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Advances in stem cell therapy for amyotrophic lateral sclerosis

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Article Highlights

- *In vitro* and *in vivo* preclinical studies demonstrate the efficacy of both neural stem cells and mesenchymal stem cells in targeting pathogenetic mechanisms and slowing disease progression in *in vitro* and animal ALS models.

- The field lacks experiments in large animal models that better simulate human anatomy and physiology.

- Phase I/II cell-based clinical trials demonstrate the safety of both neural stem cells and mesenchymals stem cells but lack definitive evidence of efficacy in ALS patients. The field strongly requires well-designed, randomized, robust clinical trials.

- The protocols for cellular expansion in most clinical studies are not reported or are suboptimal. Hence, some negative clinical results may be explained by the poor quality of the transplanted cell products.

- Comparative studies addressing main issues such as the types and numbers of cells, the modes of delivery and the appropriate populations of selected patients that might best benefit from cell-based therapies are essential.

- *In vivo* molecular imaging, advances in tissue engineering and the use of nanomaterials are promising technologies for improving future clinical trials.

- Substantial effort should be made by basic and clinical researchers to communicate to patients and others realistic expectations arising from scientific results. This can eliminate unrealistic hopes that drive the phenomenon of stem cell tourism.

ABSTRACT

Introduction: Amyotrophic Lateral Sclerosis (ALS) is a progressive, incurable neurodegenerative disease that targets motoneurons. Cell-based therapies have generated widespread interest as a potential therapeutic approach but no conclusive results have yet been reported either from pre-clinical or clinical studies.

Areas covered: This is an integrated review of pre-clinical and clinical studies focused on the development of cell-based therapies for ALS. We analyze the biology of stem cell treatments and results obtained from pre-clinical models of ALS and examine the methods and the results obtained to date from clinical trials. We discuss scientific, clinical and ethical issues and propose some directions for future studies.

Expert opinion: While data from individual studies are encouraging, stem-cell-based therapies do not yet represent a satisfactory, reliable clinical option. The field will critically benefit from the introduction of well-designed, randomized and reproducible, powered clinical trials. Comparative studies addressing key issues such as the nature, properties and number of donor cells, the delivery mode and the selection of proper patient populations that may benefit the most from cell–based therapies are now of the essence. Multidisciplinary networks of experts should be established to empower effective translation of research into the clinic.

Keywords: Amyotrophic Lateral Sclerosis, Stem Cells, Transplantation, clinical trials, Animal models, Cellular models

1.INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is a progressive, paralytic disorder characterized by degeneration of motoneurons (MNs) in the brain and the spinal cord. It begins insidiously with focal weakness but spreads relentlessly to involve most muscles, including the diaphragm. Typically, death due to respiratory paralysis occurs within 3-5 years after onset of the first symptoms [1].

ALS is currently incurable and only two disease-modifying therapies are available, with modest beneficial effects: Riluzole, which is reasonably safe and prolongs median survival by about two to three months [2], and Edaravone, which shows in early stage ALS patients a smaller decline of ALSFRS-R score compared with placebo [3].

Cell-based therapies have generated substantial interest as a potential treatment option. The intent of this review is to highlight critical elements that need to be addressed to proceed with large efficacy clinical trials, and also to suggest possible strategies derived by integrating the results obtained from over two decades of studies encompassing stem cell culture and characterization, preclinical *in vitro* and *in vivo* models, up to the most recent clinical applications in small Phase I and II trials. We analyze the underlying biology of stem cell (SC) treatments and the results obtained in pre-clinical models of ALS and examine the methods and the results obtained so far in clinical translation to patients. We discuss scientific, clinical and ethical issues and propose some directions for future studies. The review is based on an extensive literature search and our own basic, preclinical and clinical experiments related to cell-based therapies for ALS, and on discussions among basic researchers and clinicians participating in the COST Action (**CA16122)** entitled “Biomaterials and advanced physical techniques for regenerative cardiology and neurology” (BIONECA).

2. THERAPEUTIC TARGETS FOR STEM-CELL-BASED TREATMENTS

Disease mechanisms and factors underlying the selective vulnerability of somatic MNs in ALS are not yet completely understood. In addition to those previously identified in superoxide dismutase 1 (SOD1) mutants, namely protein misfolding, oxidative stress and impaired axonal transport, recent genetic advances have focused on pathways related to RNA and protein homeostasis, mitochondrial and cytoskeletal dysfunction and nucleocytoplasmic trafficking [4]. It is also well established that other cell types intimately associated with MNs, including astrocytes, oligodendrocytes, microglia and immune cells, or, more broadly, the cellular microenvironment, play a critical role in disease onset and progression [5, 6, 7, 8, 9] (Figure 1). Cell therapy focused on MN replacement is a daunting challenge, given the system complexity and anatomically distributed sites of degeneration. In contrast, non-cell-autonomous disease mechanisms are becoming an appealing substrate for cell-mediated modulation (Figure 1). We briefly describe here some pathogenic mechanisms that support the use of non-neuronal cellular therapies with the aim of modifying disease onset and progression. In the future, stratification of ALS patients according to the underlying molecular pathogenic profile will facilitate selecting appropriate patients and predicting the efficiency of SC treatments, thus improving the chance of success of this approach.

2.1 Excitotoxicity due to deficient astrocytic glutamate uptake was proposed to mediate MN death in ALS more than 20 years ago. Downregulation of *SLC1A2* (EAAT2), the sodium-dependent transporter that clears glutamate from the synaptic cleft, leads to inefficient uptake of glutamate, increased firing and calcium intake in MNs and subsequently to mitochondrial and endoplasmic reticulum (ER) stress. This has been demonstrated in murine *SOD1* mutant models [10] and in cells and tissues derived from familial and sporadic ALS patients. In addition, ALS-*SOD1* mutant astrocytes appear unable to induce neuronal upregulation of metabotropic (m)GLUR2 which normally buffers excess glutamate and protects the neurons from glutamate excitotoxicity, thus rendering them more susceptible to neurodegeneration.

Besides excitotoxicity, ALS astrocytes have been proposed to release some (as yet) unidentified factors toxic to MNs (conceivably including mutant misfolded proteins or metabolites) [11]. This view is also supported by available evidence that wild-type astrocytes can delay MN death [11, 12, 13].

