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Advances in stem cell research for Amyotrophic Lateral Sclerosis

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Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disorder characterized primarily by motor neuron loss in the motor cortex and spinal cord leading to progressive disability and death. Despite the relative selectivity of motor neuron loss, recent studies have implicated other cell types including astrocytes and microglia as contributors to this cell death. This understanding has resulted in stem-cell-replacement strategies of these cell types, which may result in neuroprotection. In addition to cell-replacement strategies, the development of induced pluripotent stem cell (iPSC) technologies has resulted in the establishment of motor neuron cell lines from patients with ALS. The use of iPSCs from ALS patients will allow for potential autologous cell transplantation, drug discovery, and an increased understanding of ALS pathobiology.

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Introduction

Amyotrophic Lateral Sclerosis (ALS) is a neurodegenerative disease caused by the progressive loss of motor neurons in brain and spinal cord, resulting in progressive paralysis and death within two to five years after diagnosis. The vast majority of ALS cases are sporadic (sALS), with approximately 5–10% of cases inherited (familial; fALS). In 20% of fALS patients, there is a clear genetic link to point mutations in the gene encoding for Cu/Zn superoxide dismutase 1 (SOD1) [1]. This has led to the development of transgenic rodents that carry mutant human SOD1 genes (i.e. with amino acid substitutions G93A, G85R, and G37R) and show many of the clinical and histopathological features of familial as well as sporadic ALS [2–5]. To date, the cause of the relatively selective death of motor neurons in ALS remains elusive; however, numerous mechanisms that likely contribute to disease pathogenesis have been proposed [6]. These include oxidative damage, glutamate excitotoxicity, mitochondrial dysfunction, cytoskeletal abnormalities, impaired neurotrophic support, mutant SOD1 and neurofilament protein aggregation, axonal transport defects, activation of apoptotic pathways, altered glial function and, more recently, impairment of blood–brain/spinal cord barrier [7,8]. However, over the past two decades, a recurring theme suggests that cell death in ALS is not only dependent upon motor neuron abnormalities but that other cell types participate in disease development. In light of these observations, stem cells provide enormous potential for understanding and treating the disease.

The development of relevant therapies for ALS has proven particularly challenging due to: firstly, the lack of understanding of the underlying cause(s) of ALS; secondly, the spatially diffuse death of motor neurons throughout the neuraxis; thirdly, the selective disruption of both short and long distance axonal connections between local and projection interneurons in the CNS; and lastly, the chronic, insidious, neurodegenerative course of the disease which begins before the time of formal diagnosis.

Much attention has been placed on cellular therapy as a promising new treatment for ALS. Cellular therapy is an attractive approach given the possibility that donor cells might replace dead motor neurons or provide protection to surviving host motor neurons. Stem cells, generated from either embryonic or adult tissues, are lucrative candidates for donor cells given their ability to divide for indefinite periods in culture and give rise to multiple cell types. Here we discuss recent progress in stem cell research for transplant-based cellular therapies in animal models of ALS and human ALS patients, along with challenges to the development of such therapeutic applications. In addition, we discuss induced pluripotent stem cells (iPSCs) as a novel resource to study ALS disease mechanisms, screen potential candidate drugs, and develop new therapies.

Stem cell transplantation strategies

Motor neuron replacement

The hope for those patients with ALS is that stem cell transplantation will replace motor neurons and result in the eventual recovery of neuromuscular function to premorbid levels. With that goal in mind, many initial strategies in ALS focused on motor neuron replacement and regeneration. Past *in vitro* and *in vivo* studies have successfully generated motor neurons from both mouse and human pluripotent embryonic stem cells (ESCs) that maintain typical motor neuron phenotype and show functional engraftment after transplantation into the spinal cords of developing chicks and adult rodents with motor neuron deficiencies [9–12]. A common theme to these strategies is that the beneficial effects seemed to be dependent on motor neuron replacement in more static models of motor neuron loss rather than a progressive disorder as is seen in the mutant SOD1 rodent models of ALS.

