Stem cells in amyotrophic lateral sclerosis: state of the art

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Amyotrophic lateral sclerosis (ALS) is a devastating incurable neurodegenerative disease that targets motor neurons, manifesting as a linear decline in muscular function and leading to death within 2 – 5 years of diagnosis. The vast majority of ALS cases are sporadic, the aetiopathology of which is incompletely understood. Recent data have implicated the microenvironment of the motor neuron as a primary target of the pathophysiology. Any experimental therapeutic approach to ALS is very difficult because of some peculiarities of the disease, such as the unknown origin, the spatial diffusion of motor neuron loss and the paucity of animal models. Despite such daunting challenges, in experimental models a number of potential benefits of stem cells in ALS therapy have been demonstrated: by providing non-compromised supporting cells such as astrocytes, microglia or growth factor-excreting cells, onset can be delayed and survival increased. Moreover, in animal models of acute or chronic motor neuron injury, neural stem cells implanted into the spinal cord have been shown to differentiate into motor neurons, with some evidence of axonal sprouting and formation of neuromuscular junctions with host muscle. Here we summarise and discuss current preclinical and clinical evidence regarding stem cells application in ALS, particularly focusing on methodological issues.

Keywords: amyotrophic lateral sclerosis, animal models, cell therapy, stem cells

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1. Introduction

Amyotrophic lateral sclerosis (ALS) is a devastating incurable neurodegenerative disease that targets motor neurons (MN) and their connections to muscle. The decline in the number of MNs in ALS is very rapid compared to other neurodegenerative diseases [1] leading to death due to respiratory failure within 2 – 5 years from the clinical onset. Notwithstanding the improved knowledge about the mechanisms of cell death in the disease, ALS still represents one of the most frustrating diagnoses for a neurologist and one of the most dramatic diseases for a patient: it is also considered one of the great conundrums of clinical neuroscience [2]. ALS etiology is unknown and the pathogenesis remains elusive. Recent data have implicated the microenvironment of the MN, rather than the MN itself, as a primary target of the pathophysiology.

Putative mechanisms of toxicity targeting MNs include oxidative damage, accumulation of intracellular aggregates, mitochondrial dysfunction, defects in axonal transport, impairment of growth factor trophic support, altered glial function, aberrant RNA metabolism and glutamate excitotoxicity. The convergence of these events is likely to promote the onset and the progression of the disease; the question remains on which is the cause and which is the epiphenomenon [3]. All these
mechanisms represent a potential therapeutic target and many clinical trials have been developed even though currently none of the candidate compounds has been demonstrated to be effective [4].

Stem cells represent a promising therapeutic strategy since they potentially target several of these putative mechanisms. Moreover, stem cells can be a good means for studying disease-specific cellular pathways and can represent a model system to test new therapeutics, and finally to achieve direct cell-based therapy.

2. Principles of stem cell transplantation in ALS

ALS is characterised by the extensive degeneration of MNs of spinal cord, brain stem and cerebral cortex. Therefore, due to the diffuse distribution of affected MNs, a relevant aspect in cellular therapy is to select the best implantation site, in order to rescue and eventually replace the greatest number of host MNs.

There are two main strategies for transplantation: the systemic and the local. The rationale for the systemic injection consists of the possibility for stem cells to extravasate and cross the blood brain barrier (BBB), especially in the pathological nervous system. In addition, it has been suggested that the pathological brain can attract stem cells. And finally, there is evidence that systemic injection of stem cells can be effective even though they do not enter the CNS [5]. Systemic injections require larger amounts of stem cells to transplant, and can require immunosuppression, even though stem cells can be immunomodulatory themselves [6,7].

Local injections of stem cells, close to the anterior horn of the spinal cord, have the advantage that the operator can place the cells close to their target. CNS is an immune-privileged tissue, and there is evidence that transplant of stem cells does not require immunosuppression. The adverse effects consist of the need of multiple injections due to the length of the spinal cord and in the risk of lesion of the spinal cord. Intramuscular injections of stem cells can also be performed either as an alternative by the axon to the cell body and systemic injections, stem subarachnoidal space or the administration allows them to the risk of lesion of the CNS. Administration through the ventricular system is not trivial, since there are continuity of these cavities and ventral stem cells can cross the pia over the nervous tissue.

The type of stem cells to be differentiated stem cells are, the differentiation of MSCs has been questioned [13,14]. Therefore, as yet, the neurotrophic and immunomodulatory roles seem more relevant than the potential for cell replacement. In fact, as discussed in the following sections, stem cells express immunomodulatory and neuroprotective substances, which can play a fundamental role in protecting MNs in ALS.

3. Stem cell types

Stem cells can be defined as cells able to self-renew giving rise to additional undifferentiated stem cells and to differentiate into committed mature cells. According to their developmental more they can proliferate and give rise to different cell types. On the other hand, adult stem cells display a lower potential for developing into tumours following transplantation than highly pluripotent cells such as embryonic stem (ES) and induced pluripotent stem (iPS) cells that can give rise to teratoma [8]. An alternative strategy consists of orienting and differentiating stem cells in vitro, before transplantation. In the CNS, the most obvious cell type to be transplanted are neural stem cells (NSCs); in addition, immortalised cell lines, mesenchymal stem cells and genetically engineered stem cells can be used.

In order to assess the efficacy of treatment, it is necessary to obtain a general consensus on the outcome to be achieved. Fundamentally, the outcomes should be positive on two sides: anatomical and functional. The anatomical and morphological outcome consists first of the higher number of MNs compared with vehicle-treated diseased animals. A future, very ambitious, goal will be to increase the number of MNs by differentiating stem cells. Relative to the functional outcome, it will consist of a less rapid decrease in motor performance as assessed with a battery of behavioural tests, such as the rotarod test, paw grip endurance test and hanging wire test in animal models. Similarly, a battery of motor and behavioural tests must be used in patients to investigate the functional outcome of stem cell transplantation. Very sensitive and reliable electrophysiological protocols must be developed to detect changes in the progression of the disease related to collateral reinnervation or preservation of MNs such as Motor Units Number Estimations (MUNEs). Moreover, objective standardised measures of muscular strength [9,10] could help in monitoring the functional changes in the transplanted areas.

