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Stem Cell-Derived Motor Neurons: Applications and Challenges in Amyotrophic Lateral Sclerosis

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Abstract

Amyotrophic lateral sclerosis (ALS) is a neurodegenerative disease caused by the selective loss of both spinal and upper motor neurons. One strategy in treating ALS is to use stem cells to replace lost spinal motor neurons. However, transplanted stem cell-derived motor neurons may not survive when exposed to the harsh microenvironment in the spinal cord of ALS. In particular, dysfunctional astrocytes and overactivated microglia in ALS may limit the survival of motor neurons generated from cell replacement therapy. On the other hand, stem cells may provide large quantities of motor neurons that can be used for studying glia-mediated toxic mechanisms and potential therapies in ALS. Here we will review methods and molecular factors for directed differentiation of stem cells into spinal motor neurons, the potential uses of these models for dissecting the mechanisms underlying glia-induced motor neuron degeneration and screening for new therapeutics aimed at protecting motor neurons in ALS, as well as discuss challenges facing the development of motor neuron replacement-based cell therapies for recovery in ALS.

Keywords

amyotrophic lateral sclerosis; stem cell; motor neuron; microglia; astrocyte; transplantation

INTRODUCTION

Amyotrophic lateral sclerosis (ALS) is the most common motor neuron disease in adults with an average age of disease onset being in the sixth decade of life. Most ALS patients develop limb weakness initially, which progresses gradually to generalized muscle atrophy and paralysis. Death often occurs within 5 years, usually due to respiratory failure [1]. Unfortunately, ALS is diagnosed very late in the course of disease progression. At the time of diagnosis, symptoms have manifested as a large number of motor neurons have already been lost or degenerated. Thus, in order to restore muscle function and recover from ALS, lost motor neurons will ultimately need to be replaced.

Most cases of ALS are sporadic and not associated with known risk factors. However, 5–10% of cases are inherited [1]. Although many genes have been linked to familial ALS [2], mutations in superoxide dismutase 1 (SOD1) are the most common primary causes and represent 1–2% of total ALS patients [3,4]. Studies on transgenic rodent models overexpressing human mutant SOD1 [5–8] have significantly advanced our understanding of ALS disease mechanisms and allowed testing of a variety of therapeutic strategies in animal models [9]. Despite our improved understanding of ALS pathogenesis from the use of transgenic animal models, there are still

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no effective treatments or preventive strategies in humans. Many potential therapies for ALS, ranging from drugs for anti-inflammation, anti-oxidation and anti-apoptosis, to providing trophic factors, have been unsuccessful in human clinical trials [10]. The only FDA-approved medicine for ALS, Riluzole, acts as an anti-excitotoxicity agent and provides a marginal effect by prolonging lifespan for approximately two to three months. As such, ALS patients are still in desperate need of new therapies.

Recent advances in stem cell differentiation (for motor neuron differentiation methods, see Table 1) and transplantation techniques combined with the need of ALS patients for new therapies prompted the exploration of stem cells for ALS [11,12]. Many types of stem cell therapies have been tested and provided some benefit in transgenic ALS rodent models, including fetal human neural stem and progenitor cells, human umbilical cord blood stem cells, human mesenchymal stem cells, rodent bone marrow and mesenchymal stem cells, mouse olfactory bulb neural progenitor cells and mouse neural stem cells (for details on stem cell therapy in ALS rodent models, see Table 2). The moderate improvement in motor function and slightly delayed disease progression by stem cell transplantation in some of these pre-clinical studies are hypothesized to be, at least, partially due to neuroprotection of endogenous motor neurons through the release of trophic factors that directly promote survival or decrease inflammation [11–14].

Several clinical trials with stem cells have also been reported. Initially, two small trials using autologous peripheral blood cells administered intrathecally or mesenchymal stem cells injected intraspinally showed few or no adverse effects, but the efficacy of such transplantation was not determined [15,16]. More recently, another pilot study confirmed the potential for the autologous transplantation of peripheral blood stem cells that were mobilized with granulocyte-colony stimulating factor, collected and then reinfused into ALS patients [17]. Although the procedure was well-tolerated, no therapeutic benefits were observed. Another clinical trial allografted hematopoietic stem cells in six sporadic ALS patients, but like the autologous transplant study, no clinical benefits were observed [18]. However, transplanted cells in this study did locate into areas of degenerating motor neurons, which suggests their potential to be genetically modified and used to deliver factors to modulate motor neuron injury pathways in ALS patients. Finally, autologous transplantation of mesenchymal stem cells intraspinally into the thoracic cord of nine ALS patients was well tolerated and four patients actually exhibited a slowing in the decline of their forced vital capacity [19]. The exact underlying mechanisms remain unknown. Although neuroprotection may be a viable approach to delaying disease progression in ALS, the ultimate goal of stem cell therapy is still to replace lost motor neurons in order to improve muscle function.

Hence, additional applications of stem cells for treating ALS include replacing dysfunctional astroglia, overactivated microglia or degenerated and lost upper and spinal motor neurons. For the latter, the therapeutic efficacy of stem cell replacement would depend greatly on the survival of grafted stem cell-derived motor neurons in the microenvironment of the ALS spinal cord. Furthermore, transplanted stem cell-derived astrocytes or microglia may become activated and dysfunctional in the inflammatory and oxidative environment of the ALS cord, thus, rendering them toxic to endogenous motor neurons. The environment in ALS degenerating areas, especially after disease onset, may be hostile to grafted stem cell-derived motor neurons and glia due to progressive and exacerbated neuroinflammation, oxidative stress and glutamate excitotoxicity. Moreover, already arduous tasks such as incorporation into the neural circuitry, targeted axonal growth and reinnervation of denervated muscle fibers may be further hampered in ALS where transplanted stem cell-derived motor neurons may degenerate rather than prosper. This article will begin with a brief review of glial cells and particularly oxidative stress in ALS that create a hostile microenvironment to both endogenous and transplanted motor neurons. It will then focus on the current knowledge of spinal motor neuron generation during

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development and from stem cells. Finally, the article will discuss potential uses and challenges of stem cell-derived motor neurons for dissecting ALS disease mechanisms, screening novel drug-based therapeutics aimed at protecting motor neurons in ALS and developing motor neuron replacement-based cell therapies for recovery in ALS.

AMYOTROPHIC LATERAL SCLEROSIS

Pathogenesis and Motor Neuron Susceptibility

Mutations in SOD1 result in a toxic gain of function for which the exact mechanisms remain unclear. However, several hypotheses have been proposed for the pathogenesis of mutant SOD1-mediated ALS, many of which may also be applicable to non-SOD1 linked familial and sporadic ALS, since all forms of ALS share striking similarities in pathology and clinical symptoms. These include mitochondrial dysfunction, oxidative damage, glutamate excitotoxicity, protein aggregation, proteasome dysfunction, cytoskeletal and axonal transport defects and inflammation [1,20–24]. These factors may be linked in that one factor could be the cause or consequence of the other factors. Furthermore, multiple cell types have been implicated in mutant SOD1-mediated ALS pathogenesis. The latest evidence suggests that mutant SOD1-mediated ALS is initiated due to mutant SOD1 expression within motor neurons and other yet unidentified non-motor neuron cells excluding astrocytes, microglia and oligodendrocytes [25,26], while disease progression is accelerated by dysfunction or activation of surrounding mutant SOD1-expressing non-neuronal cells such as astrocytes and microglia [25,27–29].

The selective motor neuron degeneration observed in ALS pathogenesis is speculated to be, at least, partially due to the vulnerability of motor neurons. These cells may be at a higher risk due to their high metabolic activity, low levels of reduced glutathione and high levels of unsaturated lipids on their large membrane surfaces along axons. These risk factors presumably contribute to an increased susceptibility to oxidative damage. In addition, high levels of glutamate input are also present partly due to a decreased expression of the excitatory amino acid transporter 2 (EAAT2) in astrocytes, the main mediator of extracellular glutamate removal, in combination with high levels of AMPA-receptor expression on motor neurons. This increase in glutamate input is accompanied by low levels of intracellular calcium-binding proteins, which may result in a toxic level of intracellular calcium [30–33]. Although still controversial, motor neuron death in ALS seems to be attributed to caspase-mediated apoptosis [34–37]. The susceptibility of motor neurons to the hostile microenvironment generated by overactivated astrocytes and microglia during disease progression becomes particularly relevant in terms of post-transplant survival and function of stem cell-derived motor neurons.

Astroglia and Microglia Involvement in ALS

Activated microglia and astroglia are found in the degenerating areas in both ALS patients and human mutant SOD1 transgenic mice [38–43]. It is known that these glial cells play important roles in progressive motor neuron degeneration in transgenic ALS rodent models [25,29]. Although astrogliosis and microgliosis are pathological hallmarks of ALS [44–46], the critical roles of microglia and astroglia in ALS pathogenesis have only recently been revealed. Accumulated evidence shows that the ALS phenotype is only induced by the expression of human mutant SOD1 in all cell types in the central nervous system (CNS), but not by targeted expression within motor neurons, astroglia or microglia alone [47–50]. However, high levels of neuron-specific mutant SOD1 expression in transgenic mice resulted in motor neuron degeneration, likely due to the mutant SOD1 aggregation-induced disruption of the cytoskeletal structure and trafficking within dendrites [51]. Furthermore, selectively decreasing the expression of human mutant SOD1 in motor neurons delays the onset of the disease whereas selectively decreasing the expression within astroglia or microglia delays the progression of

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the disease after disease onset [25,29]. In addition, the presence of wild-type non-motor neuronal cells in chimeric mice expressing mutant SOD1 in all motor neurons and oligodendrocytes also results in a significant delay in disease onset [26]. Cell types other than motor neurons that may determine disease onset, but have not yet been tested, include interneurons, Schwann cells, and the endothelial cells that make up the vasculature of the brain-spinal cord barrier [26]. Interestingly, there is evidence that mutant SOD1 causes endothelial damage and disruption of the blood-spinal cord barrier prior to disease onset [52]. Accordingly, mutant SOD1-mediated ALS progresses through a non-cell autonomous mechanism in which the disease is initiated by mutant SOD1-acquired damage and protein aggregation [25,26,53–61] within motor neurons as well as other unidentified non-motor neurons, while disease progression is accelerated by dysfunction or activation of the surrounding mutant SOD1-expressing non-neuronal astroglia and microglia [25,27,29,50]. In other words, mutant SOD1-aquired damage within motor neurons and other non-motor neuronal cell types may cause initial degeneration and retraction of axons from neuromuscular junctions, thus resulting in disease onset, while overactivated astroglia and microglia hasten disease progression by producing a microenvironment toxic to motor neurons through increased inflammation, oxidative damage and glutamate excitotoxicity.

Under normal conditions, microglia, which are the principal immune cells in the CNS, protect neural cells against invading pathogens and neoplastic cells, while astroglia support neurons by providing neurotrophic factors, regulating glutamate levels and synaptic transmission, secreting reduced glutathione and providing precursors for reduced glutathione synthesis in motor neurons. However, in ALS, astrocytes and microglia may be pathologically activated by proinflammatory cytokines and increased oxidative stress. Overactivated astroglia and microglia then produce high levels of neurotoxins, including proinflammatory factors [36,46, 62–66], reactive oxygen species and nitrogen species (ROS/RNS), and glutamate [67,68], which exacerbate inflammation and perpetuate a vicious cycle that results in the degeneration and loss of motor neurons. Reactive astroglia may also lose their ability to regulate synaptic transmission, take up excess glutamate in the cerebrospinal fluid, maintain normal glutathione levels in motor neurons [69] and provide neurotrophic support, which may all contribute to motor neuron degeneration. In addition, mutant SOD1-expressing astroglia secrete toxic factors, including prostaglandin D2, which selectively kill motor neurons [70–72].

Activated microglia also accelerate ALS disease progression. Their activation in ALS is accompanied by increases of inflammatory cytokines such as TNFα, IL-1β and IL-6 [36,63, 64,66,73–75]. Furthermore, microglial activation is initiated before significant motor neuron loss and disease onset [36,62]. The broad suppression of microglial activation is neuroprotective as it delays disease onset and prolongs lifespan in transgenic ALS models [76–80]. However, conditioned media from microglia derived from neonatal ALS mice show no toxicity to mouse primary or embryonic stem cell-derived motor neurons [71]. On the other hand, brain microglia from neonatal ALS mice caused a significant loss of primary mouse motor neurons when the cells were cocultured [68].