The major mechanistic limitation to motor neuron replacement and regeneration is that ALS (and the models which mimic the disease) is a progressive disorder where death ultimately comes from diaphragmatic failure. Any motor neuron replacement strategy would first have to recapitulate the synaptic inputs from upper motor neurons and interneurons and then extend axons to an appropriate target muscle which, at a rate of 1–3 mm/day, would require months to years (in humans) before target muscle innervation would be adequate. This realization is one of the most underappreciated limitations to neuronal replacement for this disease. Because of this limitation, among others, motor neuron replacement is not currently considered a good treatment strategy.

New observations suggest that the differentiation of human NSCs may provide potential support to host motor neurons in the $SOD1^{G93A}$ rat model through their differentiation, not into motor neurons, but rather into other neuronal subtypes including inhibitory GABAergic neurons with synaptic connections between transplanted and host motor neurons (Table 1). This may provide a rationale for the neuroprotective effects seen in previous studies despite the absence of any axon outgrowth into target muscle in this model [13].

Astrocyte replacement

A more practical approach to motor neuron replacement might be to deliver a stem cell population that migrates to sites of motor neuron degeneration, replacing nearby support cells (i.e. glia) to provide a protective environment to help remaining motor neurons survive and function. Converging data suggest that motor neuron death in ALS is non-cell autonomous, identifying microglia and astrocytes as key drivers of disease progression. Studies with chimeric mice showed that increasing the proportion of healthy, wildtype non-neuronal cells in proximity to mutant human SOD1-expressing motor neurons reduces mortality of those motor neurons and extends survival in these animals [14••]. More recently, it was found that a reduction in mutant human SOD1 selectively from microglia or astrocytes using a CRE-lox system in mice prolongs disease progression but has no effect on disease onset [15,16].

In light of this, Lepore *et al.* [17•] transplanted rodent glial restricted precursor (GRP) cells — tripotential astrocyte-restricted and oligodendrocyte-restricted precursor cells derived from developing embryonic spinal cord — into the spinal cords of $SOD1^{G93A}$ rats (Table 1). Multiple, targeted injections were aimed at specific motor neuron pools of the cervical spinal cord involved in respiratory function, as respiratory failure is the main cause of death in ALS [18]. Transplantation of GRP cells led to extensive differentiation of grafts into mature astrocytes that prevented host motor neuron loss and reduced microgliosis. This neuroprotective effect was partially attributed to the ability of these grafts to maintain normal levels of the glutamate transporter GLT-1, an astrocyte specific protein reduced in both animal models of ALS as well as human ALS [4,19]. GRP grafts also extended survival and disease duration, and slowed declines in forelimb motor and respiratory physiological functions. These results demonstrate that stem cell transplantation-based astrocyte replacement is a potentially viable option for ALS therapy. Focal delivery into the cervical spinal cord, as performed in that study, presents a new therapeutic strategy to target phrenic motor neurons that innervate the diaphragm and ultimately affect the survival of ALS patients.

Microglial replacement

The target for cell-replacement strategies is not limited only to neural subtypes. Two notable studies have shown that transplantation of adult murine bone marrow (BM) cells — a source rich in mesenchymal and hematopoietic stem cells (MSCs and HSCs, respectively) — via intraperitoneal injection into irradiated $SOD1^{G93A}$ and $SOD1^{G93A}/PU^{-/-}$ mice (born without CNS microglia or peripheral immune cells) leads to efficient differentiation of these cells into microglia [20•,21]. BM transplants also slowed motor neuron loss and prolonged disease progression and survival in these transgenic animals. The ability of BM stem cells to develop into mature CNS microglia, promote neuroprotection, and possibly suppress inflammatory factors (i.e. free radicals such as nitric oxide and superoxide anion) [20•] in CNS of ALS model mice prompted a recent clinical study in which peripherally harvested, donor-derived HSCs were intravenously administered into irradiated patients with sALS [22] (Table 1). HSCs give rise to a variety of blood and immune cells and can differentiate into microglia when introduced into a neural environment [23], thus having the capacity to replace damaged microglial cells with healthy microglia in patients with ALS. Unfortunately, no significant clinical benefit was found following HSC treatment. However, transplanted HSCs infiltrated areas of motor neuron injury and neuroinflammation, and engrafted as immunomodulatory cells. On the basis of this finding, the authors concluded that such cells could potentially provide a cellular vehicle for viral vector-mediated gene delivery to the degenerating CNS.

