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Title: Current Developments in Gene Therapy for Amyotrophic Lateral Sclerosis

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Article highlights:

- Types of ALS and the recent genetic discoveries.
- No effective treatment for ALS is available.
- Gene therapy approaches to modulate mutant ALS genes by using siRNA or antisense oligonucleotide (ASO) therapy.
- AAV or LV-based vectors driving the expression of neurotrophic factors to support motor neuron survival.
- A summary of the previous and the ongoing gene therapy clinical trials for ALS.

Abbreviations

Acetylcholinesterase enzyme (AChE)

Amyotrophic Lateral Sclerosis (ALS)

Antisense Oligonucleotide (ASO)

Blood-Brain Barrier (BBB)

Central Nervous System (CNS)

Double stranded RNA (dsRNA)

Fused-in-sarcoma (FUS)

Granulocyte-Colony Stimulating Factor (G-CSF)

Hepatocyte Growth Factor (HGF)

Human Mesenchymal Stem Cells (hMSC)

Induced Pluripotent Stem Cell (iPSC)

Insulin-like Growth Factor 1 (IGF-1)

Intracerebroventricular (ICV)

Lentiviral (LV)

Mesenchymal Stem Cells (MSCs)

Motor neuron disease (MND)

Recombinant Adeno-Associated Virus serotype 9 (rAAV9)

Repeat-associated Non-ATG (RAN)

RNA interference (RNAi)

RNA-induced Silencing Complex (RISC)

Short hairpin RNAs (shRNAs)

Small interfering RNAs (siRNAs)

Superoxide Dismutase 1 (SOD1)

TAR DNA-binding Protein (TARDBP)

Vascular Endothelial Growth Factor (VEGF)

Zinc Finger Protein Transcription Factor (ZFP-TF)

Abstract

Introduction: Amyotrophic Lateral Sclerosis (ALS) is a devastating adult neurodegenerative disorder characterised by motor neuron degeneration and death around 3 years from onset. So far, riluzole is the only treatment available, although it only offers a slight increase in survival. ALS' complex aetiology, with several genes able to trigger the disease, makes its study difficult.

Areas covered: RNA-mediated or protein-mediated toxic gain-of-function leading to motor neuron degeneration appear to be likely common pathogenic mechanisms in ALS. Consequently, gene therapy technologies to reduce toxic RNA and/or proteins and to protect motor neurons by modulating gene expression are at the forefront of the field. Here we review the most promising scientific advances, paying special attention to the successful treatments tested in animal models as well as analysing relevant gene therapy clinical trials.

Expert opinion: Despite broad advances in target gene identification in ALS and advances in gene therapy technologies, a successful gene therapy for ALS continues to be elude researchers. Multiple hurdles encompassing technical, biological, economical and clinical challenges must be overcome before a therapy for patients becomes available. Optimism remains due to positive results obtained in several *in vivo* studies demonstrating significant disease amelioration in animal models of ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS), also commonly known as motor neuron disease (MND) or Lou Gehrig's disease, is a progressive, relentless and unfailingly fatal neurodegenerative disorder. ALS most commonly presents as a late-onset adult disorder and death from respiratory failure typically occurs around 3 years from onset of symptoms.

Although primarily characterised by the degeneration of upper and lower motor neurons, ALS displays wide phenotypic heterogeneity amongst patient populations and a significant number of patients (~10-15%) will develop fronto-temporal dementia in addition to widespread, debilitating muscle atrophy and weakness [1, 2].

The majority of ALS cases (90%) are sporadic, with the remaining 10% having an identifiable hereditary component (familial ALS) [3]. Beginning in 1993 with the discovery of the first mutations in the gene encoding superoxide dismutase 1 (SOD1) [4], the genetic aetiology of ALS has expanded dramatically to include more than 20 different genes [3, 5] (Figure 1). Advances in technology combined with the concerted efforts of ALS researchers to identify new pathogenic genes have led us to the point where it is now possible to identify the underlying genetic cause of approximately 60% of familial ALS cases and 11% of sporadic cases [6].

The only treatment currently available to patients, other than symptomatic care, is the drug Riluzole. Approved in 1996, Riluzole offers only a modest increase in lifespan of approximately 3-4 months and subsequent clinical trials for a number of therapeutic agents have failed to show any significant benefits for ALS patients [7].

Gene therapy, the process of delivering genetic material to correct a faulty or missing gene, shows great promise for the treatment of several forms of ALS. In humans, gene therapy is

still in its infancy and currently (not taking into account antisense oligonucleotide therapies) there are no FDA/CBER-approved gene therapy products available in the US and only one gene therapy product has been granted market authorisation by the European Medicines Agency: Glybera®, in 2012. However, a number of early phase clinical trials for a small number of diseases have shown that gene therapy can be both a safe and effective treatment for patients [8-10].