Activated astroglia and microglia contribute not only to the degeneration of endogenous motor neurons, but may also underlie the degeneration of grafted stem cell-derived motor neurons[1, 2]. It has previously been shown that human neural stem cell (NSC)-derived motor neurons, when grafted into an axotomy model of motor neuron degeneration, innervated peripheral muscles and improved motor functions [81]. However, human NSC-derived motor neurons

[1]Data published as abstract (Thonhoff JR, Jordan PM, Gao J, Wu P. Effect of normal and ALS rat astroglia on human fetal neural stem cells. Society for Neuroscience Annual Meeting, 2007).

[2]Data published as abstract (Thonhoff JR, Gao J, Wu P. ALS rat microglia are toxic to human fetal neural stem cell-derived motor neurons through oxidative stress. Society for Neuroscience Annual Meeting, 2008).

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showed signs of oxidative damage and degeneration when grafted into ALS rat spinal cords, indicating that an oxidative harsh environment in the spinal cord may affect the survival and maturation of transplanted cells[1,2]. This result correlates with reports that neonatal astroglia from ALS mice are toxic to embryonic stem cell-derived motor neurons [70–72], and that adult astroglia and microglia isolated from ALS rats after disease onset kill human NSC-derived motor neurons[1,2]. Further studies are required to determine the mechanisms underlying ALS microglial and astroglial neurotoxicity specifically targeting motor neurons from both endogenous and exogenous sources.

ALS Glial Cells and Oxidative Stress

One of the main consequences of microglial overactivation in ALS is the production of oxidative stress. The elevation of reactive oxygen and nitrogen species (ROS/RNS) beyond endogenous antioxidant capacities plays a role in neurodegeneration by destroying cells through oxidation of proteins (protein carbonyl and protein nitration), lipids (toxic aldehydes) and DNA (mutation). Evidence of redox perturbation has been revealed in both ALS patients and transgenic animal models, including increased protein carbonyl levels [82,83], protein nitration [84–87], and lipid peroxidation [85,88–94]. Oxidative stress has also been linked to excitotoxicity in ALS. The lipid peroxidation product, 4-hydroxynonenal, may interfere with normal transport of the astrocyte glutamate transporter [95]. The impaired glutamate transport may then contribute to excitotoxicity and the ensuing degeneration of motor neurons.

Despite the indisputable presence of oxidative stress in ALS, the initiating cause of the increased ROS/RNS production is ambiguous. The expression and activity of inducible nitric oxide synthase (iNOS), which stimulates nitric oxide production, and nicotinamide adenine dinucleotide phosphate-oxidase (NADPH oxidase or NOX), which generates superoxide, in activated astroglia and microglia are increased in the spinal cords of transgenic ALS animal models and patients [41,44,96–98]. ALS astroglia and microglia also show enhanced iNOS expression and nitric oxide production in response to inflammatory signals [44,50,66,68]. Thus, RNS may play a critical role in ALS microglial neurotoxicity. Increased levels of ROS, including hydrogen peroxide and hydroxyl radicals, have also been observed in the spinal cords of transgenic ALS animals [99]. The most abundant source of ROS in the CNS is generated from the respiratory burst system of activated microglia [100,101]. This system is an assembly of 5 subunits of the NADPH oxidase complex, which includes gp91^{phox} (NOX2), p22^{phox}, p47^{phox}, p67^{phox} and Rac1/2 [102]. Cytokines stimulate normal microglia to produce superoxide [103,104], while microglia from transgenic ALS mice are more sensitive and thus, release more superoxide upon proinflammatory stimulation [50,68]. Increased expression of NADPH oxidase and production of superoxide in the spinal cords of ALS patients and transgenic ALS mice indicate the potential involvement of microglia-generated ROS in the pathogenesis of ALS [98]. Furthermore, inhibiting NADPH oxidase in transgenic ALS mice dramatically prolongs their lifespan [105,106]. As such, ALS microglial toxicity due to increases in ROS and RNS may contribute to a hostile ALS environment detrimental to both endogenous motor neurons and stem cell therapies aimed at replacing motor neurons.

Astroglia are critical in maintaining reduced glutathione levels in neurons [107–109]. Glutathione, a tripeptide containing glutamate, cysteine and glycine, is synthesized by glutamate cysteine ligase and glutathione synthetase. It is the most abundant non-protein thiol and the main antioxidant in the CNS [110]. Astrocytes release reduced glutathione into the extracellular fluid to protect neurons from oxidative stress by ultimately providing the cysteine necessary for neurons to synthesize reduced glutathione [107,110–115]. Reduced glutathione is decreased in the spinal cords of ALS transgenic mice and depleting reduced glutathione in a motor neuron cell line results in motor neuron death [69], but the administration of reduced glutathione was shown not to be effective in a small, randomized clinical trial in ALS patients

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[116]. Furthermore, it is unknown whether the glutathione deficiency is due to the inability of dysfunctional ALS astroglia to maintain sufficient levels of extracellular glutathione, thus, decreasing their capacity to protect motor neurons from increases in oxidative stress. Actually, the factors rendering ALS astroglia dysfunctional and toxic to motor neurons have not been completely elucidated. Some evidence in astrocytes extracted from transgenic ALS rats suggested that mutant SOD1 caused mitochondrial dysfunction and enhanced superoxide generation in these astrocytes, which resulted in motor neuron toxicity through unidentified secreted factors, and that this toxicity was prevented by mitochondrial-targeted antioxidants [117]. On the other hand, interactions between ALS microglia and astroglia have not been thoroughly explored, and it is possible that microglia-generated oxidative stress and proinflammatory cytokines could also cause or enhance astroglia toxicity *in vitro*[2]. Furthermore, it remains to be determined whether ALS astrocytes exert toxicity through these *in vitro* mechanisms in the transgenic ALS animal models and patients.

In summary, both primary and stem cell-derived motor neurons have been shown to be susceptible to mutant SOD1-expressing astroglia- and microglia-induced toxicity *in vitro*, irrespective to mutant SOD1 expression within motor neurons. These findings indicate that after disease onset has occurred in which toxicity driven by non-neuronal microglia and astroglia becomes crucial, transplanted stem cell-derived motor neurons may also be vulnerable to the toxic microenvironment that ensues in the ALS spinal cord. Based on observations that stem cell-derived motor neurons are susceptible to toxic ALS glia, *in vitro* models designed to mimic the ALS microenvironment together with stem cell-derived motor neurons may be used for studying interactions between ALS glial cells and motor neurons. Such models would also allow high-throughput screening for novel ALS therapeutics. Developing methods for generating large quantities of motor neurons from stem cells is the first step in designing experiments to test mechanisms of motor neuron degeneration and potential ALS therapeutics. Identifying causes and mechanisms underlying stem cell-derived motor neuron death will allow us to develop strategies to prevent endogenous motor neuron degeneration and enhance the therapeutic efficacy of replacing lost motor neurons using stem cells in ALS.

SPINAL MOTOR NEURON DIFFERENTIATION

Generation of Spinal Motor Neurons during Development

The specification of cell fate in the CNS is largely dictated by both rostrocaudal and dorsoventral signaling systems [118,119]. According to their position along these two axes, neural progenitor/stem cells (NP/SCs) are exposed to different concentrations of morphogens, which modify their transcriptional profile [120,121]. While the rostrocaudal signaling establishes the main subdivisions of the CNS (the forebrain, midbrain, hindbrain and spinal cord), the dorsoventral signaling system determines the cell types within each of these rostrocaudal subdivisions. Our current understanding of spinal motor neuron generation during embryonic development is based largely on studies in chicks and mice, and has previously been reviewed in details [118,122,123]. Here, we provide a brief review of motor neuron development with the focus on the extrinsic morphogens and intrinsic transcription factors important for motor neuron specification during development *in vivo*, since they are likely to be involved in the fate determination of motor neurons from stem cells *in vitro*.

In the spinal cord, the ventral half of the neuroepithelium gives rise to the floor plate and five populations of neurons: V0, V1, V2 and V3 interneurons and motor neurons [123]. Two important gradient molecules influence the fates of these cells: Sonic hedgehog (Shh) that is initially produced by the notochord and later by the floor plate, and retinoic acid (RA) released from the paraxial mesoderm [123]. Shh signaling induces the more ventral class II homeodomain (HD) transcription factors (TFs) such as Nkx6 and Nkx2.2 and represses the more dorsal class I TFs such as Pax6, Irx3 and Dbx2 [122]. In contrast, RA signaling induces

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the expression of class I TFs. Class-I and Class-II HD TFs cross repress each other, establishing boundaries that define the different cell types along the dorsoventral axis of the spinal cord [118]. Thus, the cells that express Pax6 and Nkx6 become primed to induce the basic helix-loop-helix (bHLH) TF Olig2, which is required for motor neuron differentiation.

Olig2 allows motor neuron progenitors (pMNs) to become motor neurons by inducing the expression of the TFs Ngn2 and Lhx3 [124]. However, Olig2 also antagonizes the premature expression of motor neuron genes such as Hb9, while Ngn2 counteracts this effect [125]. Thus, when Olig2 levels are high the cells are maintained in a pMN state, whereas increasing the levels of Ngn2 favors the conversion of pMNs into post-mitotic motor neurons. Ngn2 is a bHLH TF that regulates the commitment of progenitor cells to both pan-neuronal and specific motor neuron fates [126]. Ngn2 promotes neurogenesis by increasing the expression of neurogenic genes such as NeuroD and β-III tubulin. In addition, Ngn2 possesses two conserved serine residues (S231 and S234) that, when mutated to alanines, impair motor neuron differentiation without affecting general neurogenesis [127]. These serines are phosphorylated by glycogen synthase kinase 3 (GSK3). Serine phosphorylation facilitates the interaction of Ngn2 with LIM HD TFs such as Lhx3 and Isl1, which in a complex activate the transcription of motor neuron-specific genes such as Hb9 [127,128].

In addition to a general motor neuron fate specification, spinal motor neurons also acquire discrete columnar identities as a function of their position along the rostrocaudal axis of the spinal cord [129]. Motor neurons in each column innervate particular target muscles, which is required for proper locomotor function. This patterning of motor neurons into columns is influenced by extrinsic morphogens. Recent evidence indicates that a gradient of fibroblast growth factor 8 (FGF8) is critical for the specification of columnar motor neuron subtypes through FGF8-mediated induction of various Hox-c genes [130]. The posterior Hox genes inhibit the expression of the anterior Hox genes and vice versa. Thus, cell autonomous repressor and activator functions of Hox-c proteins define the boundaries of MN columns. In addition, the forkhead domain transcription factor Foxp1, through its activity as a Hox accessory factor, is required to establish the pattern of LIM-HD protein expression that defines the columnar identity of motor neurons [131,132].

Generation of Spinal Motor Neurons from Stem Cells

Stem cells are cells that self-renew, give rise to differentiated progenies and maintain these properties over a long period of time. For the generation of motor neurons, two major types of stem cells have been used as starting undifferentiated cell sources: embryonic stem cells (ESCs) and neural stem cells (NSCs). ESCs are pluripotent cell lines obtained from the inner cell mass of the blastocyst and have the potential to differentiate into cells of all three embryonic germ layers (i.e. endoderm, mesoderm, and ectoderm). On the other hand, NSCs are multipotent cell lines isolated from nervous tissue, commonly from fetal and adult brains or spinal cords of rodents and humans. These cells have the potential to generate neural cells, including neurons, oligodendrocytes and astrocytes [133–135]. Here, the studies thus far on the generation of spinal motor neurons from both ESCs and NSCs will be reviewed (see Table 1).

Generation of spinal motor neurons has been reported first from mouse ESCs, which apparently recapitulates the embryonic development of motor neurons that requires two steps: general neural induction and motor neuron specification [136]. Under non-adhesive growth conditions, mouse ESCs aggregate forming small spheres called embryoid bodies. Efficient generation of neurons from mouse embryoid bodies requires RA, which promotes neural and represses mesodermal gene expression [137]. Further motor neuron specification from mouse embryoid bodies requires the neuralizing and caudalizing activity of RA together with the ventralizing action of Shh [136]. When treated with RA, but not Shh, less than 0.5% of mouse ESCs differentiate into $Hb9^{+}/Isl1^{+}$ motor neurons [138]. In contrast, treating mouse ESCs with a

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combination of RA and Shh or a Shh agonist generates spinal motor neurons at 20–30% efficiency [136,139,140]. When transplanted into embryonic chick or adult rat spinal cords, mouse ESC-derived motor neurons are able to extend axons into the periphery and form neuromuscular junctions [136,141].

