Molecular Therapy
Review

Gene therapy for ALS: A review

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Amyotrophic lateral sclerosis (ALS) has historically posed unique challenges for gene-therapy-based approaches, due to a paucity of therapeutic targets as well as the difficulty of accessing both the brain and spinal cord. Recent advances in our understanding of disease mechanism and ALS genetics, however, have combined with tremendous strides in CNS targeting, gene delivery, and gene editing and knockdown techniques to open new horizons of therapeutic possibility. Gene therapy clinical trials are currently underway for ALS patients with SOD1 mutations, C9orf72 hexanucleotide repeat expansions, ATXN2 trinucleotide expansions, and FUS mutations, as well as sporadic disease without known genetic cause. In this review, we provide an in-depth exploration of the state of ALS-directed gene therapy, including antisense oligonucleotides, RNA interference, CRISPR, adeno-associated virus (AAV)-mediated trophic support, and antibody-based methods. We discuss how each of these approaches has been implemented across known genetic causes as well as sporadic ALS, reviewing preclinical studies as well as completed and ongoing human clinical trials. We highlight the transformative potential of these evolving technologies as the gene therapy field advances toward a true disease-modifying treatment for this devastating illness.

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a uniformly fatal disease characterized by degeneration of upper and lower motor neurons, leading to progressive paralysis, respiratory failure, and death in 2–5 years. It is also the most common adult motor neuron disease, with a prevalence of 5 per 100,000[1] and a lifetime risk of 1:400–1:800.[2] The search for disease-modifying therapies has long been hampered by a poor understanding of disease mechanism. In fact, out of >80 human clinical trials,[3] only riluzole[4,5] and edaravone[6–8] have slowed progression, both of them modestly and by targeting non-specific factors such as excitotoxicity or oxidative stress. Recent advances in our understanding of ALS pathophysiology, including the discovery of many of its genetic underpinnings, have enabled the development of increasingly targeted therapies, with today's clinical trials holding growing promise and hope for efficacious treatments.

A pathologic hallmark of ALS, affecting over 97% of patients, is ubiquitinated cytoplasmic inclusions consisting largely of Tar-DNA binding protein of 43 kDA (TDP-43).[9,10] TDP-43 pathology is also found in 50% of frontotemporal dementia (FTD) patients and is seen in nearly all ALS-FTD spectrum cases.[9–13] Normally found in the nucleus, TDP-43 broadly mediates transcription, translation, and splicing,[14–17] interacting with ~30% of the transcriptome.[14] Its cytoplasmic mislocalization is thought to be detrimental on two fronts: (1) toxicity of cytoplasmic aggregates,[18,19] and (2) loss of normal nuclear function.[20–24] In motor neurons from ALS patients, TDP-43 associates with cytoplasmic stress granules (SGs),[25,26] conglomerates of proteins and RNA that become maladaptive in disease. SGs additionally bind a host of nuclear import, export, and transcription and translation factors, resulting in loss of nucleocytoplasmic shuttling and eventual cell death.[25–27] This intracellular cascade provides several therapeutic targets aimed at pathologic elements common to most ALS cases.[14]

Another approach, and one particularly amenable to gene therapy techniques, is targeting genetic mutations known to cause ALS. Ten percent of ALS can be classified as familial, with ~70% of familial cases explained by known gene mutations.[28] The first ALS-associated gene, superoxide dismutase-1 (*SOD1*),[29] was described in 1993 and accounts for 12%–20% of familial and 1%–2% of sporadic ALS.[28,29] For the decade and a half following its discovery, this remained the only major known heritable cause and therefore was the basis of all preclinical ALS models.[30,31] However, unlike the 97% of cases described above, *SOD1*-ALS patients do not demonstrate TDP-43 pathology at autopsy, raising concern that *SOD1* animal models similarly do not represent most ALS pathology, and studies conducted in these models may not broadly translate.[30,31] Indeed, most early studies failed in human clinical trials.[32,33] It was not until 2008 that a second contributor to heritable ALS, *TARDBP*—which encodes TDP-43—was discovered.[34,35] *TARDBP* mutations account for 4% of familial and 1% of sporadic cases, and the discovery of both this direct genetic cause and the identification of TDP-43 in motor neuron inclusions yielded additional mouse models.[30,31,36,37] A year later, mutations in the fused in sarcoma (*FUS*) gene were discovered, accounting for another 4% of familial ALS,[38,39] although, like *SOD1*, *FUS* patients lack TDP-43 pathology. In 2010, intermediate-length CAG trinucleotide repeat expansions in the ataxin-2 (*ATXN2*) gene were found to be associated with up to 4.7% of all ALS.[40] The most significant genetic discovery to date has been that of *C9Orf72* in 2011;[41,42] G_4C_2 hexanucleotide repeat expansions in this gene have

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been found in 40% of familial and 8% of sporadic cases, or 11% of all ALS,[28,43] and some mouse models recapitulate ALS pathophysiology.[44,45] More than 20 additional ALS-associated genes together account for an additional 12%–15% of familial ALS,[28,46] leaving 25%–30% of familial cases unexplained. Aside from *SOD1* and *FUS*, TDP-43 pathology is seen across all mutations, with nearly all known ALS-associated genes also being identified in FTD or acting as FTD phenotype modifiers, *SOD1* being a notable exception.[10]

Gene therapy involves the delivery of genetic material to cells in order to introduce functional copies of dysfunctional genes, introduce trophic factors and other disease-modifying genes, or silence harmful gene expression using antisense oligonucleotides (ASOs), RNA interference (RNAi), or gene-editing technology such as CRISPR. In order to target the central nervous system (CNS), this material can be delivered naked, as is often the case for ASOs; by viral vectors including adeno-associated virus (AAV) and others; or by physical or chemical systems such as nanoparticles.[2,47] Methods to access the CNS across the blood-brain barrier include intravenous (i.v.), intracerebroventricular (i.c.v.), intrathecal (i.t.), or intraparenchymal delivery. An ALS-specific challenge is the need to reach both the motor cortex and the spinal cord.