2.2 Local and systemic inflammation*.* Microglial activation in ALS patients has been demonstrated both *in vivo*, using positron imaging tomography (PET) imaging, and in post-mortem tissue. Local

infiltration of macrophages and T cells and a peripheral increase in several cytokines and chemokines including TNFα, IL6, IL1β and VEGF, recently confirmed in a meta-analysis, strongly support an enhanced inflammatory activity in ALS [14]. Experimental evidence (mostly *in vitro*) supports the presence of microglia-activating pro-inflammatory substances in serum and/or cerebrospinal fluid (CSF) that induce neurotoxic features in astroglial cells.

2.3 The blood-brain barrier (BBB) in ALS has been reported to be dysfunctional. Phenotypic changes in astrocytes forming the outer rim of the BBB include retraction and swelling of their end-feet. In the hSOD1^{G93A} ALS rat model we have described overexpression of aquaporin-4 channels and a decrease of Kir4.1 potassium channel activity in cortex and brainstem astrocytes [15]. The ensuing disrupted ion homeostasis could contribute to MN death. Reactive astrocytes and a faulty BBB can facilitate leakage of peripheral molecules and infiltration of immune cells.

2.4 Oxidative stress and abnormal reactive oxygen species (ROS) metabolism, associated with neuro-inflammation, are present in different cell types in ALS [16]. Cell-based treatments could be paradigmatic here, acting to restore cellular homeostasis by preserving physiological ROS signaling while eliminating the disease-related component of oxidative stress. In addition, it is known that perineuronal nets, a specialized form of extracellular matrix (ECM) in the CNS, protect from oxidative stress and related neurodegeneration [17] and it has been shown that treatment with mesenchymal stem cells (MSCs) preserved these ECM structures in an ALS animal model [18].

3. DYSREGULATION OF NEURAL STEM CELL NICHES AND NEUROGENESIS IN ALS

The existence of neural stem/progenitor cells (NSCs/NPCs) in adult brain is a well-accepted concept. Although the magnitude of endogenous adult neurogenesis (eNG) is debated, its manipulation is regarded as a potential strategy to promote functional recovery in neurodegenerative disorders, including ALS.

Whereas eNG is well documented in the subventricular zone (SVZ) and the hippocampal subgranular zone (SGZ) of rodents [19] the presence of NSCs/NPCs in the spinal cord has been controversial [as reviewed by 20, 21]. Here, NSCs/NPCs reside in the periventricular region lining the central canal and are characterized by relative quiescence, likely a consequence of both cell autonomous and non-cell autonomous limitations, including a non-permissive neurogenic microenvironment. Indeed, several studies document that although NSCs/NPCs in the spinal cord can react to injury, they primarily generate astrocytes [22, 23, 24]. Those few immature neurons

that are newly generated migrate preferentially to the superficial layers of the dorsal horn [20]. Altogether, at present, the adult spinal cord is regarded as a scarcely neurogenic niche.

Compared to other neurodegenerative disorders, experimental data on eNG in ALS are limited. Activity of the TGF-β system, a common feature of several neurodegenerative disorders, is upregulated in the spinal cord of ALS patients [25]. In parallel, NSC/NPC activity is diminished while expression of doublecortin, a marker of neuroblasts, is increased. These data suggest that altered TGF-β activity might promote disease progression by inducing an imbalance of neurogenesis and neurodegeneration [25].

Whereas in humans the neurogenic potential of the SVZ is limited, it is generally believed that hippocampal eNG can be physiologically relevant for functions such as cognition. In recent years there has been increasing appreciation of non-motor-system contributions to the ALS clinical phenotype. In ALS patients, hippocampal volume loss correlates with the severity of verbal memory impairment, strongly suggesting hippocampal network involvement [26]. In mSOD1 mice a reduction of hippocampal parvalbumin $(PV)^{+}$ interneurons (which contribute to modulating eNG), and cognitive alterations temporally precede motor symptom onset [27]. Recently a group evaluated postmortem brain tissue from ALS patients with and without associated frontotemporal degeneration (FTD). An immunohistological study reported a statistically significant increase of proliferation in the SVZ of ALS brains, more pronounced in cases associated with dementia [28]. Conversely, markers of proliferation were decreased in the dentate gyrus [28]. These alterations showed a positive and direct correlation with the percentage of pTDP-43 in the SVZ, and a negative correlation with that percentage in the SGZ. Years before, it was suggested that ALS mice may have abnormalities in forebrain NSC/NPC. Bromodeoxyuridine labeling of cells was reduced in both presymptomatic and symptomatic hSOD1 G93A (mSOD1) mice [29]. Interestingly, gender-specific effects of the mutant SOD1 transgene have been described in rat NPC, with significantly reduced proliferation of NPC obtained from male, but not female rats. Moreover, the number of newly generated neurons was reduced only in male mSOD1 mice [30].

In summary, several preclinical and clinical data suggest a deregulation of eNG in ALS. Although at present the pathophysiological significance of eNG deregulation in ALS is unclear, this topic deserves further investigation. A better understanding of perturbed eNG in ALS may also potentially help refine therapeutic strategies based on stem cell transplantation.

4. **PRECLINICAL IN VITRO AND IN VIVO STUDIES USING STEM CELLS**

To sustain a clinical translation of SCs, a large variety of *in vitro* and *in vivo* studies have been made in the past two decades to define safety, reproducibility and efficacy issues related to cell production and therapeutic utilization.

4.1 Neural stem and progenitor cells

Human neural stem cells (hNSCs) or neural progenitor lines, suitable for allogeneic transplantation, can be obtained from the fetal CNS [31, 32, 33]. Currently, only two such neural cell lines have been used in Phase I and II trials pursuing intra-spinal cord delivery for ALS patients:

- human neural progenitors from fetal spinal cord, grown as an adherent monolayer with FGF-2 as single growth factor [32], produced by Neuralstem Inc. [34, 35]
- hNSC lines derived from fetal brain and expanded as floating neurospheres by means of EGF and FGF-2 [33], produced by Azienda Ospedaliera Santa Maria in Terni [36].