Generation of spinal motor neurons from human ESCs has also been successful with approaches both similar to and different from those used for mouse ESCs. In terms of the initial neural induction, human ESCs, unlike mouse counterparts, require additional signals such as FGF2 or those generated from cocultured feeder cells [142–144]. Neuroepithelial cells derived from human ESCs are then treated with a combination of Shh and RA to generate spinal motor neurons. These neuroepithelial cells show two developmental stages during their differentiation: an early primitive stage where the columnar epithelial cells express several neuroectodermal TFs, but not the definitive neuroectodermal TF Sox1 and a later definitive stage where Sox1 is expressed [145]. It is only in the early stage when neuroepithelial cells are more responsive to RA caudalization and Shh ventralization and thus generate motor neurons efficiently. RA at concentrations from 0.1 to 1 μM induces the expression of Hox genes and downregulates the expression of the rostral TF Otx2. Shh acts on the caudalized neuroepithelial cells to activate the expression of the TFs Olig2 and Hb9 through modulation of other TFs (e.g. Pax6, Nkx6). Using similar protocols with minor variations, several groups reported the generation of $Hb9^+/ChAT^+$ motor neurons from human ESCs with a 20–58% efficiency [142–144,146]. Most recently, a 50% efficiency of motor neuron differentiation through treating human and primate ESCs with purmorphamine, a small molecule that activates the Shh pathway, has been reported [147]. These human ESC-derived motor neurons form synapses on cocultured myoblasts/myotubes and are electrophysiologically functional [142].

As with ESCs, NSCs have also been used to generate motor neurons *in vitro* and *in vivo*. During development, NSCs are found initially in the neural plate and then in the ventricular zone (VZ) of the neural tube where they are thought to acquire a radial glia phenotype [148]. NSCs are also found in the peripheral nervous system such as neural crest stem cells [149]. In the adult brain, NSCs are mainly found in two regions, the subventricular zone (SVZ) of the lateral ventricles where they adopt an astrocyte-like phenotype and the subgranular zone of the hippocampal dentate gyrus [150]. NSCs can also be found in the spinal cord [151] and in areas outside the CNS such as the olfactory epithelium [152]. Spinal motor neurons can be generated not only from spinal cord NSCs that naturally produce this type of neuron during embryonic development, but also from NSCs derived from the regions that do not usually give rise to spinal motor neurons.

Several groups have previously reported the successful generation of spinal motor neurons from rodent embryonic and adult spinal cord NSCs. The isolated NSCs are usually expanded as neurospheres in the presence of basic fibroblast growth factor (bFGF) and/or epidermal growth factor (EGF). By withdrawing growth factors, some of the mouse embryonic spinal NSCs differentiate into $ChAT^+/Isl1^+$ motor neurons [153]. Additional treatment with RA seems to increase $Hb9^+$ motor neuron differentiation from rat embryonic spinal NSCs via the activation of RA receptor β [154]. Another method to obtain motor neurons is to add both Shh and RA in the culture medium. Using this protocol, mouse embryonic spinal NSCs generated significantly more $ChAT^+$ motor neurons (27.6%) than those derived from adult spinal cords [155]. However, the efficiency of generating motor neurons from human spinal cord NSCs has yet to be determined.

Interestingly, spinal motor neurons can also be produced from NSCs isolated from other regions of the CNS, where there is no development of spinal motor neurons *in vivo*. Human fetal brain-derived NSCs, when primed with bFGF, heparin, and laminin for 4–5 days and further differentiated in B27, generated cholinergic motor neurons *in vitro* [156]. Activation of FGF

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receptors (FGFR) by bFGF during priming is required to generate a high percentage (50%) of $Hb9^+/ChAT^+$ spinal motor neurons [157]. Unlike spinal NSCs and ESCs, RA and Shh are not required for caudalizing and ventralizing human brain-derived NSCs toward a spinal motor neuron phenotype. Instead, FGF signaling seems sufficient to guide these NSCs to change their fate [157]. Using a similar priming technique, but adding RA and Shh, 25% $Hb9^+$ motor neurons were generated from a subpopulation of cortical NSCs that were isolated from adult mouse brain and expressed the cell surface markers, Lewis X and chemokine receptor CXCR4 [158]. When grafted into adult rodent spinal cords, human and mouse cortical NSCs as well as NSCs derived from the adult mouse olfactory bulb differentiated into $Hb9^+$ and/or $ChAT^+$ spinal motor neurons [13,81,156,158]. Interestingly, NSCs derived from patients or animal models with motor neuron disease have also been shown to generate spinal motor neurons. These include fetal cortical NSCs derived from patients with lethal congenital contracture syndrome [159] and adult olfactory bulb NSCs from transgenic mice expressing the human mutant SOD1 gene [13].

In addition to the use of mitogens and other factors on wild-type stem cells, genetic modifications have taken place in attempts to increase the efficiency of spinal motor neuron generation. Fetal rat spinal cord neural precursor cells have been genetically engineered to coexpress the transcription factors Hb9, Ngn2 and Nkx6.1, which make them responsive to Shh and RA, and direct their differentiation into cholinergic motor neurons at 4–8% efficiency. *In vitro*, these motor neurons project axons that form contacts with cocultured myotubes [160]. When transplanted into the injured adult rat spinal cord, the engineered cells transiently proliferated, reached the ventral horn, projected their axons into the ventral root and expressed motor neuron markers. Using a similar strategy, enforcing the expression of Hb9 together with either Olig2 or Ngn2 drove human adult olfactory neuroprogenitor cells to become $Isl1/2^+$ and $ChAT^+$ motor neurons [161]. Furthermore, these stably transfected neuroprogenitors uniformly expressed Hb9 (>95%), among which 40–60% coexpressed NeuN and Isl1/2. Genetic modifications have also been used to reprogram human fibroblasts from two elderly ALS patients to generate motor neurons. The genes Klf4, Sox2, Oct4, and c-Myc were introduced into the fibroblasts by retroviral transduction. The forced expression of these genes reprogrammed the fibroblasts to form induced pluripotent stem (iPS) cells. In culture, these iPS cells aggregated into embryoid bodies, which were subsequently differentiated into motor neurons by treatment with Shh and RA [162]. Finally, enrichment of the motor neuron population from ESCs has been achieved through the stable transfection of human and mouse ESCs with Hb9 promoter-driven green fluorescent protein genes, and a further isolation of the green cells by fluorescence-activated cell sorting (FACS) [163].

In summary, stem cell-derived motor neurons or their progenitors may be used for therapeutic purposes. However, in order to choose the right type of stem cells, both the advantages and limitations involved in their applications must be taken into consideration. ESCs display unlimited growth in culture, an undifferentiated state and great differentiation potentials. However, the risk of these cells to form teratomas is a major concern. Fetal brain NSCs can be expanded for long term *in vitro*, exhibit multiple differentiation potential and do not form teratomas. Unlike ESCs and fetal NSCs, adult stem cells can be used without ethical and immunological constraints, and thus, allow for autologous transplantation. However, their potential to become spinal motor neurons remains to be elucidated. Induced pluripotent stem cells also circumvent the issue of immune rejection. The drawback of these genetically modified cells is the high risk of cancer formation, mainly due to the lack of control on where the transgenes integrate when using retrovirus as the gene delivery method. To reduce the cancerous risk, techniques such as homologous recombination could be used to gain control of the integration sites. In addition, iPS cells should be fully characterized for a period of time during their *in vitro* growth before being transplanted. Another alternative would be to use

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plasmids instead of viruses to deliver the transgenes, so that these genes would be transiently expressed and thus would not integrate [164].

APPLICATIONS OF STEM CELL-DERIVED MOTOR NEURONS IN ALS

Stem Cell Transplants in Transgenic ALS Animal Models

Stem cell transplantation may be used to treat ALS by replacing lost motor neurons or protecting endogenous motor neurons through the replacement of dysfunctional non-neuronal glial cells such as astrocytes and microglia. Stem cells modified to secrete vital factors aimed at preserving surviving motor neurons through neurotrophic support or ameliorating inflammation is also an attractive strategy [11,12,165]. Several groups have transplanted stem cells into transgenic ALS animal models and reported various outcomes (see Table 2).

A few studies have examined the effect of rodent bone marrow cells (BMCs) on the disease progression when transplanted at presymptomatic ages in irradiated ALS mice. The cell delivery routes varied in each study, including intravenous and intraperitoneal injections. The delivery of mBMCs into the tail vein showed no significant effects on survival or motor performance [166], although many transplanted mBMCs migrated to the spinal cord and most of these cells expressed markers typical of microglia at the disease end-stage. However, when mBMCs were delivered intravenously through a retro-ocular injection, an increase in lifespan was observed, but evaluation of cell fate and differentiation in the CNS was not performed [167]. Mouse BMCs, transplanted intraperitoneally with 6 times more cells than the intravenous/retro-ocular delivery method, also showed an improvement in lifespan and motor performance [168]. Furthermore, the mBMCs protected endogenous motor neurons and maintained axons exiting the ventral root. The majority of the cells differentiated into microglia/macrophages, while a few cells expressed neuronal markers possibly through a fusion process with endogenous neurons. No transplant-derived cells in any study showed motor neuron morphology or extension of processes down ventral roots. Similarly, mesenchymal stem cells (MSCs) extracted from transgenic GFP-expressing rats were delivered intrathecally into the fourth ventricle in presymptomatic $SOD1^{Leu126delTT}$ mice [169]. Although there was no delay in disease parameters, except when evaluating the female population only, transplanted cells were found in the brain and spinal cord parenchyma. Thus, intrathecal transplantation into the fourth ventricle may represent a suitable delivery route for potentially more efficacious grafts of stem cells either specifically modified to secrete vital trophic factors or primed to differentiate into specific lost or dysfunctional cell types. The beneficial effects on disease progression in two of these studies may be due to the replenishment of the microglia population in the ALS animals with non-mutant SOD1-expressing, and thus non-compromised microglia. The mBMCs may have also exerted a positive effect through secretion of factors influencing motor neuron survival directly or indirectly through ameliorating the inflammatory process ensuing in the ALS spinal cord. The lack of effect when cells were given through the tail vein or intrathecally is unknown, but may be due to a low number of stem cells, particularly transplant-derived non-mutant SOD1-expressing microglia, actually reaching pathological areas of the CNS. Positive benefits of mBMC transplantation, on the other hand, were not due to motor neuron or neuronal replacement.

Several studies have also evaluated the effect of human mesenchymal stem cells (hMSCs) on disease progression after transplantation into presymptomatic transgenic ALS animal models. Human MSCs injected intravenously into irradiated mice delayed the disease onset, extended lifespan, prolonged motor performance and protected endogenous motor neurons [170]. Implanted cells were located in the brain, brainstem and spinal cord, but only a few expressed neuronal or astroglial markers. Surprisingly, when hMSCs or hMSC-derived neural stem-like cells (hMSC-NSCs) were delivered intrathecally into the cisterna magna to presymptomatic ALS mice, no efficacious effects on lifespan, disease onset, motor performance and

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endogenous motor neuron survival were observed [171]. However, widespread cell migration within the subarachnoid space was evident shortly after transplantation and transplanted hMSCs were found in the brain parenchyma. The differentiation fate of the transplanted cells was not determined. Thus, cell survival and migration to degenerating areas in the CNS are suggested to be a major concern when cells are injected intrathecally as compared to the intravenous route [171]. On the other hand, direct injection of hMSCs into specific lumbar spinal segments did delay the disease progression [172]. Furthermore, the transplanted cells seemed to prevent the microgliosis and astrogliosis typically seen in ALS cords, while preserving endogenous motor neurons in the transplanted areas. Less than 1% of hMSCs differentiated into neurons or astrocytes and differentiation into microglia was not observed. There was also no evidence of motor neuron differentiation. Data from this study supports the hypothesis that intraspinal hMSC transplantation provides efficacy not through replacing motor neurons, but through neuroprotection by limiting microgliosis and astrogliosis and possibly direct neurotrophic support, although this was not verified in their study. Finally, hMSCs have been genetically modified to secrete glial cell line-derived neurotrophic factor ($hMSC^{GDNF}$) and transplanted into muscles affected in the transgenic ALS rats, including the tibialis anterior, forelimb triceps brachii and long muscles of dorsal trunk [173]. No effect on disease onset was observed, but endogenous motor neurons as well as muscular innervations were maintained and lifespan was significantly extended. The differentiation fates of these cells were not determined, although the survival rate was high throughout the disease process. When assessing the potential of hMSC therapy based on these studies, intramuscular transplantation with hMSCs designed to secrete neurotrophic factors is an attractive approach that may be combined with intravenous or intraspinal hMSC therapy. In this manner, both axonal projections and connections with neuromuscular junctions will be maintained by the intramuscular transplant, while transplanted cells, potentially also modified to secrete neurotrophic factors, in the degenerating areas of the spinal cord will protect the cell bodies of surviving motor neurons. Intrathecal administration of hMSCs shows less promise than intravenous or intraspinal delivery. Although stem cells reach the CNS more quickly when delivered directly into the cerebrospinal fluid (CSF), the cell survival, homing capability and migration to pathological areas seem to be inferior compared to stem cells placed into the circulatory system.