Viral vectors have the advantage of conferring sustained expression of genetic material in transduced cells after a single treatment. Among these, AAV has emerged as the lead vector for CNS delivery, based on its ability to transduce terminally differentiated cells and to establish nuclear episomes without incurring the risk of insertional mutagenesis.[48,49] Equally advantageous is the selective tropism of its many different serotypes, both naturally occurring and modified.[47,50] Thus far, the serotypes that have dominated ALS preclinical studies and clinical trials are the naturally occurring AAV9 and AAVrh10, due to their ability to target motor neurons.[47,51,52] In particular, the discovery that self-complementary AAV9 (scAAV9) vectors cross the blood-brain barrier[51] led to their use in treatment of spinal muscular atrophy (SMA), an inherited lower motor neuron disorder in which patients lack a functioning copy of the SMN1 gene. i.v. delivery of scAAV9 encoding SMN1 rescued SMA mouse models,[53–55] and subsequent human clinical trials showed dramatic efficacy in SMA patients,[56] leading to US Food and Drug Administration (FDA) approval of Zolgensma in 2019. This served as an important proof of principle for ALS therapeutics, which also must target lower motor neurons.[49] Recently, novel technologies have used guided evolution to create novel AAV serotypes with increased CNS tropism and expression while de-targeting peripheral tissues,[57–59] granting the potential to broaden the efficacy and increase the specificity of future AAV-mediated therapeutics. Of note, lentiviral approaches have also been used with some success in preclinical models of ALS,[49,60] but their translatability is limited by their small area of transduction, low viral titers, and broad tropism, as well as their integration into the host genome with associated risk of mutagenesis.[47,61] For this reason, the majority of preclinical ALS lentiviral studies, reviewed elsewhere,[49] have focused on delivery to muscle or *ex vivo* gene transfer,[49,62] including one approach combining both strategies[62] that recently completed a phase I/II clinical trial (ClinicalTrials.gov: NCT02943850).

In this manuscript, we provide an overview of the most promising gene-therapy-based approaches for ALS to date, including ASO therapy, AAV-mediated gene silencing, and AAV-mediated gene delivery. We focus primarily on recent studies and review how these strategies have been applied in preclinical models and human clinical trials for each of the most common familial forms of ALS, as well as for sporadic disease.

ASOs

ASOs are single-stranded, 8- to 50-base sequences of synthetic oligonucleotides that can be designed to complement target mRNAs for RNase H enzyme-mediated target degradation or designed against primary transcripts to induce alternative splicing (Figure 1A).[63–65] Recently, various modifications have been made to increase ASO stability, protect against nuclease degradation, improve cellular uptake, recruit RNase H, and reduce immunogenicity.[64] Although they do not cross the blood-brain barrier, ASOs achieve widespread CNS distribution after i.t. delivery,[63,65] making them an important tool in the arsenal against ALS. Indeed, ASOs were successfully developed to treat SMA, providing an important proof-of-concept for ALS. For SMA therapy, delivery of a splice-altering ASO resulting in the conversion of SMN2 to SMN1 showed efficacy in mouse models of SMA as well as distribution to affected areas of the spinal cord in nonhuman primates (NHPs).[66] This was followed by clinical trials showing marked improvements in strength and longevity in children with SMA,[67–69] leading to FDA approval of nusinersen in 2016 and its current widespread use in the clinic.

SOD1

Mutations in *SOD1* are believed to cause ALS through toxic gain of function caused by aggregation of misfolded SOD1 protein.[30,70] Although more than 150 ALS-associated *SOD1* mutations have been described, the G93A mutation, rare in humans, is the most commonly studied in preclinical models, as there exists a readily available $SOD1^{G93A}$ mouse[71,72] and rat.[73] In a key preclinical study, a 20-mer ASO targeting *SOD1* was delivered via continuous infusion in the lateral ventricle of rats and the lumbar spine of NHPs and demonstrated significant and widespread ASO concentrations throughout the brain and spinal cord with deep tissue penetration.[74] In $SOD1^{G93A}$ rats treated pre-symptomatically, there was substantial *SOD1* mRNA and protein knockdown in the brain and spinal cord that was associated with slowed disease progression and extended survival.[74] This led to a first-in-human phase I clinical trial (ClinicalTrials.gov: NCT01041222), where ASO ISIS 333611 was delivered via a single, 11.5-h i.t. infusion to patients with *SOD1*-associated ALS.[75] While the study demonstrated safety and tolerability as well as establishing the use of cerebrospinal fluid (CSF) *SOD1* as a pharmacokinetic marker, there were no reductions in CSF *SOD1* protein at the conservative concentrations used (maximum dose, 3 mg). A subsequent trial (ClinicalTrials.gov: NCT02623699) delivered the ASO, now named BIIB067/Tofersen (IONIS-SOD1Rx), via serial lumbar i.t. injections over 12 weeks, with patients cohorted into dosing groups ranging from 20–100 mg.[76] The trial demonstrated safety at these higher doses, as well as reductions in CSF *SOD1* protein of

