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# Advances, Challenges, and Perspectives in Translational Stem Cell Therapy for Amyotrophic Lateral Sclerosis

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## Abstract

Finding an effective therapeutic approach is a primary goal for current and future research for amyotrophic lateral sclerosis (ALS), a fatal neurological disease characterized by degeneration and loss of upper and lower motor neurons. Transplantation approaches based on stem cells have been attempted in virtue of their potential to contrast simultaneously different ALS pathogenic aspects including either the replacement of lost cells or the protection of motor neurons from degeneration and toxic microenvironment. Here, we critically review the recent translational research aimed at the assessment of stem cell transplantation safety and feasibility in the treatment of ALS. Most of these efforts aim to exert a neuroprotective action rather than cell replacement. Critical aspects that emerge in these studies are the need for the identification of the most effective therapeutic cell source (mesenchymal stem cells, immune, or neural stem cells), the definition of the optimal injection site (cortical area, spinal cord, or muscles) with a suitable administration protocol (local or systemic injection), and the analysis of therapeutic mechanisms, which are necessary steps in order to overcome the hurdles posed by previous in vivo human studies. New perspectives will also be offered by the increasing number of induced pluripotent stem cell-based therapies that are now being tested in clinical trials. A thorough analysis of recently completed trials is the foundation for continued progress in cellular therapy for ALS and other neurodegenerative disorders.

**Keywords** Amyotrophic lateral sclerosis · Motor neuron · Mesenchymal stem cells · Regulatory T cells · Neural stem cells · Induced pluripotent stem cells · Stem cell transplantation

## Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating neurodegenerative disorder which provokes the progressive degeneration of upper and lower motor neurons [1]. The disease starts with insidious focal muscle weakness, frequently in one hand, and then disseminates relentlessly to affect most skeletal muscles, leading to complete paralysis. Death occurs approximately 3–5 years after symptoms onset, mainly because of respiratory failure [2]. So far, no therapy was shown to provide a substantial clinical benefit for ALS patients. Up to now, FDA has approved only two treatments, riluzole, which prolongs median survival by about only 2 to 3 months [3] and edaravone, which slightly reduces the rate of decline in the early stages of disease [4–6].

Since ALS still represents a devastating disease with a significant impact on patients, caregivers, and society, effective treatments are urgently needed. The lack of therapeutic tools could be ascribed, at least in part, to incomplete knowledge of the pathogenetic basis of ALS motor neuronal degeneration. In this context, why is stem cell therapy so fascinating and potentially useful for ALS? Stem cell transplantation could potentially tackle the multifaceted and largely unknown ALS disease pathogenesis through multiple mechanisms, such as by replacing lost or diseased cells, by introducing factors that will provide neuroprotective effects or by modulating the pathogenetic pathways linked to toxic microenvironment [7]. Regarding paracrine delivery, growth factors have been shown to exert neuroprotective effects when delivered in a variety of motor neuron models [8]. However,

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trials focusing on the peripheral delivery of these factors in ALS patients yielded disappointing results, possibly because of the interference of the blood–brain barrier (BBB) [9]. Conversely, stem cells of various origin have the potential to secrete growth factors directly in the CNS when delivered intrathecally [8]. A further bystander effect of stem cells is immunomodulation, in particular when using anti-inflammatory molecules-producing immune cells or mesenchymal stem cells [8, 10, 11]. These findings are especially relevant considering the growing evidence for the role of neuroinflammation in ALS pathogenesis [6, 11].

However, despite the abundance of preclinical data concerning stem cells manufacturing, engineering or transplantation, no stem cell-based therapy has been approved for ALS yet, and there are relatively few rigorous safety and efficacy trials of stem cell transplantation conducted in this field (already reviewed in [12, 13]).

This article will critically review the advances and results of recently performed or undergoing cell transplantation trials in ALS, considering those of larger size and with planned further development, included in clinicaltrials.gov, offering a basic research perspective on the rationale of using stem cells as well as discussing the hurdles to advance this approach towards a clinically meaningful therapeutic strategy.

## Therapeutic Mechanisms of Stem Cell Transplantation

Several preclinical in vitro and in vivo studies already pointed out the beneficial effects of NSCs, MSCs, and immune cells in addressing ALS pathogenetic events and moderately reducing the rate of progression (already reviewed in [7, 12]). Surely, the identification of therapeutic strategies directed against the pathogenic hits in ALS is essential to find therapies that halt disease progression. However, the only possibility to restore motor neurons function, once lost, would be cell replacement. Transplantation of motor neurons derived from human pluripotent stem cells and neural stem cells (NSC) in order to boost motor neuronal differentiation has been already explored in murine models [14–18], but different practical and physiologic obstacles limit the feasibility of direct motor neuron replacement. In fact, transplanted motor neurons have to connect with both the corticospinal tract and the periphery, projecting their axons through the adult white matter, and reaching the muscular compartment to form functional neuromuscular junctions. Indeed, the diseased host spinal cord might not properly aid the survival of engrafted motor neurons [19]. For all these reasons, direct motor neurons replacement by cell transplantation remains an unpractical therapeutic solution for ALS.

However, stem cells may act not only through direct replacement of diseased cells but also through the modulation of a variety of mechanisms which contribute to the survival and functionality of resident cells (Fig. 1). For example, replacement of interneurons, which have shorter connections and might modulate motor neuron function, represents a more suitable approach, although it has not been extensively explored yet. In addition to that, transplantation of stem cells committed to a glial phenotype has been tested as well. During the last decades, several studies have pointed out that the process of motor neuronal degeneration does not spring from intrinsic cellular defects, but it is crucially determined by diseased astrocytes and microglia, through mechanisms that likely include lack of trophic support, defective neuronal–glial communication, and secretion of toxic substances [11]. Evidence from experimental models suggests that the presence of a sufficient number of healthy glial cells around diseased motor neurons can halt ALS progression [20]. Astrocytes or microglia replacement appears more technically feasible since these cells have a less complex organization, network, and soma extension. It was observed that, following intrathecal transplantation, both rodent and human astrocytes were capable of surviving and integrating into the spinal cord of familial ALS rodents, without being negatively influenced by adverse environmental conditions [21]. Grafted cells exerted several beneficial effects, spanning from an increase in survival to the protection of resident motor neurons and, in some cases, an amelioration of motor symptoms [22].