Human umbilical cord blood cell (hUCBC) transplantation has also revealed mixed outcomes in regards to efficacy depending on the route of transplantation and number of cells transplanted. Human UCBCs administered into the venous system through a retro-ocular injection to presymptomatic irradiated ALS mice at relatively large doses extended the lifespan of the animals in a dose-dependent manner [167,174]. Thus, when administering hUCBCs intravenously, the number of cells transplanted directly affects the rate of the disease progression. Histological analysis in these studies was not performed to verify the presence and differentiation of transplanted hUCBCs in the CNS, but hypothetically, the more transplanted cells reaching the affected areas of the CNS, the better the improvement will be. Whether improvement in these studies is due to replacement of cells or a trophic effect on surviving motor neurons directly or indirectly through ameliorating inflammation is not known. In another study, hUCBCs were injected intravenously into presymptomatic mice and also showed a delay in disease progression and extension in lifespan [175]. Upon morphological analysis, transplanted hUCBCs were found to express leukocytic CD45, uncommitted Nestin, neuronal Tuj1 and astroglial GFAP in the brain as well as the lumbar and cervical spinal cord. A few cells showed morphology typical of neuronal and glial cells. The optimal therapeutic dose of hUCBCs administered intravenously has also been determined by the same group to be approximately 25 million cells [176]. Surprisingly, the highest dose tested of 50 million hUCBCs did not provide an efficacious outcome in this study, which possibly indicates that the beneficial effects on neuroprotection may be overturned by toxic effects produced by introducing a large number of potentially new inflammatory cells into the host. One strategy

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in improving the efficacy of hUCBC transplantation without increasing the total number of grafted cells to potentially toxic levels is through the genetic modification of these cells to secrete neuroprotective trophic factors. Human UCBCs have previously been transiently transfected with VEGF and L_1CAM, a cell adhesion molecule, through electroporation and were transplanted retro-orbitally into presymptomatic mice [177]. These modified grafted cells showed homing capability to the lumbar spinal cord and differentiation into endothelial cells within the lumbar cord blood vessel walls. Finally, when hUCBCs and hUCB-derived neural stem-like cells (hUCB-NSCs) were intrathecally administered into the cisterna magna of presymptomatic mice, no effect on disease parameters was observed [171]. These cells migrated within the subarachnoid space shortly after the transplant, but were not found in the brain or spinal cord. As in the case of intrathecally administered hMSCs and mBMCs, the survival and migration of hUCBCs after intrathecal administration seems to be very poor in affected areas of the CNS. Similarly to mBMCs and hMSCs, the hUCBCs showed potential for an efficacious treatment for ALS. However, the beneficial effects were more than likely due to neuroprotection rather than replacement of motor neurons and dependent upon a sufficient number of surviving cells that homed into degenerated regions of CNS.

The therapeutic potential of neural stem and progenitor cells, both mouse and human, have also been tested in ALS animal models. Two studies have stereotaxically transplanted genetically modified human neural progenitor cells (hNPCs) that secrete GDNF ($hNPC^{GDNF}$) into specific lumbar regions of the spinal cord [178,179]. In both studies, there was no effect on disease progression, but $hNPC^{GDNF}$ transplants caused an upregulation in ChAT expression and an increased soma size in surviving $ChAT^+$ motor neurons [178], and protected endogenous motor neurons, but did not prevent denervation [179]. Most of the transplanted cells were $Nestin^+$ and only 5–10 % of the cells expressed astroglial GFAP in these studies. There were no motor neurons or neurons of any type that had differentiated from these progenitor cells. Thus, this approach of using genetically modified stem cells that secrete neurotrophic factors to save the cell bodies of surviving motor neurons may be useful in ALS patients when combined with cell therapies, as described above, aimed at maintaining neuromuscular junctions from these surviving motor neurons. Two additional studies have examined the effects of intraspinally injecting human NSCs isolated from the cervical spinal cord of an 8 week human fetus, without genetic modifications, into the lumbar region of presymptomatic ALS rats [14,180]. These human spinal NSC transplants extended the lifespan and delayed disease onset with FK-506 immunosuppression therapy [14], but prolonged the lifespan considerably longer when grafted in combination with multiple immunosuppressive drugs or CD4 antibodies [180]. Furthermore, the human NSCs protected endogenous motor neurons possibly through the secretion of GDNF and BDNF in the CSF and lumbar spinal cord [14]. In both studies, the majority of the human NSCs differentiated into $Tuj1^+$ neurons, although motor neuron specification was not observed. Some $Nestin^+$ grafted cells were present and $GFAP^+$ astroglial differentiation was rarely seen. This efficacious effect on lifespan is quite astounding considering the restriction of the grafted cells mainly to the lumbar area of the spinal cord. The secretion of neurotrophic factors from grafted human NSCs, and thus neuroprotection, may underlie the beneficial outcome in both studies. Evidence of grafted neuronal cell integration into the host circuitry was also observed [180], however, the effect of which on the beneficial delay in disease progression is not known. Insights from these studies do demonstrate, however, the potential for strategic intraspinal injections of human NSCs into pathological areas of the spinal cord to protect endogenous motor neurons and differentiate into new neurons that may aid in replacing affected neuronal connections.

Since abnormal astrocytes contribute to disease progression in ALS, it is logical to test the efficacy of replacing the dysfunctional astroglial cells surrounding endogenous motor neurons with human NSC-derived normal astrocytes. Interestingly, however, none of the human NSCs or NPCs provided a significantly higher population of $GFAP^+$ astrocytes when grafted

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intraspinally [14,178–180]. Conversely, when the transplantation of lineage restricted glial progenitor cells into the cervical spinal cord, which gives rise to the phrenic nerve and regulates diaphragm muscle function, was tested in presymptomatic $SOD1^{G93A}$ rats, significant lifespan extension was observed [181]. Although there was no effect on disease onset, weight loss or hindlimb grip strength, the cervical transplant delayed the decline in forelimb grip strength, maintained the phrenic nerve compound muscle action potential amplitude, protected endogenous motor neurons, attenuated the loss of the astroglial glutamate transporter, GLT1, and limited the microglial response in the cervical spinal cord. The positive effects of the grafted glial progenitor cells were likely due to the replacement of dysfunctional astrocytes and their actions at synaptic sites particularly in removing excess glutamate, rather than neuronal or microglial replacement or the secretion of neurotrophic factors such as BDNF, IGF-1 and VEGF. This study, thus, represents a proof of principle that stem cell therapy aimed to provide normal astrocytes is a feasible strategy for treating ALS.

As discussed in the studies above, replacing overactivated microglial cells or dysfunctional astrocytes and providing trophic support through stem cell transplants are only likely to have short-term benefits in delaying the progression of ALS. In order to reverse paralysis and regain muscle strength, motor neurons will need to be replaced. To this end, several groups have generated cholinergic spinal motor neurons from embryonic and neural stem cells (see Table 1 for details). These methods of generating relatively high quantities of spinal motor neurons may prove useful when attempting to replace motor neurons in ALS animal models and hopefully, ALS patients. Three groups, thus far, have demonstrated stem cell therapies that show $ChAT^+$ spinal motor neuron differentiation in the transgenic ALS spinal cord. The first group used mouse NSCs (mNSCs) isolated from adult transgenic β-actin-GFP-expressing mice or spinal motor neuron specific Hb9-GFP expressing mice, which were further subjected to fluorescent activated cell sorting (FACS) to purify Lewis $X^+/CXCR4^+$ stem cells [158]. These cells were primed in the presence of RA and Shh among other factors to enhance their capacity for spinal motor neuron differentiation. Intraspinally injecting these cells into the lumbar region of the spinal cord of presymptomatic ALS mice resulted in the extension of lifespan, a delay in disease onset and an increase in endogenous motor neuron survival possibly due to IGF-1 and VEGF secretion. On morphological analysis, 45 % of the β-actin-GFP^+ cells became $MAP2^+$ neurons, 26 % $GFAP^+$ astrocytes, 4 % $O4^+$ oligodendrocytes and 20 % $ChAT^+$ motor neurons. On the other hand, 77 % of the Hb9-GFP^+ transplanted cells expressed ChAT, indicating a spinal motor neuron phenotype. Furthermore, based on stereological analysis, the investigators estimated that approximately 20% of the surviving motor neurons in the transplanted area were graft-derived motor neurons, many of which exhibited neuritic outgrowth. The improved outcome is thought to be due to not only trophic support and alteration of the toxic environment, but also the addition of a new cell population that may have integrated into the host spinal cord circuitry within degenerating areas [158]. In this study, the transplanted spinal motor neurons adopted typical motor neuron morphology and seemed to have large somata at the end-stage of the disease. The second group used multipotent neural precursor cells (NPCs) from the mouse olfactory bulb (OB-NPCs) and showed that bFGF-cultured OB-NPCs became motor neuron-like cells when transplanted into the lumbar spinal cord of presymptomatic ALS mice [13]. Transplantation of these cells extended lifespan, delayed disease onset and maintained motor performance by protecting endogenous motor neurons. Grafted OB-NPCs showed a 30 % differentiation into $ChAT^+$ motor neurons, 15 % $GFAP^+$ astrocytes and 5 % $O4^+$ oligodendrocytes. Integration of grafted GFP^+ cells in host neural circuitry and extension of processes down ventral roots and into the sciatic nerve were also observed. However, injecting a retrograde tracer into the hindlimb muscle did not reveal any retrogradely labeled grafted stem cell-derived motor neurons. Upon evaluation of the neuromuscular junctions, grafted GFP^+ cell axons showed no signs of innervation and in fact, displayed irregular swelling and dystrophy at axon terminals. Thus, the beneficial therapeutic effects in this experiment may be due to a variety of factors including neuroprotection through

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amelioration of excitotoxicty by forming new EAAT2-expressing astrocytes, reducing inflammation through immunomodulatory effects, direct neurotrophic effects on motor neuron survival and integration of potential spinal interneurons into the neural network within the host environment. Delays in disease progression were not due to reinnervation of distal muscle targets by new stem cell-derived motor neurons, however. This is the first *in vivo* evidence illustrating that transplanted motor neurons could not form new connections at neuromuscular junctions and exhibited morphology of degenerating distal axons after transplantation into the hostile ALS microenvironment. Evidence of stem cell-derived motor neuron susceptibility to the toxic ALS microenvironment was observed by our group when human NSC-generated motor neurons were transplanted into the lumbar and cervical spinal cord regions of presymptomatic ALS rats[1,2]. Prior to transplantation, human NSCs were primed in the presence of bFGF to enhance their capacity to differentiate into spinal motor neurons *in vivo* [81,156,157]. ALS rats receiving the human NSC transplants displayed a prolonged lifespan as well as delays in the decline of weight and motor function. Although a majority of the grafted human cells expressed ChAT, most of the surviving $ChAT^+$ grafted cells showed a degenerated morphology indicated by a small soma size at the end-stage of the disease. Furthermore, almost every grafted cell within the ventral horn showed protein nitration damage and some of the transplanted cells had undergone lipid peroxidation as well. A few axons were found in the L5 ventral root at disease end-stage, but not to the same degree as described in other animal models [81,156]. This study showed that human $ChAT^+$ motor neurons can be generated from NSC transplants in the ALS spinal cord, but adopt a degenerated, shrunken shape rather than the typical morphology of the motor neuron soma. The degeneration of human NSC-derived motor neurons is possibly due to the harsh oxidative environment that ensues in the spinal cord during the progression of the disease. Thus, stem cell-derived motor neurons may be susceptible to the same toxic cues that drive disease progression as endogenous motor neurons. To combat the potential vulnerability of stem cell-derived motor neurons to the ALS toxic environment, other approaches, either pharmacological or cell-mediated, will need to be developed to significantly change or even halt the persistent toxic ALS environment prior to initiating a motor neuron replacement therapy.