Two major objectives can be aimed at in applying stem cells for ALS therapy: replacement and neural protection. Replacement consists of differentiating MNs either in vitro or in vivo from embryonic or adult stem cells; these newly generated MNs should send their axons through the anterior branch of the spinal nerve to their appropriate target muscle. MNs have been obtained from embryonic neural stem cells [11] and several authors have claimed that neurons to be retrogradely transported of the MN.

Somewhat in between local implantations, cells can be injected into the lateral ventricles; this way of injection bypass the BBB, reducing the time-course. However, the diffusion of cells and the subarachnoidal space is frequent interruptions in the CNS, since it is unclear what extent the ependymal layer to enter.

Another fundamental issue is the origin of the transplanted. The more undifferenti
status, stem cells can be divided into embryonic and adult cells; the former are isolated from the inner layer of the blastocyst, formed 4–5 days after fertilisation; the latter are specialised cells found within many tissues of the body (brain, bone marrow, liver, skin, gastrointestinal tract, cornea, retina and dental pulp, for example), that are in a dynamic state even in absence of injury and are able to differentiate into several different cell types [15].

According to their differentiation potential, stem cells can be further classified as totipotent, pluripotent, multipotent and unipotent.

Totipotent stem cells derive from early (1–3 days) embryos and can give rise to all differentiated cell types in an organism; pluripotent cells derive from the 5–14-day-old blastocyst and can originate almost any cell type; fetal tissue, cord blood, peripheral blood and bone marrow for example contain multipotent stem cells, that can differentiate into cell types characteristic of only one specific tissue; finally, unipotent cells can self-renew and originate only one cell type [16,17].

For their potential to originate new cells of any type, stem cells are ideal candidates for regenerative medicine, tissue engineering, gene therapy and cancer therapies.

In fact, in vitro studies have demonstrated that embryonic stem cells can grow indefinitely depending on culture conditions, and can differentiate into somatic and somatic-like cells, as neurons, cardiomyocytes, hepatocytes and others [17]. Moreover such cells have been employed in numerous studies in animal models, of conditions such as Parkinson’s disease, diabetes, spinal cord injury, Parkinson’s disease, Duchenne muscular dystrophy, liver and heart failure, and osteogenesis imperfecta [18].

Also adult stem cells can differentiate in vitro into several cell types, such as osteoblasts, chondrocytes, endothelial cells, glia, neurons, skeletal and cardiac myocytes, according to their origin [17]. However, the main source from which adult stem cells can be isolated remains bone marrow, containing hematopoietic stem cells and stromal mesenchymal cells. Hematopoietic stem cells can produce cell types and blood cells, and for this reason are successfully used in the treatment of blood cancers and disorders; on the other hand, mesenchymal cells can give rise to bone, cartilage, adipose tissue and muscle cells, and represent a promising tool for the treatment of bone defects, ischemic heart and liver diseases [16].

Ethical issues have been raised relative to the use of embryonic cells, which are not relevant to adult stem cell research. However, recently it has been shown that adult differentiated cells, such as mouse fibroblasts, can be reprogrammed to an embryonic-like state by introducing four factors, Oct3/4, Sox2, c-Myc and Klf4 [19], even if following studies demonstrated development of tumours correlated with the presence of these oncogenes.

Stem cells can be used for both in vitro disease modelling and therapeutic applications.

4. Stem-cell-derived motor neurons from the patient as a diagnostic tool and a source of cells to transplant

Human cell culture is an essential complement to research with animal models of disease. Murine models of ALS, in fact, do not fully mimic human disease. Stem cells can provide human samples from the patients themselves, which are not transformed or genetically modified, allowing creation of reliable disease models for laboratory studies and drug research [20].

Several groups have generated MNs from human embryonic stem cell (hESC) lines [21] ESCs can be used effectively to identify factors involved in motor neuron degeneration as well as small neuroprotective molecules. Genetically defined ESCs from animal models of amyotrophic lateral sclerosis can give important insights into the pathophysiology of MN deterioration [22]. Human ESC-derived MNs genetically manipulated to express SOD1 mutants exhibit typical signs of MN degeneration linked to ALS, such as reduced cell survival and shortened axonal processes [23].

Moreover, in co-culture experiments, human MNs were selectively sensitive to the toxic effects of glial cells harbouring a mutant allele of the SOD1 gene, while interneuron populations produced from embryonic stem cells were unaffected, in association with several significant changes in glial gene expression. These cultures were used to screen several candidate molecules, possibly involved in the toxic effect of mutant glia, identifying prostaglandin D2 as a toxic molecule for motor neurons [24].

Another considerably interesting approach is to generate iPS lines from patients affected by ALS. iPS cells are the product of somatic cell reprogramming to an embryonic-like state. This occurs by the introduction of a defined and limited set of transcription factors and by culturing these cells under ESC conditions. The method was first described by Shinya Yamanaka and colleagues [25] and recently MNs have been derived from an old patient bearing a familial form of ALS [26]. Human iPS cells might represent an ideal cell source for cell therapy given that iPS cells can be derived from the patients themselves thus preventing immune rejection. However, human iPS cells have not yet been directed to differentiate into a specific functional tissue [27].

5. Stem cell therapy for ALS

Any experimental therapeutic approach to ALS is complicated by the unknown origin, the spatial diffusion of the MN loss and the paucity of animal models. Despite such daunting challenges, a number of the potential benefits of stem cells in ALS therapy have been demonstrated in experimental models.

First, transplanted stem cells of both neural and bone marrow lineages improve survival and function of endogenous glial and neural precursors by producing neurotrophic and
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growth factors [28,29]. The efficacy of this approach could be improved by genetically modifying the stem cells to secrete molecules that promote motor neuron survival (reviewed in [30]).