Furthermore, transplanted stem cells may also act via a powerful bystander effect, secreting trophic factors, scavenging toxic ones, and promoting neoangiogenesis and axonal sprouting (Fig. 1) [8]. Given that one key event in ALS degenerative cascade is the denervation of the neuromuscular junction and the retraction of the distal part of axon [23], the delivery of neurotrophic factors (i.e., GDNF or IGF1/2) or extracellular vesicles on the body or axons of anterior horns motor neurons might stabilize the neuromuscular junction and promote collateral axonal sprouting [24–26]. In this perspective, the administration of stem cells that secrete trophic factors inside the central nervous system (CNS) or in the muscles could be a potential approach to offer a bystander neuroprotective effect for ALS motor neurons. In addition to that, inflammation likely plays a central role in this disease, and some stem cells might have the ability to modulate inflammatory pathways and to promote the tissue repair mechanisms through endogenous stem cell activation or suppression of microenvironment neurotoxic activity [6].

## Essential Steps in Clinical Translation of Stem Cell Research

Moving stem cells research from bench to bedside requires a thorough knowledge of their mechanism of action and of their expected interactions with human organism. Otherwise, it would be difficult to predict with a relative degree of certainty

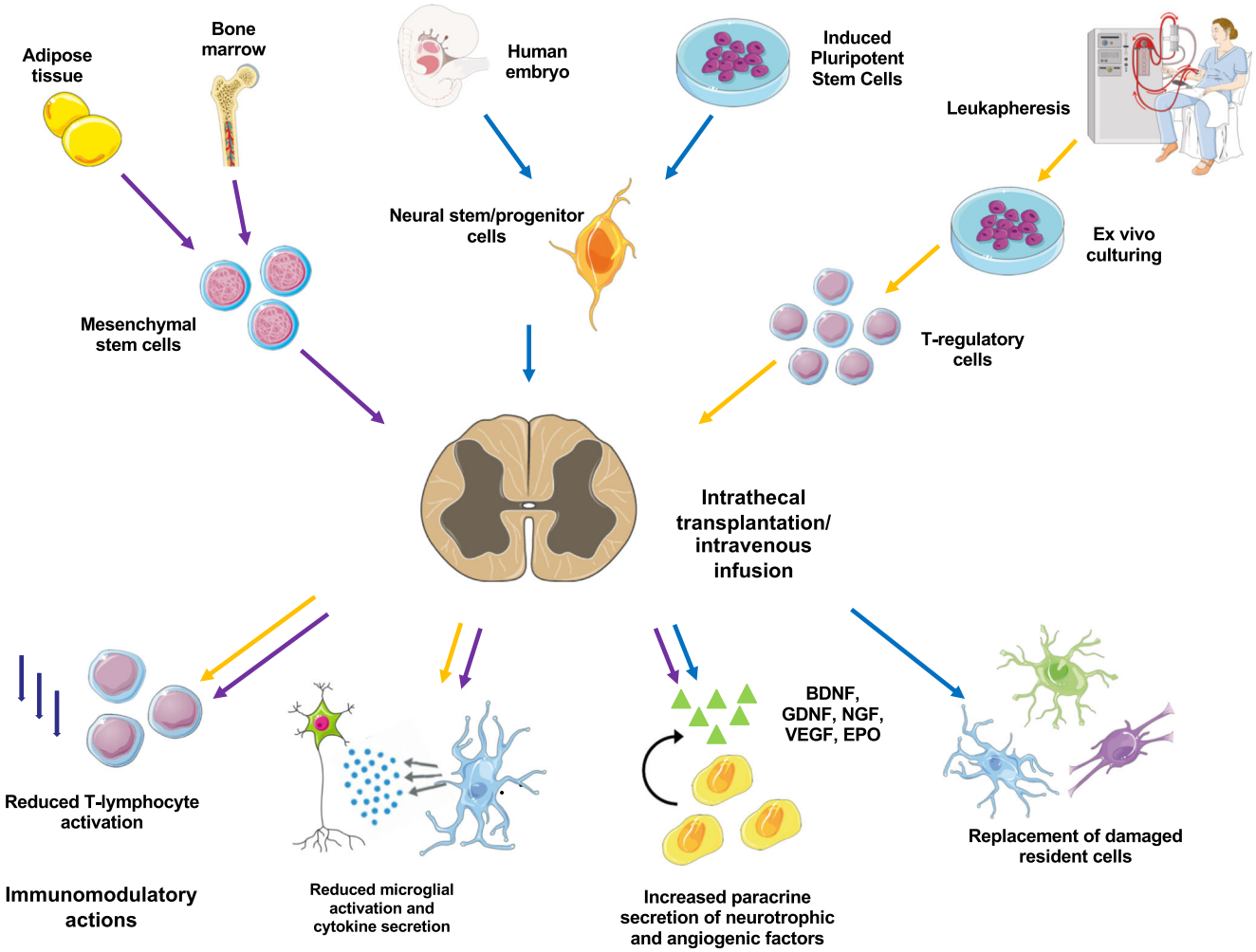


**Fig. 1** Recent clinical trials of cell transplantation in ALS tested three major cell sources: mesenchymal stem cells, neural stem/precursor cells, and immune cells. All of these cells have different origin and properties and potentially exert a different therapeutic action (indicated by arrows of different colors). Immune and mesenchymal cells have been injected locally, into the brain or spinal cord, but also in the blood and cerebrospinal fluid, whereas NSCs can undergo only local delivery as they are not able to cross the blood–brain barrier. Transplanted cells exert their beneficial effects through paracrine secretion of neurotrophic and angiogenic factors, reduction of inflammation, i.e., by reducing effector T lymphocytes proliferation and microglial activation, and replacement of resident interneurons and glial cells

how transplanted cells could migrate, interact with endogenous cell population and contribute to tissue repair. These data are then essential to develop effective delivery strategies and pharmacological protocols in order to achieve desired clinical outcomes. To reach this goal, we thereby need appropriate in vitro and in vivo models, which accurately reproduce human anatomy and the molecular and cellular mechanisms of ALS disease.