Pluripotent SCs such as ESCs and, more recently, iPSCs, represent a novel source of hNSCs [37]. However, standardization of protocols and reproducibility of iPSC-derived hNSC lines should be improved.

hNSCs have been shown to be resilient to transformation and genetically and functionally stable [38, 36], although others have reported a moderate degree of chromosomal instability [39]. hNSCs can be expanded (for over 30 passages) without transformation and do not generate tumors in long-term transplantation studies [36]. Whether NSCs can survive in the long-term within tissue or are subject to immune-mediated rejection is still a matter of debate. Preclinical studies have shown that hNSCs can survive up to several months when transplanted into the CNS of immune-competent animal models in which rejection has been controlled by short-cycle immune-suppressive treatment [40].

Preclinical experiments employing intraparenchymal implantation of hNSCs near MNs suggests that this may be a promising potential strategy for ALS treatment. Several studies using SOD1G93A rodent models [41, 42, 43] have shown that implanted hNSCs can integrate into the tissue, differentiate into astrocytes, oligodendrocytes and neurons and form synapses with host neurons [41, 43]. These experiments have also demonstrated that transplantation of NSCs can slow MN degeneration, ameliorate motor symptoms and prolong animal life span in a dose-dependent manner. Strategies that include multiple injections along the spinal cord including cervical and lumbar levels have been more effective in prolonging animal survival [42]. Interestingly,

transplantation of GDNF-producing astrocyte precursors into the motor cortex has also been shown to be neuroprotective and prolonged survival in an SOD1 rat model [44].

Intravascular delivery of hNSCs [45] yields a more widespread distribution of cells. Cells accumulate in the anatomical regions affected by disease (e.g. ventral horns of the SC) or in permissive CNS niches (e.g. hippocampus). However, this approach is associated with a high loss of cells [45].

Mechanisms underlying positive effects may include direct replacement of neurons [41, 45], enhanced functional synaptogenesis [41, 43], maintenance of neuromuscular function [41, 42, 43], neuroprotection [41, 42, 46] and stimulation of endogenous neurogenesis [42, 43]. Importantly, animal studies have shown that, in addition to cell replacement and neurotrophic actions, hNSCs can reduce astrogliosis and microgliosis [42] and inflammation (rev in [47]).

4.2 Mesenchymal stem cells

Whereas the best-known source of MSCs is bone marrow (BM-MSCs), MSCs obtained from adipose (AD-MSCs) or umbilical cord tissues (Wharton´s Jelly: WJ-MSCs) have been used more often in ALS therapy. MSCs derived from different sources possess characteristic features possibly related to their developmental origin, the age of the donor, and the isolation method [48].

Due to their near-fetal origin, neonatal or early postnatal MSCs (WJ-MSCs and umbilical cord blood (UCBs) permit allogeneic transplantation without the need for burdensome immunosuppressive treatment. Allogeneic transplantation also avoids reintroducing the disease-related genetic defects of the patient [49]. Compared to BM-MSCs, WJ-MSCs have been shown to overexpress genes involved in neurotrophic support, neuronal maturation [50], cell adhesion, proliferation, and immune system function [51].

The other valuable source of therapeutic MSCs is adipose tissue. Isolation of MSCs from fat is highly efficient, minimally damaging and well tolerated by patients, even those who are cachectic (BMI< 20). Compared to BM-MSCs, AD-MSCs are more effective suppressors of immune responses, probably due to their enhanced ability to stimulate dendritic cells to secrete IL-10. In addition, AD-MSCs express genes highly involved in cellular communication (CCL3, FGF9, IL1R2, KDR) and in transcriptional control (PAX3, SPI1, ZNF45) [52]

Recent studies have shown that both AD-MSCs and WJ-MSCs, cryopreserved and cultivated *in vitro* for lengthy periods (up to 3 months or longer), maintain genetic proliferative stability, with low

rates of senescence and chromosomal aberrations, especially under physiological (~5%) oxygen conditions [46, 53].

Since procedures for isolation, maintenance and differentiation of MSCs may vary and influence clinical outcome, there is an urgent need for standardization of general protocols to achieve a more unified therapeutic effect [46, 54].

The most efficient route of administration seems to be intraspinal injection with subsequent cell migration towards the damaged tissue. Intraspinal transplantation of UBCs at an early stage of progression in an animal ALS model improved motor function, attenuated MN loss and astrogliosis and improved survival [55]. Similar results were obtained by intra-cerebroventricular (icv) injection of UCBs [56], WJ-MSCs [57] or AD-MSCs [58]. Since migration of grafted cells to the spinal cord was not well documented in these studies, the observed protective effects were linked rather to adjuvant cell properties and increased levels of neurotrophic factors such as nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), insulin-like growth factor (IGF-1), vascular endothelial growth factor (VEGF), glial derived neurotrophic factor (GDNF) and fibroblast growth factor-2 (FGF-2). WJ-MSCs that express increased levels of VEGF and FGF may improve the microenvironment around MNs and neuromuscular transmission. The potential of WJ-MSCs to induce immunomodulation and neuroprotection was shown to be mediated by the secretion of neurotrophic factors [54] or release of extracellular vesicles [59]. In addition, recent evidence has shown that WJ-MSCs may develop tunnelling nanotubes (TNTs), which mediate intercellular transfer between neighboring cells [60]. Although differentiated *in vitro* WJ-MSCs [61], and AD-MSCs [62] have been shown to express markers typical for neuronal cells, including MN-like cells, there is still a lack of protocols enabling targeted and complete functional differentiation of these MSCs along the neuronal lineage that would permit integration into functional neural networks after transplantation *in vivo.*

5. **NOVEL PRECLINICAL ALS MODELS AND THERAPEUTIC TOOLS**

5.1 Animal and cellular models of ALS

Preclinical testing of new drugs and other molecularly targeted disease therapies using animal or cellular models are typically required before entering clinical trials. Growing focus is being placed on the utilization of increasingly sophisticated human cellular models to better mimic the human *in vivo* situation. Here we provide an overview of established and innovative animal and human

cellular models that will facilitate more rapid screening of potential treatments and almost certainly contribute to the development of new clinical trials.