Second, both neural and non neural stem cells can differentiate into glial lineages [31,32] replacing the surrounding cells, which nurse and protect neurons. Moreover neural stem cells seem to be neuroprotective against glutamate-induced spinal MN neurotoxicity [33], which represent a well established pathogenic mechanism in ALS. More recently Lepore and colleagues [34] reported an improvement of survival and disease duration in SOD1<sup>G93A</sup> transgenic mice engrafted with astrocyte precursors, mediated by the primary astrocyte glutamate transporter GLT1.

Third, both neural precursor cells [35] and mesenchymal stem cells [23,36] promote 'bystander' immunomodulation, as they can release soluble molecules and express immuno-relevant receptors that are able to modify the inflammatory environment. Although ALS aetiology is not yet well understood, an inflammatory reaction characterised by increase of immunoglobulins and lymphocytes has been demonstrated [37]. Therefore inflammation could represent a possible therapeutic target, eventually exploiting stem cells ability. When transplanted intravenously [38,39], stem cells were also found in the spleen parenchyma and improved disease outcome, probably through immune modulation. Similar results were obtained in a model of intracerebral haemorrhage followed by intravenous graft of neural stem cells: NSCs reduced cerebral inflammation, modulating the splenic inflammatory pathway [5]. Therefore these cells seem able to promote a bystander modulation, changing inflammatory environment both by release of cytokines and chemokines, and by expression of immuno-relevant receptors [39].

Differentiation toward a neural-like morphology has been demonstrated for both non-neuronal stem cells and neural progenitors [32,40,41], with some evidence of axonal sprouting in the second ones [42,43]. Xu et al. recently demonstrated that fetal neural stem cells grafted into the spinal cords of normal and SOD1-mutated rats differentiate into interneurons and form structurally mature synapses [43]. When induced to differentiate, neural stem cells can give rise both in vitro and in vivo to cholinergic motoneurons, even though in a small percentage [44].

In light of all these stem cells properties, future stem cell therapies for MN diseases could include a synergic combination of strategies aimed at both neuroprotection of host MNs and cellular replacement of neurons and glia. Many experiments have been performed in animal models of the MN diseases [45], however no conclusive data can be drawn because of the heterogeneity of the approaches (Table 1). The translation of preclinical studies in mutant SOD1 rodent models to humans is limited by different sources of stem cells, routes of delivery, timing of therapeutic intervention and relevance of the animal model of familial ALS to human patients.

The mobilisation of endogenous precursors from germinal niche could be an attractive technique, but proliferation of endogenous progenitors in the adult spinal cord in response to ALS neurodegeneration cannot compensate for the pathogenic loss of MNs [46]. Moreover, the NSC/neuroprogenitor niche in the adult forebrain of ALS mice displays altered proliferation and phenotypic characteristics [47].

Candidate cell types for stem cell therapy in ALS must be able to survive and influence the pathological tissue environment, including inflammatory and immune reactions, and migrate into the sites of diffuse neurodegeneration. Moreover, it is fundamental for clinical application that stem cells are safe, and can be easily isolated and expanded.

ESCs display a great plasticity [48]. In models of acute MN death, ESC-derived MNs implanted into the spinal cord can extend axonal processes to innervate muscle targets [49]. Nevertheless, the differentiation in vivo of uncommitted embryonic and fetal stem cells is conditioned by environmental cues, and how such differentiation can be directed to generate specific subtypes of MNs still remains to be elucidated [50]. Moreover, in addition to ethical considerations concerning the source, the clinical use of ESCs in humans is limited by some key issues, such as their unlimited in vitro proliferation and in vivo teratocarcinoma formation [51].

Neural stem cells might represent a ready-to-use cell source for cell-based therapies, because, when placed in culture, they can be grown and extensively expanded for months, allowing the generation of stem cell lines which maintain stable and constant functional properties, and they have been used in vivo without tumour formation or overt toxic or other side effects [51].