However, considering the current preclinical scenario, such a model is still lacking, especially for sporadic-onset ALS. In fact, in contrast to the genetic forms of ALS, the absence of a known gene defect makes this disease extremely puzzling to reproduce in vitro and in vivo. Furthermore, its complex neuropathologic features and the involvement of multiple cellular lineages make it even more difficult to test the efficacy of potential therapies at a preclinical level.

Another crucial issue is the reduced survival of cells after transplantation, particularly into the hostile ALS environment [8], which makes targeted stem cell manipulation to promote survival an essential target for intervention. To achieve that, different methods of stem cell engineering and manipulation have been developed, such as pharmacological or hypoxic preconditioning, delivery of NSCs within bioengineered scaffolds, and ex vivo genetic manipulation of cells prior to transplantation, for example, to increase expression of neurotrophic genes or to suppress stress responses (Fig. 2) [8]. Immunosuppression protocols might as well be helpful in critically promoting cell survival [8].

Another challenge to be addressed is the confirmation of graft survival. In animal models, this information is collected through immunohistochemical identification with human-specific markers [7]. In the perspective of clinical translation, alternative methods should be developed. In vivo molecular imaging might allow tracking of transplanted cells and of the modification of key pathogenetic events after treatment, like

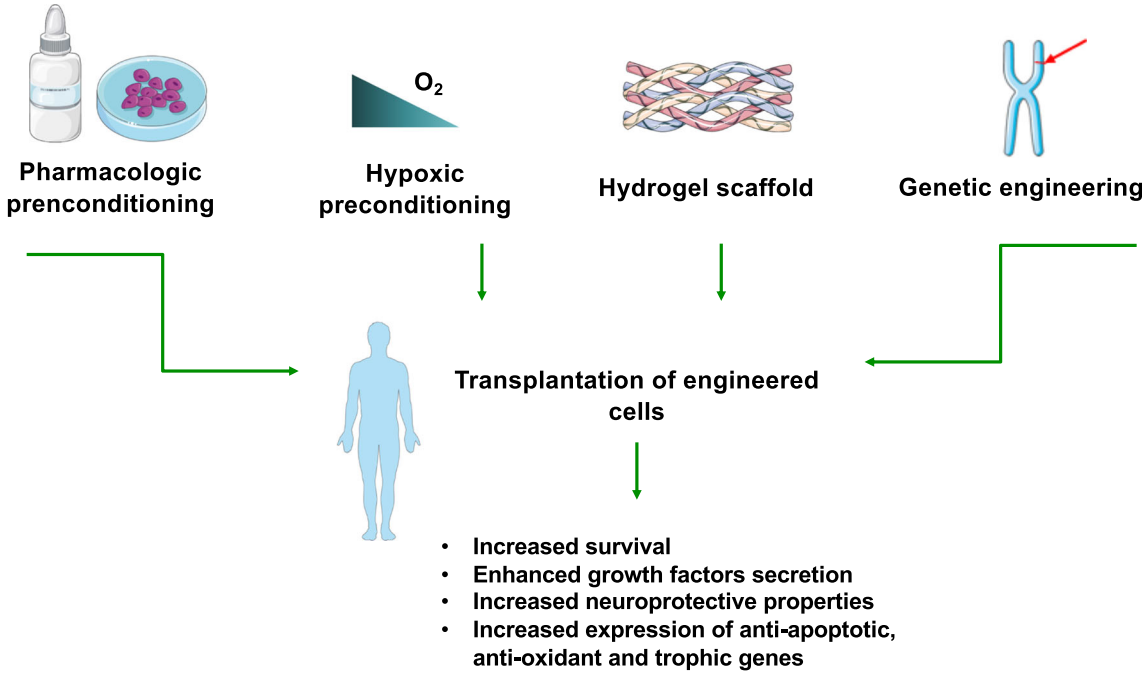


**Fig. 2** Engineering strategies to boost graft integration and survival within the host tissue include hypoxic and pharmacological (minocycline, adjudin, interleukin-6, BDNF) preconditioning, scaffolding of stem cells within biological matrices, and genetic modification to overexpress pro-survival or trophic genes. Upon transplantation within the CNS, engineered cells display lower apoptosis rates and greater proliferative abilities. Moreover, they demonstrate increased neuroprotective properties, as they enhance endogenous neural progenitors' proliferation and colonization of diseased tissues. Furthermore, treated cells also express greater levels of anti-apoptotic (HIF1a, Bcl-2), anti-oxidant (iNOS, SOD2, catalase, Nrf2), and trophic (VEGF, EPOR) genes. Notably, these cells also suppress cytokines release and microglial activation, while stimulating the secretion of neurotrophic factors

inflammation. These techniques have been extensively studied and validated in the context of myocardial stem cell transplantation and are based on the detection via different imaging modalities (positron emission tomography, single-photon emission tomography, magnetic resonance imaging) of in vitro-labeled cells [27]. Another possibility is the use of genetic engineering strategies, such as reporter gene imaging, which is based on the use of reporter genes (vectors) transduced into stem cells, which are translated into mRNA and then to a reporter protein with specific affinity to an imaging reporter probe [27]. Another option is the use of MRI-trackable scaffolds [28].