Furthermore, as our knowledge of the genetic causes of ALS continues to grow and the pathogenic mechanisms underlying ALS continue to be unravelled, new therapeutic targets for gene therapy will undoubtedly begin to emerge.

Gene therapy for ALS has become a very exciting topic in the last few years. Published review articles, for instance Federici et al. and Nizzardo et al. in 2012 summarised the most promising advances at that point [11, 12]. Here, we will seek to summarise and critically evaluate current developments in gene therapy targeting ALS by examining the main techniques that are being utilised, with attention to the potential for their future clinical application.

2. Genetics and pathophysiology of ALS

As previously stated, over 60% of fALS cases and 11% of sALS cases may now be explained by mutations in various genes (Figure 1). Four of the most prominent genetic subtypes of ALS are summarised below (C9orf72, SOD1, TDP-43 and FUS), along with current hypotheses as to the possible pathogenesis of each genetic variant. In addition to these prominent genes, mutations in many other genes have been associated with ALS, including: VCP, OPTN, UBQLN2, SQSTM1 and PFN1 [3]. This is by no means an exhaustive list of the genes involved

in ALS but is intended to serve as an indication of both the diversity of ALS genetics and to provide a context in which the challenges of treating ALS can be appreciated.

Since the topic of this review is not the in depth analysis of ALS pathophysiology, we will briefly summarize the most relevant aspects relating to ALS pathophysiology published so far. Some recent papers broadly review this topic [5, 13, 14]

2.1. **C9orf72**

Although its function is unknown, it is believed that the *C9orf72* gene encodes a protein homologous to a DENN (differentially expressed in normal and neoplastic cells) protein structure, which is likely to regulate membrane traffic in conjunction with Rab-GTPase switches [15]. In 2011, it was discovered that the most common genetic cause of both fALS (~40%) and sALS (~7%) is the large intronic GGGGCC-hexanucleotide repeat expansion in the *C9orf72* gene (Figure 1) [1, 6]. Along with ALS, *C9orf72* expansions are found in 25% of familial frontotemporal dementia (FTD) cases [16]. Unaffected individuals can carry up to 30 GGGGCC copies, while up to several thousand repeat expansions have been found in ALS patients [1, 5, 6].

The pathogenesis of *C9orf72*-linked ALS is currently unknown, although a number of causal mechanisms have been suggested, including a loss of function due to haploinsufficiency caused by reduced *C9orf72* expression or a gain of function prompted by the sequestration of RNA-binding proteins by toxic RNA species resulting from the expanded repeat and toxic dipeptide proteins formed as a result of repeat-associated non-ATG (RAN) translation [2, 5, 17-19].

The levels of C9orf72 transcripts in the central nervous system (CNS) are downregulated in patients carrying the expansions in comparison to healthy controls [1, 17]. Although C9orf72 haploinsufficiency still remains to be elucidated in mammalian models, studies in zebrafish have already shown how loss of C9orf72 transcripts causes behavioural deficits and cytopathological changes as well as major morphological abnormalities [20].

Aberrant RNA foci, which accumulate due to the deficient transcription of the GGGGCC expansion, have been found in the brain of C9orf72 repeat expansions carriers [1, 5]. These abnormal transcript accumulations contain stable guanine quadruplexes which could sequester essential RNA-binding proteins triggering nucleolar stress and other downstream effects [21, 22]. A gain of toxic function hypothesis is also supported by the presence of repeat-associated non-ATG dependent translation (RAN translation) to generate dipeptide repeats (DPRs), which have been detected in C9orf72-ALS/FTD tissue [18, 23]. It is known that RAN translation of the intronic GGGGCC C9orf72 repeat expansions in both sense and anti-sense directions can generate up to five different DPRs, which can be toxic [18, 23-26]. Although it is not fully understood yet how C9orf72 DPRs potentially lead to toxicity, some mechanisms have been proposed. Poly-PR and poly-GR seem to impair the biogenesis of ribosomal RNA [26] and poly-GA mediates cytotoxicity by endoplasmic reticulum stress, increased release of lactate dehydrogenase and caspase-3 activation [25]. A recent study has shown the arginine-rich DPRs are neurotoxic causing nuclear and nucleoli disruption, reduction in the number of processing bodies and formation of granules [24].

It is unclear whether both mechanisms - loss and gain of C9orf72 function - can exert neurotoxic effects independently or whether they have to act in tandem to trigger

neurodegeneration. The pathophysiological evidence suggests that both mechanisms are relevant and, therefore, a contribution of both is likely to generate cytotoxicity.