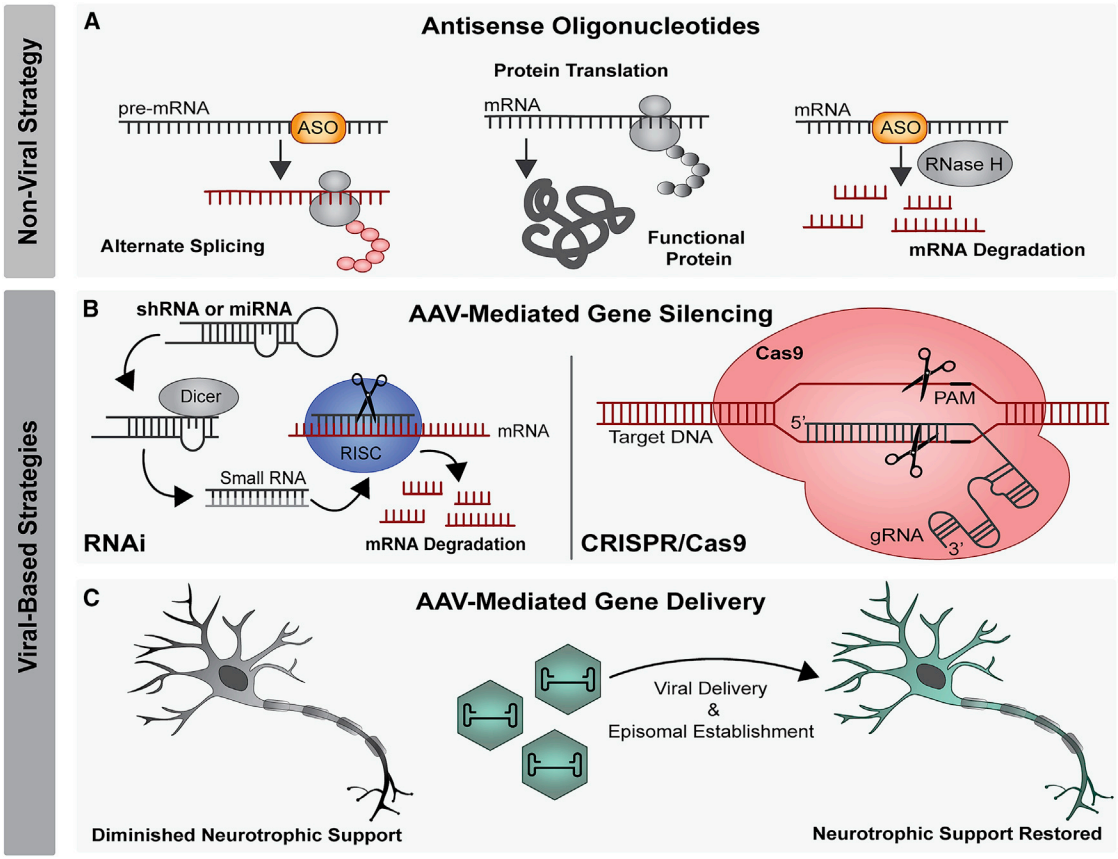

Figure 1. Summary of gene therapy strategies

(A) Non-viral strategies include using ASOs to induce alternate splicing or RNase H-mediated degradation. (B and C) Viral strategies include (B) AAV-mediated gene silencing, through RNA interference or CRISPR-Cas9 or (C) AAV-mediated gene delivery including neurotrophic factors. AAV, adeno-associated virus; ASO, antisense oligonucleotide; Cas, CRISPR-associated system; miRNA, microRNA; PAM, protospacer adjacent motif; RISC, RNA-induced silencing complex; RNAi, RNA interference; shRNA, small hairpin RNA.

33% in the highest-dose group. Exploratory analyses showed promising reductions in rate of decline as measured by the revised ALS functional rating scale (ALSFRS-R) in the high-dose group, especially among fast progressors.[76] Safety and efficacy are currently being evaluated in a phase 3, randomized, double-blind, placebo-controlled trial (ClinicalTrials.gov: NCT02623699) and its extension study (ClinicalTrials.gov: NCT03070119). In addition to the traditional delivery of naked ASOs described in these studies, another group reported a novel approach using AAV to achieve targeted and sustained ASO expression. AAVrh10 expressing antisense sequences targeting *SOD1* embedded in a U7 small-nuclear RNA was delivered both i.v. and i.c.v. to *SOD1*^{G93A} mice, at birth or in pre-symptomatic adulthood, leading to nonsense-mediated *SOD1* decay and markedly increased survival, strength, and weight in both age groups.[77]

C9orf72

Although the full mechanisms by which *C9orf72* hexanucleotide repeat expansions cause ALS are unknown, studies suggest a prominent role for a toxic gain of function, mediated in part by the G_4C_2 repeat RNA and in part by dipeptide repeat proteins produced by repeat-associated non-ATG (RAN) translation.[78,79] The first ASO-based strategies focused on reducing this toxicity in induced pluripotent stem cells (iPSCs) derived from humans with *C9orf72* expansions. Three such studies published in tandem in 2013 found that ASO-mediated reduction of the *C9orf72* transcript—whether through binding within the repeat expansion or within the surrounding regions—reduced nuclear RNA foci in *C9orf72*-associated ALS.[78,80,81] Two of the groups found additionally that ASO treatment reversed aberrant gene expression and reduced susceptibility to excitotoxicity in iPSC-differentiated neurons,[78,81] while the third group demonstrated persistence of aberrant RNA expression after sense-strand targeting and evidence suggesting the importance of simultaneously targeting the antisense strand.[80] This group also performed the first *in vivo* studies, demonstrating sustained efficacy and tolerability of knocking down *C9orf72* RNA in wild-type mice for up to 18 weeks after a single injection of ASO into the lateral ventricle.[80] In a subsequent study, the authors used a single i.c.v. injection to deliver ASOs targeting G_4C_2 repeat-containing sense-strand RNAs to pre-symptomatic adult

mice expressing up to 450 repeats. They demonstrated sustained reduction in RNA foci and dipeptide repeat proteins in the cortex and spinal cord, as well as amelioration of cognitive deficits.[82] This work has ultimately led to a phase I clinical trial of the ASO BIIB078 (ClinicalTrials.gov: NCT03626012) for *C9orf72*-ALS patients.