## Cell Sources and Delivery Routes

Thus far, different stem cell types have been assessed in preclinical and clinical trials for ALS, despite some of them do not possess the bona fide properties of stem cell, namely self-renewing and pluri/multipotency, but are more properly classified as precursors or even differentiated cells. Here, we will consider recent clinical trials testing three major cell sources: mesenchymal stem cells (MSC), immune system cells, and neural stem/precursors cells (Fig. 1). All of these cells have different properties and potentially exert different therapeutic action. Indeed, the same label, for instance "neural stem cells," may include many different cell populations, depending on their origin (i.e., fetal CNS or in vitro differentiation from pluripotent stem cells) and on derivation and culture protocols [7]. Furthermore, bulk culture consists of different cell populations with various degrees of stem cell properties and differentiation that can influence the characteristics and quality of the final therapeutic products.

Recently, induced pluripotent stem cells (iPSCs) obtained from somatic cells of adult patients have raised hopes as an alternative, autologous source for cell transplantation, overcoming ethical concerns related to the use of fetal, or embryonic tissues [29]. Furthermore, iPSCs databases reporting iPSCs' HLA signature could represent a promising technique to obtain quality-controlled cellular products and abate the risk of graft rejection. The use of human iPSC-derived neural stem/progenitor grafts has been already explored in ALS rodents, showing some degree of efficacy [30–32], but not in humans. Recently, the first patient affected by Parkinson's disease has been transplanted with iPSC-derived dopaminergic neurons and an increasing number of iPSC-derived cellular therapies are being tested in clinical trials [33]. The first iPSC-based clinical trial in ALS patients is going to start in 2019 [12].

Furthermore, differentiation of iPSCs into small CNS-like structures, the so-called brain organoids, provides the

unique opportunity not only to reproduce the human brain development and disease but also to test a novel source for cell transplantation. Intracerebral grafting of human brain organoids in rodents has been successfully attempted. Upon implantation, engrafted mini-brains integrated into the host CNS, showing progressive neuronal and glial differentiation, developing a vascular network, and outgrowing axonal connections in multiple regions of the host brain [34]. It has yet to be evaluated whether transplantation of tridimensional cultures or cells dissociated from tridimensional CNS organoids present advantages compared with conventional cellular grafts.

In addition to cell type selection, one of the most critical aspects of the transplantation approach is the method of cell delivery, which should balance the need for a minimally invasive injection strategy with the necessity of a widespread cell distribution along the neuraxis. The ideal route of delivery would allow to obtain the best therapeutic effect with the minimal invasiveness. To achieve a meaningful effect, cells should distribute uniformly along the CNS and reach both upper and lower motor neurons. Despite the reduced incidence of side effects, however, less invasive methods might be unable to warrant sufficient engraftment. Intrathecal and systemic intravenous strategies are both non-invasive, repeatable administration methods, and they could fit the needs of ALS researchers and patients. Nevertheless, some cellular lineages might not be able to pass the blood–brain barrier when delivered intravenously. Immune and mesenchymal cells have been injected locally, not only into the brain or muscle but also in the blood and cerebrospinal fluid (CSF) [7]. However, the real ability of these cells, particularly MSCs, to cross the BBB or the meninges, has not been fully demonstrated yet [35]. Indeed, it is possible that the vast majority of MSCs after injection into the CSF remains on the meninges, possibly releasing soluble factors. Theoretically, T cells can cross the BBB, but this event is highly regulated by a series of adhesion molecules and chemokines and in health, the rate of this passage is minimal [36]. However, a subset of hematopoietic stem cells, with specific cell adhesion molecules or chemokines receptors, might migrate more efficiently into the parenchyma after systemic delivery [37].

Conversely, local brain or spinal cord injection is the most tested route for NSC/NPC therapies, which could not penetrate into host CNS after blood or CSF administration. However, subset of NSCs, with specific cell adhesion molecules, might migrate into the parenchyma after intrathecal delivery and warrant more extensive investigation [31, 32].

A novels alternative is the use of new stereotaxic devices, perhaps MRI-guided, that could facilitate neural progenitor cell (NPC) delivery [38]. It was suggested that the guide of a robot could improve the speed and reduce the invasiveness of intraspinal injection. One of the robotic systems proposed is the so-called SpinoBot, which, under MRI guidance, is capable of performing percutaneous injection into the spinal cord [39]. The development of these devices could prove very helpful for research in this field. However, focal delivery might not be able to achieve a successful distribution around a sufficient number of motor neurons across neuraxis to have a therapeutic impact.

Another important aspect to consider is the optimal number of cells to be delivered since a dose-dependent effect is expected.

## Design of Stem Cell-Based Clinical Trials

In the perspective of clinical translation, the selection of an appropriate patient population, which might receive the major benefit from cell-based therapies, is essential. As in ALS pharmacological studies, disease duration for inclusion could be 2 years from onset of symptoms at longest, with an age range of 18 to 65 years. Younger patients may benefit more from these treatments, as already pointed out in Parkinson's disease, because of higher CNS reparative ability and plasticity [40]. In order to establish the magnitude of treatment efficacy with a small number of patients, enrolled subjects should belong to a homogeneous group with regard to disease progression (e.g., a similar decline in the ALSFRS-R and FVC in the 3–6 months before treatment). To this purpose, patients with rapid progression would be more likely to experience a significant modification in outcome measures after effective treatment. On the other hand, in these patients, the disease could be too aggressive and barely modifiable by treatments. Thus, ALS patients with intermediate progression appear to be the most suitable patient group for these trials.

Another important issue is the design of the study. Given the rare incidence of the disease and the absence of alternative treatments, the majority of trials conducted so far did not include a placebo-treated arm for measuring efficacy. Therefore, a consistent placebo effect is likely present, as expectations regarding stem cell treatments are high and 63% of patients reported a dose-independent subjective amelioration [41].

Phase I/II cell-based clinical trials showed an overall general safety of MSCs, immune cells and NSCs transplantation. Some studies detected a possible transitory clinical benefit after transplantation, generally indicated by modifications in the progression rate of the ALS Functional Rating Scale-Revised (ALSFRS-R) score and forced vital capacity (FVC), but they do not provide definite evidence of efficacy in ALS patients, being open label and uncontrolled. Therefore, there is a need for rigorous well-designed, randomized clinical trials, based on robust preclinical efficacy data.