Cellular strategies for the delivery of neuroprotective factors

As our understanding of the pathways relevant to ALS pathobiology becomes more sophisticated, targeted stem

Table 1

Current progress (2007–2009) in stem cell transplantation therapies for ALS

	Stem cell source	Conditioning regimen	Delivery method	Dose	Cells identified post-transplant	Outcome	Reference
Animal models							
Presymptomatic SOD1^{G93A} rats (P56[a])	Human NSCs (from eight-week-old fetuses)	FK-506 (1 mg/kg i.p. daily)	Bilateral lumbar SC injections	2×10^4 cells/site, 8 sites	GABAergic neurons	Formed functional synapses with host MNs in ventral horn but not NMJs with host muscle	[13]
Presymptomatic SOD1^{G93A} rats (P90)	Rat GRPs (from 12.5-week-old fetuses)	Cyclosporin A (10 mg/kg i.p. daily)	Bilateral cervical SC injections	1×10^5 cells/site, 6 sites	~88% GFAP$^+$ astrocytes; ~9% RIP$^+$ oligodendrocytes; ~3% nestin$^+$ cells; no mature neurons	Decreased GLT-1 levels; prevented MN loss; increased lifespan (~17 d) and disease progression; delayed declines in forelimb motor and respiratory functions	[17]
Presymptomatic SOD1^{G93A} rats (P65)	Human NPCs (from fetal brain) infected with lenti-GDNF	Cyclosporin A (10 mg/kg i.p. daily)	Unilateral lumbar SC injections	120–180,000 cells/site, 4 sites	>95% Nestin$^+$ and <10% GFAP$^+$ migratory cells; no mature neurons	Released GDNF; prevented MN loss; did not innervate muscle end plates; no functional recovery of ipsilateral hindlimb	[24]
Presymptomatic SOD1^{G93A} rats (P80)	Human MSCs (from neonatal BM) infected with lenti-GDNF	Cyclosporin A (10 mg/kg i.p. daily); focal muscular injury with BVC (0.35 mg)	Bilateral muscle injections (TA, forelimb triceps brachii, long muscles of dorsal trunk)	120,000 cells/site; spaced 1 week apart/muscle group	Skeletal muscle	Released GDNF; increased number of NMJs and MN cell bodies; prevented loss of proximal MNs; increased lifespan (~28 d) and disease progression	[25•]
Presymptomatic SOD1^{G93A} mice (P196)	Human MSCs (adult BM)	None	Unilateral lumbar SC injection; intrathecal injection (did not work)	100,000 cells/site	<1% GFAP$^+$ and <1% MAP2$^+$ cells	Prevented astrogliosis and microglial activation; delayed MN loss; improved motor performance	[26]
Presymptomatic SOD1^{G93A} mice (P49–56)	Human mononuclear UCB cells	Cyclosporin A (10 mg/kg i.p. daily)	i.v. injection	10×10^6 cells, 25×10^6 cells or 50×10^6 cells per mouse	No histological analysis performed	25×10^6 cells most effective dose; decreased proinflammatory cytokines; reduced microgliosis; restored leukocyte profiles in peripheral blood; increased lifespan (20–25%) and delayed disease progression (15%)	[28]
Presymptomatic SOD1^{G93A}/PU.1$^{-/-}$, SOD1^{G93A}/RAG2$^{-/-}$ mice (P1)	Mouse BM (from wildtype, SOD1^{G93A}, or CCR2$^{-/-}$ adult mice)	γ-Irradiation (400 rads)	i.p. injection, SOD1^{G93A}/PU.1$^{-/-}$ mice; i.v. injection, SOD1^{G93A}/RAG2$^{-/-}$ mice	1×10^7 cells per SOD1^{G93A}/PU.1$^{-/-}$ mouse; 3×10^7 cells per SOD1^{G93A}/RAG2$^{-/-}$ mouse	CD4$^+$ T cells at all stages of disease; CD8$^+$ T cells at terminal stages (within ventral gray matter)	Reconstituted CD4$^+$ T cells; prolonged survival, suppressed cytotoxicity, and restored glial activation	[32]

Table 1 (*Continued*)