2.2. SOD1

Cu/Zn SOD1 is a homodimeric, ubiquitously expressed, primarily cytosolic protein that serves to protect cells from toxic free-radical superoxide species produced during oxygen metabolism. Cu/Zn SOD1 catalyses the dismutation of two superoxide radicals (O_2^-) into hydrogen peroxide (H_2O_2) and oxygen (O_2). Eleven missense mutations in the gene encoding SOD1 were the first to be linked to fALS [4] and since then more than 140 other mutations have been identified in the gene and linked to ALS [27]. SOD1 mutations account for ~12% of fALS and ~1-2% sALS cases (Figure 1). SOD1-linked ALS displays broad phenotypic heterogeneity. After two decades of investigation, the current consensus is that multiple pathophysiological mechanisms contribute to motor neuron injury in the presence of mutant SOD1. The most prominent of these are briefly summarised below.

2.2.1. *Loss of Dismutase Activity*

Currently, the pathogenesis of SOD1-linked fALS is generally believed to be a toxic gain of function [28]; however studies in SOD1 null mice have suggested the possibility of loss of dismutase activity to be a potential modifier in ALS [29].

2.2.2. *Oxidative Stress*

It has been suggested that mutant SOD1 may generate toxic products due to the enhanced accessibility, and subsequent reaction of aberrant substrates with the Cu/Zn active site of the enzyme. Other contributors to oxidative stress in SOD1 beyond the enzymatic activity of SOD1 include disruption of redox-sensitive Rac regulation of NADPH oxidase [30] and dysregulation of the Nrf2-anti-oxidant response signalling pathway [31].

2.2.3. Protein Misfolding and Aggregation

Misfolded SOD1 accumulates as aggregated protein inclusions in both human fALS patient tissue [32] and astrocytes and motor neurons of ALS mouse models expressing human SOD1 containing SOD1-linked fALS mutations [32-35]. Although most often associated with SOD1-linked fALS mutations, wild-type SOD1 immunoreactive inclusions have also been found in motor neurons, microglia, oligodendrocytes and astrocytes in sALS patients [36-38] and motor axons of non-SOD1 linked fALS patients [38]. Whether or not the aggregates themselves are a major contributor to disease is contentious, as some studies have shown that large aggregates appear only after transgenic mice begin to display signs of motor dysfunction [39], and others have shown that SOD1 protein complexes can be detected in the spinal cord of SOD1-G93A mice at P30, a pre-onset stage [35]. The mechanism behind aggregate mediated toxicity is unknown but may be due to sequestration of essential proteins, such as HSC70, a chaperone protein involved in endocytosis, and KAP3, which is involved in the axonal transport of ChAT [40, 41]. It also remains to be determined exactly what triggers misfolding in SOD1, which is an inherently stable protein in its native state.

2.2.4. Excitotoxicity

Glutamate is the primary excitatory neurotransmitter in the CNS. Despite the role of glutamate in neurotransmission, it is itself highly toxic to neurons. Elevated levels of glutamate have been observed in the CSF of ALS patients, suggesting a role of excitotoxicity in ALS pathogenesis [42]. Further evidence implicating excitotoxicity in ALS is the fact that the drug riluzole acts to ameliorate excitotoxicity at least in part through reduction in pre-synaptic glutamate release [43]. Excitotoxicity in ALS may be exacerbated by a number of potential mechanisms. SOD1 fALS mutations have been shown to inhibit the glutamate uptake function of excitatory aminoacid transporter -2 (EAAT2 or GLT1 in rodents) [44]. Aberrant release of glutamate from pre-synaptic vesicles has been identified in the SOD1-G93A mouse model, where increased glutamate release appears to be an early event in the disease course of the model [45]. Studies in the SOD1-G93A mouse model have also shown that metabotropic glutamate receptors are highly expressed compared to wild-type mice and can induce abnormal glutamate release [46]. Further to this, knockdown of metabotropic glutamate receptor 1 in SOD1-G93A mice reduced astrogliosis and microgliosis, extended survival and increased the numbers of motor neurons surviving in the spinal cord [47].

2.2.5. Mitochondrial Dysfunction

Mitochondria exhibiting abnormal morphology such as vacuolation, swelling and membrane degeneration can be seen in the dendrites and axons of motor neurons in SOD1-G93A and SOD1-G37R ALS mouse models [48, 49]. Damage to mitochondria has also been linked to the accumulation of aggregated mutant SOD1, where the presence of the mutant protein correlates with an increase in mitochondrial volume and increased production of toxic superoxide free radicals [50] as well as potentially promoting apoptosis [50, 51].

2.2.6. The Role of Non-neuronal Cells

Non-neuronal cells have been also implicated in fALS. In an attempt to identify which cell types contributed to the ALS phenotype, mutant SOD1 was selectively expressed solely in the neurons [52, 53] or astrocytes [54] of mice. None of the models developed motor neuron disease and demonstrated the need for mutant SOD1 to be expressed in multiple cell types to generate a phenotype i.e SOD1-linked fALS is non-cell autonomous.