Their derivatives, human neural progenitor cells (NPCs), can be expanded in culture for long periods; they survive and continue to proliferate after transplantation into the adult rodent central nervous system [52]. NSCs constitutively produce neurotrophic factors, in particular GDNF, that can exert a potent protective and neurite outgrowth-promoting effect on motor neurons [53]. Moreover, NSCs are neuroprotective against glutamate-induced spinal MN neurotoxicity in a model of MN apoptosis induced by facial nerve axotomy [28]. Similarly, NSCs can be neuroprotective in SOD1 mice, delaying onset and progression of disease, and extending survival [44]. Neural precursors can also be genetically modified [53] to release GDNF, and following unilateral transplantation into the spinal cord of SOD1-G93A rats there was robust cellular migration into degenerating areas, efficient delivery of GDNF and remarkable preservation of MNs at early and end stages of the disease within chimerical regions [54,55]. Interestingly, this robust MN survival was not accompanied by continued innervation of muscle end plates and thus resulted in no improvement in ipsilateral limb use. Behavioural effects after grafting neuron-like hNT cells (human teratocarcinoma cell-line cultured with retinoic acid) at level of the L4 – L5 spinal segment of transgenic mice have been observed [56].
<table>
<thead>
<tr>
<th>Cell source</th>
<th>Disease model</th>
<th>Route of delivery</th>
<th>Number of cells</th>
<th>Proposed therapeutic mechanism</th>
<th>Outcomes</th>
<th>Ref.</th>
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<tbody>
<tr>
<td>Human UCBs (pooled donors)</td>
<td>Presymptomatic, irradiated sod1 (g93a) mice</td>
<td>Intravenous</td>
<td>34.2 - 35 × 10⁶</td>
<td>Immunomodulation/ providing non mutant (functional) sod1 enzyme</td>
<td>Delay in disease onset (22 days) and increased lifespan (21 days)</td>
<td>[38]</td>
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<tr>
<td>Wild-type mice BMCs</td>
<td>Presymptomatic, irradiated sod1 (g93a) mice</td>
<td>Intravenous</td>
<td>5 × 10⁶</td>
<td>Immunomodulation/ providing non mutant (functional) sod1 enzyme</td>
<td>Delay in disease onset (7 days) and increased lifespan (12 - 13 days)</td>
<td>[38]</td>
</tr>
<tr>
<td>Human UCBs</td>
<td>Presymptomatic, sod1 (g93a) mice</td>
<td>Intravenous</td>
<td>10⁶</td>
<td>Neuroprotection by modulation of autoimmune processes</td>
<td>Delayed disease progression (at least 2 - 3 weeks) and modestly increased lifespan</td>
<td>[39]</td>
</tr>
<tr>
<td>Human UCBs</td>
<td>Presymptomatic, sod1 (g93a) mice</td>
<td>Intravenous</td>
<td>10 × 10⁶, 25 × 10⁶, 50 × 10⁶</td>
<td>Modulating the host immune inflammatory system response</td>
<td>Dose of 25 × 10⁶ cells increased lifespan by 20 - 25% and delayed disease progression by 15%</td>
<td>[97]</td>
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<td>Wild-type mice versus SOD1 (g93a) BMCs (mesenchymal)</td>
<td>Presymptomatic, irradiated SOD1 (g93a) mice</td>
<td>Intraperitoneal</td>
<td>3 × 10⁶</td>
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<td>Delay in onset (14 days) and increased lifespan (12 - 13 days) of wild-type BMCs, no effect of SOD1 mice BMCs</td>
<td>[98]</td>
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<td>Human bone marrow MSCs from adult donors</td>
<td>Presymptomatic, sod1 (g93a) mice</td>
<td>Bilateral lumbar spinal cord injection, different levels</td>
<td>Total amount 10⁶</td>
<td>Increasing neuron survival and preventing astrogliosis and microglia activation</td>
<td>Increased motor neuron count, decreased astrogliosis and microglia activation</td>
<td>[29]</td>
</tr>
<tr>
<td>NSCs from spinal cord of human embryo (8 weeks old)</td>
<td>Presymptomatic, immunosuppressed sod1 (g93a) mice</td>
<td>Bilateral lumbar spinal cord injections</td>
<td>Four sites, 5 × 10³ cells/site</td>
<td>Differentiation of NSCs into neurons, initial networks with host nerve cells, release of growth factors</td>
<td>Delay in onset (7 days) and increased average lifespan (11 days)</td>
<td>[116]</td>
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<tr>
<td>Wild-type mouse embryonic stem cells</td>
<td>Adult rats with chronic diffused motor neuron deficiency (sindbis virus)</td>
<td>Bilateral lumbar spinal cord injections, one site</td>
<td>6 × 10⁴</td>
<td>Motor neurons differentiation, forming junctions with host muscle</td>
<td>Partial recovery from paralysis</td>
<td>[49]</td>
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<tr>
<td>Wild-type adult mouse NSCs (purified from adult brain and primed into a motor neuron phenotype)</td>
<td>Presymptomatic, immunosuppressed sod1 (g93a) mice</td>
<td>Bilateral lumbar spinal cord injections, one site</td>
<td>10⁴</td>
<td>Neuronal and glial differentiation, release of growth factors (trrophic support)</td>
<td>Delay in onset (21 days) and increased average lifespan (22 - 23 days) (unchanged progression), delayed loss of lumbar motor neurons</td>
<td>[44]</td>
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<tr>
<td>Wild-Type Mice NSCs from embryonic spinal cord</td>
<td>Presymptomatic mmd mice (animal model of SMA1)</td>
<td>Intrathecal delivery</td>
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<td>Neuronal and glial differentiation, release of growth factors (trrophic support)</td>
<td>Delayed onset and increased average lifespan (18 - 19 days) Decreased loss of motor neurons</td>
<td>[99]</td>
</tr>
</tbody>
</table>

BMC: Bone marrow cell; GRP: Gliial-restricted precursor; h: Human; MSC: Mesenchymal stem cell; MSC (GDNF): MSC engineered to secrete glial cell line-derived neurotrophic factor; NSC: Neural stem cell; NSC (GDNF): NSC genetically modified to release GDNF; SMA1: Spinal muscular atrophy with respiratory distress type 1; UCB cell: Umbilical cord blood cell.
Table 1. Preclinical transplantation studies of stem cells in animal models of ALS (continued).