Mutant SOD1 transgenic rodents are the most widely used animal models of ALS and have given us important insights into the potential of stem cell therapy. However, genomic, anatomical and physiological difference between rodents and humans may preclude the translation of results obtained in rodents to the treatment of ALS patients. Large animal models may overcome some of these issues, in particular the short life span of rodents. Recently, transgenic pigs expressing mutant G93A hSOD1 have been generated [63]. These animals present, in addition to classical ALS motor symptoms and anatomical hallmarks, specific pathogenic features seen in ALS patients but not in mouse models, such as nuclear accumulation and ubiquitinated nuclear aggregates. This large animal model thus represents a valuable tool for enabling a more accurate preclinical evaluation of the therapeutic potential of stem cell treatments.

In vitro cellular models provide opportunities to investigate disease mechanisms and test potential therapies in a more accessible manner than in *in vivo* models. Disease endpoints can be assessed in large numbers of cells simultaneously, thus increasing the statistical power of analyses. The obvious drawbacks are that *in vitro* cell cultures are limited in their anatomical and physiological complexity, and thus underrepresent the *in vivo* situation.

iPSC technology provided for the first time ALS patient-derived cells that could be expanded and differentiated into the cell types involved in the disease. Since 2008, iPSCs have been generated from ALS patients with mutations in *SOD1* [64, 65, 66], *VAPB/C* [67], *TARDP* [68, 69], *C9ORF72* [70, 71, 72, 66] and *FUS* [73, 66] genes, and from sporadic ALS patients [74, 75]. Differentiation of these iPSCs into MNs and/or astrocytes have provided some insight into pathogenetic mechanisms, including intracellular aggregation of the TDP-43 protein [69, 74], sequestration of RNA binding proteins by C9orf72 hexapeptide repeats [70, 71, 72], increased sensitivity to glutamate excitotoxicity [71], electrophysiological abnormalities [72, 66] and mitochondrial dysfunction [75].

As an alternative approach, the direct conversion of fibroblasts into neural progenitors (iNPCs) or neurons (iNeurons), has permitted the generation of MNs and astrocytes that display typical ALS features such as electrophysiological impairments [76] or astrocyte-induced toxicity [13]. Direct conversion is faster and maintains the epigenetic signatures of cellular aging, promoting the

expression of neurodegenerative diseases *in vitro*. However, iNPCs and iNeurons exhibit more limited proliferation and a more restricted differentiation potential than iPSCs.

Investigations of astrocyte-mediated MN cytotoxicity have also employed primary astrocytes obtained from postmortem ALS patient brains and spinal cords [11, 77]. Muscle cells, microglia [8, 9] and oligodendrocytes [5] are also involved in ALS pathogenesis and can be obtained respectively from muscle progenitor cells, iPSCs and either iPSCs or by direct conversion of fibroblasts.

The assessment of functional phenotypes in *in vitro* models can be a challenge. Electrophysiological abnormalities in MNs may be an early hallmark of ALS disease progression. Hence, assays that can be used to test electrical properties and functions are likely to be pivotal in the characterization of disease mechanisms. Compared to patch-clamp recording and microelectrode arrays, optical recording of electrical events provides several advantages. For example, voltage sensitive dye imaging permits the recording of membrane potential at submillisecond resolution, sufficient to distinguish the fine temporal structure of electrical signals, and can be used to assess both excitatory and inhibitory synaptic interactions and depolarizing and hyperpolarizing neurotransmitter and drug actions [78].

To date, *in vitro* ALS models have been limited to 2D cultures of MNs and astrocytes. Current efforts indicate that the evolution of ALS modeling *in vitro* will include multiple cell type co-cultures, additionally elaborated by 3D bioprinting and microfluidics, providing a richer and more faithful context in which to elucidate pathogenetic mechanisms and to evaluate novel molecular and cellular therapies, including therapies utilizing stem cells.

5.2 Novel therapeutic tools to support cell–based therapy

5.2.1 Physical factors for inducing cell differentiation

The use of physical as opposed to chemical factors to induce cell differentiation is one of the most interesting developments in the field of regenerative medicine. For example, the PC12 pheocromocytoma cell line can be induced to develop into dopaminergic neurons when exposed to the physical waves of the Radio Electric Asymmetric Conveyer (REAC TO-RGN). Such effects can be useful for implementing standardized protocols to differentiate stem and progenitor cells towards a neuronal phenotype [79, 80, 81, 82, 83, 84].

5.2.2Nanomaterials

In the context of SC-based therapy for ALS, nanomaterials can: i) provide a substrate for SC migration and integration, ii) function as carriers for agents promoting SC survival and differentiation, either directly interacting with SCs or by modifying tissue-derived factors, and iii) provide a biomimetic 3D matrix for organoid-based *in vitro* disease models [85].

5.2.3 Nanofibers are spun from biodegradable polymers and can be embedded with guidance and trophic molecules [86], providing a suitable 3D matrix for the preparation of SCs prior to treatment and for supporting their survival and tissue integration after transplantation. Nanofibers have also been used to create organoids representing early (but not yet mature) stages of spinal cord development [87].

5.2.4 Nanocarrier technology has been used only to a limited extent in relation to ALS [88]. Mesoporous silica nanoparticles (MSPs) have been shown to provide an effective delivery system for morphogenes and growth factor peptide mimetics to achieve functional differentiation of ESC-derived MNs and axonal growth *in vitro* [89] and *in vivo* after transplantation [90]. MSPs can be administered systemically, cross the BBB [91], and can be decorated with target recognition molecules facilitating specific drug delivery to MNs [92]. Alternatively, MSPs can be incorporated into mesenchymal SCs and piggyback on these as they home to disease-affected areas [93].