2.2.7. Cytoskeletal Elements and Axonal Transport

Aberrant accumulation of neurofilaments in the soma and axons of motor neurons are pathological hallmarks in both sporadic and familial ALS, as well as animal models of ALS [55-57]. A possible mechanism for neurofilament involvement in ALS is that accumulation of neurofilaments disrupts axonal transport of essential proteins in motor neurons [58].

2.3. TAR DNA-Binding protein (TARDBP) and Fused-in-sarcoma (FUS)

The TAR DNA-binding Protein (TARDBP) gene encodes the 43kDa protein TDP-43, a nuclear protein involved in multiple aspects of RNA processing including a major role in the splicing of genes relevant to the functioning of the CNS. Mislocated ubiquitinated wild-type TDP-43 is the major component of cytoplasmic aggregated protein inclusions seen in the CNS of >95% ALS patients (both sporadic and familial) and ~45% FTD patients [59, 60]. Mutations in TDP-43 are also the cause of ~4% of fALS cases and ~1% of fALS cases (Figure 1), although whether this is due to a toxic gain-of-function, loss-of-function or both mechanisms is unclear [3, 61, 62].

Mutations in the gene Fused-in-sarcoma (FUS) are responsible for 4% of fALS cases and 1% sALS cases (Figure 1) [3, 63, 64]. FUS is predominantly a nuclear protein but it is also found in cytoplasmic inclusions in some cases of ALS and FTD, where it is aggregated and sequestered [65].

3. RNA-mediated therapy for ALS/MND

Current developments in RNA-mediated therapy for ALS fall into two major categories: RNA interference (RNAi) and antisense oligonucleotide (ASO) therapy.

RNA interference (RNAi) is an endogenous mechanism of post-transcriptional gene regulation. RNA transcribed from nuclear DNA can form double stranded RNA (dsRNA) molecules and hairpin structures which are then cleaved by enzymes to produce short dsRNA duplexes of ~21nt. The dsRNA duplexes are then loaded onto the RNA-induced silencing complex (RISC), a complex of proteins which preferentially retains one of the RNA strands (termed the guide strand) whilst the other strand (termed the passenger strand) is degraded. Binding of the RNA guide strand-loaded RISC complex to mRNA transcripts of partial complementarity results in repression of gene expression due to blocking of the translational machinery or, in the case of full complementarity, directs degradation of the mRNA transcript.

This mechanism has now been engineered to provide a powerful tool for selective gene repression. RNAi can be achieved either by direct delivery of therapeutic RNA duplexes called small interfering RNAs (siRNAs) or by expression of hairpin structures that are then processed in a similar way to endogenous microRNA transcripts, often termed short hairpin RNAs (shRNAs). Exogenous si- and shRNAs can be engineered to be fully complementary to

the mRNA of a target gene, resulting in degradation of the transcript and a robust gene knockdown effect.

ASO's are single-stranded oligonucleotides which are designed to interact with complementary RNA transcripts. Binding of the oligonucleotides to pre-mRNA regulates gene expression through a number of different mechanisms including DNA-RNA duplex degradation by endogenous enzymes such as RNaseH, inhibition of correct splicing of pre-RNA and inhibition of translation of mRNA [66]. Therapeutic ASOs have been approved by the FDA, as for example Fomivirsen and Mipomersen, which are used to treat cytomegalovirus retinitis and homozygous familial hypercholesterolemia respectively.

3.1. RNAi for SOD1-linked fALS

As previously mentioned, over 150 ALS causing mutations in SOD1 have been discovered to date and these account for ~12% of fALS cases (Figure 1) [3]. RNAi mediated reduction in the level of both wildtype and mutant SOD1 protein by lentiviral (LV) delivery of shRNA targeting human SOD1 has been shown to delay disease progression and extend survival in mice carrying the SOD1-G93A mutation [67, 68]. This finding has been confirmed by Foust et al., (2013) who exchanged the lentiviral vectors for a recombinant adeno-associated virus serotype 9 (rAAV9) vector [66]. The group succeeded in slowing disease progression in the SOD1-G37R mouse model with treatment post- disease onset. Lifespan was extended by up to 39% when treatment was initiated at birth in G93A mice and SOD1 protein levels in motor neurons and glial cells were significantly reduced in non-human primates treated with the therapeutic shRNA construct. The group also demonstrated the ability of AAV9 to

efficiently transduce motor neurons and astrocytes *in vivo*, a property essential to any viral gene therapy delivery system aimed towards translation into human trials [69].

These findings suggest a strong potential for translation of AAV9 delivered shRNA-mediated SOD1 knockdown to the clinic, although undoubtedly the safety profile of this therapy will need to be extensively investigated before human clinical trials.