<table>
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<tbody>
<tr>
<td>Human NSCs (GDNF)</td>
<td>Presymptomatic, immunosuppressed SOD1 (G93A) rat</td>
<td>Unilateral lumbar subcutaneous injections, one site</td>
<td>$12 - 18 \times 10^4$</td>
<td>Trophic support</td>
<td>Efficient delivery of GDNF, motor neuron preservation, no improvement in ipsilateral limb use</td>
<td>[55]</td>
</tr>
<tr>
<td>Human MSCs (GDNF) from neonatal bone marrow</td>
<td>Presymptomatic, immunosuppressed SOD1 (G93A) rat</td>
<td>Bilateral injection into three skeletal muscle groups</td>
<td>$12 \times 10^5$</td>
<td>Trophic support</td>
<td>Increased number of neuromuscular connections and motor neuron cell bodies in the spinal cord; increased overall lifespan by up to 26 days</td>
<td>[92]</td>
</tr>
<tr>
<td>Glial-restricted precursors (GRPs)</td>
<td>SOD1 (G93A) rat</td>
<td>Transplantation around cervical spinal cord</td>
<td>$9 \times 10^5$</td>
<td>GRPs, efficiently differentiated into astrocytes and reduced microgliosis</td>
<td>Extended survival and disease duration, attenuated motor neuron loss and slowed declines in forelimb motor and respiratory physiological functions</td>
<td>[34]</td>
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<tr>
<td>Wild-type rat mesenchymal stem cells (MSCs)</td>
<td>14 weeks transgenic SOD1-Leu126delTT mice</td>
<td>Intrathecal transplantation via the fourth cerebral ventricle</td>
<td>$3 - 4 \times 10^5$</td>
<td>Neuroprotection, modulation of the neural environment</td>
<td>Females, but not males, showed a statistically longer disease duration</td>
<td>[100]</td>
</tr>
<tr>
<td>Mouse olfactory ensheathing cells (OECs)</td>
<td>14 weeks transgenic SOD1-Leu126delTT mice</td>
<td>Intrathecal transplantation via the fourth cerebral ventricle</td>
<td>$3 - 4 \times 10^5$</td>
<td>No benefits</td>
<td>No significant differences in clinical evaluation</td>
<td>[101]</td>
</tr>
<tr>
<td>Human bone marrow-derived mesodermal stromal cells (hMSCs)</td>
<td>Pre-symptomatic ALS mouse model overexpressing G93A</td>
<td>Intrathecal transplantation (via cisterna magna)</td>
<td>$10^5$</td>
<td>No benefits</td>
<td>Negative outcome</td>
<td>[100]</td>
</tr>
<tr>
<td>Umbilical cord blood cells (hUCBs)</td>
<td>Pre-symptomatic ALS mouse model overexpressing G93A</td>
<td>Intrathecal transplantation (via cisterna magna)</td>
<td>$10^5$</td>
<td>No benefits</td>
<td>Negative outcome</td>
<td>[100]</td>
</tr>
<tr>
<td>Wild-type rats MSCs</td>
<td>Symptomatic hSOD1G93A</td>
<td>Intrathecal delivery (lumbar level)</td>
<td>$2 \times 10^6$</td>
<td>MSCs; substantial infiltration into the ventral horn; massive differentiation into astrocytes; decreased motor neuron loss</td>
<td>In treated rats the first signs of paralysis were detected 14 days later compared with sham animals; the life expectancy was increased by 16 days</td>
<td>[117]</td>
</tr>
</tbody>
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BMC: Bone marrow cell, GRP: Gliial-restricted precursor, h: Human, MSC: Mesenchymal stem cell, MSC (GDNF): MSC engineered to secrete glial cell line-derived neurotrophic factor, NSC: Neural stem cell, NSC(GDNF): NSC genetically modified to release GDNF, SMARD1: Spinocerebellar atrophy with respiratory distress type 1; UCB: Umbilical cord blood cell.
Physiologically MNs and glial cells mutually affect their function and survival, but in a degenerative disease like ALS a disturbance of this balance between glial cells and neurons occurs, influencing disease onset and leading to the death of MNs. In the light of this evidence, therapeutic strategies involving glial cells could represent a feasible approach for developing new ALS treatments [57,58].

Olfactory epithelium represents another easily accessible source of stem-like progenitors, that can differentiate both into supporting cells or neurons [59]. Experimental trials in various different models of acute or chronic rodent spinal cord injury have suggested the ability of olfactory ensheathing cells (OECs), taken from olfactory bulb or mucosa, to stimulate tissue sparing and neuroprotection, to enhance outgrowth of both intact and lesioned axons, activate angiogenesis, change the response status of endogenous glia after lesion and remyelinate axons after demyelinating insults. Their ability to stimulate regeneration in specific tracts appears, however, limited (reviewed in [60]).

6. Mesenchymal stem cells and ALS

Numerous reports demonstrate the plasticity of non-neural cells, such as bone marrow stromal and cord blood adult stem cells (reviewed in [61]). Pluripotent hematopoietic stem cells (HSCs) from adult bone marrow may give rise to neurons, oligodendrocytes and astrocytes after transplantation into newborn brains [62]. When grafted into the spinal cord, HSCs express GDNF (glial cell line-derived neurotrophic factor) and induce its production by the host cells [63]. Healthy bone marrow transplantation in mutant SOD mice demonstrated that functional CD4+ T cells, either directly or indirectly, can modulate microglial and astrogial activation, attending trophic/cytotoxic balance of glia and, in this way, assuring neuroprotection and prolonging survival [64]. Autologous transplantation of bone marrow stem cells (BMSCs) fully circumvents the problem of immune rejection, does not cause the formation of teratomas and bears no ethical or political concerns. Pre-clinical studies have shown the effectiveness and positive safety profile of treatment with autologous bone marrow cells [65].

Mesenchymal stem cells (MSCs) are very attractive multipotent stem cells for ALS cell therapy because of their great plasticity [66] and their ability to provide the host tissue with growth factors or to modulate the host immune system [67]. They can be easily isolated from bone marrow (BM) and expanded in culture. Although MSCs lack unique cell markers, minimal criteria for their characterisation, including immunophenotype and differentiating potential, have been established [68]. Despite evidence that MSCs can transdifferentiate into multiple cell types in vitro and in vivo, their real contribution to tissue repair is still unclear [69].

Actually, human MSCs (hMSCs) under specific culture conditions can express neural markers, such as GFAP, Nestin, Tuj-1, tyrosine hydroxylase and MAP2 [70-73] and display at electrophysiology K+ channels usually expressed in cerebral cortex [40]. Our experiments in vivo [29], in agreement with others [12,62,74-75], seem to support a neural differentiation of hMSCs. On the other hand, neural differentiation has been questioned [41,42]; the morphological changes in culture might be consequent to cellular toxicity and related cytoskeletal changes [76-78] and even undifferentiated MSCs express markers for different cell lines at very low levels which can be increased [79,80]. Therefore, as for neural stem cells, the neurotrophic and immunomodulatory roles seem more relevant than the potential for cell replacement. The production of trophic factors might support the survival, migration and differentiation of endogenous precursors [81]. Moreover, the immunomodulatory properties of stem cells, rather than transdifferentiation potential, could be relevant for experimental results.

MSCs have been tested with success in rodent models to treat diseases such as multiple sclerosis and diabetes where immunomodulation is thought to be the main operative mechanism [82,83]. MSC transplantation increases neuron survival and prevents astrogliosis and microglia activation [29]. Astrocytes are both the target and cause of neuroinflammation, since when stimulated by mediators released from microglia they downregulate the expression of neurotrophic factors and release additional inflammatory mediators, which, in turn, further activate microglia [84]. Reactive astrogliosis is present in the presymptomatic stage and gradually increases to end-stage ALS [85]. Several studies demonstrated that the expression of proinflammatory mediators is an early event in murine ALS, even preceding the development of clinical signs. A role in preventing astrogliosis and microglial activation has been suggested also for neural stem cells (reviewed in [86]).