5.2.5 Extracellular vescicles

Extracellular vescicles (EVs) provide a novel mechanism of intercellular communication via the transfer of biological information between cells [94]. According to their origin and size, EVs can be classified as exosomes or microvescicles. Exosomes originate from endosomal compartments known as multi-vesicular bodies and are 30-150 nm in diameter, whereas microvescicles are larger (150-1000 nm) and released through budding from the plasma membrane. EVs are enclosed by the phospholipid bilayer and can contain many different proteins, nucleic acids, lipids and metabolites. Importantly, the cargo content of EVs depends on cell type and physiological state [94]. Accordingly, EVs derived from healthy cells may have a therapeutic potential comparable to the cells themselves [95], whereas EVs from diseased tissues may contribute to the maintenance and spread of pathological processes [96, 97]. Most importantly, exosomes can cross the BBB [98] and can therefore be delivered into the CNS without neurosurgical interventions. Recently, several studies have demonstrated a neuroprotective potential of EVs in different *in vitro* and *in vivo* models including ALS models [95, 99, 100].

However, we still have limited knowledge about the molecular mechanisms underlying neuroprotective actions of EVs. Since EVs carry complex and variable cargo, it is likely that neuroprotection is achieved by the simultaneous action of several miRNAs and/or proteins making identification of these mechanisms a difficult task. It is not unlikely that EVs exert neuroprotective actions by simultaneously affecting MNs directly and by modulating microglial and astroglial responses to pathological insults.

The best cellular source of EVs for potential treatment of ALS has yet to be determined. From the therapeutic perspective, the most promising sources are MSCs, dendritic cells, iPSCs and iPSC-derived macroglial and microglial cells.

There are many key challenges that need to be addressed before EVs can enter clinical development. First, new technologies are needed for quick and reliable evaluation of different lots of EVs to ensure better reproducibility and therapeutic efficacy. Finally, an optimal delivery route of EVs to ALS patients still needs to be determined.

6. CLINICAL TRANSLATION

The number of ALS clinical trials employing SCs is very small. As of January 2018, a search of ClinicalTrials.gov (using the search terms “stem cell” and “ALS”) identified 34 trials. Five trials have not been concluded or have been withdrawn. Sixteen have been completed but only the results of 9 of them have been published in peer-reviewed scientific journals. The remaining 13 trials are listed as active. All completed trials are phase I/II and recruited very few patients (median=18). Among the 13 active trials, only 2 are randomized double-blind placebo-controlled phase III trials.

6.1 Clinical results to date

Recently published clinical studies are summarized in Table 1. Most of these studies were small and single-center, with different recruited patient populations, therapeutic protocols, and outcome measures. Assessing safety rather than efficacy was the major aim in most. All studies have reported that both the cells and the procedures used were safe, but these results are still inconclusive because most studies lacked long-term follow-up and well-defined outcomes, and the cohorts of patients were small, heterogeneous and tended to have more advanced disease.

Although these studies were primarily targeted to assess safety, most provided some indications of a possible transitory clinical benefit induced by the treatment, generally reflected by changes in

the progression rate of the ALS-FRS-R score and FVC. Both safety and efficacy results seem to be independent of the type and number of cells and the mode of delivery.

6.2 Key questions and issues

6.2.1 Cell number and types. Most of clinical trials (86%) have been performed with autologous bone marrow (BM) SCs, particularly BM-MSCs, because they can be obtained easily from the patient and expanded, thus bypassing ethical constraints and avoiding immune rejection. However, patient-derived stem cells may maintain epigenetic footprints, which may not be appropriate for therapeutic purposes.

The first clinical trial using autologous BM-MSCs in ALS patients was published by Mazzini et al [101]. BM-MSCs have since been tested in most phase I and II clinical trials for ALS. Intraspinal, intrathecal, intracerebral, intravenous and intramuscular transplants were found to be safe and feasible and showed no evidence of ectopic tissue formation [102, 103, 104, 105, 106, 107, 108] also in the long-term [109]. BM-derived hematopoietic stem cells have also been investigated in patients [110, 111, 112,113, 114] and deemed almost free from significant adverse events (AEs).

A new mesenchymal cell type trademarked as "NurOwn™" has been developed by BrainStorm Cell Therapeutics and tested in 2 small phase I/II clinical trials in ALS patients [115, 116]. These cells can differentiate into specialized neuron-supporting cells capable of stably secreting neurotrophic factors (MSC-NTFs). These clinical studies concluded that NurOwn cells are safe and feasible and resulted in the reduction of disease progression rates [115, 116].

Autologous MSCs have also been administered in combination with T-cell vaccination. No serious AEs were reported and some patients experienced a transient improvement in symptoms [117].

Endogenous mobilization of hematopoietic SCs by granulocyte colony-stimulating factor (G-CSF) has also been tested in clinical trials. The results generally supported the feasibility and safety of the procedure and no severe adverse events occurred even after long-term administration [118-119-120-121]. These studies demonstrated mobilization of hematopoietic SCs into the CSF. However, no clinical benefit was reported in controlled trials [118-119-120-121].

Olfactory ensheating cells (OECs) have also been tested in two studies in China. The cells were injected either into the corona radiata bilaterally [122] or both intraspinally and intracortically [123]. These studies concluded that the procedures were safe and resulted in the reduction of

disease progression rates. However, one American and 7 German patients who underwent surgery in China but were later evaluated by their local hospitals exhibited serious side effects [124-125].

In 2009, the US Food and Drug Administration (FDA) approved the first in-man phase I clinical trial testing the feasibility and safety of direct transplantation of a single concentration of human spinal cord-derived neural SCs (HSSCs) into the spinal cord of ALS patients [126, 34]. This study was implemented as a phase II clinical trial that tested the safety of escalating doses of stem cells delivered in a combination of increasing numbers of cells per injection, numbers of injections and numbers of procedures [35]. In 2011, the Italian Istituto Superiore di Sanità and the Agenzia Italiana del Farmaco approved a Phase I clinical trial on ALS in which multipotent hNSCs were isolated and expanded from human foetal tissues obtained from spontaneous miscarriages and implanted using stereotaxic and surgical apparatuses and injection procedures similar to those used by Glass et al [126]. Each of these studies demonstrated the safety and feasibility of HSSCs/hNSCs and the associated procedures [35, 36]. Recently, iPSC-technology has created a new scenario in stem cell research due to the major potential advantage of obtaining patient-specific or MHC-defined allogeneic SCs for transplantation. The first in-man clinical trial with iPSCs in ALS patients is planned in 2018.

Cell doses adopted up to now have been empirical in most clinical trials and no demonstration of the potential effects of repeated infusions have been tested.