3.1.1. *Antisense oligonucleotides in SOD1-linked fALS*

A therapeutic ASO targeting SOD1 has previously been shown to increase survival in a SOD1-G93A mouse model from 122 ± 8 days to 132 ± 7 days, despite having no effect on disease onset or early disease (defined as the peak animal weight and the point at which the animals had lost 10% of their peak weight, respectively) [70]. The therapeutic oligonucleotide - ISIS 333611 - was the focus of a phase I human clinical trial completed in July 2012 (Table 1). The study, registered in Clinicaltrials.gov as NCT01041222, demonstrated that intrathecal delivery of ISIS 333611 to the CNS was well tolerated by patients and produced no adverse effects beyond those experienced by patients receiving placebo [71]. Measured concentrations of ISIS 333611 in both the CSF and plasma post-injection were in agreement with predicted values determined by pharmacokinetic studies in Rhesus monkeys, allowing future doses to be selected based on body weight scaling and CSF volume. SOD1 protein concentrations measured in the CSF of patients did not decrease dramatically as a result of the treatment, however the authors identify the intentionally low dose administered during the initial phase 1 safety study as the cause of this. Further to this, the authors predict that reduction of SOD1 mRNA and protein in the spinal cords of patients would necessitate 4 days continual administration of the highest dose of ISIS 333611 used in the study [71]. Despite the preliminary nature of these results, they do provide encouraging evidence that ASOs delivered intrathecally in patients is a tolerable and effective route of

delivery for ALS therapy, an important point to consider as ASOs are unable to cross the blood-brain barrier, in contrast to viral vector delivery of RNAi molecules. The absence of adverse effects when using ASOs could therefore be seen as a positive aspect of this approach.

3.1.2 *Antisense oligonucleotides in C9orf72-linked ALS*

ASOs have been used in a number of *in vitro* studies in an attempt to rescue pathological features of the C9orf72 expansion. Donnelly et al., (2013) showed that ASOs targeted towards different regions of C9orf72 mRNA were able to mitigate RNA toxicity in induced pluripotent stem cell (iPSC) -differentiated neurons from C9orf72 ALS patients through several mechanisms. ASOs targeted towards the expansion region were predicted to either disrupt the expansion region by preventing the formation of stable G-quadruplexes or to target the expansion for RNase-H-mediated cleavage. Neither of these approaches result in a reduction in cellular C9orf72 mRNA [17]. ASOs were also targeted to the coding region of C9orf72, resulting in RNase-H mediated cleavage and a reduction in both wildtype and expanded C9orf72 RNA. Treatment with these ASOs reduced the number of GGGGCC-RNA foci in the cells, normalised dysregulated gene expression for a number of candidate biomarker genes and rescued the glutamate excitotoxicity that had been observed in the iPSC cells [17]. Lagier-Tourenne et al., (2013) were also able to develop ASOs that selectively reduced GGGGCC (i.e. sense) RNA foci in C9orf72 ALS patient fibroblasts without affecting the overall level of C9orf72 encoding RNA [72]. The reduction in sense RNA foci however did not correct the observed pathological gene expression RNA signature described in patient fibroblasts by the group. The authors speculated that this may be a result of pathogenic CCCC GG (i.e. antisense) transcripts forming foci which were not degraded by the sense-

targeting ASOs. Lagier-Tourenne et al., (2013) also contrast their work with the finding that siRNA targeting C9orf72 mRNA resulted in a reduction in non-pathogenic RNA but no corresponding reduction in the number of nuclear RNA foci [72].

Both of these studies suggest a future role for ASOs in the treatment of C9orf72 mediated ALS, although a deeper understanding of the pathogenesis underlying the disease is needed to enable ASOs to be targeted against the most relevant mechanisms.

3.1.3 *Other potential targets for antisense oligonucleotide therapies in ALS*

The acetylcholinesterase enzyme (AChE) has been implicated in motor neuron injury and denervation of muscle in ALS [73-75]. It is known that the sera of ALS patients show elevated levels of AChE [74, 76], and this protein could potentially represent a new therapeutic target for ALS. Indeed, ASO therapy against AChE mRNA in SOD1-G93A pre-symptomatic mice was shown to slightly prolong their lifespan, with an attenuation of motor neuron loss [74].

Dysregulation of RNA processing is emerging as a major pathophysiological mechanism in ALS [Insert Hautbergue ref]. There is also increasing evidence for dysregulation of miRNAs in this disease [77]. In 2013 it was reported that endogenous miRNA-155 is significantly upregulated in the spinal cord tissue of both ALS rodent models and human ALS patients [78, 79]. In this study, specific ASOs designed to decrease the levels of miRNA-155 in SOD1-G93A mice prolonged survival in this murine model in comparison to scrambled control anti-miRNA [78].