ATXN2

Although long trinucleotide repeat expansions in *ATXN2* have been known to cause spinocerebellar ataxia type 2,[83–86] intermediate-length expansions were discovered in 2010 to be a relatively common cause of heritable ALS.[40,87–89] Ataxin-2 serves diverse functions in the cell, including RNA processing and receptor endocytosis, but its critical functions in the formation of stress granules[27,90] and induction of aberrant TDP-43 cleavage by caspase 3[91,92] are of particular relevance in ALS. In 2018 it was demonstrated that a host of nucleocytoplasmic transport factors are bound in ataxin-2-containing SGs upon induction of stress in HEK293T cells. Further, delivery of ASOs targeting ataxin-2 to neuronal-differentiated iPSCs from *C9orf72*-ALS patients reversed cytoplasmic mislocalization of nuclear proteins.[27] Seminal work *in vivo* used a rapidly progressive TDP-43 ALS mouse model to demonstrate that a one-time delivery of *Atxn2*-ASO to the lateral ventricle at birth resulted in sustained, marked reduction of *Atxn2* mRNA as well as prolonged survival and improved gait.[93] While this strategy can be of benefit to patients with *ATXN2*-ALS, importantly, it also offers a therapeutic avenue for the broader ALS population, as TDP-43 localization to ATXN2-dependent stress granules is a shared pathologic endpoint.[25] A phase I clinical trial of the ASO BIIB105 (ClinicalTrials.gov: NCT04494256) is currently underway, enrolling patients both with and without CAG repeat expansions in *ATXN2*.

FUS

FUS is the most commonly mutated gene found in juvenile and pediatric ALS, with the p.P525L mutation causing a particularly aggressive and early-onset form[94,95] through a toxic gain-of-function mechanism. In 2019, Ionis Pharmaceutical, in conjunction with Columbia Medical Center, developed an ASO targeting this mutation via i.t. delivery and obtained FDA approval for experimental use in a young woman, for whom the therapy, jacifusen, is named.[96] Jaci has since passed away, but jacifusen has been used to treat three additional *FUS*-ALS patients through the FDA's compassionate use protocol, and 8 more patients are approved for investigational treatment with funding from the ALS association and Project ALS.[95,97,98]

AAV-MEDIATED GENE SILENCING

AAV-mediated silencing has the advantage over ASOs of eliminating the need for readministration, which is of use when targeting both the brain and spinal cord. AAVs are frequently used to deliver small noncoding RNAs to achieve RNAi, a naturally occurring process in which double-stranded RNA molecules regulate the expression of mRNA through homologous base pairing and subsequent cleavage via the RNAi-induced silencing complex (RISC; Figure 1B).[2,99] Two types of small noncoding RNAs, small hairpin RNA (shRNA) and artificial microRNA (miRNA), are in increasingly widespread use as a therapeutic strategy against neurodegenerative disease, particularly miRNA due to its more favorable safety profile.[100,101] Further refinement has been achieved using bioinformatic tools to minimize passenger strand loading[102] and optimize promoter, serotype, and dosing choices,[99] leading to greatly decreased off-target effects. The first AAV-RNAi human clinical trial for a neurodegenerative disease (ClinicalTrials.gov: NCT04120493) is currently in phase I/II and uses AAV5 to deliver a miRNA targeting Huntingtin to treat Huntington's disease.[2,103] This study will provide important safety and feasibility data for the ALS field.

An alternative approach to treat gain-of-function disorders is gene editing, which can be achieved using CRISPR-associated (Cas) systems, the most widely used of which is the RNA-guided Cas9 endonuclease[104] (Figure 1B). CRISPR-Cas9 enables specific targeting of genomic sequences and induction of double-stranded DNA breaks, causing frameshift-inducing base insertions or deletions (indels) with diverse applications,[105] although its use in the ALS field is relatively nascent.[106] Additional CRISPR-based systems have been developed that have transformative potential.[107–110] These include CRISPR-interference (CRISPRi), which uses a catalytically dead Cas9 to repress DNA without the potential mutagenic effects associated with cleavage;[111] the Cas13 family, which targets RNA for degradation[112,113] and variants of which are small enough for AAV packaging;[114] and prime editing, which is a method of introducing specific genomic insertions or deletions without inducing double-stranded breaks.[115] The application of these technologies to the ALS field is highly anticipated as they are further developed.

SOD1

As the first-identified form of ALS, *SOD1* has been the target of the majority of AAV-RNAi-based approaches, with increasingly promising results. The first AAV9 studies delivering shRNA against *SOD1* compared i.v. administration in *SOD1*^{G93A} mice at differing ages and found a 39% increase in survival after P1-injection that decreased substantially with age and disease progression;[116] they noted increasingly preferential glial targeting over neurons with later injections.[116,117] They also found markedly increased survival in the slower-progressing *SOD1*^{G37R} mouse model even when treated after disease onset, and they demonstrated robust *SOD1* mRNA knockdown throughout the spinal cord in NHPs after lumbar i.t. injection.[116] Another group used bilateral direct cortical injection of AAV9-shRNA targeting *SOD1* in presymptomatic adult *SOD1*^{G93A} rats and found that upper motor *SOD1* suppression alone was able to preserve motor function and increase survival by 20 days.[118] A 2016 study used AAV9 to deliver an artificial miRNA against *SOD1* (mi-*SOD1*) to the ventricles in neonatal *SOD1*^{G93A} mice. The authors found a 50% extension in survival with significant delay in hindlimb paralysis, as well as improvements across multiple histopathologic parameters including number of spinal motor neurons.[119] Notably, neonatal treatment has limited translatability, as most patients present in adulthood after symptom onset, and the authors therefore conducted a separate study using rAAVRh10 to deliver mi-*SOD1* systemically to adult *SOD1*^{G93A} mice. They found substantially delayed