6.2.2 Method and route of cell delivery. Local injections of SCs have the obvious advantage of placing the cells close to their therapeutic target and favor the diffusion of trophic and immunomodulatory factors to both the target and surrounding cells, thereby enhancing the likelihood of obtaining therapeutic effects. Intraparenchymal delivery has been adopted in all trials with hNSCs and in most studies with BM-derived SCs, using different surgical approaches. All studies reported few side effects, and most of these were related to surgery, including transitory local pain and paresthesia (Table 1). No AEs related to SC implantation per se were reported. Few studies assessed the integrity and survival of the grafted cells in post-mortem analyses. Tadesse et al. [127] analyzed the post-mortem spinal cords of 6 patients recruited in the Neuralstem, Inc. trial [34-126] and found that the transplanted HSSCs survived up to 2.5 years and that some differentiated into neurons, while others maintained a SC phenotype. Necroscopy was also performed on the cadavers of three patients that had received intramedullary injections of

autologous bone marrow mononuclear cells (BM-MNCs). Pathological analysis showed a greater number of MNs in the targeted spinal cord segments compared to untargeted segments. In the targeted segments, MNs were surrounded by CD90+ cells and did not show degenerative ubiquitin deposits [128], providing evidence of a neurotrophic activity exerted by the BM-MNCs.

Several clinical trials with MSCs have also utilized intrathecal delivery and in a few cases intravenous or intramuscular administration. Few minor AEs were reported with these administration routes (see Table 1). Low invasive delivery methods may be safer and facilitate repeated administration of large numbers of cells. However, in pre-clinical and clinical studies it has been shown that only a low percentage of administered cells enter the CNS [129-130] and following intravenous injections many cells are trapped in the lungs [131].
At present, in the absence of comparison studies, the optimal route of delivery remains uncertain.

6.2.3 Immunosuppression is indicated when using allogeneic cells but at present optimal protocols for immunosuppressant treatment have not been ascertained for SC transplantation in the human CNS. Moreover, chronic immunosuppression poses severe risks particularly in the late stages of ALS and must be discontinued in rapidly deteriorating patients [132].

The fetal hNSC transplantation trials [34, 36] included empiric immunosuppressive treatment. The Italian trial adopted a temporary immunosuppressive treatment by the low expression profile of HLA determinants in hNSCs and the limited immunological reaction that these cells appear to elicit. In the American trial, the immunosuppressive treatment was prolonged as long as tolerated by the patient. One patient in the Italian trial and 4 patients in the US trial stopped the treatment due to AEs.

6.2.4 Selection of appropriate patients. Given that safety rather than efficacy has been the major issue in initial clinical trials, a risk-escalation paradigm was adopted in most of these trials. Hence, recruited patients had advanced disease. In such patients it may be difficult, if not impossible, to measure efficacy because at this stage most MNs are already lost and cannot be influenced by the treatment.

It is well known that ALS has diverse genetic causes and phenotypic expression patterns and patients with the same severity of disease at the time of recruitment can exhibit dramatically different progression. A potential strategy to overcome this problem is to include patients with a similar progression of ALSFRS-R and FVC during the 3-6 months prior to intervention. Patient

selection is also intimately connected with study end points. As the focus of clinical trials advances from safety to efficacy, study hypotheses focus on demonstrating significant delay or reversal of disease progression and the potential improvement of the pathophysiological targets. Hence, defining *in vivo* biomarkers of cell activity is a major challenge in clinical research using SCs. An important unresolved issue in the design of SC efficacy in clinical trials is the use of the sham surgical arm. The risks of sham procedures are high and must be balanced against scientific value. Issues concerning clinical trial design are discussed extensively in a recently published consensus document [132].

6.2.5 Regulation of cell-based therapies. In the past decade, genes, cells and engineered tissues have been extensively studied as potential new therapeutic tools in Europe and worldwide for many different diseases, particularly neurodegenerative diseases. These innovative products were collectively defined as Advanced Therapies Medicinal Products (ATMP) according to the European Directive 2001/83/EC [133], amended by Commission Directive 2009/120/EC [134]. Complex therapeutic products such as ATMPs require a precise legal definition. Each of these products should possess specific pharmacological, metabolic, and immunological activities. For these reasons, ATMPs, prepared on a routine basis, must meet the same stringent conditions required for "traditional" drugs before they are used on patients or placed on the market. Their activity, efficacy, safety, and required dose must be defined. Furthermore, they must be produced according to Good Manufacturing Practice (GMP), tested preclinically according to Good Laboratory Practice (GLP), and clinical trials must be conducted according to Good Clinical Practice (GCP) (Eudralex see applicable EU legislation [135]. *Regulation (EU) No 536/2014* [136] on clinical trials on medicinal products for human use, and repealing Directive 2001/20/EC [137].

6.2.6 Ethical Issues. Specific guidelines for SC research, including the most recent revision of international guidelines in 2016 [138], have been published also in a version intended for patients. These guidelines and a recent Lancet Commission of Experts report [139] stress the ethics of procurement, derivation, banking, distribution, and use of cells and tissues and helps assure patient safety and the integrity of the research process.

Cell-based therapies for ALS are still only experimental, yet commercial SC "clinics" operate worldwide, often without any regulation. The proliferation of these "clinics" has led to 'medical tourism' by ALS patients who are particularly vulnerable and feel that they have "nothing to lose".

Such unregulated clinics are usually private organizations promoting the therapeutic benefits of MSCs. They generally offer little if any assurance of expertise, quality of care or ethical standards [140].

Another important ethical issue in ALS stem cell research is represented by the appropriate communication with patients. Clinical studies must be preceded by an exhaustive discussion with the patient to avoid misleading expectations. A careful and accurate presentation of the state of research and of results achieved with SC therapy must be provided to patients by expert clinicians in ALS centers. Informed consent should clearly reflect acknowledgement that the procedure is experimental and potentially harmful [140].

Patients planning to undergo unproven SC interventions must be informed about the many risks and undefined benefits of these procedures. Researchers and media but also legislators have the responsibility to protect vulnerable patients from charlatans and "stem cell clinics" [138].

