NSC line to deliver a conditionally replication-competent adenovirus (CRAd-Survivin-pk7) driven by the surviving promoter. This virus therefore replicates specifically in cells that overexpress *survivin*, which is highly expressed in glioma cells (upregulated by radiation) but not in normal differentiated cells (Tobias et al., 2013). In other words, once seeded into the tumor, the conditionally replication-competent adenovirus will continue to reproduce only in tumor cells, amplifying the effect in neighboring tumor cells until normal tissue is reached and the effect ceases. A first-in-human trial of CRAd-S-pk7 NSCs with temozolomide and radiation combination therapy (TMZ + XRT) is being conducted at Northwestern University and City of Hope for newly diagnosed GBM patients (ClinicalTrials.gov Identifier: NCT03072134).

NSC antibody delivery

The treatment of human epidermal growth factor receptor 2 (HER2)–overexpressing breast cancer has been revolutionized by the drug trastuzumab (Herceptin). However, increased survival time now predisposes these patients to developing brain metastases, as the therapeutic antibodies cannot effectively cross the BBB. We therefore modified our NSC line to stably secrete a full-length anti-HER2 antibody (HER2Ab), which is functionally equivalent to trastuzumab (Kanojia et al., 2015). Preclinical *in vivo* experiments using HER2Ab-overexpressing NSCs in a breast cancer brain metastases mouse model demonstrated that intracerebral injection of HER2Ab-NSCs significantly improves survival (Kanojia et al., 2015). In effect, these NSCs provide tumor-localized production of HER2Ab, minimizing potential off-target side effects.

NSC nanoparticle delivery

Because intravenously administered chemotherapies do not efficiently penetrate the BBB or reach hypoxic tumor regions, intratumoral infusions of therapeutic NPs have been combined with convection-enhanced delivery for the treatment of brain tumors (Allard et al., 2009). Obstacles to successful treatment include poor NP tumor penetration and distribution, as well as poor NP retention at the tumor site. Decades spent modifying NPs with targeting peptides and stimuli-responsive activation elements have not yielded adequate tumor-selective deposition and penetration. Thus, NSCs are appealing for use as NP carriers in order to overcome these biodistribution challenges. We have shown that NSCs maintain their tumor tropism when transporting either surface-bound or internalized NPs (Mooney et al., 2014c) and are exploring two NSC-NP treatment strategies for brain tumors.

NSC-NP conjugates for small-molecule drug delivery

Although the importance of GBM-selective chemotherapy has long been appreciated, patients today still take oral TMZ, a drug approved in 1995. TMZ is one of < 2% of small-molecule drugs that can cross the BBB (Ferber, 2007); however, brain concentrations reach only ~30–40% of plasma levels (Tentori and Graziani, 2009), and drug toxicity limits dosing. Because 80–90% of GBM recurrences are located within 2 cm of the primary resection cavity (Wallner et al., 1989), newer treatment strategies deliver drugs from within the primary resection cavity. In 2002, biodegradable hydrogels were approved by the FDA to release high carmustine concentrations from the GBM resection cavity. However, only modest improvements in patient survival (~2 months) were achieved because drug penetration into surrounding tissue was limited to ~1 mm (Zhou et al., 2013). The combination of NSCs and NPs offers a platform for the release of anticancer drugs and other agents selectively at tumor sites, where NSCs can deliver and retain NPs at invasive tumor sites for either triggered or slow drug release.

NSC-AuNRs plus NIR light for thermal-ablative therapy

Photothermal ablative therapy uses inert gold nanorods as tumor-localized antennae that convert NIR light into heat to essentially "burn" the tumor tissue, regardless of chemoresistance or molecular phenotype. Although advances in image-guided procedures and nanotechnology have improved synergy with other treatments, heating the entire tumor mass requires good distribution and retention at the tumor site to be effective and to avoid collateral damage to surrounding healthy tissue. NSCs can internalize AuNRs and maintain their viability and tumor-homing properties. Compared with free AuNRs, NSC-AuNRs improve distribution and retention selectively. They can also improve localized heating on exposure to NIR light to kill surrounding tumor cells while minimizing damage to nontumor tissue (Schnarr et al., 2013; Mooney et al., 2014a).

NSC-secreted exosomes as carriers for oligonucleotide therapeutics

Oligonucleotide therapeutics (ONTs) can overcome the limitations of small-molecule inhibitors of many undruggable molecular targets, such as oncogenic transcription factors. However, ONT delivery, short circulatory half-life, and intratumoral penetration are still major hurdles in their clinical application. NSCs are known to secrete large amounts of exosomes, which may enable the transfer of endogenous microRNAs as well as antigenic peptides (Han et al., 2016). NSCs can also be used as carriers for synthetic ONTs, providing an opportunity to deliver these reagents into the GBM microenvironment. The major hurdle in this strategy remains packaging ONTs into exosomes. Methods such as electroporation or lipofection can damage exosomes and do not guarantee that the oligonucleotide cargo becomes encapsulated rather than aggregated with exosomal surface (Kooijmans et al., 2013). An attractive alternative is intracellular expression of short hairpin RNA (shRNA) (Alvarez-Erviti et al., 2011) or targeted delivery of synthetic ONT using conjugation with target-cell-selective ligands. TLR9 ligands and CpG oligonucleotides permit cell-selective delivery of short interfering RNA (siRNA) or decoy DNA to glioma cells (Kortylewski and Moreira, 2017). Preliminary studies suggest that NSCs can rapidly internalize exosome-encapsulated oligonucleotides for ≤ 3 d. Further studies will verify whether NSC-delivered, ECV-encapsulated ONTs improve the penetration of glioma and tumor-associated myeloid cells to generate direct cytotoxic and immune-mediated antitumor effects.