disease onset and 21% extended survival, with preserved strength and respiratory function.[120] They then tested the vector in marmosets via i.t. injection and found significant lowering of *SOD1* in lower motor neurons throughout the spinal cord without short-term toxicity.[120] Subsequent studies of rAAVRh10.mi-*SOD1* in macaques, delivered i.t. with the head down at 30 degrees, demonstrated robust silencing at the mRNA level in laser-captured motor neurons throughout the spinal cord that was proportionate to the strength of the promoter used.[121] The authors demonstrated a low off-targeting profile and found no adverse events up to 92 days post-infusion, paving the way for a human study published in July 2020. In that study, two patients with *SOD1*-ALS were treated with i.t. infusion of rAAVRh10.mi-*SOD1*. The first patient had transient improvement in strength in his right leg and slightly lowered CSF SOD1 levels without other signs of benefit, and his course was complicated by a severe meningoradiculitis with sensory symptoms. These sensory symptoms were not seen in the second patient, who was treated with immunosuppression at the time of infusion. At autopsy, *SOD1* levels were lower in the spinal cord of patient 1 when compared to those in untreated *SOD1* patients, and neurons were depleted in bilateral dorsal root ganglia (DRG) with a T-lymphocytic infiltrate of nerve roots. Patient 2 maintained stable strength and vital capacity throughout the 12-month period reported.[122]

The DRG toxicity seen in the above clinical trial has also been seen with AAV9 when administered systemically to neonatal pigs and juvenile primates,[123] although the NHPs did not show clinical signs of toxicity. Further meta-analyses of CSF and systemic administration across 33 NHP studies (a total of 256 NHPs) showed DRG toxicity in 83% of CSF-treated and 32% of systemic-treated NHPs that was independent of serotype, sex, promoter, or transgene; importantly, however, pathology was dose dependent and overall mild, and no clinical sequelae were seen.[124] Interesting alternative approaches to avoid DRG toxicity have been tried with varying degrees of success. For instance, the AAV-Rh10-mediated mi*SOD1* treatment described above also resulted in 50-day survival prolongation after direct injection of the tongue and intrapleural space of adult presymptomatic *SOD1*^{G93A} mice,[125] areas in which weakness is the lead cause of death in ALS patients. Recent studies successfully used DRG-specific miRNAs to downregulate transgene expression in these cells while preserving intended CNS transduction, an efficient detargeting strategy that can be employed across any AAV-based therapeutic.[126] Finally, one notable study used a novel subpial delivery method, administering AAV9-shRNA to the narrow space between the spinal cord parenchyma and the innermost layer of the meninges. The authors achieved long-term suppression of motor neuron disease in *SOD1*^{G37R} mice treated pre-symptomatically as well as potent blocking of disease progression in mice treated after disease onset. They then used this delivery method in adult pigs and NHPs and found homogeneous and robust transgene expression throughout the spinal cord, including motor neurons.[127] Although technically challenging, the demonstration of feasibility in these larger animal models speaks well to the promise of subpial AAV delivery in humans, who share comparable spinal anatomy and size.

The first study using CRISPR in ALS was published in 2017. The authors used a modified AAV9 to deliver *Staphylococcus aureus*-derived Cas9 (SaCas9) and a single-guide RNA (sgRNA) targeting the h*SOD1* gene via the facial vein to neonatal *SOD1*^{G93A} mice.[128] They found concentrated SaCas9 expression throughout the ventral horn cells of the spinal cord, with >2.5-fold decrease in *SOD1* protein levels. Treated mice showed preserved motor neurons with improved motor function, a 37% delay in disease onset, and a 25% increase in survival, with a low off-target indel rate across spinal cord transgenes.[128] Another group used AAV9-SaCas9-sgRNA to treat neonatal *SOD1*^{G93A} mice via i.c.v. injection and also found reduced *SOD1* in anterior horn cells. These studies also reported an increase in motor neurons and greatly improved motor function, as well as a notable 54.6% increase in survival, with a low off-target indel rate in predicted off-target sequences.[129] In a subsequent study by the first group, an intein-mediated *trans*-splicing system was devised for *in vivo* single-base editing of *SOD1*, in order to minimize the potential mutagenic outcomes of double-stranded DNA breaks. The authors used dual AAV9 vectors to deliver this system i.t. to pre-symptomatic adult *SOD1*^{G93A} mice and found a 40% reduction in *SOD1* inclusions accompanied by improved neuromuscular function and muscle bulk, as well as 11% increase in survival,[130] despite astrocyte-predominant targeting. These CRISPR-based strategies hold great promise as refinement continues.

C9of72

Due to the challenges of targeting intronic, GC-rich repeat expansions[131] and achieving knockdown in the nucleus, where *C9orf72*-mediated RNA foci are predominantly found, RNAi-based approaches for *C9orf72* have been challenging compared to *SOD1*. In 2015, a group was able to engineer duplex RNAs to overcome these hurdles and effectively target both sense and antisense G_4C_2 expansions in ALS-patient-derived fibroblast cells.[132] By introducing mismatches in key regions, they destabilized the parent duplex to allow entry into the RISC complex, with resultant 40%–60% reduction in nuclear RNA foci from both strands. The authors subsequently engineered single-stranded silencing RNAs (ss-siRNAs) that function like ASOs but mediate degradation through RNAi, and found that these achieved even more potent inhibition of sense and antisense G_4C_2 repeat expansions in human mutant *C9orf72* fibroblast cells.[133] The biotech company uniQure developed a different bidirectional miRNA-based approach, using concatenated miRNA hairpins targeting *C9orf72* (miC), and achieved 50% reduction of sense and antisense nuclear RNA foci in $(G_4C_2)_{44}$-expressing cells. They further incorporated these miC constructs into AAV5 and effectively silenced *C9orf72* in iPSC-derived neurons from an FTD patient.[134] In a subsequent study, they used miC constructs to reduce both nuclear and cytoplasmic *C9orf72* mRNA in FTD patient-derived iPSCs.[135] They then delivered their AAV5-miC constructs to the striatum of adult transgenic *C9orf72*_3 line 112 mice, a strain harboring several tandem copies of human *C9orf72* that does not develop neurodegeneration but exhibits RNA foci and poly(GP) protein. They found 20%–40% reduction of *C9orf72* mRNA and sense intronic transcripts in transduced regions, although the mice were not followed long enough to determine poly(GP) reduction.[135]