7. Expert Opinion

This integrated review of pre-clinical and clinical studies has shown that stem-cell-based therapies successfully tested in murine ALS animal models are still not satisfactory for clinical application in the treatment of patients. We think that the main reasons for this shortcoming are: 1) a lack of experiments in large animal models which better simulate human anatomy and which can be used to better define appropriate types of cells and cell products, number of cells, routes of administration, and the timing of long-term survival; 2) an inadequate interface between basic researchers and clinical experts in ALS in establishing appropriate pathogenetic targets and expected clinical outcomes; 3) uncertain quality of cell transplantation protocols (protocols for cellular expansion in most clinical studies are not reported or are suboptimal, hence some negative clinical results can be explained by the poor quality of the transplanted products); and 4) unreliable recruitment and methods of assessment of patient populations.

After nearly 2 decades of phase I/II clinical trials with SCs in ALS we can conclude that some types of adult SCs are sufficiently safe and may be proposed for large clinical trials. However, it cannot yet be established which treatments provide significant benefit, and whether positive effects on the disease are sufficiently lengthy to justify the risks and costs of cell therapy.

Thus, as strong as the preclinical animal data may be in suggesting their therapeutic effectiveness, no cell-based clinical trials have yet provided definitive evidence for efficacy in ALS patients.

The field strongly needs well-designed, randomized, robust clinical trials.

Comparative studies addressing key issues such as the type and number of cells, the modes of delivery and the appropriate selection of patient populations that may best benefit from cell therapies are essential. Protocols of cellular expansion must be standardized to provide reliable cellular products. *In vivo* molecular imaging, advances in tissue engineering and the use of nanomaterials are promising avenues for improving future clinical trials. The costs of these trials are very high and funding agencies are reluctant to finance them. Hence, well-designed and conclusive trials are a necessity. Questionable trials represent a waste of resources and time and generate an avoidable risk for patients. Moreover, it is important that definite negative results both from preclinical and clinical experiments are also published in a timely fashion. We think that the pioneering efforts of single clinical centers need to be superseded by larger scale efforts. ALS is a rare disease with substantial phenotypic and genetic variability. Hence the only cost-effective approach to cell-based therapeutic trials will be through the creation of comprehensive multidisciplinary and multicenter networks of experts including basic researchers, cell-based therapy specialists and investigators experienced in clinical trials. Federal agencies, industry, and advocacy groups should collaborate strictly with these networks to ensure the proper use of limited funds and the achievement of reliable scientific results. In addition, international registries of transplantation protocols and outcomes should be created and carefully evaluated. Such collaborations, among different foundations, academic centers, federal agencies, industry, and advocacy groups, have accomplished successful SC therapy for other severe and complex diseases. SCs have a great potential for the treatment of ALS but the road to a stem-cell–based therapy remains long and complex.

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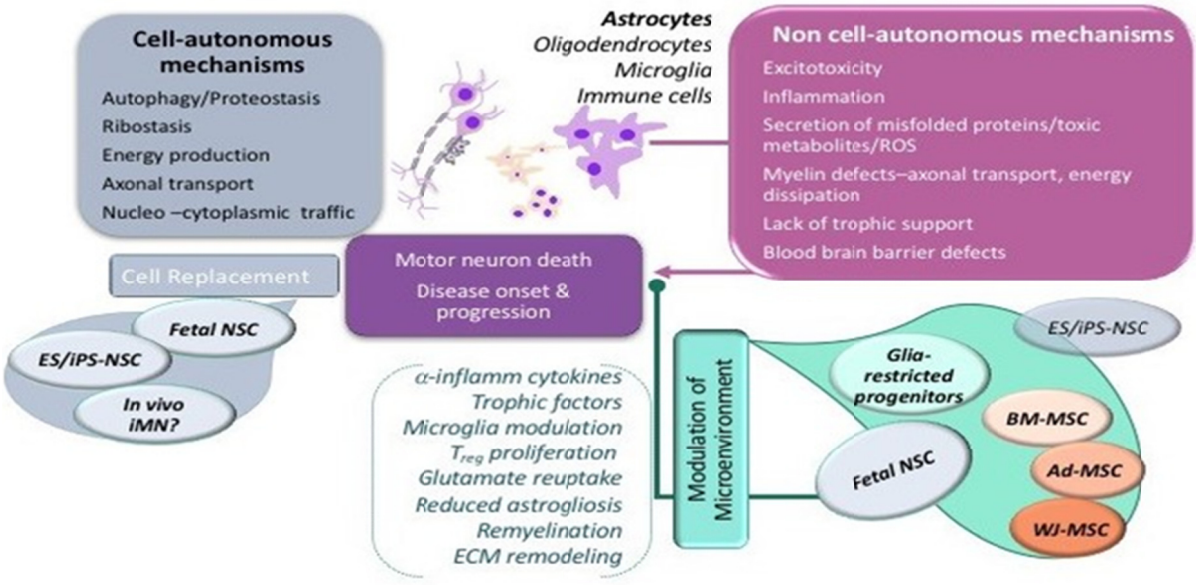

Figure 1

Phase	Stem Cell Kind	Delivery method	Number of cells	Patients number (controls)	Inclusion criteria	Planned outcome	Resu
1	autologous MSCs	intraspinal cord implantation (T4-T6 and T7-T9)	median of 75 x 10^6	7 + 10 + 2 (compassionate use)	age 20-75; FVC > 50%; spinal onset; ambulation with assitence or wheelchair bound (disease duration < 3 yrs and FVC > 50% in 2010)	ALS-FRS, FVC	Saf (4 patients a reduc ALSFRS
1	autologous BMNC	intraspinal cord implantation (T3-T4) [two infusions]	median of 462 × 10^6 BMNC (range 138.00–602.87)	11	age 20 - 65; definite ALS; spinal onset; duration of disease 6-36 months; FVC > 50%; a below 90% fall in oxygen saturation in 2% of sleep time	FVC, ALSFRS, MRC	Safe; no cl disease pr
1/2	autologous MSCs	intrathecal injection via lumbar puncture and intravenous	mean (SD): 63.2 × 10^6 (2.5 × 10^6)	10 intrathecal + 9 intrathecal/ intravenous	definite ALS; age 25-65; progressive course	immunological effects	safe; in imme immunom effe
1	autologous BM-derived mesenchymal stromal cells	two repeated intrathecal injections	1 x 10^6 cells for kg	8	age 25-75 years; probable or definite ALS; ALSFRS-R score 31-46, riluzole treatment; disease duration no longer than 5 years	ALSFRS-R	ALSFR 5 patients decreas mon
1 (dose escalation)	autologous adipose-derived mesenchymal stromal cells	intrathecal injections	from 1 × 10^7 (single dose) to 1 × 10^8 cells (2 monthly doses)	27 (5/5/7/5/5)	definite ALS; FVC >65%; symptoms of weakness lasting >1 year but <2 years	ALSFRS-R; MRI of the neuroaxis	saf