AAV and LV-based vectors show strong potential for delivering shRNA to knockdown SOD1 mRNA in ALS mouse models [67-69]. Briefly, AAV vectors provide long-term transgene

expression, show minimal pathogenicity, low immunogenicity and are easy to manufacture at high titers in large-scale production for research. These features, along with their high capacity to transduce neurons, glial and ependymal cells, make them the most promising vehicle to carry out gene therapy in the CNS. The potential toxicity associated with LV-based vectors is also low, although there is a potential risk associated with genomic integration and insertional mutagenesis. However, LV provides a long-term transgene expression, a moderate scalable production of pure virus and can infect post-mitotic cells in the CNS efficiently as well as glial cells. These characteristics make them the second most commonly used viral vector for gene therapy in the CNS [80].

The characteristics of viral vectors mentioned above are important when comparing virally mediated RNAi therapies with ASO therapies. Virally mediated RNAi is advantageous due to the long term, stable expression that can be achieved at high efficiency in motor neurons and other cells of the CNS with a single delivery of virus. ASOs, however, must be delivered directly to the CNS and require multiple rounds of administration. More investigation into methods that could increase the stability of ASOs, as well as determining the impact of potential long-term toxicological issues, would be highly advantageous for future ASO therapies.

4. Delivery of neurotrophic factors for ALS

Neurotrophic factors are a diverse group of proteins that are responsible for encouraging growth and maintaining survival of neurons. A number of neurotrophic factors have been proposed as being a promising avenue for gene therapy in light of their beneficial effects on animal models of ALS. Among the factors which have shown promise in facilitating

neuroprotection in animal models are vascular endothelial growth factor (VEGF) [81-84], hepatocyte growth factor (HGF) [85], glial-derived neurotrophic factor (GDNF) [84, 86, 87], insulin-like growth factor 1 (IGF-1) [88-90] and granulocyte-colony stimulating factor (G-CSF) [91, 92]. The delivery of these neurotrophic factors can be carried out by using different approaches, with emerging viral vector-based and cell-based therapies among some of the most promising techniques.

4.1. Viral delivery of neurotrophic factors

In this section we will focus on the ability of AAV and LV vector systems to deliver neurotrophic factors, since these viruses have been the most used in the majority of studies published so far. Engineering AAV viruses to drive neurotrophic factor expression has been successfully tested in ALS mouse models. In the early 2000s it was reported that the intramuscular delivery of GDNF or IGF-1 to SOD1-G93A mice delayed the disease onset, improving behavioural tasks and prolonging lifespan [87, 90]. Vascular endothelial growth factor (VEGF) is another promising candidate which has been used as a therapeutic tool in ALS. In 2004, Azzouz et al. reported that the injection of LV driving the expression of VEGF into various muscles delayed ALS onset, improved motor function and increased survival in SOD1-G93A mice [81]. Targeting the expression of ectopic neurotrophic factors to the CNS could enhance the efficiency of transduction, as well as avoiding undesirable side effects resulting from the expression of neurotrophic factors in peripheral tissues. This can easily be achieved by using injections to directly affected areas of the diseased CNS. This approach has been already successfully tested, for instance IGF-1 delivered by AAV vectors injected into the CNS showed a beneficial effect in SOD1-G93A mice, increasing survival and partially rescuing the phenotype [88, 89, 93]. Neuroprotective effects were also observed in male

SOD1-G93A rats after intraspinal injection of AAV1 driving IGF-1 [94]. In 2010 it was reported that the delivery of IGF-1 and/or VEGF by intracerebroventricular (ICV) injection also prolonged lifespan in the mice, delaying the failure of motor functions. However, the authors of this study did not find a synergistic effect when both neurotrophic molecules were delivered at the same time, which could mean that IGF-1 and VEGF participate in the same signalling pathway [89]. Finally, beneficial effects from administration of an uncovered neurotrophic factor, G-CSF, were reported a few years ago. The intraspinal injection of AAV driving the expression of G-CSF in SOD1-G93A mice partially recovered their phenotype and increased lifespan [91].

4.2. Delivery of neurotrophic factors using mesenchymal stem cells

Mesenchymal stem cells (MSCs) are multipotent stem cells which can be isolated from bone marrow, or other mesenchymal tissue such as adipose tissue. Some studies have reported the efficacy of using MSCs to overexpress different neurotrophic factors, which can be used as an ex-vivo gene therapy tool in ALS models [84, 86, 95]. A major milestone was reached in 2009 when Suzuki et al. demonstrated the efficacy of this method [86]. In this study, human MSCs (hMSC) isolated from neonatal bone marrow and transduced with LV encoding GDNF were transplanted into the skeletal muscle of a SOD1-G93A rat model. This work showed a neuroprotective effect, with an increase by 18 days in the lifespan, as well as a reduction in both degeneration of motor neurons in the spinal ventral horn and denervation of neuromuscular junctions [86]. The same research group reported in 2013 that the engineering of hMSCs to ectopically express both VEGF and GDNF could delay the onset of

the disease by 6 days and more relevantly could prolong the lifespan of SOD1-G93A rats by 28 days, with the dual neurotrophic factors showing a synergistic effect in the maintenance of spinal motor neurons and neuromuscular junctions [84].