There have been few CRISPR-mediated studies for *C9orf72*, but a recent study of note used CRISPR-Cas9 to generate deletions in the promoter region of iPSCs derived from patients harboring *C9orf72* expansions. They found that these deletions reduced the expression of the *C9orf72* variant containing the repeat expansion, which in turn nearly eliminated production of all dipeptide repeats and markedly reduced axonal degeneration.[136] Another recent study identified Ku80 as a DNA repair protein that is overactivated in in *Drosophila* harboring G_4C_2 expansions and in *C9orf72*-ALS patient-derived iPSCs, leading to premature apoptosis. The authors used CRISPR-Cas9 to reduce Ku80 and found reductions in pro-apoptotic pathways; they also found decreased nuclear RNA foci after direct CRISPR-mediated deletion of the G_4C_2 repeat expansion.[137] These are promising approaches for future *in vivo* studies.

ATXN2

To date there are no published RNAi or CRISPR-based approaches targeting *ATXN2*. However, the potential for this approach can be inferred from related studies in the spinocerebellar ataxia field and from the ASO-mediated reduction of Atxn2 in mouse models of SCA2[138] and ALS[93]. Viral delivery of RNAi-based approaches has demonstrated success in mouse models of SCA1,[139,140] SCA3,[141,142] and SCA7,[143] while a CRISPR-based approach in patient-derived iPSCs has shown early promise for SCA3.[144] Additional ASO, RNAi, and CRISPR-based approaches for spinocerebellar ataxia are reviewed extensively elsewhere[145] and demonstrate the feasibility and promise of this approach if adapted to motor neuron-targeting AAV delivery methods.

AAV-MEDIATED GENE DELIVERY

The most long-established application of gene therapy is delivery of a therapeutic transgene. Unlike in the case of Zolgensma for SMA, however, no ALS-causing mutations have been found to have an exclusive loss-of function mechanism, and so therapeutic transgene delivery has focused on neurotrophic factors to provide support for degenerating neurons (Figure 1C). Neurotrophins are typically secreted proteins involved in neuronal growth or survival, and several neurotrophins have been found to decline over time in ALS patients and animal models,[49] suggesting a potential role in prolonging neuronal health. While nonspecific and unlikely to be curative, these methods are attractive for their potential to prolong survival across all forms of ALS. Non-gene-therapy-mediated growth factor studies[32,146] have unfortunately not been successful in clinical trials,[147] which is felt to be in large part due to inadequate dosing to affected areas of the nervous system. For this reason, gene therapy approaches have been pursued by numerous groups in an effort to improve CNS delivery and dosing. Early gene therapy studies for neurotrophic support purposes have been extensively reviewed elsewhere.[47,49] Here, we focus on recent AAV-mediated work, most of which was conducted in *SOD1*^{G93A} mice. We also discuss AAV-mediated delivery of non-trophic factors, including neuromuscular junction modulators and aggregation-directed therapies.

Intramuscular delivery of insulin-like growth factor 1 (IGF1) using AAV9 was shown in two separate studies to preserve spinal motor neurons and modestly extend lifespan in *SOD1*^{G93A} mice treated pre-symptomatically or in early post-symptomatic adulthood.[148,149] Similar results were obtained using systemic AAV9-IGF1 delivery.[150] In a different approach, i.c.v. delivery of AAV4 was used to target ependymal cells for IGF1 secretion into the CSF in early symptomatic *SOD1*^{G93A} mice. The authors found improved motor function and modestly improved survival,[151] effects that were slightly greater with delivery of AAV4-mediated vascular endothelial growth factor (VEGF), which has been hypothesized to act in the same pathway as IGF1.[152] Delivery of both AAV4-IGF1 and AAV4-VEGF did not provide further benefit.[151] VEGF has been adapted for ALS therapy at both the gene therapy and protein levels.[153] Using i.t. delivery of AAV9, one group demonstrated that VEGF expression in adult symptomatic *SOD1*^{G93A} mice improved motor function and extended survival.[154] When AAV1 and AAV9 were used to deliver VEGF to a feline model of lower motor neuron disease via i.c.v., i.v., or intracisterna magna (i.c.m.) injection, only i.c.m. delivery resulted in sustained, high levels of VEGF expression in the spinal cord; however, there was no therapeutic benefit.[155]

Although early studies of AAV-mediated glial-derived neurotrophic factor (GDNF) delivery showed promise,[156,157] effects were modest and seen only after intramuscular delivery. A 2017 study of a more translatable, systemic AAV9-GDNF delivery in pre-symptomatic *SOD1*^{G93A} rats showed modest improvement in strength and delay of forelimb paralysis. However, there was no extension in survival, and adverse side effects were seen, including decreases in working memory, activity levels, and weight .[158] Another neurotrophic factor, granulocyte-colony stimulating factor (G-CSF), was found to preserve motor units and extend survival by 10% when delivered to adult pre-symptomatic *SOD1*^{G93A} mice via direct intraspinal injection of AAV1.[159] Hepatocyte growth factor (HGF) has also been evaluated, using i.t. AAV1[160] or intramuscular AAV6[161] to treat adult pre-symptomatic *SOD1*^{G93A} mice, with modest improvements noted in strength and survival using both delivery methods.