When assessing the affects of each of the studies described above on lifespan, each stem cell type showed a similar beneficial effect, regardless of the route of delivery or number of stem cells. The lone exception was intrathecal transplantation, which did not show an efficacious effect in any of the studies, but was only tested by two groups. Thus, neuroprotection through an alteration of the ALS environment most likely represents the major beneficial mechanism in most, if not all, of the studies that reported an efficacious outcome on lifespan. In addition, all transplants described in these pre-clinical studies were initiated well before observable motor deficits, which would not currently be relevant in the vast majority of human ALS cases where disease onset is unpredictable due to the lack of diagnostic biomarkers. Thus, when a cell therapy could be initiated in most ALS patients, many motor neurons have already been lost and an unknown percentage of motor neuron axons have already retracted from neuromuscular junctions. Furthermore, at this time, the microenvironment in the cord is toxic due to persistent inflammation, oxidative stress and excitotoxicity. The effect of this hostile ALS environment on the long-term efficacy of any cellular therapy is currently unknown. It is possible that cells transplanted into the pathological areas of the spinal cord at disease onset may not work as predicted, since stem cell-derived motor neurons may degenerate and stem cell-derived microglia or astroglia may become overactivated and dysfunctional. Enhancing endogenous motor neuron survival and maintaining axonal connections with muscle through the use of modified trophic factor secreting stem cells may be the most appropriate therapeutic approaches until the microenvironment in degenerating areas can be altered well enough to halt the progression of the disease, at which time a cellular therapy to replace lost motor neurons and irreversibly dysfunctional non-neuronal cells can be initiated.

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Stem Cell-Derived Motor Neurons and *in Vitro* Coculture Systems

Glial cells are posited to create a hostile environment that contributes to motor neuron toxicity in transgenic ALS animal models and as such, may also adversely affect the survival and maturation of transplanted stem cell derived-motor neurons. In support, several studies have recently shown that stem cell-derived motor neurons are susceptible to toxicity arising in the spinal microenvironment of ALS animals in organotypic slice cultures and from primary ALS glial cells in coculture [70,71,182]. Based on these results, stem cells may provide an excellent source of motor neurons to study the contribution of ALS glial cells to disease mechanisms and screen potential therapeutics aimed to protect motor neurons from glial-mediated toxicity. Furthermore, since primary human motor neurons are unattainable, stem cells can be utilized to provide insights into the vulnerability as well as cell injury mechanisms specifically of human motor neurons in an *in vitro* ALS disease setting.

Microglial Toxicity to Motor Neurons *in Vitro*

ESC-derived motor neurons from Hb9-GFP expressing transgenic mice were first shown to be susceptible to the ALS microenvironment after they were transplanted onto tissue slices isolated from presymptomatic mutant SOD1 transgenic mice [182]. After 7 days in culture on mutant slices, no surviving GFP-expressing transplanted motor neurons could be found, whereas many motor neurons survived in cultures on wild-type spinal cord and mutant SOD1 and wild-type hippocampal organotypic slices. Hence, motor neuron-sensitive toxic factors, a lack of trophic factors or a combination thereof must persist in the microenvironment of the ALS spinal cord. When mouse ESC-derived motor neurons were cultured with mutant slices across a semi-permeable membrane, secreted soluble factors caused neurite shortening after 3–5 days and contributed to the death of motor neurons after 7 days. Further assays confirmed that mutant slices secreted much higher levels of nitric oxide, IL-1β, IL-6, IL-12p70 and lower levels of VEGF. Not surprisingly, culturing ESC-derived motor neurons on mutant spinal cord slices in combination with a treatment consisting of neutralizing antibodies to all three proinflammatory cytokines, a nitric oxide scavenger and exogenous VEGF provided significantly more neuroprotection than any one treatment alone. These data indicate that not only will a combination treatment that blocks multiple cell injury pathways leading to motor neuron death be needed to protect endogenous motor neurons in ALS, but a combination therapy will also be needed to reduce the hostile nature of the ALS spinal cord in order to increase the efficacy of stem cell therapies aimed to replace lost motor neurons. To determine whether mutant SOD1-expressing microglia may contribute to ESC-derived motor neuron toxicity, a microglia cell line, BV-2, was stably transfected with inducible mutant SOD1 or wild-type SOD1 [182]. Cocultures across a semi-permeable membrane did not induce motor neuron toxicity. However, when the microglia were stimulated with lipopolysaccharide (LPS), the mutant SOD1-expressing microglia dramatically increased the secretion of proinflammatory cytokines and nitric oxide, and induced a significant shortening of motor neuron neurite length compared to controls. Another study found that LPS-activated primary mutant SOD1-expressing microglia isolated from adult transgenic $SOD1^{G93A}$ mice, but not neonatal transgenic mice, released more TNF-α and less IL-6 compared to non-transgenic controls [65]. Thus, microglia that express mutant SOD1 are more responsive to inflammatory signals and their overactivation during disease progression may underlie the hostile oxidative and inflammatory environment contributing to motor neuron toxicity that is observed in the ALS spinal cord of mutant SOD1 transgenic animals.

As further evidence for microglial toxicity to motor neurons in ALS, several studies have indicated that isolated primary microglia become toxic to primary motor neurons when activated with either proinflammatory LPS or IgG immune complexes isolated from human ALS patients [50,67,68,183]. Non-transgenic microglia activated by these stimulatory factors initiate motor neuron death through nitric oxide and superoxide generation as well as glutamate

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release [67]. Treatment of these activated non-transgenic microglia with an anti-inflammatory cytokine, IL-4, suppressed nitric oxide and superoxide generation and provided neuroprotection in coculture, which indicates that suppressing the overactivated microglia in ALS may preserve motor neurons by ameliorating microglia-mediated toxicity [183]. Furthermore, primary motor neurons cocultured in direct contact, particularly with primary mutant SOD1-expressing microglia, showed decreased survival and shortened neurite length [50,68]. One mechanism is that inherent overexpression of mutant SOD1, as opposed to wild-type SOD1, specifically caused primary microglia to secrete more superoxide and nitric oxide and less IGF-1. Such higher concentrations of mutant SOD1-expressing microglia-generated nitric oxide have been correlated with a further reduction in motor neuron survival in coculture. In addition, stimulation of mutant SOD1-expressing microglia with the proinflammatory LPS caused enhanced nitric oxide and superoxide release, decreased IGF-1 secretion and greater motor neuron toxicity, which was partially abrogated by inhibiting microglial iNOS [50,68]. This indicates that mutant SOD1-expressing microglia inherently possess increased reactivity to proinflammatory stimulation. On the other hand, when conditioned media from primary mutant SOD1-expressing microglia was added to the culture medium for ESC-derived motor neurons [71] or primary mutant SOD1-expressing microglia were cocultured in transwells 1 mm away from NSC-derived motor neurons[2], a substantial toxic effect to motor neurons was not observed. Thus, mutant SOD1-expressing microglia require either direct contact or close proximity to exert a toxic effect.

Based on these data that primary motor neurons are susceptible to mutant SOD1-expressing microglia-mediated toxicity, it is important to determine the vulnerability of human stem cell-derived motor neurons to ALS microglia prior to initiating a stem cell treatment to replace motor neurons in ALS. Indeed, human NSC-derived motor neurons are susceptible to primary mutant SOD1-expressing microglia-mediated toxicity and human motor neuron death is partially ameliorated through inhibition of both microglial iNOS and NADPH oxidase[2]. One potential mechanism through which mutant SOD1 directly increases ALS microglial toxicity to motor neurons is through regulating NADPH oxidase by binding and inhibiting the GTPase activity of the Rho-GTPase subunit, Rac1, and thus, maintaining NADPH oxidase in a persistently activated and superoxide-generating state [105]. Inhibiting NADPH oxidase not only protects human NSC-derived motor neurons in coculture[2], but also dramatically prolongs the lifespan of transgenic $SOD1^{G93A}$ mice when administered in their drinking water [105].

Astroglial Toxicity to Motor Neurons *in Vitro*

Primary mutant SOD1-expressing astrocytes also exhibit toxic properties to both primary and stem cell-derived motor neurons *in vitro*. However, the identity of all the astrocytic toxic factors involved has remained elusive. First, mutant SOD1-expressing astroglia inherently display a “neuroinflammatory phenotype” [184]. Even when these astrocytes are isolated from mice at young and thus presymptomatic ages, the basal expression levels of proinflammatory cytokines, eicosanoids, iNOS and protein carbonylation are significantly increased. Upon proinflammatory stimulation, mutant SOD1-expressing astroglia were more responsive and became overactivated in regards to these inflammatory factors and protein carbonylation damage as compared to non-transgenic controls. It will be important to discern the mechanisms of how this “neuroinflammatory phenotype” leads to selective motor neuron degeneration in ALS. For this purpose, stem cell-derived motor neurons can be used to dissect whether inhibiting these astroglia inflammatory processes prevents motor neuron death in cocultures. Reactive astrocytes that were activated with either LPS or peroxynitrite treatment secreted significantly more nerve growth factor (NGF), which contributed to primary motor neuron death through $p75^{NTR}$ activation only in the presence of low levels of nitric oxide [185]. Furthermore, mutant SOD1 may induce oxidative damage and dysfunction of astroglial mitochondria leading to enhanced superoxide radical release and development of an astroglial

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neurotoxic phenotype that requires nitric oxide synthase activation [117]. Mitochondria-specific antioxidants reduced mitochondria-generated superoxide, reversed mitochondrial respiration abnormalities and abrogated primary mutant SOD1-expressing astroglial toxicity to primary motor neurons. On the other hand, uncoupling the mitochondria electron transport chain in non-transgenic, normal astroglia resulted in astroglia-acquired toxicity to primary motor neurons [117]. Functional consequences induced in motor neurons, irrespective to neuronal mutant SOD1 expression, by primary mutant SOD1-expressing astrocyte-released substances included a reduced mitochondrial redox state, decreased resting mitochondria membrane potential, elevated mitochondrial Ca^{2+} levels and diminished cytoplasmic Ca^{2+} levels, which may cumulatively enhance motor neuron vulnerability to potential toxic factors that have been implicated in ALS disease progression [186]. Stem cell-derived motor neurons, specifically human motor neurons, should be tested in coculture to determine whether these abnormalities described in ALS astroglia and primary motor neurons are relevant to human motor neuron degeneration. As such, drug-screening can be initiated in coculture paradigms to determine whether inhibiting these potential cell injury pathways promotes human motor neuron survival.

Two reports first indicated that mouse ESC-derived motor neurons were susceptible to soluble toxic factors secreted from primary mutant SOD1-expressing astroglia [70,71]. In the first study, ESCs were isolated from Hb9-GFP expressing rats that either overexpressed mutant SOD1 or wild-type SOD1 and were differentiated into motor neurons by treating the embryoid bodies with RA and Shh [70]. Both ESC-mutant SOD1- and ESC-wild-type SOD1-derived motor neurons died over a time period of weeks when cocultured with primary mutant SOD1-expressing astrocytes as compared to wild-type SOD1 astrocytes. Additionally, the toxicity of mutant SOD1-expressing astrocytes was greater for mutant SOD1-expressing motor neurons than for wild-type motor neurons. Hence, autonomous mechanisms caused by mutant SOD1 expression increased the vulnerability of motor neurons to non-cell autonomous toxicity mediated by mutant SOD1-expressing astrocytes. In a similar study, non-transgenic primary motor neurons and motor neurons derived from ESCs, isolated from transgenic Hb9-GFP expressing mice, were lost when cocultured on mutant SOD1-expressing primary astrocyte monolayers as compared to non-transgenic or wild-type astrocytes [71]. Furthermore, conditioned medium from mutant SOD1 astrocytes killed both primary and ESC-derived motor neurons, which indicated that mutant SOD1-expressing astrocyte-mediated toxicity involved soluble and stable toxic factors. Mutant astrocyte toxicity was shown to be specific for motor neurons, since primary GABAergic neurons, dorsal root ganglion neurons and mouse stem cell-derived interneurons were not vulnerable when cocultured with primary mutant SOD1-expressing astrocytes. Moreover, inhibiting Bax-mediated apoptosis prevented motor neuron death in coculture.