4.3. Neurotrophic factors being tested in clinical trials

Although much success has been observed in animal models, therapeutic delivery of neurotrophic factors has yet to be translated to success in human clinical trials. Most often delivered systemically as a drug, gene therapy techniques delivering neurotrophic factors have been subjected to clinical trials and will be summarised here.

4.3.1. VEGF clinical trials

SB-509 is a plasmid developed by Sangamo BioSciences for intramuscular injection that encodes a zinc finger protein transcription factor (ZFP-TF) which acts to upregulate endogenous VEGF. SB-509 showed an acceptable safety profile in a 2010 Phase II clinical trial (NCT00748501), where delayed deterioration in ankle and toe muscle strength was observed in 40% of treated subjects compared to 23% of baseline matched historical controls and 27% of the global control population [96, 97] (Table 1). Despite these results, as of 2014 Sangamo BioSciences appear to have pulled back from SB-509 in order to focus on ZFP-TF therapies for HIV/AIDS, with SB-509 not appearing in the “Product Pipeline” section of their website.

4.3.2. HGF gene therapy

A phase I/II safety study (NCT02039401) is currently recruiting for the purpose of testing the tolerability and safety of VM202, an intramuscularly delivered plasmid expressing multiple isoforms of HGF (Table 1). Developed by ViroMed Co., Ltd, VM202 was granted Orphan-Drug designation by the FDA on 14th February 2014 and the trial is being conducted at Northwestern University, Illinois [98]. In addition to ALS, VM202 is also being investigated as a treatment for critical limb ischemia and painful diabetic peripheral neuropathy.

5. Gene therapy to mitigate the ALS cell response

One of the main problems in treating ALS is its complex pathophysiology. As previously mentioned, the course of the disease can be strongly affected by different pathophysiological mechanisms including excitotoxicity, mitochondrial and endoplasmic reticulum stress, defects in RNA processing, defective axonal transport, protein misfolding/aggregation and oxidative stress. Some gene therapy studies have focused on trying to reverse or mitigate these pathophysiological elements of motor neuron injury.

To decrease oxidative stress, Nanou et al., in 2013 [99] showed promising results after delivering antioxidant genes, such as PRDX3 and NRF2, into cellular models of ALS using an LV vector system. However, the intramuscular delivery of these genes driven by AAV serotype 6 failed to rescue the SOD1-G93A mice phenotype. Low levels of transduction were observed in the CNS and the poor efficiency of AAV-6 in crossing the blood brain barrier could explain the disappointing lack of efficacy *in vivo* [99]. However, a better strategy to deliver these antioxidant genes may open a new therapeutic option *in vivo*.

A gene therapy strategy to block misfolded SOD1 protein has recently been published. AAV vectors were engineered to deliver a single chain fragment variable of the D3H5 antibody,

which is able to block the toxic misfolded SOD1 protein produced in the SOD1-G93A mouse model [100, 101]. The increase in lifespan was up to 40 days with an average of 16, and the levels of misfolded SOD1 protein in the spinal cord were decreased, along with a reduction in neuronal stress [101].

Inflammatory responses are upregulated in the spinal cords of ALS patients as well as in SOD1-G93A mice. The use of anti-inflammatory molecules to protect injured motor neurons was recently tested. In a study published in 2015, intraspinal injection of AAV driving the expression of murine interleukin 10, an anti-inflammatory cytokine, in newborn SOD1 G93A mice prolonged their expected lifespan [102].

One of the most relevant clinical consequences of ALS is muscle weakness and eventual paralysis. Recently, Jackson *et al* demonstrated rescue of TDP43-induced forelimb paralysis in rats by viral delivery of human upframeshift protein 1 (hUFP1), a protein involved in nonsense-mediated decay of mRNA transcripts containing premature stop codons [103].

The cause(s) of many cases of sporadic ALS (sALS) is currently unknown (Figure 1). Although the variability between cases can be broad, it seems that the presence of typical TDP43 positive cytoplasmic inclusions in motor neurons is a common pathological hallmark in the majority of sALS cases [104]. TDP43 pathology can correlate with a reduction in the levels of ADAR2, a pre-mRNA editing enzyme which is involved in the Ca²⁺ entry through AMPA receptors channels [105]. In 2013, Yamashita *et al.*, engineered AAV to drive the expression of ADAR2 in ADAR2 knockout mice, a mechanistic mouse model for sALS. In this study, motor neurons were rescued by normalizing the expression of TDP43 and the progression of motor dysfunction was prevented, which may be considered in the future as a new gene therapy approach for the treatment of sALS [106].