Factors that act at the neuromuscular junction have also been assessed, including intramuscular delivery of AAV1-neuregulin to promote collateral sprouting and electrophysiologic function in adult symptomatic *SOD1*^{G93A} mice. However, there were no effects on strength or survival.[162] Systemic administration of AAV9 delivering the gene encoding the neuromuscular junction protein DOK7 preserved the neuromuscular junction, improved locomotion, and modestly increased lifespan in early symptomatic *SOD1*^{G93A} mice, without affecting motor neuron counts.[163] D-amino acid oxidase (DAO) is thought to modulate excitotoxicity, and i.t. delivery of AAV9-DAO to early symptomatic *SOD1*^{G93A} mice resulted in motor neuron preservation and a modest survival extension.[164]

Factors with direct effects on misfolded ALS-associated proteins are also being studied. For example, the cytosolic chaperone, macrophage migration inhibitory factor (MIF), inhibits mutant *SOD1* misfolding.[165] AAV9-mediated expression of MIF after direct intraspinal delivery to neonatal *SOD1*^{G93A} mice or the slower-progressing

Table 1. Gene therapy-based therapeutic strategies for ALS

Gene therapy	Date	Model	Route	Outcome	Citation
Antisense oligonucleotides					
SOD1	2006	rat; NHP SOD1^{G93A}	i.c.v. (rat), i.t. (NHP)	increased survival; good biodistribution in NHP	Smith et al.[74]
	2013	human SOD1-ALS phase I	i.t.	safe; no benefit	Miller et al.[75]
	2017	mouse SOD1^{G93A}	i.c.v. + i.v.	increased survival, strength, and weight	Biferi et al.[77]
	2020	human SOD1-ALS phase I/II	i.t.	safe; reduced CSF SOD1	Miller et al.[76]
	current	human SOD1-ALS phase III	i.t.	TBD	ClinicalTrials.gov: NCT02623699
C9orf72	2013	human iPSC C9-ALS		reduced nuclear RNA foci and excitotoxicity; reversed aberrant gene expression	Donnelly et al.[78]
	2013	human iPSC C9-ALS		reduced nuclear RNA foci and excitotoxicity; reversed aberrant gene expression	Sareen et al.[81]
	2013	human iPSC C9-ALS; mouse (WT)		reduced nuclear RNA foci; persistent aberrant expression; safe in mice	Lagier-Tourenne et al.[80]
	2016	mouse C9$_{(450)}$	i.c.v.	sustained reduction in nuclear RNA foci and DPRs in motor neurons; cognitive benefit	Jiang et al.[82]
	current	human C9-ALS phase I	i.t.	TBD	ClinicalTrials.gov: NCT03626012
ATXN2	2017	mouse hTDP-43	i.c.v.	reduced Atxn2 mRNA; increased survival; improved gait	Becker et al.[93]
	2018	human iPSC C9-ALS		reduced nuclear RNA foci and excitotoxicity; reversed aberrant gene expression	Zhang et al.[27]
	Current	human ATXN2-ALS & sporadic ALS phase I	i.t.	TBD	ClinicalTrials.gov: NCT04494256
FUS	2019	human FUS-ALS	i.t.	patient passed; further clinical data not reported	Arnold[96]
	Current	human FUS-ALS	i.t.	TBD	Figueiredo[97]
AAV-mediated gene silencing					
AAV-RNAi					
SOD1	2013	mouse SOD1^{G93A} & SOD1^{G37R}; NHP	i.v. (mice), i.t. (NHP)	increased survival, decreasing with age of administration; reduced *SOD1* mRNA in NHP spinal cord	Foust et al.[116]
	2014	rat SOD1^{G93A}	cortex	delayed disease onset and increased survival; preserved motor function	Thomsen et al.[118]
	2016	mouse SOD1^{G93A}	i.c.v.	increased survival; delayed paralysis; increase in motor neurons	Stoica et al.[119]
	2016	mouse SOD1^{G93A}; NHP	i.v. (mice), i.t. (NHP)	delayed onset; increased survival; preserved motor and respiratory function in mice; reduced *SOD1* mRNA in NHP spinal cord	Borel et al.[120]
	2018	NHP	i.t.	reduced *SOD1* mRNA in spinal cord	Borel et al.[121]
	2019	mouse SOD1^{G93A}	tongue, subplural	increased survival; lowered *SOD1* mRNA in muscle, tongue, and diaphragm	Keeler et al.[125]