In the following paragraphs, we will present some of the major recent ALS human trials, classified by cell type and route of administration.

## Mesenchymal Stem Cells Transplantation into the CSF or Muscles

The term "mesenchymal stem cell" mainly refers to bone marrow stromal cells. When bone marrow single-cell suspensions are cultured, a subset of rapidly adherent fibroblast-like cells originating from bone marrow connective tissue rapidly adheres to the plate and starts to replicate. These are non-hematopoietic cells and are able to form bone, cartilage, connective tissues, and adipocytes [35, 42]. It was hypothesized that MSCs could differentiate into other mesodermal cells and perhaps even into cell types with a different embryonal origin. However, even if these cells show numerous differentiation capacities, i.e., into bone/cartilage, they cannot differentiate into neurons [35, 42]. Contradictory findings of the so-called MSCs, including their origins, developmental capacity biological functions, and possible therapeutic uses, have prompted biologists, clinicians and scientific societies, and even the author that first identified MSCs to recommend the term to be revised [43]. In fact, scientific confusion about MSCs could facilitate the sale of unproven treatments to patients outside clinical trial and drug agencies' approval [43].

The relative ease of in vitro harvesting and expansion of MSCs from patients' bone marrow, thus allowing autologous transplantation with no or minimal immunosuppression required, prompted their application in cell transplantation in neurologic diseases including ALS [44, 45]. The possible drawback of using autologous cells is that they may retain key disease epigenetic signature, with a possible negative influence on end therapeutic effect. MSCs exert their beneficial effects through paracrine secretion of neurotrophic and angiogenic factors, reduction of inflammation, i.e., by reducing T lymphocytes activation, and reduction of CNS apoptotic cell death [Fig. 1] [44, 45]. Furthermore, MSCs seem to modulate the immune system also by increasing M2 phenotype. The rationale for using these cells in ALS include preclinical safety and efficacy data in wild-type and rodent ALS models, even if with a limited impact on disease progression [44, 45].

MSC-based clinical trials of phase 1 and 2a were primarily focused on evaluating safety and tolerability, with some efficacy measures included (Table 1). A further phase 2 trial of intrathecally injected of autologous adipose-derived MSCs in 60 ALS patients is planned to start at the Mayo Clinic. The study was designed as an open-label trial with safety, ALSFR, FVC, and MRI as outcome measures. The chief conclusion that emerges from these studies is that intrathecal MSCs injection seems overall safe and well tolerated, even if it can occasionally result in transient infusion reactions. Lumbosacral radiculitis (associated with CSF protein elevation and pleocytosis or with nodularities and enhancement in MRI study) was reported as a side effect in one study which used the highest dose of adipose tissue-derived MSCs rather than bone marrow-derived MSCs and monitored patients with spine MRI and lumbar puncture [41]. Imaging and CSF follow-up were not performed in other studies, thereby warranting the inclusion of these measures in future trials.

In a recent unblinded study performed in South Korea [48], a reduction in the rate of decline of ALSFRS-R scores following treatment could be observed. Moreover, the treated group displayed reduced levels of proinflammatory cytokines and elevated levels of anti-inflammatory cytokines. Notably, good responders also showed an inverse correlation of transforming growth factor β1 with monocyte chemoattractant protein-1. However, there was no significant difference in long-term survival between groups. Overall, this study suggests that repeated intrathecal injections of MSCs are safe and could possibly exert beneficial effects lasting at least 6 months, in ALS patients. MSCs might explicate their therapeutic action by favoring the transition from pro- to anti-inflammatory conditions. A future randomized, double-blind, large-scale phase 3 clinical trial with additional MSC treatments is needed to assess long-term efficacy and safety.

Here, we present in extensive details one of these MSC transplantation strategies, selected because of the study size and possible future development. This approach is based on the transplantation of patient-derived bone marrow MSCs stimulated with growth factors and small molecules (such as dibutyryl cAMP, basic FGF, PDGF, and heregulinb1) in vitro to enhance their survival [46] and promote secretion of neurotrophic factors (GDNF, BDNF, VEGF, and HGF). It has been reported that treated cells can be distinguished from their MSCs of origin by a unique miRNA expression profile [49], but the persistence of a specific committed secretory phenotype has not been demonstrated yet.

This cell product, called MSC-NTF or NurOwn, was developed in 2007 from BrainStorm Cell Therapeutics. The results of phase 1 and 2a open-label studies on autologous MSCs-NTF transplantation in subjects with ALS were recently published [46, 50]. MSCs were injected into the patients either intrathecally (in late-stage patients), intramuscularly (in early-stage patients), or both (in early-stage patients), with a single administration. The primary end point was assessing the safety and tolerability of this method. Secondary end points comprised the impact of therapy on different clinical measures, such as the ALSFRS-R score and the FVC. Results showed that the treatment was generally secure, despite the relatively large number of cells injected, and well tolerated over the follow-up period. The majority of adverse reactions were bland and transitory, the most common being headache and fever after infusion, and no treatment-related serious adverse event was reported. IT and IT+IM-treated patients displayed a slight decline in the progression of the FVC and of the ALSFRS-R score (from − 5.1 to − 1.2%/month percentage predicted FVC, $p < 0.04$ and from − 1.2 to 0.6 ALSFRS-R points/month, $p = 0.052$) in the subsequent 6 months if compared with the pretreatment period. Interestingly, this decrease