1/2	BM-derived hematopoietic progenitor stem cells	intraspinal injection (C3-C4) with C2 laminec-tomy	?	13	2-5 years from disease onset; age 34-71; moderate or severe symptoms with bulbar involvement; 3 points ventilation bounded	ENMG	safe; one was stable any de
1/2	autologous MSC	intravenous and then intralumbar injection	0.5-1.5 x 10^6 for kg	10 (+ 15 controls)	symptoms of central and peripheral motor neurons (at least ¾ levels); progressive course	ALSFRS-R and quotient of disease progression rate	safe; no ben
1/2	ex vivo-expanded autologous BM-MSCs	intrathecally administered	15 +/- 4.5 x 10^6	26	FVC > 70%; riluzole-naive or on a stable dose for at least 2 months; age 18-65 years; life expectancy of more than 2 years.		reduc stabiliza ALSFRS de months application
1/2 (dose escalation)	autologous MSC-NTF	intrathecally and intramuscularly	lowdose: 1x10^6cells/kg IT and 24 x 10^6 cells IM; mid-dose: 1.5 x 10^6 IT and 36x10^6 IM; high dose: 2 x 10^6 IT and 48x10^6 IM	12 (6 IM + 6 IT) + 14 (IM + IT)	age 20-75; definite or probable ALS; history of ALS of less than 2 years' duration, FVC > 50%	ALS-FRS-R, FVC%	rate of pro of FVC% a FRS-R in patient redu during th mon

1 + 2 (dose escalation)	human spinal cord-derived stem cells	intraspinal cord implantation (C3-C5 and L2-L4)	numbers of injections: from 10 to 40; numbers of cells injected: from 2 million to 16 million	18 + 15 (historical control group)	age > 18 yrs; within 24 months of symptoms onset; all participants were ambulatory with some extremity weakness but not less than antigravity strength; FVC >60%; weakness of neck extensor muscles was exclusionary	ALS-FRS-R, FVC, grip strength	safe (all d difference of progr
1	fetal neural stem cells	intraspinal cord implantation (lumbar tract)	750,000 cells per injection site (uni or bilateral)	6	Age 20 to 75 years, non ambulatory patients; FVC > 60%; absence of sleep apneas or hypopneas with blood oxygen saturation lower than 90%	ALSFRS-R, FVC, MRC	safe; 2 p showed a t improver subsc ambulatio FRS
2	PBSC	injected subcutaneously	5 mg/kg/day for 4 days every 3 months for 4 times	17 (+ 18 placebo)	age 18-85 years; clinically definite or probable ALS; limitation of motor function in at least one limb; FVC > 50%	ALS-FRS, FVC, MMT and CMAP megascore, QoL, death	no cha
1/2	mobilization of autologous PBSC with G-CSF	endovenous injection	5–6 days with dose of 300–600 μg dependent on weight	8	time interval from onset: 3 months – 4 years; FVC 50-150%	FVC, MMT, ALSFRS-R, MRI with spectroscopy	no cha
2	fetal olfactory ensheathing cells (OECs)	injection in corona radiata bilaterally	2 million cells	15 (+20 controls)	age 20–70 years; probable or definite ALS	ALS-FRS-R	slow the progressi first 4 mo
1/2	Allogenic HSC	total body irradiation;peripheral blood HSCT infusion	G-CSF + irradiation 30 mg/m^2 + ATG	6 (historical control group)	age 20-65 years; rate of disease progression between 0.5 and 5.0 Appel ALS	CD83, MCP1	no be

					(AALS) points per month; has HLA–identical related donor		
1/2	autologous blood purified CD133(+) stem cells	bilateral implantation in frontal motor cortex	300 µg for three days and than 2.5-7.5x10^5	10 (+ 13 controls)	age 38-62 years; 18-42 months from diagnosis; no severe bulbar involvement	ALSFRS-R	the surv treated w (P=0.01 untre
1/2	G-CSF	subcutaneous injections	5 lg/kg every 12 hours for 4 consecutive days every three months for 12 months	26 → 24	definite, probable, or probable-laboratory-supported ALS; age 40 – 65 years; disease duration <12 months; moderate disability; FVC >80%; disease progression in the last 3 months	safety, efficacy, immunological changes	no clinical reductio levels of n chemoatt protein-1
1/2	G-CSF	subcutaneous injections	5-day with 2 mg/kg once a day	13	diagnosis of definite or probable ALS with duration less than 3.5 years; FVC > 45%	CMAP, ALSFRS-R	saf
2	autologous blood purified CD133(+) stem cells	bilateral implantation in frontal motor cortex	300 µg for 3 days and than 2.5-7.5 x10^5 or 3-5 x 10^6	67	confirmed ALS; no structural damage to the brain or spinal cord; FVC > 30%; appropriate nutritional status; BMI > 19 kg/m2.	ALSFRS-R, MRC, FVC	safe an toller
1/2	autologous BM-derived stem cells	Intrathecal (L2-L3 and L3-L4)	BM aspiration (30-100 ml)	10	age > 18; diagnosis of clinically definite, probable or probable lab-supported ALS	ALSFRS-R, survival	Trend t stabiliza ALSFI
1/2	autologous BMNC	intrathecal transplantation and intramuscularly	80.5 x 10^6	37 (+ 20 controls)	definite ALS	survival	survival c was high subgr

Table 1 Clinical trials with stem cells in ALS