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Table 1. *Continued*

Gene therapy	Date	Model	Route	Outcome	Citation
	2020	human SOD1-ALS phase I	i.t.	possible clinical stabilization in 1 patient; lowered spinal cord SOD1 in second, with DRG toxicity and no clear clinical benefit	Mueller et al.[122]
	2020	mouse SOD1^{G37R}; pig; NHP	lumbar subpial	stopped disease progression in mice; strong LMN targeting in large animals	Bravo-Hernandez et al.[127]
C9orf72	2015	human iPSC C9-ALS		marked reduction in nuclear foci	Hu et al.[132]
	2017	human iPSC C9-ALS		marked reduction in nuclear foci	Hu et al.[133]
	2019	(G4C2)44 cells; human iPSC C9-FTD		reduced nuclear foci; silenced *C9orf72* in iPSC	Martier et al.[134]
	2019	human iPSC C9-FTD; mouse C9orf72_3	striatum	reduced nuclear foci in iPSCs, reduced *C9orf72* mRNA in mouse striatum	Martier et al.[135]
AAV-CRISPR					
SOD 1	2017	mouse SOD1^{G93A}	i.v.	increased survival and strength with decreased SOD1 protein in spinal cord	Gaj et al.[128]
	2020	mouse SOD1^{G93A}	i.c.v.	increased survival and strength with decreased SOD1 protein in spinal cord	Duan et al.[129]
	2020	mouse SOD1^{G93A}	i.t.	increased survival and strength with decreased SOD1 inclusions in spinal cord	Lim et al.[130]
C9orf72	2019	Drosophila C9; human iPSC C9-ALS		reduced apoptotic pathway activation; reduced nuclear foci	Lopez-Gonzalez et al.[137]
	2020	human iPSC C9-ALS		reduced *C9orf72* expression; near elimination of dipeptide repeats; reduced axonal degeneration	Krishnan et al.[136]
AAV-mediated gene delivery					
Neurotrophic support					
IGF	2010	mouse SOD1^{G93A}	i.c.v.	improved motor function and increased survival	Dodge et al.[151]
	2016	mouse SOD1^{G93A}	i.m.	preserved motor neurons; increased survival	Allodi et al.[148]
	2018	mouse SOD1^{G93A}	i.m.	preserved motor neurons; increased survival	Lin et al.[149]
	2018	mouse SOD1^{G93A}	i.v.	preserved motor neurons; increased survival	Wang et al.[150]
VEGF	2010	mouse SOD1^{G93A}	i.c.v.	improved motor function and increased survival	Dodge et al.[151]
	2013	feline Lix1$^{-/-}$ (LMN disease)	i.c.v., i.v., or i.c.m.	only i.c.m. delivery resulted in sustained VEGF in spinal cord, without therapeutic benefit	Bucher et al.[155]
	2016	mouse SOD1^{G93A}	i.t.	improved motor function and increased survival	Wang et al.[154]
GDNF	2017	rat SOD1^{G93A}	i.v.	improved strength, but no effect on survival; worsened cognitive function, decreased activity	Thomsen et al.[158]
G-CSF	2011	mouse SOD1^{G93A}	intra-spinal	preserved motor units; increased survival	Henriques et al.[159]
HGF	2019	mouse SOD1^{G93A}	i.t.	improved motor function and increased survival	Lee et al.[160]
	2019	mouse SOD1^{G93A}	i.t.	improved motor function and increased survival	Lee et al.[161]
Neuromuscular junction modulators					
Neuregulin	2016	mouse SOD1^{G93A}	i.m.	no effect on strength or survival	Mancuso et al.[162]

(*Continued on next page*)

Table 1. *Continued*

Gene therapy	Date	Model	Route	Outcome	Citation
DOK7	2017	mouse SOD1^{G93A}	i.v.	preserved neuromuscular junction, improved locomotion, increased survival; no effect on motor neuron counts	Miyoshi et al.[163]
DAO	2017	mouse SOD1^{G93A}	i.t.	preserved motor neurons, increased survival	Wang et al.[164]
Targeting misfolded protein					
MIF	2019	mouse SOD1^{G93A} + loxSOD1^{G37R}	intra-spinal	improved strength and increased survival; reduced misfolded SOD1 in SOD1^{G93A} mice	Leyton-Jaimes et al.[166]
SOD1 Ab	2014	mouse SOD1^{G93A}	i.t.	increased survival, reduced misfolded SOD1, reduced neuronal stress	Patel et al.[167]
TDP-43 Ab	2019	mouse TDP-43^{G348C}	cortical	reduced TDP-43 proteinopathy, cognitive impairment, motor defects & neuroinflammation	Pozzi et al.[168]

CSF, cerebrospinal fluid; i.c.m., intra-cisterna magna; i.c.v., intracerebroventricular; i.m., intramuscular; i.t., intrathecal; i.v., intravenous; NHP, nonhuman primate; WT, wild type.

loxSOD1^{G37R} mouse model significantly reduced misfolded *SOD1* levels in the spinal cord of the SOD1^{G93A} mice and improved strength and survival in both models.[166] i.t. delivery of AAV1 expressing a monoclonal antibody targeting misfolded *SOD1* to pre-symptomatic adult SOD1^{G93A} mice resulted in a notable 28% extension of lifespan accompanied by reduced misfolded spinal *SOD1* and attenuated neuronal stress signals and gliosis.[167] A similar strategy was tested in symptomatic TDP-43^{G348C} mice, which manifest cytoplasmic accumulation of TDP-43 as well as memory impairment. AAV9-mediated cortical delivery of an antibody targeting an aggregation-prone region of TDP-43 reduced TDP-43 proteinopathy, cognitive impairment, motor defects, and neuroinflammation.[168]

CONCLUSIONS AND PERSPECTIVES

The ability to provide targeted, sustained treatment gives gene therapy the potential to permanently alter the therapeutic landscape for diseases of the CNS, as it has already done in the case of SMA. ALS in particular is in dire need of impactful, disease-modifying approaches capable of reaching some of the body's most protected regions, the cerebral cortex and the anterior horn cells of the spinal cord, which can increasingly be achieved through minimally invasive means by harnessing the tremendous potential of gene therapy. Once delivered to their target, these therapies can act through knockdown-mediated amelioration of gain-of-function mechanisms or through delivery of protective agents, enabling approaches ranging from targeting a specific ALS-causing mutation to modifying a common pathologic endpoint across sporadic cases. As we continue to hone our delivery methods, vector specificity, and cargo efficacy, there is great hope that truly impactful treatments for ALS will emerge in the near future.

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AUTHOR CONTRIBUTIONS

D.A.A. and B.L.D. planned the manuscript. D.A.A. wrote and coordinated the draft and designed the figure and Table 1. D.A.A. and B.L.D. reviewed and revised the manuscript.

DECLARATION OF INTERESTS

B.L.D. is a founder of Spark Therapeutics and Spirovant Sciences and is on the SAB of Patch Bio, Resilience, Saliogen Therapeutics, Panorama Medicines, Homology Medicines, and Spirovant Sciences.

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