**Table 1** Recent clinical trials of mesenchymal stem cells transplantation

| Cell source | Dose | Mode and site of delivery | Number of patients | Follow-up (months) | Adverse events | Refs. |
|---|---|---|---|---|---|---|
| Autologous bone marrow | $1 \times 10^6$/kg × 2 doses | Lumbar intrathecal infusion | 8 enrolled, 7 treated | 12 | Flu-like illness (62.5%), back pain (50%), headache (25%) | Oh et al. [44] |
| Autologous bone marrow | Phase 1/2: $1 \times 10^6$/kg<br>Phase 2a: $1 \times 10^6$/kg, $1.5 \times 10^6$/kg, $2 \times 10^6$/kg | Lumbar intrathecal infusion ± intramuscular injection | Phase 1/2: 6 intrathecal, 6 intramuscular<br>Phase 2a: 14 intrathecal + intramuscular | 6 | Headache (50%)<br>Fever (42%)<br>Back pain (30%) | Petrou et al. [46] |
| Autologous adipose tissue | Escalating $1 \times 10^7$ to $1 \times 10^8$ × 2 monthly infusions | Lumbar intrathecal infusion | 27 enrolled and treated | 24 | Dose-dependent lumbosacral polyradiculitis (80% of patients receiving the highest dose) | Staff et al. [43] |
| Autologous bone marrow | $1.5 \pm 4.5 \times 10^7$ | Lumbar intrathecal infusion | 26 enrolled and treated | 12 | Headache (30%) | Sykova et al. [47] |

in disease progression rate was observed only in patients who received IT administration, but as the study did not include a placebo group, no conclusions about efficacy could be derived.

A placebo-controlled, randomized, double-blind multicentric phase 2 study (NCT02017912) of autologous MSC-NTF cells in patients with ALS (NurOwn) took place in the USA between 2014 and 2016 and enrolled 48 participants. Autologous MSCs delivered with a combined intramuscular (24 injections, 2 million cells each into biceps and triceps) and intrathecal injection (125 million cells in a 4-mL volume). Findings are still unpublished.

A pivotal randomized, double-blind, placebo-controlled phase 3 trial, which uses repetitive dosing, is currently being conducted at multiple US sites (NCT03280056), funded by a grant from the California Institute for Regenerative Medicine (CIRM CLIN2-0989). The study plans to recruit 200 ALS patients assigned in a randomized fashion into two groups to receive NurOwn or placebo and will use ALSFRS-R analysis a primary efficacy outcome measure. Autologous NurOwn (MSC-NTF cells) are transplanted intrathecally through standard lumbar puncture. Enrolled patients must be younger than 60 years old, with disease duration less than 2 years and rapid progression.

Even if transplantation of MSC seems overall safe and well tolerated at given doses, provided that the therapeutic product is properly prepared, there are several important questions to be addressed, including the definition of the precise biodistribution after CSF injection, the persistence of the graft after transplantation, the maintenance of the desired ex vivo induced phenotype, and the duration of its functional effects. These aspects will influence also the frequency of MSC administration. Overall, it is of paramount importance to clarify the real therapeutic meaning of MSCs in ALS, also by means of further preclinical studies, and through the identification of in vivo biomarkers in order to define their effect in ALS disease.

## Intravenous Infusion of Immune Cells

The most consolidated and widely used SC-based treatment in humans is the transplantation of hematopoietic stem cells (HSC) for the treatment of hematological disorders. Indeed, bone marrow transplantation has been used for clinical purposes for more than 40 years [51]. Furthermore, transplantation of T cells genetically modified with chimeric antigen receptors (CAR-T cells) had recently raised hopes as a novel immunotherapeutic approach for the treatment of hematologic malignancies, particularly B cells tumors [52]. Unique and favorable properties of HSCs or their progeny include the ease of collection from peripheral blood or bone marrow and the facility of in vitro expansion and ex vivo modification, as well as the convenient non-invasive administration with an intravenous injection for autologous transplantation. As concerns

their use in ALS research, it was found that intravenous administration of bone marrow or microglial cells delayed disease progression and increased motor neuron survival by reducing macro- and microgliosis in ALS mice [53, 54].

An increasingly recognized key pathogenetic event of disease progression in ALS is neuroinflammation, characterized by activation of microglial and astroglial cells and by the infiltration of peripheral monocytes and lymphocytes, including T cell populations [55]. In particular, regulatory T lymphocytes (Tregs) are a subset of immunosuppressive T lymphocytes which appear quantitatively and qualitatively impaired as the disease progresses in ALS models and patients [55]. Tregs play a key role in modulating the balance between pro and anti-inflammatory effectors and preserve self-tolerance, and endogenous Tregs transplantation has already been explored for the treatment of autoimmune diseases [56].

Transplantation of autologous Tregs in an ALS rodent significantly lengthened disease duration and survival [57]. Although it is yet unclear how Tregs act beneficially to suppress inflammation, it was demonstrated that they are able to reduce the proliferation of responder T lymphocytes (Tresp) and activation of microglia [58].

Recently, transplantation of autologous Tregs was investigated with a human phase 1 clinical trial in ALS patients, demonstrating its safety and feasibility, although tested in a small patient cohort. Three ALS subjects, with a different disease progression rate, were repeatedly infused with autologous Treg cells, collected with leukapheresis and expanded ex vivo [59]. IL-2 was concomitantly administered subcutaneously 3× per week with the aim to keep Treg identity post-transfusion. Hematologic analyses demonstrated that the Treg numbers and suppressive function increased after infusions. Functional rating scales (ALSFRSR and the Appel ALS scales) and respiratory function, measured as maximal inspiratory pressure, showed a transient stabilization. Notably, all subjects presented an increase of fasciculations soon after Treg infusions, an event of unclear origin and meaning. Mild to moderate infections were reported by all participants during the study. The small sample size and the open-label design limit the ability to formulate conclusions. Important questions that have to be answered include the possibility that Treg protective cells might become unstable after transplantation shifting to a proinflammatory phenotype, as well as the mechanism of action of Treg infusions and whether it can be more effectively modulated using a pharmacological approach, such as drugs or small molecules. Interestingly, the reduced number of Tregs in ALS patients could represent a possible disease biomarker.

## Fetal Neural Progenitor Cells

While MSCs and immune cells appear able to exert a positive impact on ALS disease through a bystander effect, they do not have the capacity to replace affected CNS cells. Conversely, NSCs have the potential not only to provide neurotrophic support but also to replace resident cells, at least glial cells or interneurons. Moreover, they could also exert a possible neuroprotective effect via the secretion of several molecules and growth factors (Fig. 1).