MSCs can rescue neurons and oligodendrocytes from apoptosis through the release of trophic and anti-apoptotic molecules, resulting in the induction of a neuroprotective microenvironment. In addition, MSCs can promote the proliferation and maturation of local neural precursor cells, leading to their differentiation into mature neurons and oligodendrocytes [63,66]. MSCs can be considered as trophic mediators [87] via the production of an assortment of cytokines, of the angiogenic VEGF and of the prosurvival gene Akt1, and can be genetically modified to produce and deliver neurotrophic factors [88,89] or angiogenic factor [90] respectively to protect neurons and favour revascularisation in neurodegenerative diseases [91]. Subclones of MSCs already produce brain-derived neurotrophic factor and β-nerve growth factor [28]. Trophic factors produced by MSCs such as VEGF [87] or BDNF [28] can support motoneuron survival both at a distance and by local interaction with motoneurons. In addition, transplanted hMSCs can provide motorneurons with wild type SOD. Similar results were reported by authors using neural stem cells (reviewed in [45]) or hMSCs [91,92] that secrete GDNF.

In addition to their potential therapeutic effects, BM-derived MSCs are almost free from significant adverse
effects. When put in culture, they do not display malignant transformation [89] and maintain a stable profile of mRNA expression of tumour suppressor genes (p53, p16 and RB) and oncogenes (H-RAS) [84]. Most importantly, in vitro transplantation of long-term cultured hMSCs in nude mice did not result in tumour formation [78].

Since they originate from a different lineage from MNs, MSCs are potentially less vulnerable to the pathological mechanisms underlying ALS. We have demonstrated that MSCs isolated from the bone marrow of ALS patients maintain all their peculiar characteristics and when expanded in vitro do not display chromosomal alterations or cellular senescence. Moreover they acquire, under specific culture conditions, new morphological characteristics and neural markers which are suggestive of neural differentiation as well as those obtained in healthy donors [41].

Umbilical cord blood samples, collected from placenta and umbilical cord blood (UCB), are a source of stem and progenitor cells as well. Interestingly, CD34+ and CD45− mesenchymal-like stem cells could be isolated and propagated through adherent cluster from both cord blood and the cord stroma and exhibited a potential to differentiate into neuron-like cells in culture [74,75]. Moreover, Garzuzzo-Davis et al. [39], showed that a single intravenous administration of mononuclear cells from hUCB into pre-symptomatic G93A SOD1 mice delayed disease symptom progression and extended lifespan.

7. Administration routes

In view of the different approaches to stem cell therapy, it is mandatory to develop feasible and reliable methods of delivery. Proposed approaches include direct cell transplantation into the CNS parenchima, intravenous or intrathecal delivery and combined strategies. The route of cell administration, which represents another constraint for stem cell therapy in neurological diseases, is very much dependent on the CNS lesion sites. Given the widespread cell loss in ALS a 'systemic' route of administration could be the most effective therapeutic approach.

Intravenous delivery represents the less invasive approach, but is it uncertain whether the infused stem cells can successfully cross the BBB. In SOD1 mice BBB is damaged [95], even though it has been reported that the tight junctions of the capillary endothelial cells, that prevent the passive diffusion of circulating cells from blood, appear intact [96]. Nevertheless, intravenous administration of mononuclear cells from human UCB into pre-symptomatic SOD1 [98] mice delayed the progression of the disease and extended lifespan [39,97]. Similar positive results were obtained after injection of hMSC into retro-ocular space or into murine tail vein [98] or intraperitoneally [98]: in fact, in this last case bone marrow cells were found in brain, cerebellum, spinal cord, heart and skeletal muscles, contributing to ameliorating the disease phenotype of SOD1 mice. Although intravenous and intraperitoneal transplantations are feasible, not invasive and without side-effects, they require a large number of grafted cells (up to $35 \times 10^6$ cells in mice) and immunosuppression in the case of non-autologous cells. The therapeutic effect is probably related to decreased pro-inflammatory cytokines in the brain and spinal cord, reduced microglia density in the spinal cord, and restored leukocyte profiles in the blood of treated mice, even in absence of a significant cell penetration in the CNS.

Intrathecal delivery is characterised by the low invasiveness and allows multiple engraftments at required intervals. This administration route is already used for effective delivery of other substances such as neurotrophins and neurotrophic molecules [such as, ciliary neurotrophic factor (CNTF); brain-derived neurotrophic factor (BDNF), IGF-1] or drugs.

Intrathecal transplantation of MSCs in SOD1 rats with a catheter directed from the cisterna magna towards the lumbar enlargement prolonged survival, decreased inflammation and showed neuroprotective effects on MNs. This administration route was used also in other neurodegenerative pathologies, such spinal muscular atrophy with respiratory distress type 1 (SMARD1), delaying disease progression and increasing lifespan [99]. On the contrary, no or poor effect where obtained with intrathecal transplantation of human bone marrow stromal cells (hMSCs), umbilical cord blood cells (hUBCs) and their neuroectodermal derivatives [100], olfactory ensheathing cells (OECs) and BM-MSCs [101]; grafted cells often remain on the surface, without invading the spinal cord parenchima. As yet, no clinical effects have been observed in ALS patients following intrathecal of peripheral blood stem cells [102].

Positive outcomes have been achieved in ALS animal models with both neuronal and non neuronal stem cells by spinal intraparenchymal implantation [29,56,103-105]. It has been suggested that the proximity of grafted cells favours the diffusion of trophic and immunomodulatory factors to MNs and surrounding glia. This holds true especially when targeting specific neurons of the spinal cord, even though in some cases multiple injections do not seem to provide advantages relative to single-site transplant [29,56,103-105].