	Stem cell source	Conditioning regimen	Delivery method	Dose	Cells identified post-transplant	Outcome	Reference
Humans							
sALS patients (20–65 years)	Peripheral blood $CD34^+$ HSCs from HLA-matched sibling donors	Total body irradiation (450 cGY); tacrolimus (0.3 mg/kg/d IV) and methotrexate (5 mg/m^2 IV)	Intravenous injection	Absolute neutrophil count $>0.5 \times 10^9\ L^{-1}$	$CD68^+$ macrophage-monocytes in spinal cord	No clinical benefit; increased MCP-1 expression	[22]
ALS patients (21–75 years)	Autologous MSCs (from BM)	None reported	Multiple intraspinal thoracic SC injections	$\sim 57 \times 10^6$ cells total	No histological analysis performed	Decelerated linear decline of the forced vital capacity and of the ALS-FRS score in some patients	[27]
ALS patients (32–62 years)	Autologous CD 133^+ cells (from peripheral blood)	None reported	Bilateral injection into frontal motor cortex	$2.5–7.5 \times 10^5$ cells/site	No histological analysis performed	Transplanted patients survived a mean of 47 months more than control patients (from time of diagnosis to end of follow-up)	[31]

SC, spinal cord; BVC, bupivacaine hydrochloride; TA, tibialis anterior; i.v., intravenous; i.p., intraperitoneal; GFAP, glial fibrillary acidic protein; MAP-2, microtubule-associated protein 2; MN, motor neurons; HLA, human leukocyte antigen; MCP-1, monocyte chemotactic protein 1.

[a] Age at transplantation time.

cell transplantation strategies will be designed to deliver trophic and/or immunomodulatory factors to areas of motor neuron degeneration. Suzuki *et al.* [24] successfully engineered neural precursor cells (NPCs) — comprised of multiple classes of dividing cells including NSCs and lineage-restricted precursors — to genetically express and continually secrete glial-derived neurotrophic factor (GDNF) and transplanted them directly into the lumbar spinal cord of $SOD1^{G93A}$ rats (Table 1). Engraftment of these cells resulted in robust cellular migration into degenerating regions, efficient delivery of GDNF, and remarkable preservation of host motor neurons. Interestingly, motor neuron survival was not accompanied by continued innervation of muscle end plates, and therefore resulted in no improvement in ipsilateral hindlimb use. To maintain these neuromuscular connections, Suzuki *et al.* [25•] subsequently engineered human MSCs, which give rise to skeletal muscle, to secrete GDNF and transplanted them bilaterally into three muscle groups in $SOD1^{G93A}$ rats (Table 1). The cells survived within muscle, released GDNF, and significantly increased the number of neuromuscular connections and motor neuron cell bodies in spinal cord at mid-stages of disease. In addition, intramuscular transplantation of these cells ameliorated motor neuron loss within the spinal cord where it connected with limb muscles receiving transplants, and delayed disease progression but not onset, increasing the overall lifespan of animals. Together, these studies have provided an initial framework for the future development of a combinatorial stem cell/growth factor delivery method for humans, which could potentially target both skeletal muscles (i.e. nerve terminals of motor neurons) and spinal cord (i.e. cell body) to slow progression of ALS. GDNF is only one potential candidate for study. One can imagine that cell-based delivery of other neuroprotective factors may be realized as our understanding of disease mechanisms expands.

Potential immune modulation by transplanted cells

The transplantation of MSCs has also resulted in neuroprotection in SOD1 animal models with proposed mechanisms including the elaboration of trophic factors and immunomodulatory properties [26] (Table 1). This finding culminated in a recent pilot clinical trial which involved the intraspinal injections of autologous *ex vivo* expanded human MSCs into the thoracic spinal cord of ALS patients [27].

Similarly, HSC-rich human umbilical cord blood (UCB) cells have been shown to have neuroprotective and therapeutic benefit in $SOD1^{G93A}$ mice [28–30] possibly through the active involvement of these cells in inhibiting the host immune/inflammatory response (i.e. cytokines) (Table 1). In humans, autologous transplantation of peripherally derived CD133+ HSCs into the frontal cortex was recently undertaken in a small cohort of ALS patients [31].