NSC Manufacturing
Cell source

Tumor-tropic NSCs are a platform technology that could improve the delivery of a wide repertoire of therapeutics to a wide variety of tumors. Early in their development process, the most critical issues to solve were their source, the ability to expand a sufficient supply of NSCs, and ways to characterize their stability over time and passages. Although the self-renewing NSCs present in developing brain tissue could be used as a renewable cell population, culture conditions have yet to be identified that reproducibly permit continuous propagation of primary NSCs. One common approach is to expand NSC pools by repeated subculture of polyclonal neurospheres. However, using this technique, progressive passages lead to decreased capacity for cellular self-renewal and differentiation potential and increase the accumulation of chromosomal and functional instabilities (Kallos and Behie, 1999). Thus, a new source of primary tissue must be obtained for each production cycle, making process scale-up, regulatory approval, and clinical translation substantially more difficult and costly.

We took the approach of generating stable, immortalized NSC lines using both retroviral transduction with a v-*myc* gene into early gestational NSC pools (Kim et al., 2008) and clonal selection. Our NSC therapies have used a well-characterized clonal line, HB1.F3.CD21, that demonstrates stability of normal karyotype, tumor tropism, and stemness over time and passage, thereby enabling product standardization and scale-up. This cell line is established as a Master Cell Bank at City of Hope's Center for Biomedicine and Genetics GMP Facility and can be further engineered with various therapeutic cargo to generate multiple off-the-shelf allogeneic cell banks for treating patients.

Scale-up GMP manufacturing

As cell-based therapies move from phase I to phase II–III clinical trials and commercialization, we need to address methods for scaling up the manufacture and production of GMP-grade stem cells in a cost-effective, safe, and reproducible manner. We have used both multilayer cell factories and hollow fiber bioreactors for our adherent NSCs and tested each clinical bank release for sterility, mycoplasma, endotoxin, identity, viability, therapeutic cargo levels, and karyotype. Such stringent quality control is necessary to ensure patient safety when using an allogeneic cell line. The FDA has created guidance documents that address the various controls and safeguards, starting with donor eligibility, initial

collection of the source tissue under current good tissue practices, and subsequent manufacturing steps under current GMP standards (Burger, 2003).

From Bench to Bedside: Perspective and Lessons Learned

For cell-based therapies, translation has typically been the purview of industry. However, academic researchers are increasingly driven to bring their findings to bear on patient treatments. In so doing, they face challenges in the knowledge gap and resource access accentuated by the unique financial, manufacturing, scientific, and regulatory aspects of cell therapy (Aboody et al., 2011). By "translation," we mean advancing scientific discoveries from the laboratory to the clinic for patient benefit, i.e., "bench to bedside." This requires a comprehensive collaborative team approach: Research scientists and clinicians must work closely with regulatory agencies, patient advocacy groups, ethics bodies, cell manufacturing facilities, and industry to achieve the study quality and necessary funding to ensure success. This effort requires new partnership models for research in which traditional silos are broken down, translational teams are created, and new mechanisms for effective hand-off from nonprofit to for-profit organizations are generated.

Despite these difficulties, steady progress toward this goal is being spearheaded by industry, academic institutions, and nonprofit foundations, in conjunction with a recent focus by the National Institutes of Health (NIH) and the FDA in the United States on both translational research and regenerative medicine. Recognizing the "valley of death" where progress is halted by lack of funding, several private foundations are targeting and supporting translational research for specific neurological diseases. The NIH has created the National Center for Advancing Translational Sciences and a Center for Regenerative Medicine. The FDA has issued guidelines for preclinical safety evaluation and assessment of investigational cellular and gene therapy products. Human cellular products such as stem cells and progenitor cells have unique requirements for characterization, manufacturing, and testing that are regulated by the Center for Biologics Evaluation and Research and its Office of Cellular, Tissue and Gene Therapies. In addition to demonstrating preclinical proof of concept and then performing efficacy and safety/toxicity studies required for filing an IND with the FDA, initiating clinical study requires approval from the Institutional Review Board, Institutional Biosafety Committee, and Stem Cell Research Oversight Committee.

Conclusions

With sufficient development, NSC-mediated therapies can revolutionize the way brain cancer patients are treated and promise to significantly improve quality of life during treatments. Further avenues for development include optimizing NSC delivery (e.g., route, dose, treatment schedule) to maximize NSC viability and tumor coverage. In addition, we are actively exploring delivery of other therapeutic payloads to different tumor types, and combination regimens, including coupling NSC-mediated treatments with immunomodulatory strategies. Much work is still needed to realize the full potential of NSC-mediated cancer treatments.

Acknowledgments

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