Recently local injections produced a neuroprotective effect, using neural progenitors [55] and glial restricted precursors [34]. Reactive astrocytes can affect the survival of healthy MNs; in fact, astrocytes carrying the SOD1 mutation release toxic factors to motor neurons [106]. Thus, replacement of glial cells via stem cell transplantation could dilute the toxic effects of host astrocytes and release neuroprotective factors [30]. Astrocyte precursors, transplanted into the cervical spinal cord close to respiratory MN pools of transgenic SOD1 rodents, survive, differentiate into astrocytes and reduce microgliosis. Moreover, they extend survival, attenuate MN loss and slow the decline in forelimb motor and respiratory functions [34].

All these experimental data seem to suggest that targeting multisegmental cell delivery to the cervical spinal cord might
be a promising therapeutic strategy for slowing focal MN loss associated with ALS.

Intraparenchymal delivery in patients requires a technology capable of a safe targeted, localised administration: a stabilised platform can be used to achieve accurate targeting of infused cells to the ventral horn with the use of MER and motor evoked potentials as guidance tools [106].

Finally, while ALS is characterised by progressive loss of neurons and their connections to muscles, and moreover while recent studies have demonstrated that skeletal muscle is a primary target of SOD1G93A-mediated toxicity [107], intramuscular transplantation could represent another helpful therapeutic approach: in particular, grafting of stem cells (myoblasts or MSCs) genetically modified to produce GDNF significantly delayed progression of disease, probably acting as 'mini-pumps' delivering growth factors directly into affected muscles [92]. These data referred to transplantation in gastrocnemius, tibialis anterior muscle, forelimb triceps brachii and long muscles of dorsal trunk, but in the future the aim could be targeting muscles of the diaphragm in order to protect respiratory MNs.

8. Association of stem cell therapy to delivery of trophic factors

Furthermore, treatment might combine cell transplantation and administration of growth factors [95]. We can, in fact, suggest that there is a synergy between stem cells and the growth factors release when administered together. Some studies where expression of GDNF in the lumbar spinal cord was achieved using direct lentiviral expression had no effects on motor neuron survival [108] while in other studies using wild-type hNPC, those secreting GDNF had a highly significant protective effect on motor neuron cell death in chimaeric regions of theSOD1G93A rat spinal cord [55]. One report showed that myoblasts modified to secrete GDNF can prevent motor neuron loss in a model of ALS [109] while hMSCs engineered to secrete glial-cell-line-derived neurotrophic factor (hMSC-GDNF) and transplanted into the skeletal muscles of SOD1G93A rats, significantly increases the number of neuromuscular connections and motor neuron cell bodies in the spinal cord at mid-stages of the disease and delays disease progression, increasing overall lifespan [92].

9. Clinical trials

Questions related to the source and optimal number of cells to engraft and the ideal way of delivery to guarantee stem cells availability in the affected regions of the central nervous system are still open. However, given the absence of a primate model and the lack of any effective treatment, it is mandatory to undergo clinical trials when the major requirements for cell therapy in animal model are satisfied and successful. This position is also reported in the Guidelines for the Clinical Translation of Stem Cells, by the International Society for Stem Cell Research [110].

Strict cooperation between clinicians and basic science researchers is mandatory to develop reliable protocols. Clinics where harvesting, culture, purification and storage of the human stem cells are performed in authorised and highly specialised laboratories and the patients are recruited, treated and monitored in a tertiary ALS centre are the ideal sites for well managed clinical trials.

Over the past few years stem cell research has greatly expanded in clinics in order to develop innovative therapies for treating incurable neurodegenerative diseases. Some clinical trials have been developed for ALS (Table 2).

We tested the feasibility of menenchymal stem cells transplantation in ALS patients in two Phase I clinical trials. MSCs were isolated, expanded and analyzed as described elsewhere in detail [111], according to the GMP conditions (European Medicines Agency,1999). Surgery was uneventful in all patients. Mild (OMS grade I – II) and reversible symptoms were reported by patients: pain in the trunk, light-touch sensory impairment and tingling sensation in one lower limb, sensory light-touch impairment in the sacral region, in the absence of structural parenchymal changes and syringomyelia at the MRI both at short and long term.

Recently, another trial was conducted by collecting and re-infusing G-CSF-mobilised peripheral blood stem cells (PBSC) in ALS patients [112], without adverse effects, but with no significant changes in disease progression. However it was concluded that the results paved the way for a properly powered therapeutic trial with an optimised regimen of G-CSF.

Allogeneic transplantation of hematopoietic stem cells (HSC) from human leukocyte antigen identical matched sibling donors in sporadic ALS [113] following total body irradiation show that HSC enter the human CNS primarily at pathological sites acting as immunomodulatory cells. Even though the transplant did not extend survival of patients, such cells might provide a cellular vehicle for future CNS gene therapy.

Bone marrow-derived hematopoietic progenitor stem cells, transplanted into the caudal brain stem and the cranial spinal cord (C1 – C2) in patients affected by sporadic ALS, have been reported to improve symptoms in half patients [114]. In another clinical trial, autologous blood CD133+ stem cells transplanted into the frontal motor cortex were reported to be safe and well-tolerated (at the one-year follow-up) and to delay disease progression, improving quality of life [115].

In sum, stem cell therapy from different cell sources and employing different routes of administration is safe and well tolerated, even though the follow up in several studies is probably too short to exclude possible delayed complications, such as tumour formation. On the other hand, only some studies report clinical benefits: anyway, in our opinion, given the small number of clinical trials and some methodological issues, the results do not allow clear cut conclusions.
Table 2. Clinical trials of stem cells in ALS.