In developing a stem cell therapy for ALS patients, it is important to determine whether human motor neurons are also vulnerable to ALS astroglial toxicity. Indeed, motor neurons generated from human ESCs have also recently been shown to be susceptible to mutant SOD1-expressing astroglia-secreted toxic factors [72,187,188]. Human ESC-derived motor neurons were lost when cocultured in direct contact with mutant SOD1-expressing astroglia or cultured in conditioned media from mutant SOD1-expressing astroglia for 10–20 days, but spared in cocultures with wild-type or non-transgenic astroglia, or mutant SOD1-expressing fibroblasts [72]. Interneurons derived from human ESCs were unaffected in cocultures with ALS astroglia, which indicated that the toxic factors were selective for human motor neurons. A microarray analysis was performed, which specified numerous genes that were upregulated in ALS astroglia as compared to non-transgenic and wild-type astroglia [72]. Many of these genes are involved in inflammatory pathways. Astroglia were then treated with selected upregulated inflammatory factors and human ESC-derived motor neurons were used to screen for a potential toxic phenotype induced by these factors in previously non-lethal astroglia.

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Pretreatment of wild-type astroglia with prostaglandin D2 (PGD2) resulted in astroglial-mediated human motor neuron toxicity. Sure enough, inhibiting the PGD2 receptor on mutant SOD1-expressing astroglia partially abrogated human motor neuron loss in coculture. In a related study, human ESC-derived motor neurons were shown to be susceptible to toxicity arising from human astrocytes transduced with mutant $SOD1^{G37R}$ using a lentiviral vector [188]. Once again, human ESC-derived non-motor neurons were resistant to mutant SOD1-expressing astroglia-secreted toxic factors while transduced mutant SOD1-expressing fibroblasts did not exhibit toxicity to human motor neurons. Increased expression of several proinflammatory factors occurred, including iNOS and NOX2 expression, in human mutant SOD1-expressing astrocytes compared to wild-type astrocytes. A NOX2 inhibitor and two antioxidants were shown to reduce ROS levels in mutant astrocytes and NOX2 inhibition in coculture was able to save stem cell-derived human motor neurons. Our own studies have also shown that human NSC-derived motor neurons were susceptible to mutant SOD1-expressing astroglial-mediated toxicity[1,2]. Additionally, primary mutant astroglia isolated from transgenic $SOD1^{G93A}$ rats after disease onset were significantly more toxic to human NSC-derived motor neurons than mutant astroglia isolated from one month presymptomatic rats.

Based on the above studies, one can predict that in clinically relevant studies performed at the time of disease onset, human stem cell-generated motor neurons transplanted into the spinal cords of post-disease onset transgenic animals or ALS patients may encounter much higher levels of astroglia-secreted toxic factors and thus a more hostile environment. This may severely limit the maturation and survival capability of grafted stem cell-derived motor neurons compared to transplantations performed presymptomatically. These studies also indicate that stem cells can be utilized to generate an endless supply of motor neurons that can be used to elucidate non-cell autonomous mechanisms of motor neuron death and screen therapeutics rationally designed to protect motor neurons from injury cascades. These data also provide evidence that grafted stem cell-derived motor neurons will be at risk to an ALS microenvironment made hazardous by toxic astroglia. It will be important to identify other astrocyte-secreted lethal factors using these experimental paradigms prior to initiating a motor neuron replacement stem cell therapy, not only to save remaining endogenous motor neurons, but also to protect transplanted motor neurons. Given that stem cells provide a renewable and easily obtainable source of motor neurons, potential combination therapies with stem cell grafts can be rationally designed using *in vitro* coculture paradigms prior to initiating stem cell pre-clinical *in vivo* studies with the ultimate goal of developing a combined therapy to translate into human clinical trials.

Interaction between Microglia and Astroglia *in Vitro*

In vitro paradigms using stem cell-derived motor neurons may be designed to test motor neuron death involving interactions between several cell types critical to ALS pathogenesis. Crucial to understanding ALS disease progression is dissecting the relationships between microglia, astroglia and motor neurons. Addition of astrocytes to cocultures between activated primary microglia and motor neurons ameliorated microglia-induced motor neuron death by taking up glutamate and possibly through the secretion of reduced glutathione or neurotrophic factors as astrocyte condition medium also exerted a minimal, albeit not significant, protective effect [67]. However, pre-treating astrocytes with hydrogen peroxide prevented their beneficial effect in cocultures with microglia and motor neurons by reducing their capability to take up glutamate. Furthermore, oxidative and excitotoxic insults in primary neuronal-glial cultures caused astrogliosis, a decrease in astroglial EAAT activity and motor neuron loss [189]. Increasing astrocytic antioxidant defense mechanisms by increasing glutathione synthesis through overexpression of Nrf2 prevented primary mutant SOD1-expressing astrocytes from developing toxicity to primary motor neurons *in vitro* and increased the lifespan of transgenic mutant $SOD1^{G93A}$ mice [190].

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Astrocytic activity is influenced by the pathological milieu in the ALS spinal cord. Increases in inflammatory factors, oxidative stress and glutamate levels may transform astrocytes from neuroprotective to neurotoxic phenotypes. It is possible that overactivated microglia may drive disease progression by modulating the function and activation of astrocytes through the release of inflammatory factors, ROS/RNS and glutamate. In fact, coculturing primary mutant SOD1-expressing microglia with non-transgenic astroglia one day prior to initiating coculture with human NSC-derived motor neurons caused these previously non-toxic, normal astroglia to adopt ALS-like astroglial toxicity to human NSC-derived motor neurons through secreted toxic factors[2]. This adopted neurotoxicity was partially blocked by inhibiting NADPH oxidase or iNOS during the microglia-astroglia coculture and throughout coculture with human motor neurons, which indicates that oxidative damage to astrocytes from overactivated microglia can convert normal astrocytes to lethal cells. Accumulated data from these studies indicate that astroglial functions are sensitive to the pathological microenvironment in the ALS spinal cord. Oxidative damage, either from intracellular sources such as mitochondria or exogenous sources such as microglia, increased glutamate levels and proinflammatory cytokines, may transform astrocytes rendering them dysfunctional and toxic. Thus, exposing transplanted stem cell-derived astrocytes to the ALS spinal cord milieu, especially after disease onset has occurred, may not be very efficacious for long-term treatment as grafted astrocytes may become dysfunctional and toxic in this harsh environment.

In summary, stem cell-derived motor neurons provide a useful means to test whether ALS microglia or astroglia-mediated oxidative stress, proinflammatory cytokine release, glutamate release, NGF release, etc., is detrimental to motor neuron maturation, function and survival. High throughput screening of potential therapeutics to protect motor neurons, especially human motor neurons, from injury and death cascades can easily be performed in these *in vitro* coculture systems. Interactions between several neural cell types can also be dissected to determine whether their detrimental impact on stem cell-derived motor neurons is through direct damage such as microglial-mediated oxidative damage and astroglial secretion of toxic factors or indirect effects such as microglial-generated superoxide and nitric oxide induced damage to astrocytes resulting in astrocytic production of toxic factors. The relative importance of blocking each potential injury pathway in motor neurons or overactivation mechanisms of glial cells can be determined and combination therapies inhibiting the multi-factorial pathogenesis of ALS can be developed *in vitro* prior to initiating pre-clinical experiments in animal models. Results from these types of studies have the potential to provide essential insights into ALS glia-mediated toxicity as well as motor neuronal susceptibility and ultimately lead to the development of novel combined therapies with stem cell-derived motor neurons in order to provide motor recovery in ALS patients.

CHALLENGES TO STEM CELL THERAPY AIMED TO REPLACE MOTOR NEURONS IN ALS

Several challenges must be overcome before stem cell treatment for replacing lost motor neurons in ALS patients becomes reality. Not only will combined therapeutic methods need to be developed to protect transplanted stem cell-derived motor neurons from the hazardous ALS spinal cord microenvironment, but effective means of preventing immune rejection of the grafts will also need to be implemented. Furthermore, if long term survival of the transplanted motor neurons is accomplished, axonal propagation into ventral roots and down appropriate pathways toward denervated muscle tissue will need to be achieved. In addition, the appropriate type and amount of stem cells as well as the segmental locations and number of graft sites will need to be optimized for attaining the most efficacious transplants possible. Low numbers of motor neurons transplanted into a few localized sites within the cervical or lumbar spinal cord, as in transgenic ALS animal models, may not prove to be as beneficial in patients. ALS is a result of a rather widely spread degeneration of upper motor neurons in the

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brain, and both motor neurons and interneurons throughout the spinal cord. It is questionable that replacing spinal motor neurons in a few segments of the cord alone will be effective without a new supply of upper motor neurons and interneurons. Along this line, the route of stem cell delivery becomes one of the most critical issues. Current techniques in obtaining a significant number of motor neurons from stem cells *in vivo* are limited to those grafting the cells directly into the ventral horn of spinal cords (Table 1). One way to allow implanted stem cells to spread throughout the CNS is through intrathecal injection, which permits cells to circulate via the CSF. However, it is unknown whether stem cells grafted in this manner will efficiently generate neurons, particularly motor neurons, and locate to pathological areas in the CNS. Also, since glial cells may be irreversibly dysfunctional and overactivated, stem cell therapies aimed at incorporating functional glial cells around grafted motor neurons may need to be combined with motor neuron replacement. Finally, it is likely that each individual ALS patient will require a unique, personalized stem cell therapy based on areas of the spinal cord affected at disease onset and how wide-spread the disease has become when initiating the stem cell therapy.

Due to immune rejection of allogeneic ESC or NSC transplants, optimal immunosuppressive regimens for achieving and maintaining long-term efficacy of the grafts will need to be developed as has been shown in one pre-clinical study [180]. Reduced immune rejection will limit the number of stem cell-derived motor neurons that need to be intraspinally transplanted. One strategy to circumvent the use of allogeneic transplants would be to transplant autologous iPS cells that are differentiated into motor neurons or glial cells. Somatic cells have been shown to change into iPS cells when transduced to overexpress critical genes essential for embryonic stem cells [191,192]. In fact, somatic cells from an elderly patient with familial ALS have been induced into the pluripotent state and then differentiated into motor neurons [162]. This technique could be invaluable, not only in providing ALS patient-specific immune compliant stem cell-derived motor neurons or glia for transplantation, but also for *in vitro* studies on drug screening, motor neuron death mechanisms and glial overactivation mechanisms. Bone marrow or mesenchymal stem cell transplants may also be useful in ALS patients to provide neuroprotection through the secretion of trophic factors or the replacement of overactivated microglial cells, especially since autologous bone marrow cells are easily obtained. A drawback to autologous transplantation, whether the cells are derived from the bone marrow or iPS cells, relies in the fact that cells that were originally predisposed to developing ALS will be transplanted into an ALS environment. Whether autologous stem cell-derived motor neurons and glial cells will reactivate ALS is unknown. One study has indicated that MSCs isolated from the bone marrow of transgenic mutant $SOD1^{G93A}$ rats displayed an impaired neuroprotective capacity, including a reduced ability to take up aspartate [193]. Thus, it is possible that autologous stem cell-derived neural cells will not be very efficacious in providing protection. On the other hand, MSCs derived from the bone marrow of human donors and sporadic ALS patients did not have any apparent differences in proliferation rates, differentiation capacity or chromosomal appearance [194], but the neuroprotective capacity was not determined *in vitro* or in transgenic ALS animal models.