ALS trials using human fetal tissue-derived NPCs expanded in cultures have been carried out in Europe [60] and in the USA [61–63] and are reviewed elsewhere [12, 13].

We hereby review the largest phase 1 and phase 2 trials of intrathecal transplantation of human fetal NSC/NPCs carried out up to now [63].

These trials used a particular human spinal cord-derived stem cell (HSSC) line, obtained from the spinal cord of an 8-week human embryo and expanded in vitro using serum-free culture medium and fibroblast growth factor 2 used to maintain proliferation and avoid differentiation [64]. Preclinical studies demonstrated that HSSCs give rise to interneurons for the major part, whereas a minority of them differentiates into astrocytes. Moreover, they are able to secrete growth factors, including BDNF and GDNF [14, 15]. Following engraftment, these cells can express glutamate transporters that possibly restore functional excitatory amino acid reuptake around diseased motor neurons [14].

Two studies of HSSC transplantation in ALS rat models showed delayed disease onset, prolonged survival, and an increase in ventral horns motor neurons compared to controls receiving dead cells transplant, although positive effects on motor symptoms were only transient [65, 66]. This suggests that performing multiple injections along the spinal cord could prove more effective in ameliorating animal survival.

In a phase 1 trial conducted at the Emory University, 15 ALS patients were enrolled at various stages of disease and HSSC were surgically injected into lumbar and/or cervical spinal cord [62]. Cells were delivered into specific spinal cord locations at cervical (C3–C5) and lumbar (L2–L4) levels. After the procedure, patients were treated with a short course of methylprednisolone, basiliximab, mycophenolate mofetil, and lifelong tacrolimus.

The study met its primary goals, demonstrating that intraspinal injections were safe and well tolerated and paving the way for a phase 2 multicentric trial with dose escalation. No evident neurological deficit linked to surgical procedure was detected [61]. Postmortem examination of transplant recipients' spinal cord revealed the persistence of vital engrafted cells. Most of the cells presented an immature neural stem phenotype, while a small fraction differentiated into neurons.

The phase 2 open-label study, funded by the National Institutes of Health included three different centers with 15 ALS participants, with the same cell product and delivery method as the previous study [63]. This trial met primary safety end point, although two patients receiving the highest dose reported serious, but not life-threatening, complications.

The commonest adverse events were related to immunosuppressant medications and transient surgical pain. There was one case of acute deterioration in neurologic function following surgery and another report of central pain syndrome. Post-treatment ALSFRSR and FVC curves of treated patients did not differ from slopes of three separate historical control groups. Thus, transplantation did not seem to benefit treated patients. However, since no control nor placebo group was included, it is not possible to draw correctly any consideration about treatment efficacy.

A recent post hoc analysis compared the 3-year survival and clinical course of ambulatory limb-onset ALS participants of phases 1 and 2 after transplant with participants in Pooled Resource Open-Access ALS Clinical Trials (PRO-ACT) and ceftriaxone trial datasets. Survival did not differ significantly between the groups. On the contrary, significant differences in the mean ALSFRS-R at 24 months and in ALS/SURV index could be detected between Ph1/2 participants and both comparison cohorts, supporting the hypothesis of improved functional outcomes transplanted patients. However, these data are limited by historical comparison.

A phase 3 trial to investigate stem cell transplantation efficacy has been planned, but it has not begun yet. Interestingly, a single ALS patient participating in this trial, who was diagnosed with El Escorial criteria and demonstrated definite disease progression prior to enrollment, experienced almost complete clinical and electromyographical disease resolution following treatment. The reason for this dramatic response is still unexplained. It was hypothesized that this effect could be related to the immunosuppression employed to prevent graft rejection. For this reason, a recent small trial of immunosuppressive therapy in ALS patients was recently carried out, showing some beneficial effects on immune modulation. However, no other "responders" were detected [67].

Several questions regarding these NSCs trials, however, remain unanswered. Above all, it is yet to be determined whether the apparently limited response of the 30 treated patients might be due to the type of cells used, to the local delivery strategy that does not allow a widespread distribution across all the affected areas, or to the limited ability of NSCs to halt the disease progression in particular in advanced stage. It is also possible that, since the disease is heterogeneous, only a fraction of the patients is a "responder." Indeed, further clinical trials using other types of neuroectodermal cells are planned.

Another source of NPCs is the fetal cortex. Upon transplantation within rodent spinal cord, these cells are able to differentiate into neuroprotective astrocytes and, if genetically modified to produce GDNF, they exert beneficial effects on motor neurons in ALS animal models [26, 68]. This way, the therapeutic activity of NPCs is enhanced by their ability to secrete GDNF.

The combination of cellular and gene therapy has been recently tested in a single-center phase 1/2a dose clinical trial, which recruited 18 ALS patients for transplantation of NPCs, engineered ex vivo to produce GDNF, within the spinal cord, assessing safety and tolerability. In this trial, cells were injected on one side of the spinal cord, so to verify treatment efficacy by comparing muscle function and innervation on the transplanted and non-transplanted side. The study is now closed until completion.

Furthermore, the FDA has recently approved the use of fetal brain-derived cells, preconditioned with growth factors to differentiate into glial precursor cells (Q cells), in an ALS clinical trial. Q cells are then able to differentiate into astrocytes and oligodendrocytes [21]. The rationale for the therapeutic use of Q cells stems from the observation that glial cells are implicated in neuroinflammation and neurodegeneration in ALS. This trial, however, has not started yet.

Phase I trials showed that spinal cord surgery and intraparenchymal cell delivery were well tolerated and safe. The most common adverse event reported in all published trials was transient pain at the surgical site. At least 3 mL of cells suspension and up to 20 injections in different segments could be tolerated by the atrophic spinal cord of ALS patients. No uncontrolled growth of tumors were detected in the long-term follow-up after transplantation of bone marrow stem cells (up to 9 years) and NSCs (up to 2 years) [69]. In all clinical trials, the reduction of stem cells teratogenic properties was achieved through the delivery of committed stem cells or the differentiation of stem cells into postmitotic cell phenotypes before transplantation. The engraftment and survival of the cells in the 2.5 years following transplant was demonstrated postmortem [69]. Cellular grafting did not appear to provoke any acceleration in disease progression. Despite these results are not definitive, they suggest that stem cell transplantation is feasible and relatively safe in ALS patients.