<table>
<thead>
<tr>
<th>Stem cells</th>
<th>Route of delivery</th>
<th>Number of patients</th>
<th>Patients characteristics</th>
<th>Outcome</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Autologous MSC (from bone marrow)</td>
<td>Injection into the central part of thoracic SC after laminectomy and mielotomy</td>
<td>9</td>
<td>Spinal onset, FVC &gt; 50%, normal polysomnography, ambulation with assistance or wheelchair bound Age 32 – 75, Months from diagnosis 8 – 60</td>
<td>Safe and well-tolerated even in long-term (4 years)</td>
<td>[111]</td>
</tr>
<tr>
<td>Peripheral blood stem cells (PBSC)</td>
<td>Mobilisation of autologous PBSC with GCSF</td>
<td>8</td>
<td>Seven patients had limb onset; Time interval from onset: 3 months to 4 years. Three patients wheelchair-bound and five ambulatory. Pre-treatment FVC range 50 – 150%</td>
<td>Safe and well tolerated. No significant changes in disease progression</td>
<td>[112]</td>
</tr>
<tr>
<td>Allogenic hematopoietic stem cell (HSCT)</td>
<td>Intravenous infusion following total body irradiation; immuno-suppression</td>
<td>6</td>
<td>Spinal cord or bulbar onset, FVC &gt; 60%, Age 35 – 59, Months from diagnosis 5 – 30</td>
<td>Tolerated (three chronic GVHD). No clinical benefits. Autopsies: spinal cord engrafted with immune cells, probably donor-derived</td>
<td>[113]</td>
</tr>
<tr>
<td>Autologous Bone marrow (BM)-derived hematopoietic progenitors</td>
<td>Laminectomy; cells injected to the anterior part of the spinal at the C1 – C2 level. (free hand?)</td>
<td>13</td>
<td>2 – 5 years from disease onset; age 34 – 71; ‘moderate or severe’ symptoms, three patients ventilation bounded</td>
<td>Nine patients ‘became much better’ (improved neck and limbs MRC; EMG findings of ‘regeneration’). One patient was stable. Three patients died (1, 5, 2 and 9 months after), of lung infection or myocardial infarction</td>
<td>[114]</td>
</tr>
<tr>
<td>Autologous blood purified CD133+ stem cells</td>
<td>Bilateral implantation in frontal motor cortex, with stereothathic or navigation guidance</td>
<td>10</td>
<td>Age 38 – 62; 18 – 42 months from diagnosis; no patients with severe bulbar involvement or malnutrition; occurrence of FVC values</td>
<td>Safe and well-tolerated (1 year follow-up). Patients survival significantly higher than control group (10 non-operated ALS patients)</td>
<td>[115]</td>
</tr>
</tbody>
</table>

ALS-FRS: ALS-functional rating scale; FVC: Forced vital capacity; GVHD: Graft-versus-host disease; MSC: Mesenchymal stem cell; SC: Spinal cord.

Stem cell clinical trials represent a new scenario in ALS clinical research. Our and other author's studies demonstrate that a surgical approach in ALS is feasible. The concerns that the surgical procedure may be harmful to the spinal cord or that general anaesthesia may cause severe complications in ALS patients are, apparently, not legitimate. Efforts should be directed to designing brief clinical trials that may provide the most meaningful information. It is important for the application and development of stem cell therapeutic approaches to develop non-invasive methods to monitor the modifications of cerebral and spinal cord tissue that lead to functional outcome. A better monitoring of transplanted MSCs and of their morphological outcomes could be obtained from ongoing neuroimaging studies (i.e., diffusion tensor imaging). Moreover, patient selection represents a fundamental issue, taking into account the location, severity, and clinical form of the disease and the adequacy of the site of injection. Delivery of stem cells to the key motor neuron pools innervating respiratory muscles and ultimately affecting survival in ALS patients might represent a helpful short-term clinical approach.

10. Expert opinion

From the study of pharmacological clinical trials we know the extremely limited results in human ALS of several agents successfully tested in animal models and that a drug can have distinct pharmacological effects in different patients. It may lead us to suggest that similar problems will occur for stem cells. Despite many rodent studies showing that cell transplantation can change the disease course the variables responsible for the success of these therapies are largely unknown. Researchers have used different cell types, rodent models, transplant target locations, time of administration, and behaviour tests to assess the transplant's efficacy. The types of cells that may prove to be safer or better remain
purely speculative and await comparison studies. Recent technologies that reprogram adult dermal cells into MNs should be extensively studied in the near future in the attempt to develop an autologous stem cell-based intervention. The route of delivery represents another main point that is under debate. A direct injection might be the most reliable to ensure cells are near the MNs but a less invasive route such as intravenous administration could lead to a clinical efficacy. Laboratory studies on cell therapy for ALS should consider the standardisation of treatment protocols in symptomatic animals and of the outcome measures and safety indices. A primary goal could be to harness a collaboration across laboratories that have extensive experience in conducting studies in animal models using a standardised set of behavioural and histological outcome measures, and demonstrating mechanisms of action for testing the potential of cell therapies in ALS. Investigation of the effect of age and sex on cell transplantation therapy should also be considered as such parameters are generally overlooked in current rodent studies but are of critical clinical significance. The fact that more laboratories will carry out identical studies will help confirm the results for each parameter, which lends an high degree of veracity that is essential for translational studies.

Although ALS is a complex condition to consider for cell therapy, moving into the clinic will be warranted when the main points of the requirements of cell therapy clinical trials are successfully met, because of its severity and the lack of any effective therapy. In order to successfully and accurately translate laboratory research to clinical practice we look forward to new clinical studies ahead to answer some main questions. What kind of patients should be recruited? When should cellular injection be contemplated during the course of the disease? Which cells should be tested in clinical trials? Do we need comparison studies? What kind of motor and behavioural tests must be used in patients to investigate the functional outcome of stem cell transplantation? How long should patients be followed in initial safety studies?

The future for cell therapy is exciting and opens a new scenario in the organisation of clinical trials in ALS. Many efforts should be addressed by clinicians expert in ALS to develop new small meaningful Phase 1 clinical trial. The physicians facing ALS patients in their clinical practice know very well that many of them are queueing to be treated with stem cells in countries where provision of such treatments is not strictly regulated. Rigorously conducted and well managed and designed controlled clinical trials might represent hope and an answer for these patients, avoiding therapeutic misconception resulting from the patient's desire for a miracle cure.

Declaration of interest

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Stem cells in amyotrophic lateral sclerosis: state of the art


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Stem cells in amyotrophic lateral sclerosis: state of the art


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