One strategy in lowering the number of stem cells needed to provide an efficacious number of motor neurons after transplantation is to develop techniques that increase the quantity of motor neuron differentiation after transplantation. This may require a pre-transplant priming stage in ESCs or NSCs to increase their motor neuron generation capacity. Higher quantities of human motor neurons following engraftment into the adult rat spinal cord have been generated by priming human NSCs in a cocktail containing bFGF [156]. The grafted human NSC-derived motor neurons were able to send axons through the ventral roots, innervated distal muscles by forming neuromuscular junctions and improved the motor function of rats with sciatic axotomy-induced motor neuron deficiency [81,195]. The possibility of a future stem cell-based motor neuron replacement therapy for ALS has also been suggested by the finding that mouse ESC-derived pre-committed motor neurons sent axons through ventral roots, reached target

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muscle tissue and partially improved paralysis when combined with molecules that augmented axonal outgrowth in a rat model of virus-induced motor neuron death [141,196]. These studies indicate that if immune rejection is prevented and long-term survival of stem cell-derived motor neurons is achieved, then motor neurons do have the potential to reach distal muscle targets. Since most ALS patients die within 3–5 years after disease onset, the effectiveness of transplanting stem cell-derived motor neurons into the spinal cord is questionable due to the long distances transplanted cells must grow to reach affected muscles. Even if the new motor neurons were resistant to the toxic ALS environment, it would theoretically take approximately 2–3 years for motor neurons to extend axons and form synaptic connections at neuromuscular junctions across these long distances based on an axonal growth of 1 mm per day. However, the hope is that combined therapies can be developed that will both protect transplanted motor neurons in the toxic ALS milieu and delay or even halt the disease progression, thus, allowing more time to establish muscle innervations from new motor neurons. It is, of course, also rational to implement stem cell therapeutic strategies aimed to regenerate the axons of surviving endogenous motor neurons potentially through the use of genetically modified stem cells that secrete vital neurotrophic factors within the spinal cord to rescue motor neuron somas and from muscle targets to attract axons as mentioned above in pre-clinical studies. Such stem cell transplantations acting as biological trophic factor pumps seem essential to slowing the disease progression and maintaining as many viable motor neurons as possible prior to or in combination with commencing a motor neuron stem cell replacement therapy.

CONCLUSION

Stem cells provide the means to replace lost spinal motor neurons and potentially recover from paralysis in ALS. However, other therapies will need to be combined with motor neuron replacement to strengthen the therapeutic efficacy, since transplanted motor neurons are likely just as vulnerable to the toxic ALS microenvironment as endogenous motor neurons. *In vitro* coculture systems utilizing human stem cell-derived motor neurons together with diseased glial cells can be used to dissect both cell autonomous and non-cell autonomous mechanisms of ALS. Thus, stem cell-derived motor neurons provide an invaluable tool to develop combined therapies as drugs can be screened for their ability to prevent motor neuron death. It is likely that multiple drugs will be required to protect motor neurons due to the multi-factorial nature of ALS pathogenesis. Optimistically, combined therapies designed to protect human motor neurons *in vitro* will also save remaining endogenous motor neurons and slow or even halt the disease progression in transgenic ALS animal models and ALS patients. Once disease progression is significantly slowed, then hopefully, the spinal cord microenvironment will be amenable to motor neuron replacement using a variety of potential stem cell sources and ALS patients may begin their road toward recovery of motor function.

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Abbreviations

ALS	amyotrophic lateral sclerosis
BDNF	brain-derived neurotrophic factor
bFGF	basic fibroblast growth factor
bHLH	basic helix-loop-helix

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BMC	bone marrow cell
cAMP	cyclic adenosine monophosphate
ChAT	choline acetyltransferase
CMAP	compound muscle action potential
CNTF	ciliary neurotrophic factor
CNS	central nervous system
CSF	cerebrospinal fluid
CXCR4	chemokine (C-X-C motif) receptor 4
EAAT2	excitatory amino acid transporter 2
EGF	epidermal growth factor
ESC	embryonic stem cell
FACS	fluorescent activated cell sorting
FBS	fetal bovine serum
FCS	fetal calf serum
FGF	fibroblast growth factor
FGFR	fibroblast growth factor receptor
GDNF	glial cell line-derived neurotrophic factor
GFAP	glial fibrillary acidic protein
GFP	green fluorescent protein
GSK3	glycogen synthase kinase 3
IGF-1	insulin-like growth factor 1
HD	homeodomain
hMSC	human mesenchymal stem cell
hNPC	human neural progenitor cell
hNSC	human neural stem cell
HNu	human nuclear antigen
hUCBC	human umbilical cord blood cell
iNOS	inducible nitric oxide synthase
iPS	induced pluripotent stem
L1CAM	L1 cell adhesion molecule
LPS	lipopolysaccharide
MAP2	microtubule-associated protein 2
mBMC	mouse bone marrow cell
MN	motor neuron
mNSC	mouse neural stem cell
mOB-NPC	mouse olfactory bulb neural precursor cell

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MSC	mesenchymal stem cell
NeuN	neuronal nuclei
NF	neurofilament
NGF	nerve growth factor
NOX	NADPH oxidase
NPC	neural progenitor cell
NSC	neural stem cell
NT3	neurotrophin 3
NT4	neurotrophin 4
PGD2	prostaglandin D2
pMN	motor neuron progenitor
RA	retinoic acid
rAAV	recombinant adeno-associated virus
rMSC	rat mesenchymal stem cell
RNS	reactive nitrogen species
ROS	reactive oxygen species
SC	spinal cord
Shh	sonic hedgehog
SOD1	superoxide dismutase 1
SVZ	subventricular zone
TF	transcription factor
Tuj1	antibody against β-III tubulin
VEGF	vascular endothelial growth factor
VZ	ventricular zone
YFP	yellow fluorescent protein

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Table 1

Spinal Motor Neurons Differentiated from Stem Cells

Type of Cells	Species	Methods to Generate Spinal Motor Neurons	Efficiency of Spinal Motor Neuron Generation	References
Embryonic stem cells (ESC)	Mouse	Embryoid bodies treated with RA for 6 days	0.5–0.8% of stem cells expressed neurofilament, Isl1 and Hb9 *in vitro*	Renoncourt et al., 1998 [136]
	Mouse	Embryoid bodies treated with RA plus Shh or Shh agonist for 5 days	20–30% of stem cells expressed Hb9 and NeuN/TuJ1 *in vitro* Following graft into stage 15–17 chick spinal cords, differentiated into $Hb9^+$ motor neurons, formed synaptic connections with intercostal muscles	Wichterle et al., 2002 [134]
	Mouse	ESCs cocultured with MS5 stromal cells, treated with Shh and RA for 4 days, followed by further differentiation for 6 days	60% of $TuJ1^+$ cells expressed Hb9 *in vitro*	Barberi et al., 2003 [137]
	Human	bFGF-differentiated ESC rosettes treated with Shh and RA for 7 days, followed by further differentiation for 1–2 weeks.	21% of rosette cells expressed Hb9 and ChAT, formed synapses on cocultured myotubes *in vitro*	Li et al., 2005 [140]
	Human	bFGF-differentiated ESC rosettes treated with bFGF, Shh and RA for 7 days, followed by further differentiation for 2 weeks	20–30% of rosette cells expressed Isl1, TuJ1 and ChAT *in vitro*	Shin et al., 2005 [141]
	Mouse	pHb9-eGFP-transfected ESCs treated with a Shh agonist and RA for 5 days, followed by further differentiation in GDNF/CNTF for 4 days	96% of Hb9-$eGFP^+$ motor neurons were $Lhx3^+$ Following graft into stage 17 chick neural tubes, projected to epaxial muscles	Soundararajan et al., 2006 [138]
	Human	Embryoid bodies treated with bFGF, RA and Shh for 2 weeks, followed by further differentiation for 3 weeks	58% of stem cells expressed Hb9 *in vitro*	Lim et al., 2006 [144]
	Mouse	ESCs treated with RA for 4 days, treated with BDNF/CNTF/NT3 and cAMP before grafting	Following graft into adult rat spinal cords, differentiated into Hb9 and ChAT motor neurons, sent axons into muscles and formed neuromuscular junctions	Deshpande et al., 2006 [139]
	Human	MS5 stromal cell-induced neural rosettes treated with Shh and RA for 15 days, followed by further differentiation in	20% of rosette cells expressed Hb9 *in vitro* Following graft into rat and chick spinal cords, differentiated into Hb9 or ChAT motor neurons, sent axons into ventral root	Lee et al., 2007 [142]

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Type of Cells	Species	Methods to Generate Spinal Motor Neurons	Efficiency of Spinal Motor Neuron Generation	References
		GDNF/BDNF/AA for 10 days		
	Human, Primate	bFGF-differentiated neuroepithelial cells treated with RA for 1 week, then RA plus Shh or purmorphamine for 1 week, followed by further differentiation in BDNF/GDNF/IGF-1	50% of rosette cells expressed Hb9 *in vitro*	Li et al., 2008 [145]
Neural stem cells (NSC)	Human	8-week forebrain-derived neurospheres primed with bFGF/heparin/laminin for 4–5 days followed by further differentiation for 9–10 days	20–50% of cells expressed Hb9 and ChAT *in vitro* Following graft into adult rat spinal cords, differentiated into $Hb9^{+}/ChAT^{+}$ motor neurons, sent myelinated axons into peripheral nerves and formed neuromuscular junction	Wu et al., 2002 [154] Gao et al., 2005 [79] Jordan et al., 2008 [155]
	Mouse	E16-18 spinal cord-derived neurospheres differentiated in BDNF/CNTF/GDNF for 7–10 days	Some cells expressed ChAT and Isl1	MacDonald et al., 2003 [151]
	Rat	E14 spinal cord-derived neurospheres treated with bFGF for 3 days, followed by RA agonists for 2 days	Activation of RA receptor β increased Isl1 expression and $Hb9^{+}$ cells	Goncalves et al., 2005 [152]
	Mouse	E13.5 or adult spinal cord-derived cells, with high aldehyde dehydrogenase activity, differentiated in low bFGF, RA, Shh, cAMP and NGF	27.6% of E13.5 NSCs expressed Hb9, 7.4% of adult NSCs expressed Hb9	Corti et al., 2006 [153]
	Mouse	LeX+/CX+ adult mouse brain-derived neurospheres treated with bFGF/heparin/laminin plus Shh and RA for 5 days, followed by differentiation in 2% FBS plus BDNF/GDNF/CNTF/IGF1/NT3	22.5% of sphere cells expressed Hb9, coexpressed Isl1 and ChAT Following graft into adult mouse spinal cords, differentiated motor neurons sent axons to ventral roots	Corti et al., 2007 [156]
NSCs from organisms with motor neuron diseases	Human	Cortical NSCs derived from patients with lethal congenital contracture syndrome, treated	Isl1+ or Hb9+ cells derived from NSCs *in vitro*	Pakkasjarvi et al., 2007 [158]

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Type of Cells	Species	Methods to Generate Spinal Motor Neurons	Efficiency of Spinal Motor Neuron Generation	References
		with Shh agonist plus 2% FCS or IGF1 for 2 weeks		
	Mouse	Adult olfactory bulb-derived neurospheres from transgenic mice expressing either wild-type or mutant human SOD1 gene, proliferated in bFGF	Following transplantation into spinal cords, differentiated into large $ChAT^+$ neurons, formed myelinated axons and grew into peripheral nerves	Martin et al., 2007 [157]
Genetically modified stem cells	Human	pHb9egfp-transfected ESCs treated with Shh and RA for 4–5 days, sorted and differentiated in BDNF/GDNF/ NT4/CNTF	90.1% of sorted cells expressed Hb9/Tuj1, 88.6% expressed Isl1/ChAT *in vitro*	Roy et al., 2005 [162]
	Human	Adult olfactory NPCs transfected with Hb9 plus Olig2 or Ngn2, then treated with Shh, RA and forskolin	Over 95% of cells expressed Hb9, 30–40% of cells expressed Isl1/2 *in vitro* Formed synapses on cocultured chicken muscle cells *in vitro*	Zhang et al., 2006 [160]
	Rat	Fetal spinal cord NPCs transduced with Hb9, Nkx6.1 and Ngn2, treated with Shh and RA	4–8% of cells expressed ChAT, formed contacts with myotubes *in vitro* Following graft into adult rat spinal cords, differentiated into $ChAT^+$ neurons, sent axons into ventral root	Bohl et al., 2008 [159]
	Human	ALS patient skin fibroblast-derived induced pluripotent stem cells transduced with Oct4, Sox2, Klf4 and c-myc, treated with Shh and RA	20% of embryoid body cells expressed Hb9 *in vitro*	Dimos et al., 2008 [161]

BDNF, brain-derived neurotrophic factor; bFGF, basic fibroblast growth factor; cAMP, cyclic adenosine monophosphate; ChAT, choline acetyltransferase; CNTF, ciliary neurotrophic factor; ESC, embryonic stem cell; FBS, fetal bovine serum; FCS, fetal calf serum; GDNF, glial cell line-derived neurotrophic factor; IGF-1, insulin-like growth factor 1; NGF, nerve growth factor; NPC, neural progenitor cell; NSC, neural stem cell; NT3, neurotrophin 3; NT4, neurotrophin 4; RA, retinoic acid; Shh, sonic hedgehog; SOD1, superoxide dismutase 1; TuJ1, antibody against β-III tubulin.