6. Expert Opinion

The delivery of therapeutic molecules to spinal motor neurons and glial cells remains a major challenge. The lack of effective neuroprotective therapy for ALS can be attributed to a combination of five key challenges: (1) Poor understanding of the molecular pathogenesis of ALS; (2) Delivery of therapeutically attractive agents has been hampered by inefficient delivery methods and other factors like the blood-brain barrier (BBB); (3) Ineffective targeting of therapeutic agents specifically to the diseased CNS site and/or cell type; (4) The fast progression of the neurodegenerative process in this condition leading to a very short window for therapy administration; (5) Existing animal models of ALS failed to translate apparently efficacious therapies into clinic. These problems must be overcome to develop fully effective treatments for ALS.

Additionally, while allele-specific targeting of certain genes in some heritable diseases is both an achievable and preferential approach (e.g. targeting of mutant huntingtin in Huntington's disease), the wide variety of both ALS-linked genes and the multiple mutations within these genes associated with ALS makes this approach difficult. The pre-clinical development and safety profiling required for each allele-specific construct would be extremely time-consuming and prohibitively expensive. This, coupled with the reluctance of pharmaceutical companies to commit large amounts of resources to therapies that may be of benefit to only a small number of patients, further hinders the development of gene therapies in ALS. Forms of ALS in which a single gene is implicated, such as SOD1-linked familial ALS, are a very attractive target for gene therapy. Investigations in SOD1-null mice, demonstrating that complete ablation of endogenous wildtype SOD1 is non-lethal and does

not lead to an ALS phenotype, lend weight to the approach of non-allele specific gene knockdown in this ALS subtype.

Several proteins including neurotrophic factors have been reported to be potential therapeutic options and have been tested in clinical trials in ALS patients. Unfortunately all of these trials failed to show efficacy prompting us to believe that inappropriate delivery approaches of these agents is part of the problem. A further confounding factor in the translation of these therapies from animal models to human trials is the use of potentially unsuitable animal models of disease. Although rigorously studied and defined, commonly used disease models may prove problematic if results generated in them cannot be replicated in patients. Complex diseases such as ALS may require significant improvements in *in vitro* and *in vivo* models before therapies are able to make a successful transition from the bench to the clinic. Major advances in multiple technologies, including gene therapy and recent discoveries of new ALS genes, offer a window of opportunity and optimism to substantially change the pace of translational research in the field of motor neuron diseases. Viral vectors have been refined to the highest level of safety and efficiency. Multiple animal studies have now been undertaken with viral vectors and major effects, in terms of amelioration of disease models, have been obtained. These studies provide great optimism for the future utility of viral vector delivered gene delivery as a therapeutic strategy for ALS in man. However, translating these strategies into human clinical trials remains a challenge due to factors including the lack of specific targeting of CNS tissue. Another major hurdle is the manufacture of the large quantities of therapeutic vectors required for clinical applications which, though achieved in several trials of viral gene delivery, remains both extremely expensive and so far only attempted in studies using a limited number of patients in early stage trials. The amount of virus needed for gene therapy trials is of course affected

by a number of factors. For example, i.v. delivery for systemic treatments requires larger quantities of virus than sub-retinal injections to treat macular degeneration. The assumption that ALS patients will be treated during adulthood rather than as children would also necessitate a greater viral dose. As it stands, the scaling-up of vector manufacturing for Phase II/III trials and potentially to the point of commercial viability remains a challenge. The hope is that as viral vector-based delivery becomes a more commonplace gene therapy technique, the burden of large-scale viral vector production and manufacturing design will be taken up by existing or developing biotechnology companies.

Of the mentioned clinical trials, it will be interesting to see the development of the SOD1 targeting ASO study (NCT01041222), as any beneficial effects seen in patients by knocking down SOD1 protein levels will also lend credibility to efforts at achieving similar results by RNAi mediated knockdown of SOD1. Positive results stemming from the ASO study will also increase confidence in the safety of this technique, which is likely to be beneficial for studies targeted towards the treatment of other forms of ALS as well as other neurodegenerative diseases using ASOs.

The study involving plasmid delivery of hepatocyte growth factor is still in the recruiting phase but it will be interesting to see if this study manages to generate the positive results that previous neurotrophic factor studies have so far failed to deliver.

Finally, the main achievements in gene targeting for ALS reviewed in this paper have been summarized in table 2, where the studies have been categorized based on the gene therapy system used in each case.

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Figure legends

Figure 1: The diagram shows the prevalence of familial and sporadic ALS cases, as well, the percentage of ALS explained by each gene in populations of European ancestry [3]. Adapted from Renton et al., [3] by permission from Nature Publishing Group.

Table legends

Table 1: Summary of gene therapy clinical trials carried out or ongoing in ALS.

Table 2: Summary of the main gene therapy studies performed so far in ALS models.

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