The major question is whether the magnitude of efficacy proven so far is sufficient to support immediate efficacy trials, or it is necessary a step back to further improve cell transplantation efficacy in a preclinical setting before proceeding to further clinical trials.

## Perspectives and Limitations of Stem Cells Clinical Translation

Despite the great potential demonstrated by stem cells at the preclinical level, their clinical use is still limited to early clinical trials. Several obstacles limit their translation into clinical practice. This widening hiatus between laboratory discoveries and clinical application has been named the "Valley of Death" and extensive efforts have recently been directed to finding novel solutions for bridging this gap [70]. One limit that was identified is the considerable amount of manufacturing expenses connected to clinical translation, which often limit the access to standard grants [70]. Recognizing this, some institutions now offer specific funding opportunities for early translational clinical studies.

Furthermore, early cross-talk between researchers, physicians, and regulators is needed in order to expedite approval of procedures and to better assess the risk/benefit ratio of every translational study. Early confrontation with regulatory agencies might help identify the type of experimental data needed in order to obtain funding for phase I trials [70].

In addition to regulatory issues, several ethical considerations arise in this field. Reducing risks for patients, ensuring safety, and warranting informed and responsible decision-making are all part of ethical and responsible study design and are especially important in early phase human trials [71]. Another risk that should be recognized and avoided is "therapeutic misconception," whereby patients, researchers, investigators, and even regulatory agencies fail to recognize or disclose that the primary aim of clinical research is to produce generalizable knowledge and individual benefits are not guaranteed [71, 72]. The hazard of therapeutic misconception is the growth of excessive expectations in both patients and professionals and a consequent bias in the perception, assessment, and interpretation of safety data. As mentioned above, a significant placebo effect is likely present, highlighting the need for placebo-controlled studies.

Moreover, the safety of the product and careful preclinical assessment before moving from bench to bedside is essential. Toxicity and oncogenic potential of stem cell products should be carefully assessed, and cells should be prepared in facilities that comply with good manufacturing practice (GMP) standards. To date, secondary neoplastic lesions after stem cell transplantation in human subjects have been observed in the context of so-called stem cell tourism in transplantation experiments outside clinical trial [73]. Another substantial matter that needs to be explored is social justice. Like other innovative technologies, the development of cell transplantation therapies requires considerable investments. As the concept of resource allocation becomes increasingly relevant in our countries, researchers must ensure that available money is invested in well-designed projects that could benefit society. To this aim, it must be taken into account both the potential application in the population of a given therapy and its impact on the single patient. Thorough costs/benefits analysis becomes especially important in the field of rare diseases, such as ALS. Furthermore, disparities in access to treatments should be contrasted. Measures intended to spread and share existing knowledge and biological materials, such as biobanks, and to standardize production may help reducing costs and increasing access [71].

As regards clinical translation, a critical aspect is the development of a stable cellular product. The survival of transplanted stem cells within the host CNS is often jeopardized by immune activation and graft rejection [74] and by the development of a toxic microenvironment during disease progression [75]. A variety of preconditioning and genetic engineering strategies have been developed to overcome these hurdles [8].

Another emerging consideration is the influence of the site of stem cell administration, as well as the efficient distribution of the cells around diseased motor neurons, on treatment efficacy. One of the potential mechanisms of stem cells beneficial action is the delivery of a variety of growth factor. Notwithstanding this observation, it is unclear whether their physiological growth factors production could be sufficient or genetic engineering is needed to achieve a therapeutic effect. Moreover, it is essential to identify clear indices of treatment success. To date, modifications in the ALSFRS-R scale and in the FVC have been used as parameters for determining treatment response. Although changes in the rate of disease progression measured by these scales remain the primary outcome measure in various trials, and such analyses take into account the baseline pre-treatment score, these measures may be somewhat limited. Thus, additional markers of disease progression could represent a useful aid in establishing an objective and reproducible index of efficacy. Serum and CSF neurofilament levels have been shown to correlate with disease progression in ALS cohort studies [76, 77]. In addition to that, quantitative electrophysiological measurements, such as motor unit number index (MUNIX) and neurophysiological index (NI), have been found to correlate with LMN degeneration better than ALSFRS-R in presymptomatic and symptomatic phase [78]. However, further studies in ALS patients are needed in order to validate these markers and to establish disease thresholds. Overall, the road to an effective, feasible stem cell therapy for ALS appears long and scattered with hindrances. However, combined efforts of researchers, clinicians, and investors might make this long-awaited goal attainable.

## Conclusions

Key issues in the safety and effectiveness of cell transplantation for ALS are the identification of the most suitable and effective cell type, cell dose, delivery route, and therapeutic mechanisms. The quality of the cell product is important and it depends on the standardization of cell harvesting, expansion in vitro, and preparation for transplantation.

Stem cells hold great promise because of their regenerative capacity, but our technological knowledge still appears inadequate to exploit their power for the therapy of neurodegenerative disease. Therefore, the chief question to be answered is whether the results of clinical trials performed so far technology are meaningful enough to support immediate efficacy trials, or if they warrant additional preclinical research in order to better understand stem cells action and properties and further improve their efficacy before attempting novel clinical trials in humans.

Stem cells hold great potential for the treatment of ALS and other neurodegenerative diseases, but the road to a stem cell-based therapy is still long and complex. As Santiago Ramon y Cajal said, "In adult centers the nerve paths are something

fixed, ended, immutable. Everything may die, nothing may be regenerated. It is for the science of the future to change, if possible, this harsh decree."

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## Compliance with Ethical Standards

**Conflict of Interest** The authors declare that they have no conflict of interest.

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