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Table 2

Stem Cell Transplants in Transgenic ALS Animal Models

Stem Cell Therapy	Mutant SOD1 Model and Age at Grafting	Transplant Parameters	Morphological Analysis	Effect on Disease Progression	References
mBMC^{GFP}, isolated from transgenic GFP-expressing mice	SOD1^{G93A} mouse, 6 weeks, 950–1100 rads irradiated	5×10^6 cells, intravenous	GFP$^+$ cells found in SC, GFP$^+$-F4/80$^+$ microglia (83%), GFP$^+$-CD11b$^+$ microglia (28%)	No effect on survival or rotarod performance	Solomon et al., 2006 [165]
mBMCs	SOD1^{G93A} mouse, 8 weeks, 8.0 Gy irradiated	5×10^6 cells, retroocular	Not performed	Extended lifespan (~11 days)	Ende et al., 2000 [166]
mBMCs, isolated from β-Actin-eGFP-, neuron-specific Thy1-YFP or SOD1^{G93A} adult mice	SOD1^{G93A} mouse, 4 weeks, 8.0 Gy irradiated	30×10^6, intraperitoneal	YFP$^+$ cells in brain, some GFP$^+$ and YFP$^+$ cells colabeled with Tuj1, NeuN and NF possibly through cell fusion, no MNs	Extended lifespan (10–13 days), prolonged rotarod performance (~14 days), protected endogenous MNs	Corti et al., 2004 [167]
rMSC^{GFP}, isolated from transgenic GFP-expressing rats	SOD1^{Leu126delTT} mouse, 14 weeks	3–4×10^5 cells, intrathecal into fourth ventricle	Some cells found in brain and SC, no analysis on differentiation	No effect on lifespan or disease onset (extended lifespan in females only), no delay in weight loss, no delay in decline of extension score or stride length	Morita et al., 2008 [168]
hMSCs	SOD1^{G93A} mouse, 8 weeks, 6.0 Gy irradiated	3×10^6 cells, intravenous	Few HNu$^+$ cells colabled with Tuj1 and GFAP in brain, brain stem and SC, no MNs	Extended lifespan (~18 days), delayed disease onset (~14 days), prolonged rotarod performance, protected endogenous MNs, preserved sciatic nerve CMAP amplitude	Zhao et al., 2007 [169]
hMSCs or hMSC-derived neural stem-like cells (hMSC-NSCs)	SOD1^{G93A} mouse, 45 days	10^5 cells, intrathecal into cisterna magna	Widespread cell migation within the subarachnoid space 10 days after transplant, cells found in brain parenchyma, no morphological analysis performed	No effect on lifespan, disease onset, endogenous MN survival and performance on running wheel	Habisch et al., 2007 [170]
hMSCs	SOD1^{G93A} mouse, 28 weeks	10^5 cells, intraspinal at L1–L2	GFAP (<1%), MAP2 (<1%), no MNs	Delayed motor score decline, protected endogenous MNs (35%), decreased microgliosis, decreased astrogliosis, prolonged rotarod performance	Vercelli et al., 2008 [171]
hMSC^{GFP}-GDNF, modified with retrovirus-GFP and lentivirus-GDNF	SOD1^{G93A} rat, 80 days	1.2×10^5 cells/injection, 3 injections at 1 week intervals, intramuscular	Cells survived, but differentiation not determined	Extended lifespan (~28 days), no effect on disease onset, maintained large endogenous motor neurons, delay denervation from	Suzuki et al., 2008 [172]

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Stem Cell Therapy	Mutant SOD1 Model and Age at Grafting	Transplant Parameters	Morphological Analysis	Effect on Disease Progression	References
				neuromuscular junction, delayed decline in BBB	
hUCBCs, combined from 11 donors according to blood type	$SOD1^{G93A}$ mouse, 8 weeks, 8.0 Gy irradiated	$34.2–35.6\times10^6$, retro-ocular	Not performed	Extended lifespan (~21 days)	Ende et al., 2000 [166]
hUCBCs, pooled from donors according to blood type	$SOD1^{G93A}$ mouse, 7 weeks, 8.0 Gy irradiated	$70.2–73.3\times10^6$ cells, retro-ocular	Not performed	Extended lifespan (~30–39 days)	Chen and Ende, 2000 [173]
hUCBCs	$SOD1^{G93A}$ mouse, 9.5 weeks	10^6 cells, intravenous	HNu^+ cells colabeled with CD45 (leukocyte antigen), Nestin, Tuj1 and GFAP in brain and lumbar and cervical SC, morphology typical of neuronal and glial cells	Extended survival (~3 weeks), delayed weight loss, preserved hindlimb extension, delayed motor decline on footprint test	Garbuzova-Davis et al., 2003 [174]
hUCBCs	$SOD1^{G93A}$ mouse, 7–8 weeks	10×10^6, 25×10^6, or 50×10^6 cells, intravenous	$Nestin^+$ cells found in cervical and lumbar SC	Optimal dose 25×10^6 extended survival (~14 days), delayed weight loss, maintained hindlimb extension, prolonged motor performance on rotarod, decreased levels of proinflammatory cytokines in the lumbar SC, decreased microglial cell density in cervical and lumbar SC	Garbuzova-Davis et al., 2008 [175]
$hUCBC^{VEGF\text{-}L1CAM}$, modified with human VEGF and mouse L_1CAM by electroporation	$SOD1^{G93A}$ mouse, 22–25 weeks	10^6 cells, retro-orbital	Cells found in blood vessel walls of lumbar SC, $CD34^+$ human endothelial cells	Not described	Rizvanov et al., 2008 [176]
hUCBCs or hUCBC-derived neural stem-like cells (hUCB-NSCs)	$SOD1^{G93A}$ mouse, 45 days	10^5 cells, intrathecal into cisterna magna	Widespread cell migration within the subarachnoid space 10 days after transplant, cells found in brain parenchyma, no morphological analysis performed	No effect on lifespan, disease onset, endogenous MN survival and performance on running wheel	Habisch et al., 2007 [170]
$hNPC^{GDNF}$, isolated from 22 week human fetus and modified with lentivirus-GDNF, treated with CNTF	$SOD1^{G93A}$ rat, 80 days	1.2×10^5 cells/injection, intraspinal bilaterally at L1 and L3	HNu^+ cells colabeled GFAP (~5%), no Tuj1, most $Nestin^+$, no MNs	No effect on lifespan and BBB scores, upregulation in ChAT expression, surviving $ChAT^+$ cells had larger soma, no effect on $ChAT^+$ cell survival, increased GDNF in SC	Klein et al., 2005 [177]
$hNPC^{GDNF}$, isolated from 15 week human fetus and modified with lentivirus-GDNF	$SOD1^{G93A}$ rat, 70 days	$1.2–1.8\times10^5$ cells/injection, 4 injection sites, intraspinal at L1-4 unilaterally	Nestin (>95%), GFAP (<10%), no neurons	No effect on lifespan, disease onset or BBB score, protected endogenous MNs, no effect on denervation	Suzuki et al., 2007 [178]

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Stem Cell Therapy	Mutant SOD1 Model and Age at Grafting	Transplant Parameters	Morphological Analysis	Effect on Disease Progression	References
hNSCs, isolated from cervical spinal cord of 8 week human fetus	$SOD1^{G93A}$ rat, 62 days	5×10^4 cells/injection, 8 injection sites, 4/side bilaterally intraspinal at L4-5	HNu^+ cells colabeled with Tuj1 (~70%), Nestin (~19%), GFAP (~1%)	Extended lifespan (~11 days), delayed disease onset (~7 days), protected endogenous MNs (~50%), delayed weight loss, prolonged performance on BBB score and inclined plane test, increased GDNF and BDNF in CSF and lumbar SC	Xu et al., 2006 [179]
hNSCs, isolated from cervical spinal cord of 8 week human fetus	$SOD1^{G93A}$ rat, 2 months	2×10^4 cells/injection, 4 injection sites, 2/side bilaterally intraspinal at L4-5	HNu^+ cells colabeled with Tuj1, some Nestin, rarely GFAP, no O4, evidence of graft integration into host circuitry	Cells with a combined immunosuppressive regimen prolonged lifespan (~2–3 weeks), delayed disease onset (~1–2 weeks) and delayed motor score decline compared to dead cell grafts and live cell grafts with FK506 monotherapy	Yan et al., 2006 [180]
mNSCs, Lewis X^+/ $CXCR4^+$, isolated from β-Actin-GFP-or Hb9-GFP-expressing adult mice	$SOD1^{G93A}$ mouse, 70 days	2×10^4 cells, intraspinal at L4-5	β-actin-GFP: ChAT (~20%), MAP2 (~45%), GFAP (~26%), O4 (~4%); Hb9-GFP: ChAT (~77%)	Extended lifespan (~23 days), delayed disease onset by prolonging rotarod performance (~21 days), protected endogenous MNs (~75%)	Corti et al., 2007 [156]
mOB-NPCs, isolated from adult mouse olfactory bulb	$SOD1^{G93A}$ mouse, 70 days	1.5×10^5 cells/injection, 4 injection sites, lumbar intraspinal	ChAT (~30%), interneurons, GFAP (~15%), O4 (~5%)	Extended lifespan (~30 days), delayed disease onset (~27 days), protected endogenous MNs (32%), delayed weight loss, prolonged performance on running wheel and inclined plane test	Martin and Liu, 2007 [157]
$hNSC^{GFP}$, isolated from 8 week human fetus and modified with rAAV-GFP	$SOD1^{G93A}$ rat, 4 months	10^5 cells/injection, 4 injection sites, Intraspinal at L4-5 bilaterally, and C4-5 bilaterally	Majority GFP^+ cells colabeled with ChAT, few GFP positive axons observed in L5 ventral root	Extended lifespan (~17 days), delayed disease onset (~8 days), delayed weight loss, delayed motor score decline, prolonged performance on wire mesh ascending test and in a photobeam activity system	Thonhoff et al., 2007; Thonhoff et al., 2008; see footnote

BDNF, brain-derived neurotrophic factor; ChAT; choline acetyltransferase; CMAP, compound muscle action potential; CNTF, ciliary neurotrophic factor; CSF, cerebrospinal fluid; CXCR4, chemokine (C-X-C motif) receptor 4; GDNF, glial cell line-derived neurotrophic factor; GFAP, glial fibrillary acidic protein; GFP, green fluorescent protein; hMSC, human mesenchymal stem cell; hNPC, human neural progenitor cell; hNSC, human neural stem cell; HNu, human nuclear antigen; hUCBC, human umbilical cord blood cell; L1CAM, L1 cell adhesion molecule; MAP2, microtubule-associated protein 2; mBMC, mouse bone marrow cell; MN, motor neuron; mOB-NPC, mouse olfactory bulb neural precursor cell; NeuN, neuronal nuclei; NF, neurofilament; O4, oligodendrocyte marker; rAAV, recombinant adeno-associated virus; rMSC, rat mesenchymal stem cell; SC, spinal cord; SOD1, superoxide dismutase 1;Tuj1, antibody against β-III tubulin; VEGF, vascular endothelial growth factor; YFP, yellow fluorescent protein

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