In a rigorously designed set of studies, Beers *et al.* [32] sought to identify the potential role of $CD4^{+}$ T cells in motor neuron injury by performing BM transplants on mutant SOD1 mice crossed with several mouse lines deficient in their capacity for immune modulation (Table 1). The results established that the lack of T-cell recruitment accelerated disease progression and death. However, the reconstitution of the T-cell population through BM transplantation resulted in neuroprotection in the mutant SOD1 models possibly through the elaboration of trophic factors and the reduction of cytotoxic factors.

Collectively, these studies highlight the potential protective capability of somatically derived adult stem cells when grafted into CNS or delivered peripherally, and illustrate another potential target pathway in ALS. Perhaps the most attractive feature of BM and human UCB stem cells is that the use of such cells avoids ethical issues associated with ESCs or more restricted-lineage precursor cells derived from fetal tissue, and provides an autologous source for deriving cells.

Caveats in interpreting human clinical data for cell transplantation in human ALS

Because ALS is a neurodegenerative disease with a poor prognosis, the potential for using cell-replacement therapies has spawned several small pilot trials using a variety of different cell types. Unfortunately, most of these studies have a number of limitations in clinical trial design. Confounding factors include the lack of uniformity of the ALS population and the number of cells transplanted, lack of a proposed mechanism of action, poor follow-up, and no inclusion of autopsy tissue to confirm engraftment, survival, ectopic engraftment, and/or tumor formation of these cells. Overinterpretation of the potential efficacy of these transplantation strategies using small patient cohorts has been problematic. More concerning, however, is the presence of anecdotal reports of clinical improvements in ALS function following stem cell transplantation which can be seen on Internet sites and has to some degree muddied the waters of meaningful interpretation.

Stem cells for disease modeling and drug discovery

Of equal, if not greater value, to the use of stem cell transplantation as a therapeutic is the long-term potential for using stem-cell-derived neural cells for understanding ALS-relevant disease mechanisms and for the development of ALS therapeutics.

In one creative experimental paradigm, investigators used cocultures of mouse or human ESC-derived motor neurons with human mutant SOD1-expressing astrocytes. This mix-and-match methodology demonstrated selective destruction of those motor neurons by toxic mutant astrocyte-secreted factors acting through a Bax-dependent mechanism [33•,34,35,36•]. Together, these creative experiments have provided an *in vitro* platform for the future use of stem-cell-derived coculture experiments in understanding cell–cell interactions in ALS.

New and exciting studies have now also made it possible to reprogram adult fibroblast cells into pluripotent stem cells using forced expression of the transcription factors Klf-4, Sox-2, Oct-4, and c-Myc [37••,38]. These iPSCs offer advantages over traditional stem cells because of their capacity to generate differentiated cells, including neurons and glia, from individual patients with ALS. In turn, iPSC-derived cells could be utilized for: firstly, the study of how different cell types are involved in ALS pathobiology, which could possibly redefine non-cell autonomous aspects of the disease; secondly, the unraveling of cellular mechanisms that may trigger familial, as well as sporadic, forms of the disease; and thirdly the discovery of candidate drugs via high throughput screening in culture. Eventually, these cells could provide an autologous cellular replacement strategy in patients with ALS, eliminating any ethical or technical concerns seen with traditional stem cells. Toward this goal, Dimos *et al.* [37••] successfully directed the differentiation of iPSCs, generated from an elderly patient with fALS and a SOD1 mutation, into motor neurons expressing appropriate motor neuron markers including Hb9 and ISLET.

Conclusion

Current preclinical studies collectively suggest that stem cell transplantation aimed toward protecting, rather than replacing/repairing, motor neurons is currently the most appealing approach to treating humans with ALS. For clinical application to be considered, however, numerous hurdles must be overcome. It is important that these putative stem-cell-based therapies pass vigorous safety testing. Optimal cell dose, source, route of delivery, and immunosuppressive regimen (to keep stem cells alive in host tissue) must be carefully considered. If these protective strategies prove safe and effective in humans, they could pave the way for improvements in hESC, fetal, BM, and iPSC-based replacement strategies. In the future, transplant-based therapies may consist of a combination of stem-cell-subtypes delivered to multiple, defined targets throughout the CNS to provide both neuroprotection and neuroreplacement/neurorepair.

More proximally, iPSC-derived neural cell subtypes have the potential for helping us to understand the interactions between cells and their respective contributions to cell dysfunction and death *in vitro* and may allow for screening of compounds for targeted ALS therapeutics.

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